Improvement of Black pepper (*Piper nigrum* L.) through induction of stress tolerance *in vitro*

Thesis submitted to the University of Calicut
In partial fulfillment for
the award of degree of

Doctor of Philosophy in Botany

by

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2004

DECLARATION

I hereby declare that this thesis entitled "Improvement of black pepper (*Piper nigrum* L.) through induction of stress tolerance *in vitro*" is a record of research work carried out by me at Indian Institute of Spices Research, Calicut, Kerala, during the course of research for Ph.D., under the supervision of Prof. (Dr.) V.J. Philip (Guide), Retd. Head & Coordinator, Dept. of Biotechnology, University of Calicut and Prof. K.V. Peter (Co-guide), Vice-Chancellor, Kerala Agricultural University, Thrissur. This thesis or part of it has not previously formed a basis for award of any degree or diploma of any other University.

(ANURADHA. Y)

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Date :

Dedicated to……..

The
love
of
My Parents and Sisters

CERTIFICATE

This is to certify that the thesis entitled "Improvement of black pepper (*Piper nigrum* L.) through induction of stress tolerance *in vitro*" is a record of bonafide research work carried out by **Ms. Anuradha.Y.**, M.Sc. at Indian Institute of Spices Research, Calicut, Kerala, under our guidance and that this has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title or recognition.

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Acknowledgements

I wish to express my profound sense of gratitude to my Guide and Teacher, Prof. V.J. Philip, (Retd.) Head & Coordinator, Dept. of Biotechnology, University of Calicut, for all his encouragement, valuable suggestions and support during the entire course of my work and also during the preparation of this thesis. I am extremely thankful to him for introducing me to the right *in vitro* system in black pepper, the somatic embryogenic cell suspension culture, which served as an excellent system for all the studies which I undertook in my Ph.D. programme.

My humble respects to The Almighty God who showered HIS grace by educating me from all the experiences he put me through. The rich experiences He gave me during my interactions with people and in my work have helped me a great deal in evolving as a person.

I profusely thank Prof. K.V. Peter, former Director of IISR and the present Vice-Chancellor of Kerala Agricultural University, Trichur, for providing the laboratory facilities at IISR and guidance. I thank the succeeding Directors of IISR, Calicut and the present Director, Dr. V.A. Parthasarathy, for their support to complete my research work. My sincere gratitude is also due to Dr. K. Unnikrishnan, Retd. Head of the Dept. of Botany, University of Calicut, for offering me the required facilities during the initial period of my Ph. D programme.

I thank sincerely Dr. K.V. Ramana, Project Coordinator (Spices); Dr. B. Chempakam, HD, Crop Production; Mr. B. Krishnamoorthy, HD, Crop Improvement and Dr. M. Anandaraj, HD, Crop Protection, IISR, Calicut, for their full support and encouragement during the course of my research.

I also thank Drs. A. Kumar, Veena Ashok and Suseela Bhai from Plant Pathology, K.S. Krishnamurthy, K. Nirmal Babu, T.J. Zachariah, S. Devasahayam, B. Sasikumar, Johnson George, R. Ramakrishnan Nair, N.K. Leela and all other scientists at IISR for their sincere help. I thank Mr. Jayaraj for his help in the statistical analysis of my results and to Mr. Sudhakaran for his skillful photography.

My heartfelt thanks to my colleagues, Mr. Biju Joseph and Mr. Dominic Joseph for training me in handling the embryogenic cell suspension culture system in black pepper, which served as an excellent raw material for all my studies. I wish to thank Mr. K. Praveen, Mr. P.A. Sheriff, who helped me immensely in collecting literature relating to my work and Mr. Gobinath whose assistance and help in various biochemical analyses helped me a great deal.

My special thanks to my friends Santhosh Bhaiyya, Jisha, Harish, B. Beena, Vijaya, Arun and Zeena, for all their moral support and friendship. I also thank my other friends, former and present, Minoo, Geetha, Maya, Nema, Ganga, Rajan, Diby, Rajeshwari, Anil and Stephen Jebakumar for their pleasant company. My sincere thanks to all the people of Plant Pathology department who helped me in one way or the other in making my stay at IISR more comfortable and pleasant. My heartfelt thanks to Mrs. Chandravally, Mr. Unni Nair, Mr. Prakash, Mr. Balakrishnettan, Mr. Divakaran, Prabitha and Sunoj for all their help during the course of my study.

My thanks to the Administrative and Accounts staff of IISR, for all their help with official matters. A BIG ThanQ for the security personnel, labour force and helpers at IISR, some of whom were mere acquaintances, but whose friendly smiles and obliging gestures made wonders to lift my spirits everyday during my stay at IISR.

Words fail me in expressing my gratitude and indebtedness to Hafiz, whose support has been overwhelming. I also thank my well-wishers, Indira aunty, Manju aunty, Vatsala aunty, Swami Adhyatmanandji and Jeeja for their words of encouragement and support.

My husband, Raj, has been a great source of support and encouragement for me throughout my PhD programme. His consistent prodding, help and patience saw me through the completion of this thesis. My sincere thanks are also due to my in-laws for their cooperation and understanding.

Finally, I thank my parents and family for **EVERYTHING**. My amma's patience and unstinted cooperation helped me greatly in my work, not having to bother much about the affairs at home. My 'Nana' stood as a pillar behind me in all my endeavours and was very particular that I do the best in everything. My sisters, Aruna and Anupama have been too good in being too cooperative and considerate with me at all times. I warmly thank them and their families for all their encouragement.

Financial support from the Council of Scientific and Industrial Research (CSIR) granted to me as fellowships is gratefully acknowledged. Finally I want to thank Mr. Sajeevji for his help in the preparation of this manuscript.

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Introduction

Introduction

Black pepper, popularly known as 'King of Spices' and also as 'black gold' is an economically important spice that is used world over. The spice produce is the mature dried fruit (- a berry) of *P. nigrum* L., a tropical and woody perennial climber belonging to the family Piperaceae.

Black pepper, valued for its characteristic pungency and flavour, is widely used as an ingredient in many food preparations in India and abroad. It also has medicinal values and finds use in many drug preparations in the traditional Ayurvedic medicine of India.

Black pepper originated in and is indigenous to Western Ghats bordering the Malabar Coast of Kerala, India. It was from here that black pepper was later taken to Indonesia, Malaysia and other Pacific Island nations, South-East Asia, Latin America, Africa and other pepper growing countries of the present day. Pepper is grown in 26 countries. India is the largest producer of the spice, closely followed by Indonesia, Malaysia and Brazil.

In India, black pepper is grown over an area of 181,530 ha in India, producing about 57.3mt of the economic produce (Source: Directorate of Economics & Statistics, Govt. of India, NewDelhi). Pepper from India accounts for nearly 40% of world production. Kerala accounts for 95% of the country's area under cultivation and production. India earned an estimated foreign exchange value of Rs. 86,497 lakhs for the year 1999-2000 by exporting about 42,100 mt of pepper that accounts for 46.48% in terms of the total value of spices. However its export came down to 16,700mt valuing Rs.1435.50 lakhs during 2003-04 (Anon,2004).

Black pepper in general is a vegetatively propagated plantation crop. After planting, rooted cuttings start flowering and yielding from the 2nd year though the yield stabilizes in the 4th or the 5th year. The climatic requirements of the crop for optimum productivity (Vasantha, 1996) are:

1. Soil:

Pepper requires a porous, friable soil with good drainage, adequate water holding capacity, rich in humus and essential plant nutrients.

2. Temperature:

   Pepper vines do not tolerate extremes of temperature. Though they survive in the range of 10-40oc., the optimum temperature for their productivity is 20-30oc.

3. Humidity:

   A dry and slightly windy atmosphere is preferred though a hot humid climate is good for growth and development of pepper. This is because high humidity interferes with normal growth and also invites diseases and pests.

4. Rainfall:

   Well-distributed heavy rainfall ranging from 1000-3000mm is required for proper growth and development of pepper.

Though India is the native home of black pepper, and has the largest area under cultivation, the productivity of the crop is lowest in the world with just 303 kg/ha (1996-97), which is about ten times lower than that of Thailand (3,594 kg/ha) and about 6 times lower than that of Malaysia (1,888 kg/ha) (Ravindran, 2000). The world consumption of black pepper in the next two decades is estimated to increase by about 100,000mt. India can meet the world demand only by increasing the productivity. This is possible by wide-scale cultivation of varieties, which can withstand both biotic and abiotic stresses (Ravindran, 2000) that are the main production constraints of the crop.

Abiotic stress

Among the different constraints limiting pepper production in India, moisture stress is the major abiotic stress. In recent years, drought has become a regular feature and a severe problem in Kerala due to the vagaries of monsoon. Drought is regarded as one of the major production constraints in increasing the productivity of black pepper (Ramadasan, 1987). Since Kerala contributes about 95% of the total pepper production of the country, the constraints the crop faces in this region has a direct bearing on the total production of the country.

The pepper-growing belt of India receives rainfall during the Southwest monsoon period (Jun-Sept.) and northeast monsoon (Oct-Nov.), the peak being in July. Following November, Kerala experiences a dry spell of 5-6 months of summer (Dec-May). During the years 1973, 1977, 1983, 1987, 1989, when India experienced severe drought, Kerala witnessed drying up of thousands of pepper vines in the Kannur district (Sadanandan, 2000). The monsoon in Kerala has been consistently poor in the recent years, affecting the black pepper production severely. Kerala experienced drought in 2002, 2003 which resulted in low production. It is being speculated that the new crop of 2003 cannot be more than 42,000 metric tones. A further failure of rains in the pepper producing areas might result in drop in the production to even 22,600 metric tones (a report by Rawther, S.A. in the website, www.peppertrade.com). Black pepper farmers of Kerala predict a production loss of 15-20% in the current season due to the dry spell (www.spizes.com). Such a decrease in the production would affect export of Indian Pepper.

Pepper in Kerala and other pepper growing areas of Karnataka, TamilNadu and AndhraPradesh , is a rainfed crop unlike that seen in other pepper producing countries, the dry spell between December and May results in severe moisture stress. Therefore, moisture stress is a serious problem in India. Most other pepper growing countries on the other hand, either receive well-distributed rainfall or have a well-developed irrigation mechanism. In Thailand for example, Pepper is grown as an irrigated crop (Sadanandan, 2000). Irrigation during summer months (Dec-May) in India have been reported beneficial (Sadanandan, 2000).

The most important developmental phase of black pepper affected by moisture stress is the flowering phase, which commences in May-June (Vasantha, 1996). It has been found that pepper yield is significantly correlated with the rainfall received during the first half of May and the cumulative total rainfall in the second half of June. Further, it was found that 70-100mm of rainfall, depending on soil type, received within a period of 20 days is adequate for triggering the flushing and flowering mechanism of the plant (Sadanandan, 2000). Delayed monsoon delays the flowering process. Once the flowering sets in, a continuous, though not heavy rainfall is essential for proper fruit set. Dry spell even for a few days during this critical phase results in spike shedding leading to substantial reduction in pepper yield.

The traditional drought management practices consisting of mulching and growing crops like *Calpagonium* around the pepper basins may fail to protect the crop from severe drought situations and also when sensitive cultivars are grown. Acute water scarcity in major pepper growing areas of Idukki, Wyanad and Kannur districts of Kerala as well as many other areas, rules out the possibility of irrigating the crop (Vasantha, 1996, Ravindran, 2000). Therefore, developing drought-tolerant cultivars assumes significance as occurrence of drought has become a regular feature in Kerala. As on date, no variety with drought tolerance has been evolved so far, though a few promising tolerant lines, namely, Panniyur5, Acc No: 1228, 1343, 1368, 4216 and 426 (Krishnamurthy, unpublished) are under evaluation at Indian Institute of Spices Research, Calicut (Sarma *et al.*, 2001).

Biotic Stress

One of the most important reasons identified for low productivity in black pepper in India is attributed to the crop losses due to diseases and pests. Diseases of pepper have been reviewed earlier (Sarma *et al.*, 1991, Anandaraj and Sarma, 1995). A commonality of spectrum of diseases in all pepper growing countries was attributed to the possible source of planting material from India (Sarma, 2003).

Many diseases affect black pepper. The major diseases affecting the crop are *Phytophthora* foot rot (quick wilt), (Sarmra and Jose 1966) slow decline (slow wilt) caused by feeder root damage by *P. capsici* (*P. palmivor* MF4), *Radopholus similis* and *Meloidogyne incognita* (Nambiar and Sarma, 1977, Ramana, 1991), anthracnose (Pollu disease) caused by *Colletotrichum necator* (Rao,1926; Thomas and Menon,1939) and stunted disease also known as little leaf disease or mosaic disease in India, caused by a virus, the identity of which has been described differently by different researchers (CMV, Sarma *et al.*, 2001), Badna virus namely *Piper yellow mottled virus,* PYMV (Lockhart *et al.*, 1977, Bhat, *et al.*, 2003). Root rot and stem blight, caused by *Fusarium solani* f.sp. *Piperis* and the mosaic disease caused by cucumber mosaic virus are the major diseases in Brazil (Duarte and Albequereque, 1991), while in India, Indonesia and Malaysia, 4A

BLACK PEPPER AND BIOTIC STRESS


Black pepper [tea-pepper] plantation


Pepper spikes


Phytophthora foot / root rot


Soil phase
[Root / foot rot]


Pepper produce


P. capsici


Foliar infections

8

Phytophthora foot rot is the major disease (Holliday and Mowat, 1963, Sarma *et al.*, 1991c, Sitepu and Karim1991, Kueh and Sim, 1992d, 1992).

Foot rot of black pepper is caused by the oomycetous fungus, *Phytophthora capsici.* The causal organism was previously thought to be *P. palmivora* MF4 (Morphological form 4) (Sarma *et al.*, 1982, Tsao and Alizadeh, 1988, Manohara and Sato, 1992) based on sporangial characters of the pathogen. Later however, *P. palmivora* MF4 was merged with *P. capsici* since most pepper isolates resembled *P. capsici* and thus the species was redescribed and currently named as *P.capsici* (Tsao and Alizadeh, 1988, Tsao, 1991). Consequently, it was decided to refer this disease as *Phytophthora* foot rot and the organism as *P. capsici.*

Symptoms of the disease

Phytophthora foot rot is characterized by one to many dark spots on leaves having a characteristic fimbriate margin, which later coalesce leading to defoliation. The infections on runner shoots that trail on the ground occur both on tender leaves and shoots. This then reaches the stem and infects the collar, resulting in sudden wilting of the plant. Infection on spikes causes blackening of developing fruits and peduncle, later leading to shedding of spikes. Infection on underground parts such as roots and collar is fatal. The feeder root infection causes their rotting and degeneration, resulting in yellowing defoliation and drying up of plants (Anandaraj, 2000). *Phytophthora* infections are severe during the monsoon and spread through rain splashes from infected leaves and stems.

Crop loss

Crop loss of about 4.5-7.5 million tons per annum has been reported at the global level due to foot rot in black pepper (De waard, 1979). Surveys on crop losses due to foot rot of pepper in major pepper growing districts of Kerala namely, Calicut and Kannur, show that 3.4% and 9.4% of the plants respectively are lost annually resulting in corresponding production loss of about 118 and 904 tonnes (Balakrishnan *et al.*, 1986, Anandaraj *et al.*, 1989a, 1989b). Findings of a recent survey report estimates a crop loss of 9.2%in Kannur district of Kerala (Prabhakaran, 1995). Foot rot takes a heavy toll in all pepper growing countries and losses of about 95% have been recorded in individual gardens in India (Anandaraj, 2000). Majority (>90%) of the farmers in Wayanad have suffered losses due to the problem of foot rot during 1994-95, resulting in large scale devastation of yielding vines (Source: Tech. Miss. Black pepper, 2001). In Kerala, vine death of 20-30% is generally noticed. However, in some areas, disease incidence up to 90% was reported with complete wipe out of pepper gardens. Though crop loss is severe in all the districts, precise crop loss figures are not available for all pepper growing areas.

Disease management

Integrated Disease Management (IDM) involving clean cultural practices, chemical and biological control (Sarma and Anandaraj, 1996) is employed for the control of the disease.

Chemical control measures include spraying of 1% Bordeaux mixture and drenching the basins with either Bordeaux mixture or copperoxychloride and the use of systemic fungicides, such as metalaxyl (Ridomil) which have been reported to be effective against foot rot of pepper (Ramachandran *et al.,* 1991). However, the high costs of the use of these fungicides have prevented their large-scale use by farmers. Moreover, doubts about the success of chemical control were expressed by Coffey (1991) while reviewing the control of four soil-borne *Phytophthora* diseases. In addition, the application of agrochemicals leads to residual toxicity leading to inferior product, often not acceptable to pepper importing countries of the world. Further, their production and persistence in the soil is harmful to the environment. It is due to these reasons that the use of eco-friendly practices like biological control has gained importance. Biological control of *P. capsici* has been achieved with great success by the use of several microorganisms like VAM (Anandaraj *et al.,* 1996) *Trichoderma* and fluorescent Pseudomonads (Sarma and Anandaraj,1998, Rajan *et al.,* 2002)

Integrated disease management including cultural, chemical and biological controls is useful in reducing the incidence and severity of the disease but is not the panacea for eliminating the disease incidence. It is in this background that research on indentification and development of disease tolerance gains relevance. Even though black pepper originated in the western ghats of Kerala, the variabilty for disease resistance appears to be low. High degree of resistance to this disease has not been obtained so far, except that a few tolerant lines have been identified.

Thus, biotic (*Phytophthora* foot rot) and abiotic (drought) stresses form the major production constraints of black pepper. A three-year study in Kannur district in Kerala has put the total loss to 43% plants due to the combined effect of drought and diseases in pepper (Prabhakaran, 1994). Though the conventional management practices adopted to contain these stresses do ameliorate the severity of the stress on the crop, the long-term answer for these problems is surely the development of resistance/tolerance traits in black pepper. The combined effect of drought and disease has resulted in higher costs of production of Indian Black pepper in turn raising the price of the crop. This has resulted in the world market favouring other spice producing countries like Vietnam, which are in a position to export cheaper black pepper. It is unfortunate that India, known to be the largest producer of the spice in the world is itself being presently dumped with imports of economically cheaper black pepper from other countries like Sri Lanka and Indonesia, affecting the indigenous producers.

Hence, there is an imminent need to improve the situation of the black pepper production in the country by every means. Research into the development of host resistance against drought and disease would form a major component in this direction. Efforts have been in progress for many years towards breeding for tolerance to drought and disease stress in black pepper (Jebakumar *et al.*, 2001; Krishnamurthy *et al.*, 1998, 2000).

One of the many approaches in the genetic improvement of a plant for a particular trait is by exploiting the genetic variability observed in plant cell cultures. Cell and tissue cultures of higher plants have been demonstrated to be novel sources of germplasm (Larkin and Scowcroft, 1981). The genetic variability observed in plant cell cultures has allowed the isolation of variant mutant cell lines that differ in their tolerance to either biotic or abiotic factors (Bressan *et al.*, 1981; Meredith, 1983; van den Bulk, 1991).

However, before the potential use of cell culture techniques like selection and gene transfer in crop improvement can be realized, the physiological and genetic basis for the characters of interest needs to be understood better. For the stable genetic improvement of a plant for any stress tolerance, it is imperative to undertake basic studies to define the fundamental basis for tolerance. Thus, an understanding of the mechanisms of the stress responses of black pepper to biotic and abiotic stresses is essential for any future work on the genetic improvement of the crop for tolerance to these stresses.

The use of whole plant systems to study certain physiological and biochemical changes occurring in the cells is limited by the unresponsive nature of various differentiated cells and the failure to distinguish between the response of growing and non-growing cells to the changing external environment (Handa *et al.*, 1982). Suspension-cultured undifferentiated plant cells offer a relatively homogenous and experimentally controllable alternative and are excellent model systems for the careful dissection of some of the physiological and biochemical alterations induced by stress and various other responses that operate at the plant cell level to abiotic stress (Lerner, 1985, Fallon and Philips, 1989; Iraki *et al.*, 1989,). Suspension-cultures have also been successfully used to study the host-pathogen interactions in plants. For experiments on the biochemical and molecular mechanisms involved in defense responses, suspension cultured cells responsive to fungal elicitors have been used to reduce the complexity of plant -pathogen interactions (Mandujano-Chavez *et al.*, 2000; Groten and Barz, 2000; Umemura *et al.*, 2002). Implicit in these studies, however, is the assumption that the response of cell cultures to any given stress is similar to response of the plant to the stress *in vivo.*

The somatic embryogenesis system in black pepper which was recently developed by Biju *et al.,* (1996) offers an excellent tissue culture system which is highly amenable for use in basic studies mentioned above. Hence, the present study was undertaken to realize the following objectives:

Abiotic stress:

1. To study the cell responses of *P. nigrum* to moisture stress *in vitro* using the cell suspension culture as a model system, and
2. *In vitro* selection of water stress-tolerant cell lines in black pepper using the embryogenic cell suspension system.

Biotic stress:

3. To study the black pepper-*Phytophthora* interactions *in vitro*, using hyphal cell wall components of *Phytophthora* and cell suspension cultures of *P. nigrum*.

1.1. Review of Literature

The burgeoning population exerts considerable pressure on agriculture since the gap between demand and supply of food and other commodities is widening at a faster rate. To bridge this gap, efforts are underway to increase the yields by development of high-yielding varieties and hybrids apart from scientific crop cultural procedures. However, plants encounter various environmental and biological stresses, which adversely affect their productivity and yield. Plants need to be protected from these yield losses due to biotic and abiotic stress by engineering plants with resistance against these stresses. This requires concerted application of biochemical and molecular approaches to understand the resistance mechanisms that plants possess to protect themselves in the event of these adverse conditions. The present work is an effort in this direction.

1.1.1. Abiotic stress - Drought

Drought or limited water availability is a major environmental stress that causes not only differences between the mean yield and the potential yield, but also instability in yield from year to year (Kent *et al.*, 1990). It is predicted that in coming years, rainfall patterns might shift due to global warming, caused by the ever-increasing burning of fossil fuels and the corresponding increase in atmospheric dioxides. Moreover, increasing urbanization has resulted on increased pressure on the marginal saline and drought prone soils for agriculture. Consequently farming communities could become increasingly dependent on drought tolerant genotypes. Increased drought tolerance has therefore been a major objective of plant breeding programmes for regions where rainfall or irrigation supply is limited.

Although conventional breeding methods have had some encouraging successes, overall progress has been slow. Increased understanding of how the interaction of chemical and physical environments reduces plant development and yield opens up new avenues for a combination of breeding, physiological and biotechnological approaches to plant modification in a comprehensive strategy for improving resistance to environmental stresses.

Due to their immobility, plants have to make necessary metabolic and structural adjustments to cope with stress conditions. To this end, the normal genetic program in plants is altered by the stress stimuli and the plants respond by activating several biochemical pathways that ensure survival. Plants undergo significant morphological and metabolic changes in response to drought. Many of these changes are believed to be adaptive responses by which plants cope with water stress. However, the biochemical mechanisms underlying these responses are poorly understood. A better understanding of the mechanisms that enable plants to adapt to water deficit and maintain growth, development and productivity during stress periods could help plant scientists to screen for resistant plants.

Plants put forth a range of responses under water deficit conditions. The fastest among these responses include stomatal closure, osmotic adjustment, and growth inhibition (Hsiao, 1973). In addition, induction of water stress proteins, antioxidant defenses, increase in ABA content and the cell wall modifications by the alterations of cell wall constituents are other major responses encountered in plants following water stress.

1.1.1.1. Responses of plants to water stress

1.1.1.1.1. Oxidative stress

One of the major consequences of water stress on plants is often injury to specific membranes due to oxidative damage caused by the production of reactive oxygen intermediates, following impairment of electron transport system (Price *et al.*, 1989; Smirnoff, 1993; Inze & Van Montagu, 1995), a secondary effect of water stress. Active oxygen species (AOS) can react very rapidly with DNA, lipids and proteins, causing severe cellular damage. These toxic molecules damage membranes, membrane-bound structures and macromolecules, especially in the mitochondria and chloroplasts (Ishizaki-Nishizawa *et al.*, 1996), resulting in oxidative stress. The chloroplast membranes are especially affected because they are the source of oxygen radical production since chloroplast metabolism under water stress conditions favours the formation of active oxygen species (AOS). Damage to vital membranes and membrane-bound proteins involved in energy producing processes, subsequently influences the metabolism of the whole plants.

1.1.1.1.1.1. Production of reactive oxygen species (ROS)

Oxidative stress, arising from the deleterious effects of reduced oxygen species is an inevitable phenomenon in green plants. Mild drought stress even for a short period is likely to lead to the formation of ROS in photosystem I and II in chloroplasts by electrons being misdirected to oxygen or by energy transfer from triplet-excited chlorophyll to oxygen (Yu and Rengel, 1999). Under water stress conditions, leaves close their stomata to prevent water loss. But this adaptive mechanism lowers the influx of CO_2, resulting in the reduction of net photosynthetic capacity. Inflow of CO_2 through stomata into chloroplasts and its subsequent fixation and reduction must be balanced with the formation of chemical energy in photosynthesis. However, under stress conditions, the decreased CO_2 influx and its fixation results in restricted carbon metabolism that leads to depletion in the natural electron acceptor, NADP+. Under osmotic stress conditions, chloroplasts are exposed to excess excitation energy (Smirnoff, 1993). And, though photosynthesis is impaired and the rate of CO_2 fixation is reduced to a great extent, photosynthetic electron transport continues and is maintained at a relatively higher rate in stressed leaves (Lawlor & Uprety, 1993; Ranjita *et al.*, 1994;). This imbalance between ē transport chain and CO_2 fixation rate combined with the resultant decrease in sufficient electron acceptors results in the over-reduction of the ē transport chain components in the chloroplasts. As a result, the electrons are transferred from Photosystem I (PS I) directly to oxygen, thereby promoting the generation of superoxide radicals ($O_2^{\cdot-}$) via ferredoxin auto-oxidation through the Mehler reaction, providing a pathway for the removal of excess electrochemical energy from the thylakoids (Furbank *et al.*, 1983).

Molecular oxygen is relatively unreactive due to its electron configuration (two unpaired electrons with parallel spins). However, activation of oxygen in biological systems occurs primarily by two methods. Active oxygen species can be formed in plants either by (a) single ē reduction of dioxygen resulting in superoxide radical, or (b) by the direct transfer of excitation energy from photosensitized chlorophyll to oxygen giving rise to singlet oxygen (Kaiser, 1987; Cornic & Briantais, 1991).

(a) Reduction of oxygen can take place with electrons being donated by transition metal ions like Fe^{2+} and Cu^{2+} or other electron donors, as mentioned above, through the Mehler reaction. This mechanism possibly is adopted by plants to protect the photosynthetic electron transport chain components from photo-damage during water stress (Stuhlfauth *et al.*, 1988). Superoxide radicals ($O_2^{\cdot-}$) formed from this univalent reduction of oxygen consequently results in the production of other reactive oxygen species such as hydrogen peroxide (H_2O_2), perhydroxy radical ($^{\cdot}HO_2$) and hydroxyl ions ($^{\cdot}OH$). .

(b) Upon absorption of photons, chlorophyll enters the excited singlet state, which normally leads to phytochemistry. However, under excessive light, accumulating chlorophyll molecules in the excited singlet state might enter the excited triplet state through inter-system crossing. The interaction of such chlorophyll molecules in the excited triplet state with O_2 leads to the generation of singlet oxygen, $^1O_2$ (Mishra *et al.*, 1993).

The two main forms of active oxygen (superoxide $O_2^{\cdot-}$ and singlet oxygen, $^1O_2$) are involved in the initiation of photo-oxidative damage in higher plants. Superoxide radical is primarily produced in the reducing side of PSI in the thylakoids and the center D or A/B and the peripheral ferredoxin is the univalent reductant of dioxygen. The $O_2^{\cdot-}$ radicals thus produced, though capable of oxidizing various cellular components directly, are suggested to exert more of their biologically damaging effects by initiating the generation of more reactive and destructive species (Halliwell & Gutteridge, 1989). $O_2^{\cdot-}$ produced in plants is dismutated either directly (non-enzymically) or via the enzyme SOD. In both cases, the dismutation results in the production of H_2O_2. The superoxide anion $O_2^{\cdot-}$ becomes protonated at low pH to yield the perhydroperoxyl radical $^{\cdot}HO_2$. Both $O_2^{\cdot-}$ and $^{\cdot}HO_2$ undergo spontaneious dismutation to produce H_2O_2.

$$H^+ + O_2^{\cdot-} \longrightarrow {}^{\cdot}HO_2$$

$${}^{\cdot}HO_2 + {}^{\cdot}HO_2 \longrightarrow H_2O_2 + O_2$$

$${}^{\cdot}HO_2 + O_2^{\cdot-} + H_2O \longrightarrow H_2O_2 + O_2 + OH^-$$

Although less reactive than $O_2^{\cdot -}$, H_2O_2 can react with $O_2^{\cdot -}$ in the presence of iron salts and transition metals to form $OH^{\cdot}$ free radicals. This is one of the most reactive species in biological systems, with a very short lifetime and may attack and damage almost every molecule in the living cell (Halliwell, 1984).

Under normal growth conditions, AOS are efficiently scavenged by both enzymatic and non-enzymatic detoxification mechanisms. Nevertheless, during prolonged stress conditions such detoxification systems get saturated and damage occurs.

1.1.1.1.2. Scavenging of ROS

Drought-induced inhibition of photosynthesis results in the exposure of plants to excess energy, which if not safely dissipated, may be harmful to PS II because of the over-reduction of reaction centres (Demmig-Adams & Adams, 1992) and increased production of reactive oxygen species in the chloroplasts that result in membrane damage and membrane-bound proteins involved in energy transfer. AOS disrupt the balance of cellular redox systems in favor of oxidized forms, which damage different aspects of cell structure and function, by inactivation of enzymes, lipid peroxidation and potential damage to DNA (Smirnoff, 1993), in the absence of any protective mechanisms. The accumulation of reactive oxygen species in cells, results in damage to membranes and build-up of lipid peroxides.

To mitigate the damage initiated by AOS, a highly efficient antioxidant system exists in plants, which effectively scavenges these toxic oxygen species as soon as they are formed in the cell compartments (Asada & Takahashi, 1987). This includes both enzymic and non-enzymic components. The anti-oxidant enzymes include superoxide dismutase, catalase, peroxidase, and the enzymes of the AGC, namely, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase. The non-enzymic component includes low molecular weight secondary products, which are either (a) lipid-soluble, membrane-associated antioxidants (eg. α-tocopherol and β-carotene) or (b) water-soluble reductants (eg. Glutathione and ascorbate). In plants, reduced membrane damage has been linked to increased enzymatic defenses against oxygen radicals, together with synthesis of free radical scavengers (Smirnoff, 1993)

1.1.1.1.2.1. Enzymatic anti-oxidant defenses

Changes in the activities of the antioxidant enzymes have been observed in plants exposed to osmotic stress and may be related to the mechanisms by which cells receive and respond to oxidative and osmotic stress (Bueno *et* al., 1998). The study on how oxidative stress affects the activities of antioxidant system may provide useful information for better understanding of the mechanisms involved in water stress injury and the plant tolerance to drought-imposed water stress. The different antioxidant enzymes involved in scavenging the toxic active oxygen species are superoxide dismutase (SOD; E.C. 1.15.1.1), catalase (CAT; E.C. 1.11.1.6); peroxidase (POD; E.C. 1.11.1.7), ascorbate peroxidase (AP; E.C. 1.11.1.11), dehydroascorbate reductase (DR; E.C. 1.8.5.1), monodehydroascorbate reductase (MR; E.C. 1.6.5.4) and glutathione reductase (GR) (Zhang and Kirkham, 1996). AP, DR, MR and GR together form the ascorbate-glutathione cycle (AGC), which operates both in chloroplasts and the cytosol (Asada, 1992).

In chloroplasts, the endogenous scavenging system is located both on the stromal thylakoid membranes and in the stroma. The membrane-bound system prevents active oxygen species from leaking from the stromal thylakoid membranes, where the superoxide radical, $O_2^{\cdot-}$, is generated by direct reduction of oxygen by PS I. On the other hand, the stromal system functions to protect several enzymes from oxidative damage caused by active oxygen species, leaking from their generation sites (Shikanai *et al.*, 1998).

1.1.1.1.2.1.1. Superoxide dismutase

The major producing site of superoxide radicals is the reducing side of photosystem I in the thylakoids and the center X or A/B. The peripheral ferredoxin would be the univalent reductant of dioxygen that results in $O_2^{\cdot-}$. The $O_2^{\cdot-}$ radicals thus produced are disproportionated into H_2O_2 and dioxygen, catalyzed by the superoxide dismutases in the stroma and also in the thylakoid-bound form. Superoxide dismutases are essential components in almost all plant antioxidant defenses, catalyzing the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2 (Miyake and Asada, 1992). H_2O_2 inhibits photosynthetic fixaton of CO_2, which necessitates an effective scavenging system of H_2O_2 in chloroplasts to maintain photosynthetic activity. In addition, the H_2O_2 reacts with superoxide to form highly active hydroxyl radicals via the transition metal-catalyzed Haber-Weiss cycle (Scandalios, 1993). H_2O_2 and $OH^{\cdot}$ are more active, toxic and destructive than the $O_2^{\cdot-}$ radical. Taken alone, the scavenging of $O_2^{\cdot-}$ by SOD merely serves to transform one destructive oxygen species to another (H_2O_2). Therefore detoxifying H_2O_2 becomes pivotal in the defense mechanisms against active oxygen species (Zhang & Kirkham, 1996). The decomposition of H_2O_2 is achieved by ascorbate peroxidase via the ascorbate-glutathione cycle.

1.1.1.1.2.1.2. Hydrogen peroxide-scavenging enzymes

H_2O_2 is generally toxic in the chloroplasts since even at low concentrations, it inhibits the calvin cycle enzymes possessing exposed sulfhydryl groups, such as $NADP^+$ -dependent glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and fructose-1, 6-biphosphatase (FBPase), by readily oxidizing these groups, hence reducing the photosynthetic CO_2 assimilation (Takeda *et al.*, 1995). Thus, the removal of H_2O_2 is extremely important to maintain photosynthetic activity of cells under adverse conditions. The H_2O_2-scavenging enzymes are catalase, peroxidase and the enzymes of the ascorbate-glutathione cycle.

1.1.1.1.2.1.2.1. Catalase

Catalase, localized in peroxisomes of higher plants functions in the decomposition of H_2O_2 which is also produced outside the chloroplasts by the H_2O_2–generating oxidases present in the peroxisomes (Tolbert, 1972). Despite its restricted localization, it may play a significant role in defending against oxidative stress, since H_2O_2 can readily diffuse across the membranes (Bowler *et al.*, 1992). Catalase reacts with hydrogen peroxide directly to form water and oxygen (Smirnoff, 1993; Winston, 1990).

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O_2 + O_2$$

Catalase does not consume reducing power during its catalysis in the decomposition of H_2O_2, in contrast to the AGC, that also scavenges H_2O_2.

SOD, peroxidases / catalases often work in concert, with SOD mainly removing the superoxide radicals, and the concomitantly produced peroxide being removed by the peroxidases and catalases.

1.1.1.1.2.1.2.2. Peroxidase

Peroxidases catalyze hydrogen peroxide-dependent oxidation of substrates (RH_2) according to the general equation

$$RH_2 + H_2O_2 \xrightarrow{\text{Peroxidase}} 2H_2O + R.$$

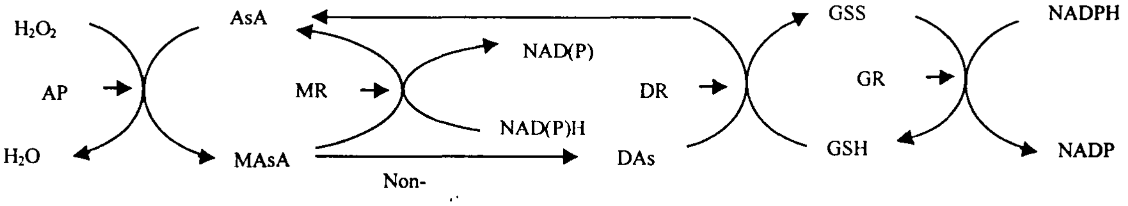
Catalase and guaiacol peroxidase in the cytosol and peroxisomes efficiently remove any hydrogen peroxide found outside the chloroplasts (Halliwell, 1982; Krans & Austin-Fletcher, 1984).

1.1.1.1.2.1.2.3. Ascorbate-glutathione cycle for scavenging H_2O_2

The rapid removal of H_2O_2 produced by SOD is important if the generation of highly destructive OH^- is to be avoided. This is efficiently done by the decomposition of H_2O_2 through a very efficient antioxidant system present both in cytosol and the chloroplasts, namely the ascorbate-glutathione cycle (AGC) which consists of four enzymes – ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase.

The AGC involves the reduction of H_2O_2 by a peroxidase reaction using a photoreductant produced in the thylakoids as the electron donor, namely the ascorbate. The peroxidase involved is ascorbate peroxidase and the primary oxidation product of the reaction is monodehydroascorabate (MDA) radical. The scavenging of H_2O_2 requires the continuous regeneration of ascorbate from MDA and/or DHA. The regeneration of

AsA from MDA is achieved from MDA directly by ferredoxin or by NAD(P)H via stromal monodehydroascorbate reductase (MR). If the MDA radical disproportionates to dehydroascorbate (DHA), the stromal dehydroascorbate reductase (DR) can catalyze the reduction of DHA using glutathione (Nakano & Asada, 1981). NAD(P)H and GSH act as the electron donors for these reductase reactions and are derived from the reduced ferredoxin in PS I. The reduced glutathione (GSH) is also derived from oxidized glutathione (GSSG) by glutathione reductase (GR), which reduces the glutathione disulphide using NADPH. (Bunklemann & Trelease, 1996). Thus, glutathione reductase (GR) plays an important role in the protection of chloroplasts against oxidative damage, maintaining a high GSH/GSSG ratio. When the amounts of AsA and GSH present in the chloroplasts are not enough, they are synthesized in the cytosol and then transported to the chloroplasts (Zhang and Kirkham, 1996), thus dioxygen is finally reduced to H_2O via $O_2^{\cdot -}$ and H_2O_2, mediated by ascorbate (Asada & Takahashi, 1987).



Ascorbate-glutathione cycle (Zhang and Kirkham, 1996; after Cakmak, 1994)

Ascorbate peroxidase occurs both in stroma and thylakoids and MDA radicals are photoreduced in the thylakoids. AP binds to the thylakoid membranes probably in a transmembrane state, and its content is higher in the stroma thylakoids than in grana thylakoids (Miyake and Asada, 1992). Localization of AP in the stroma thylakoids indicates enrichment of the scavenging enzyme in PS I where superoxide radicals and H_2O_2 are photo-regenerated. Photo-reduction of MDA radicals occurs in thylakoids in PS I. The reduction of the H_2O_2 produced in PS I by the thylakoid-bound AP and the photoreduction of the primary oxidation product of the peroxidase reaction in the thylakoids allow to scavenge the $H_2O_2$with catalytic amounts of ascorbate in thylakoids.

$$H_2O_2 + 2\ \text{Ascorbate} \xrightarrow{\substack{\text{Thylakoid} \\ \text{bound AP}}} 2\ H_2O + 2\ \text{MDA}$$

$$2MDA + 2\,(e^-) \longrightarrow 2\ \text{Ascorbate}$$

(e^-) represents the electrons generated in thylakoids in the presence of light.

Miyake and Asada (1992) suggested this short cycle, as the primary scavenging system of H_2O_2 in chloroplasts.The stromal component of the AGC is the secondary scavenging system for H_2O_2 that fails to be reduced by the primary system in the thylakoid and leaks to the stroma or that which is produced in the stroma itself. AGC is a more efficient enzymatic way to break down H_2O_2 than CAT and POD (Cakmak *et al.*, 1993). The main difference between the two H_2O_2-scavenging enzymes, AP and CAT, is the consumption of NADPH (the reducing power) in the former, so as to make $NADP^+$ more available for further reduction by electrons from ferredoxin of the electron transport chain. Since oxidation of NADPH by the ascorbate-glutathione cycle provides PS I with additional electron acceptors, i.e., $NADP^+$, AP is considered to be superior to catalase in the ability to remove excess electrons produced by the exposure of the chloroplasts to excess excitation energy. However, catalase is much superior to AP as it is more stable under oxidative stress unlike AP, and is thus a stress-resistant enzyme. Shikanai *et al* (1998) observed that the ascorbate-glutathione cycle functions only in the early steps of photoinhibition due to its lability under oxidative stress.

1.1.1.1.2.1.1. Review of work done on the anti-oxidant enzymes

Different components of the active oxygen scavenging system are modulated differently under water stress conditions and the degree of modulation depends on the degree of imposed water stress (Baisak *et al.*, 1994).

1.1.1.1.2.1.1.1. O_2^- scavenging enzyme - SOD

Quartacci & Navari-Izzo (1992) observed a decline in the activity of SOD in water stressed sunflower plants. Zhang and Kirkham (1994) reported increase in SOD and CAT activities in wheat species during the early phase of drought but the activities decreased with increase in the magnitude of water stress, favouring accumulation of H_2O_2. Baisak *et al* (1994) reported an increase in SOD activity during the imposition of polyetheylene glycol-induced water stress in wheat leaves and it was observed that the activity increased with increasing severity of the imposed water stress. The ability to maintain adequate levels of superoxide dismutase activity was shown to be more important for drought tolerance in potato than an increase in enzyme activity (Mescht *et al.*, 1998).

Bueno *et al.* (1998) studied the antioxidant defenses in tobacco cell cultures in response to PEG-induced water stress and observed an increase in SOD activity. The different isoforms of SOD behaved differently with the imposed water stress. Fe-SOD activity increased 2-fold while SOD A, the gene encoding Mn-SOD was enhanced 2-fold by moderate osmotic stress but markedly decreased in 30% PEG. Yu & Rengel (1999) reported that the activities of the 3 forms of SOD were modulated differently under water stress in narrow-leafed lupins. While the activities of total SOD, Cu-Zn SOD, Fe-SOD increased with increasing severity of water stress, resumption of water supply resulted in Cu-Zn SOD returning to the control levels while Fe-SOD activity remained high. Activity of Mn-SOD was unaffected by drought.

1.1.1.1.2.1.1.2. H_2O_2 scavenging enzymes

Increased POD activity has been reported by many workers in plants under drought (Badiani *et al.*, 1990, Dwivedi *et al.*, 1979) and salt (Siegel, 1993) stress. Zhang and Kirkham (1994) observed increase in POD activity in wheat under water stress. Mukherjee & Choudhuri, (1985, 1983) and Quartacci & Navari-Izzo (1992) reported decline in CAT activities in water stressed plants. Baisak *et al* (1994) also reported a decrease in catalase activity by the imposition of polyetheylene glycol-induced water stress in wheat leaves. Decline in CAT activity was reported in water-stressed corn (Zhang *et al.*, 1990), rice (Dwivedi *et al.*, 1979), jute (Chowdhury and Choudhuri, 1985), and sunflower (Quartacci and Navari-Izzo, 1992) with increase in the intensity of drought stress.

Elevated levels of GR (Smirnoff and Colombé, 1988; Gamble & Burke, 1984) have been reported in response to water stress. Pastori and Trippi (1992) observed a clear correlation between plant resistances to water stress and oxidative stress and the activities of antioxidant enzymes such as GR, SOD and AP. Del Longo *et al* (1993) reported higher levels of activities of SOD, CAT, AP and GR in tolerant maize, LIZA, under hyperosmotic and hyperoxygenic conditions, compared to the susceptible line, LG II. Similar observations were made by Chowdhury and Choudhuri (1985) who found lower levels of H_2O_2 in a drought-tolerant strain of jute, than in a drought-sensitive strain. Wheat leaves subjected to oxidative stress showed increased activities of SOD, AP and MR (Mishra *et al.*, 1993). Baisak *et al* (1994), reported an increase in AP and GR activities in wheat leaves under PEG-induced water stress conditions when the intensity of stress was low. However the activities declined rapidly with increase in the severity of stress. The increase in GR was found to be synthesized *de novo* during water stress imposition. Moran (1994) observed that water stress significantly decreased (72-85%) catalase, dehydroascorbate reductase and glutathione reductase activities in pea, but increased (32-42%) non-specific peroxidase and superoxide dismutase activities. Ascorbate peroxidase and monodehydroascorbate reductase activities decreased by only 15% and the two enzymes acted in a cyclic manner to remove H_2O_2, which did not accumulate in stressed leaves. Zhang and Kirkham (1996) reported higher activity of ascorbate-glutathione-cycle (AGC) in sorghum (C_4 plant) as being responsible for lower lipid peroxidation compared to that in sunflower (C_3 plant). Polyethylene glycol and free radical scavenger ascorbic acid was found to increase the ratios of SOD to CAT or AP in sunflowers, but did not affect them in sorghum suggesting that sorghum is more resistant to oxidative stress than sunflowers (Zhang *et al.*, 1996).

Antioxidant enzymes like glutathione reductase (NAD(P)H) and L-ascorbate peroxidase significantly increased under water stress in tolerant wheat genotype than the susceptible genotype resulting in lower lipid peroxidation in the tolerant variety (Sairam *et al.*, 1998). AP activity was found to increase proportionately to the intensity of the induced stress in tobacco cell cultures in response to PEG-induced water stress (Bueno *et al*, 1998). Chen *et al* (1998) observed increased activities of peroxidase, ascorbate peroxidase, superoxide dismutase and glutathione reductase, as well as the glutathione content, in tolerant litchi leaves under water stress conditions, while catalase activity and ascorbate content decreased. Li and Van-Staden (1998) treated callus tissues of drought-resistant and drought-sensitive maize were treated with brassinolide, uniconazole or methyl jasmonate. When these growth regulator-treated calli were subjected to water stress with PEG for 24 h, the activities of the antioxidant enzymes superoxide dismutase, catalase, ascorbate peroxidase, peroxidase and glutathione reductase remained higher in callus of the drought-resistant than the drought-sensitive cultivar. Differences in glutathione reductase and ascorbate peroxidase activities between potato cultivars differing in their tolerance to water stress could not be correlated with drought tolerance (Mescht *et al.*, 1998). The activity of glutathione reductase in stressed potato cultivars was consistently lower than in control treatments. The levels of ascorbate peroxidase activity were generally higher in stressed potato plants than in controls. Increased activity of glutathione reductase and a lower inhibition of ascorbate peroxidase during water stress were implicated in lower plasmalemma permeability in the drought tolerant rice cultivar compared to that in the susceptible cultivar (Lu *et al.*, 1999). Sairam and Saxena (2000) reported higher membrane stability in tolerant wheat genotypes during water stress due to increased activities of ascorbate peroxidase, glutathione reductase and non-specific peroxidases, compared to the susceptible line, indicating the role of these anti-oxidant enzymes in imparting drought tolerance. Guerrier *et al* (2000) reported that in comparison to the drought sensitive cultivar, the drought tolerant cultivar of poplar showed a decrease in catalase and glutathione reductase activities and in an enhancement of superoxide dismutase, ascorbate peroxidase, after exposure to osmotic stress.

1.1.1.1.2.2. Non-enzymic antioxidants

Several anti-oxidants such as ascorbate, glutathione (GSH), α-tocopherol, carotenoids and flavanoids are present in higher plants that contribute to the protective system against oxidative stress and deactivate the active oxygen species in multiple ways (Knox and Dodg, 1985; Asada and Takahashi, 1987; Chauhan *et al.*, 1992).

It is well documented that carotenoids are involved in the protection of the photosynthetic apparatus against photoinhibitory damage by singlet oxygen ($^1O_2$), which is produced by the excited triplet state of chlorophyll (Loggini *et al.*, 1999). Carotenoids can directly deactivate $^1O_2$ and can also quench the excited singlet state of chlorophyll, thus indirectly reducing the formation of $^1O_2$ species (Foyer & Harbinson, 1994). When drought resistant and susceptible maize calli treated with growth regulators were subjected to PEG-induced waer stress, the damage, as indicated by the levels of hydrogen peroxide and malondialdehyde, the reduction of ascorbate and carotenoids, and leakage of electrolytes from cells, was apparent in callus of both cultivars. However, the damage was less marked in the drought-resistant cultivar (Li &Van-Staden, 1998). Chlorophyll and carotenoids have been shown to impart higher membrane stability in drought tolerant wheat genotype during water stress (Sairam & Saxena, 2000). In an earlier study, Sairam *et al* (1998a) observed that the drought tolerant wheat genotype showed lowest lipid peroxidation (LPO) and highest membrane stability index (MSI) and chlorophyll and carotenoid contents under water stress compared with susceptible genotype, concluding that the relative tolerance of a genotype to water stress as reflected by its comparatively lower LPO and higher MSI, chlorophyll and carotenoid contents is closely associated with its antioxidant enzyme system.

Glutathione (GSH), a non-enzymic, low molecular weight compound of the defense system protects many cellular components and the thiol status of proteins against oxidative stress (Gilbert *et al.*, 1990). Glutathione may also metabolize H_2O_2 by participating in the ascorbate/glutathione cycle or in the reaction catalyzed by glutathione peroxidase (Drotar *et al.*, 1985).

Ascorbate functions in several enzymic and non-enzymic systems that scavenge free radicals, remove peroxide and quench $^1O_2$ (Hossain & Asada, 1984; Larson, 1988). Increase in ascorbate content in illuminated leaves may be related to the increase in MR and DR activities, since these enzymes are involved in the recycling of ascorbate from its oxidation products generated in the ascorbate peroxidase-catalyzed reaction (Hossain *et al.*, 1984). Ascorbate also increases due to its synthesis in light. Mishra *et al* (1993) observed that ascorbate and carotenoids played an important role in protection of wheat leaves subjected to oxidative stress on exposure to strong visible light. Ascorbate and carotenoid contents were reduced less markedly in maize callus of tolerant cultivar under PEG-induced water stress than those in the callus of the susceptible cultivar (Li & Van-Staden, 1998), indicating their role in water stress tolerance. Zhang *et al* (1996) observed that when water stress was induced by PEG, drought injury by lipid peroxidation could be lessened by exogenous application of free radical scavenger, ascorbic acid in sorghum and sunflower. Lu *et al* (1999) reported an initial increase in the contents of ascorbate and reduced glutathione in both tolerant and susceptible cultivars of rice under water stress, which then decreased in both cultivars, but with a greater increase in the drought-tolerant cultivar. Drought had no effect on the levels of ascorbate and oxidized glutathione in pea leaves, but decreased the content of reduced glutathione by 25% and increased vitamin E content by 67% (Moran *et al.*, 1994).

1.1.1.1.3. Lipid peroxidation

Much of the injury caused to plants by environmental stress is associated with damage inflicted by active oxygen species at the cellular level. The harmful effects of these toxic radicals are primarily due to their ability to initiate a variety of oxidative chain reactions on unsaturated fatty acids, producing a carbon-centred radical and lipid hydroperoxide that leads to lipid peroxidation and membrane destruction (Gutteridge, 1988). The occurrence of lipid peroxidation, is an indicator of the prevalence of free radical reactions in leaves. This is because the change in balance between $O_2^{\cdot}$ / H_2O_2 may facilitate the metal-catalyzed formation of highly reactive $OH^{\cdot}$ through the Haber-Weiss reaction (Bowler *et al.*, 1992), which may then initiate lipid peroxidation under severe water stress conditions. Thus, inefficient decomposition of H_2O_2 is probably responsible for the occurrence of oxidative damage and lipid peroxidation in water stressed leaves.

Polyunsaturated fatty acids (PUFA), the main components of membrane lipids, are susceptible to peroxidation. Reactive oxygen species like the hydroxyl radical or the singlet oxygen react with the methylene group of PUFA forming lipid peroxy radicals and lipid hydroperoxides. The peroxy radical formed is highly reactive and is capable of propagating the reaction by further reacting with PUFA, resulting in a continuous chain of peroxidative reactions that cause destruction of the membrane components.

$$\text{PUFA-H} + \text{A}^{\cdot} \longrightarrow \text{PUFA}^{\cdot} + \text{A-H}$$

$$\text{PUFA}^{\cdot} + \text{O}_2 \longrightarrow \text{PUFA-OO}^{\cdot}$$

$$\text{PUFA-OO}^{\cdot} + \text{PUFA-H} \longrightarrow \text{PUFA-OOH} + \text{PUFA}^{\cdot}$$

The lipid hydroperoxide formed, undergoes reductive cleavage by reduced metals such as Fe^{2+} and forms lipid alkoxyl, $\text{PUFA-O}^{\cdot}$, that can initiate more chain reactions by reacting with additional PUFA.

$$Fe^{2+} + \text{PUFA-OOH} \longrightarrow Fe^{3+} + OH^{-} + \text{PUFA-O}^{\cdot}$$

$$\text{PUFA-O}^{\cdot} + \text{PUFA-H} \longrightarrow \text{PUFA-OH} + \text{PUFA}^{\cdot}$$

Malondialdehyde (MDA), the most widely used indicator of oxidative damage or lipid peroxidation, increased upto 2-fold in 12 grass species after exposure to water stress (Price & Hendry, 1991). Zhang and Kirkham (1994) obtained similar trends while studying the antioxidant defenses in hexaploid, tetraploid and dipoloid wheat species under drought conditions. They observed increased content of MDA in the hexaploid species. Del Longo *et al* (1993) reported that when wheat leaves of tolerant and susceptible cultivars were exposed to water stress conditions, the damage due to lipid peroxidation was apparent in both the cultivars, but was less marked in the tolerant cultivar. Baisak *et al* (1994) reported that lipid peroxidation was enhanced in wheat leaves subjected to higher degree of water stress but was not observed under mild water stress. Zhang and Kirkham (1996) showed that drought-induced lipid peroxidation was more in sunflower compared to the sorghum plants, indicating that sunflower membranes are attacked more easily by oxidative stress than sorghum membranes. MDA content was shown to increase in the leaves of both drought tolerant and susceptible sweet potato cultivars, with increasing dose of PEG-induced water stress (He *et al.*, 1997). Chen *et al* (1998) reported greater increase in leaf MDA contents during water stress in the drought-sensitive litchi cultivar than the drought-resistant cultivar. Hu *et al* (1999) observed that lipid peroxidation increased with increasing severity of PEG-induced water stress, but if maintained under the stress for a longer time, protective enzymes maintained a relatively high activity and protected against damage to the plasma membrane caused by lipid peroxidation, suggesting that maple has good adaptability to drought.

1.1.1.1.4. Osmoregulation

A problem for all living organisms in dry environments is to maintain water content. The accumulation of low molecular weight osmo-protectants and osmolytes, to levels sufficient to maintain equal water potential with the environment is considered to be an important strategy that plants use to overcome cellular dehydration. The mechanism of osmotic adjustment or osmoregulation refers to the lowering of osmotic potential due to net accumulation of solutes in response to water deficits or salinity and is very important in maintaining cell turgor that enables the maintenance of plant metabolic activity and therefore, growth and productivity (Balibrea *et al.*, 1997). These substances act by raising the osmotic potential of the cell and preventing water loss to the outside environment of depleting water content, thus balancing the osmotic potential of an externally increased osmotic pressure. The solute accumulation lowers water potential of the dehydrating cells, facilitating the uptake of water from the surrounding environment even under water stress conditions. Osmotic adjustment is commonly associated with starch breakdown and concomintant increase in low-molecular weight solutes. Major contributing solutes to osmoregulation comprise inorganic ions, free amino acids, betaines, organic acids, soluble sugars and polyols (Morgan, 1984).

1.1.1.1.4.1. Inorganic ions

The ionic strength as well as pH is known to influence the salt sensitivity of enzymes. Enzymes possess a very narrow range of ion concentrations and compositions for which they have optimal activity (Schwab and Gaff, 1990). Ion binding to proteins can lead to protein dehydration with the most denaturing ion producing the greatest dehydration in the order of the lyotropic series (Hofmeister, 1888; Wyn Jones and Pollard, 1983). The cytoplasm is generally K^+ specific and Na^+ and Cl^- are sequestered in the vacuole (Wyn Jones, 1984).

K^+ has been shown to accumulate in cells of sour orange treated with PEG (Ben-Hayyim, 1987). However, there was no increase of K^+ in mannitol-treated wheat callus (Trivedi *et al.*, 1991). Santos-Diaz and Ochoa-Alejo (1994) reported positive correlation of K^+ accumulation with PEG-tolerance in chilli pepper cell cultures and was the major contributing solute to the osmotic potential in all the PEG-tolerant cell cultures. They also reported 2 to 3-fold higher accumulation of Na^+ in PEG- tolerant cell clones than in the non-selected control. The contribution of Ca^{2+} to osmotic adjustment was insignificant. Balibrea *et al* (1999) reported higher accumulation of K^+, Na^+ and Cl^- in young leaves of PEG-treated tomato seedlings, compared to the untreated plants. However, K^+ and other inorganic ions made no contribution to osmotic adjustment under water stress, a trend similar to that observed in earlier studies on *Zea mays* (Sharp *et al.*, 1990) and *T. aestivum* (Johnson *et al.*, 1984).

Kameli and Lösel (1995) observed an increase in NO^{3-} and SO_4^{2-} in wheat plants under water stress, which was explained to be due to the inhibition of nitrate reductase, a feature noted in sorghum even under mild water stress (Sivaramakrishnan *et al.*, 1988).

1.1.1.1.4.2. Organic solutes

Plant cells may generally accumulate inorganic ions, like as Na^+, Cl^-, SO_4^2, which are most commonly present in the environment, but these become detrimental to cellular biochemistry at high concentrations and must be sequestered in the vacuole. To keep the cytoplasm osmotically balanced, the plant cells usually accumulate special types of organic molecules, termed compatible solutes. They serve the primary function of maintaining osmotic balance and can accumulate to high concentrations without impairment of normal physiological functions, i.e. they do not interfere with protein structure or function. Only a few types of organic compounds (osmoprotectants) meet this requirement namely, polyhydroxylic compouonds like glycerol, sucrose, glycine betaine, polyols or sugar alcohols like sorbitol and mannitol and zwitter-ionic alkylamines like the amino acid proline and tertiary sulfonium compounds. The increase in cellular osmolarity which results from the accumulation of non-toxic (thus compatible) osmotically active solutes is accompanied by the influx of water into, or atleast a reduced efflux from, cells, thus providing the turgor necessary for cell expansion.

It has been seen that water stress sensitive tissues of *Polypodium* ferns and wheat have a poor capacity to bind water tightly (Vertucci and Leopold, 1987; Rascio *et al.*, 1994), providing evidence for the importance of bound water in tolerance to dehydration stress (Levitt, 1951). Variation in water binding strength may be due to loss or physical alteration of components to which water binds and the presence of solute in water affects its structure. If the primary function of solute accumulation is regulation of intracellular water activity (osmoregulation), it has been suggested that the role of accumulated solutes is water structure regulation (Schobert, 1977). For eg., betaine and proline may induce the formation of strong H-bonded water around the protein, preserving the native state of the cell biopolymers in the water-stressed cell. Borowitzka (1981) observed that other solutes such as inorganic ions, alter water structure and may modify its interaction with the macromolecules. Characteristically, compatible solutes, are not highly charged, but are polar, highly soluble and have a larger hydration shell (the number of water molecules surrounding and solvating each molecule) than denaturing molecules like urea or inorganic ions like Na^+, Cl^-. The technique of near infrared spectroscopy, which detects the state of water surrounding a molecule, indicates that compatible solutes are strong water structure formers (Galinski, 1993). Thus, according to the preferential exclusion model (Bartels and Nelson, 1994), such molecules would be preferentially solubilized in the bulk water of the cell rather than the hydration shell of proteins (or other labile macro-molecules) where they could interact directly with the macromolecules. They may then counteract with small, highly charged molecules (the inorganic ions), which preferentially solubilize in the water of the hydration sphere where they interact electrostatically with the macromolecule, causing damaging effects at high concentration. These osmo-protectant compounds also have the ability to stabilize membranes and/or macromolecular structures (Holmberg and Bülow, 1988). Soluble cytoplasmic compounds may stabilize biopolymers such as enzymes via solute-water-biopolymer interactions (Wyn Jones, 1984).

Water is important to organisms not only as a solvent for biochemical reactions but also as a stabilizer of structure. Hydrophilic and hydrophobic interactions impart structure to macromolecules and organelles within cells. Membrane structure, in particular, depends on these complex interactions and is often regarded as a primary site of dessication damage (Senaratna & McKersie, 1983). Since all sub-cellular structures must exist in an aqueous environment, tolerance to dehydration also depends on the ability of cells to maintain membrane integrity and prevent protein denaturation. The water replacement hypothesis suggests that the compatible solutes (mostly polyhydroxy compounds or polyols like sugars and sugar alcohols) can substitute for the evaporating water in stabilizing membrane structures in the dry state (Santarius, 1973; Webb, 1965, Clegg *et al.*, 1982) and they may also replace water when the solvation sphere around the soluble macromolecules is gradually removed (Schwab and Gaff, 1990). In the case of membranes, the hydroxyl groups can hydrogen-bind to polar head groups, providing the hydrophilic interactions necessary for membrane structure and stability. The compatible solutes also exert protective effect on the soluble enzymes in the cells of drought tolerant plants in a similar way, as they get dehydrated. In addition to protective properties of the compatible solutes to protein structure and dry membranes, a few compatible solutes at high concentrations have also been proposed to confer protection against oxidative damage by scavenging free radicals in addition to their roles in maintenance of osmotic equilibrium without perturbing macromolecular- solvent interactions (Rhodes & Hanson, 1993).

1.1.1.1.4.2.1. Sugars

Soluble carbohydrates have been known to play an important role in osmoregulation. Sugars are believed to substitute for water at the surface of membranes (water replacement hypothesis; Crowe *et al.*, 1984b), thus exerting a stabilizing effect on macromolecules. They may also alter the physical properties of membrane phospholipids (Caffrey *et al.*, 1988). The soluble sugar, sucrose accumulates in plant cells under water stress conditions. It can assume a protective role by replacing water by hydrogen binding to the phospholipid head groups of the membrane (Crowe *et al.*, 1984a). If sucrose crystallizes, as is favored by slow drying, these hydroxyls will be unavailable. Alternatively, sucrose can form an amorphous glass during drying. In this case, the hydroxyls of sugars would be free to bind to the membrane. Glass formation may be favored by the presence of oligosaccharides, which disrupt crystal matrices (Caffrey *et al.*, 1988, Smythe, 1967).

Tholakalabavi *et al* (1997) observed that soluble carbohydrate levels were higher in osmotically stressed poplar cells. Among sugars, oligosaccharides are implicated in drought tolerance. Small amounts of the oligosaccharides, stachyose and raffinose have found to be the most effective inhibitors of sucrose crystal growth (Smythe, 1967). In a study conducted by Schwab and Gaff (1986), two species of dessication tolerant grass accumulated large amounts of sugar as they dried. On the contrary a related grass species, which is drought sensitive, did not accumulate substantial amounts of sugar during drying. Koster and Leopold (1988) observed that the loss of dessication tolerance in the three species they studied, namely, soybean, pea and corn, corresponded to the loss of oligosaccharides, which may serve to prevent sucrose crystallization.

Monosaccharides are known to be associated with loss in dessication tolerance in seeds. Glucose, a reducing sugar, is known to participate in the Maillard reaction, a complex series of non-enzymic reactions that can lead to hundreds of end products. The reaction occurs with an amino group that is usually, but not always, on a protein. The Maillard reaction has been reported to cause protein inactivation and DNA damage (Fujimaki *et al.*, 1986). Koster and Leopold (1988) observed that increase in monosaccharides, especially glucose, in the axes of germinating seeds of soybean, pea and corn, coincided with loss in dessication tolerance. Bianchi *et al* (1992) reported that the acquisition of dessication tolerance in ABA treated and dessicated calli was accompanied by an increase of sucrose and concomitant decrease in the appearance of glucose and fructose. However there are reports implicating a role for glucose and fructose in osmotic adjustment in plants under water stress conditions. Handa *et al* (1982) observed reducing sugars as among the major contributors to osmotic potentials in PEG-adapted tomato cells. Similarly, leaves of cotton plants adapted to water stress showed no clear difference in sucrose content from unstressed plants (Ackerson, 1981). Kameli and Lösel (1995) observed that sugars, especially glucose, made the largest contribution to osmotic adjustment in leaves of durum wheat during water stress. However, Johnson *et al* (1984) recorded increases in non-reducing sugars and not in reducing sugars in leaves from water stressed plants of *T. aestivum*.

1.1.1.1.4.2.2. Total free amino acids

Amino acids are among the major compatible solutes of osmoregulation. The levels of the different amino acids vary with different plant species. Osmotically stressed poplar cells accumulated higher contents of tyrosine, alanine, glutamine, gluatamate, asparagines and serine compared to the control (Tholakalabavi *et al.*, 1997). Handa *et al* (1982) also reported that amino acids play an important role in osmotic adjustment in PEG-adapted cells of tomato. Kameli and Lösel (1995) reported that amino acids made no contribution to osmotic adjustment in water stressed durum wheat (*T. durum*), contrary to the observation on various other species (Wyn Jones *et al.*, 1979).

1.1.1.1.4.2.3. Proline

One member of amino acids, the imino acid, proline, is one of the minor amino acids in non-stressed plants, but accumulates very strongly during adaptation to water stress (Bornett & Naylor, 1966; Jones *et al.*, 1980). Proline has been one of the best examined metabolites under water deficits, probably because of its ubiquitous functions in the water deficit response. Proline plays an important role as a small compatible molecule, which can replace water in water-restricted conditions and stabilizes enzyme structure (Skriver & Mundy, 1990; Delauney & Verma, 1993). Proline has been considered as a stress symptom (Becker & Fock, 1986), as a nitrogen storing process for survival (Sivaramakrishnan *et al.*, 1988) and as a compatible solute. Proline as a storage compound could have significant implications for growth restoration, once stress conditions abate (Bellinger & Larher, 1987). The capacity of higher plants to utilize the stress-accumulated proline (after the release of the hyperosmotic stress) might confer some advantages for the plant after experiencing a water stress event because of the energetic and nutritional values of this nitrogenous solute (Trotel *et al.*, 1996). Proline synthesis plays an important role in potentiating pentose phosphate pathway activity by replenishing $NADP^+$ supply that helps support redox cycling, thus playing a role in anti-oxidative defense mechanism (Hare & Cress, 1997). Despite the high cost of its synthesis and growth impairment generally associated with its enhanced level, proline has been assumed to have adaptive value in plants experiencing environmental stress (Stewart & Lee, 1974). The biochemical pathways involved in the production of the compatible solutes are now better known and the genes encoding several of the relevant enzymes have been cloned (Kavi Kishor *et al.*, 1995; Igarashi *et al.*, 1997). Thus the genetic modification of selected species to increase the content of compatible solutes is now technically feasible.

Büssis and Heineke, (1998) reported that potato plants subjected to 10% PEG (6000)-mediated water stress, were able to grow under water deficit by accumulating organic solutes. The osmotic adjustment was mainly due to amino acids, especially, proline.

1.1.1.1.5. Water stress proteins

To cope with stress conditions, plants have to make necessary metabolic and structural adjustments. To this end, the genetic program in normal plants is altered by the stress stimuli to produce specific proteins and activate biochemical pathways that ensure survival (David Ho & Sachs, 1989). In addition to metabolic and physiological changes like the accumulation of low molecular weight protective compounds (osmolytes) during water deficit conditions, a large set of plant genes is transcriptionally activated, which leads to accumulation of new and unique polypeptides in vegetative tissues of plants under osmotic stress conditions (Skriver & Mundy, 1990; Chandler & Robertson, 1994). Functions of many of these genes have been predicted from the deduced amino acid sequence of the genes. Genes expressed during stress are anticipated to promote cellular tolerance of dehydration through protective functions in the cytoplasm, alteration of cellular water potential to promote water uptake, controlled ion accumulation and further regulation of gene expression. All the gene products of the genes expressed during stress do not necessarily improve the ability of the plant to survive the stress (Bray, 1993). The expression of some of the genes may result from the injury or damage that occurred during the stress, other genes may be induced, but their expression does not alter stress tolerance. Yet others are required for stress tolerance and the accumulation of these gene products is an adaptive response.

The water deficit-induced gene products and their functions have been elaborately reviewed by Bray (1993). A number of water deficit-induced proteins are predicted to protect cellular structures from the effects of water loss, based on the amino acid sequence and expression characteristics of these proteins. These genes are frequently called lea (late embryogenesis abundant) proteins as they are expressed during the maturation and dessication phases of seed development (Baker *et al.*, 1988). It has since been recognized that these genes are also expressed in vegetative tissues during periods of water loss resulting from osmotic and low temperature stress.

There are many goups of lea proteins. The majority of these gene products is predominantly hydrophilic, based on amino acid composition, lacking in cysteine and tryptophan and are proposed to be located in the cytoplasm. The individual amino acid sequences and predicted protein structures have been used to propose specific functions for each group of LEA proteins (Dure, 1993). These predicted functions include sequestration of ions, protection of other proteins or membranes and renaturation of unfolded proteins. . Group1 LEA proteins are predicted to have enhanced water-binding capacity as these proteins have a high percentage of charged amino acids and glycine. Group 2 LEA proteins have a possible chaperone function or one that preserves protein structure. Group 3 lea proteins are predicted to play a role in the sequestration of ions that are concentrated during cellular dehydration. A consensus sequence of 11-mer amino acid motif, TAQAAKEKAGC, repeated 13 times is predicted to form an amphiphilic α-helix (Bray, 1993). The hydrophobic face may be important in forming a homodimer and the outside charged face may be involved in sequestering ions whose concentration increase during water deficit. Group 4 LEA proteins may replace water to preserve membrane structure. Thus, the functions of all these gene products are those that would be needed during severe stress, when sufficient water is lost to disrupt cellular structure.

Many roles have been attributed to the newly discovered water stress-induced proteins. Some of them have a role in osmotic adjustment. These proteins have putative membrane spanning domains and a channel-like structure, which are suitable for the transport of water and ions across the membranes. Members of this protein family form water-specific, ion or solute channels. γ- TIP (tonoplast intrinsic protein) forms water-specific channels when expressed in *Xenopus oocytes* (Maurel *et al.*, 1993). As channel proteins accumulate during stress, movement of water or solutes from the vacuole to the cytoplasm could be promoted, altering either the water content or the osmotic potential of the cytoplasm. Some water stress-induced genes like ubiquitin and proteases are involved in protein degrading mechanisms. These gene products degrade proteins that are denatured during cellular water loss. There are other proteins, like chaperones and protease inhibitors, induced by water deficit, which counteract these mechanisms. In addition, genes potentially involved in regulation and signaling during periods of water deficit such as a protein kinase, nuclear proteins and an RNA-binding protein have been identified. Transcription factors that recognize DNA elements within drought-induced genes have also been identified.

The categorization and characterization of polypeptides that are synthesized during drought stress is one means to describe the drought response and to begin to determine if these polypeptides have any adaptive value. The rate of drought-induced polypeptide accumulation and depletion during recovery may be important indicators of protein function. In addition, the abundance of these polypeptides may also provide some important information about their function (Bray, 1990). As more genes are isolated and more is learned about the adaptation of plants to stress, our understanding of the functions of water deficit-induced genes is bound to improve.

The studies on induced proteins have many uses (Pareek *et al.*, 1997). These proteins can be used for sequencing and arriving at the nucleotide sequence which can then be utilized for searching homologous gene sequences connected to the stress response with the help of existing databases on stress-induced genes. The functional aspects of stress proteins can be tested in mutants for stress tolerance. Plants that are subjected to sub-lethal levels of stress acquire capability to withstand ensuing lethal stress levels, much better than plants straight away exposed to lethal stress levels. Therefore, it is likely that proteins / gene expression changes concomitant with the development of induced tolerance have a role in the induction of tolerance.

A correlation between organ survival and LEA protein accumulation during recovery from severe dehydration has been identified. In wheat seedlings, there is a correlation between dehydration survival and LEA proteins accumulation (Reid & Walker-Simons, 1993). Qualitative and quantitative changes in polypeptides under stress conditions have been studied in cell cultures of many plants (Reddy *et al.*, 1993). Borkird *et al* (1991) reported that water stressed rice cells as well as cultured organs and tissues show induction of two polypeptides encoded by hsp 70 and ubiquitin genes. Several polypeptides were induced during water stress in tomato (Bray, 1990).

In plants, several stress-induced novel proteins are found to be boiling stable. Prominent examples include cold responsive (cor) proteins of *Arabidopsis thaliana* (Lin *et al.*, 1990) and boiling stable proteins in barley (Jacobson & Shaw, 1989). All these proteins share specific sequence domains and are highly hydrophilic in nature. Such proteins are presumably associated with osmotic adjustment mechanisms by their very nature of hydrophilicity (Reddy *et al.*, 1993). Rao *et al* (1993) observed that the 23kDa polypeptide, induced by drought as well as ABA in rice seedlings, was boiling stable.

1.1.1.1.5.1. Cell wall proteins

The process of growth is sensitive to water shortage. It has been suggested that factors other than substrate availability (Bozarth *et al.*, 1987) and turgor must cause the inhibition of growth. Some of these factors may be related to cell wall metabolism, particularly to those processes involved in cell wall extensibility and hydraulic conductivity. Cell walls in higher plants are made up of complexes of cellulosic microfibrils and a non-cellulosic matrix composed of pectins, hemicelluloses and proteins (Brett & Waldron, 1990). The synthesis of the cross-linking of these wall proteins are under strict control and can be environmentally induced by a number of factors like drought stress (Fernanda *et al.*, 1997). There are many reports indicating that cell wall constituents are altered in response to biotic and abiotic stresses (Sadka *et al.*, 1991; Showalter, 1993). Bozarth *et al* (1987) described the accumulation of a 28 kD protein in the cell wall fraction of soybean seedlings exposed to low water potentials. Similar results were reported by Covarrubias *et al* (1995). There are data demonstrating that cultured cells of tomato or tobacco which do not enlarge after adaptation to water stress, have physical and biochemical alteration of the cell walls (Iraki *et al.*, 1989a, 1989b).

1.1.1.1.6. ABA in plant water stress response

ABA has been widely implicated in stress responses. The increase in the levels of this hormone has been shown to be one of the first physiological responses to various biotic and abiotic stresses in a variety of plant genera (Chandler & Robertson, 1994), thus having the potential to improve adaptation to stress. Plants respond to dessication or water stress, both developmentally and physiologically. Some of these responses are due to altered levels of plant hormones. Many of the water deficit responses in plants are at least partly controlled by ABA, a phytohormone, the concentration of which increases in plants subjected to water deficit (Zeevart & Creelman, 1988).

The rapid induction of ABA accumlation in plant tissues losing turgor is a well known phenomenon (Voesenek & van der Veen, 1994). Ried and Walker-Simmons (1993) reported the increase in ABA levels and induction of 3 LEA mRNAs in shoot and scutellar tissue in dehydrated wheat seedlings. The presence of these mRNAs resulted in resumption of growth after rehydration. However the LEA mRNAs were not observed in roots, which did not resume growth after rehydration. Thus, in severely dehydrated wheat seedlings, the accumulation of high levels of group 3 LEA proteins is correlated with tissue dehydration tolerance. In alfalfa, correlation was found between tolerance to osmotic stress and the ability to maintain high ABA contents under severe stress conditions (Ivanova *et al.*, 1997). The increased content of ABA in plants under stress is probably related to the hormonal control of the adaptive response of plants.

A substantial number of drought effects on plants can be mimicked by application of ABA. Many of the stress-responsive proteins / genes can be induced under control, unstressed conditions, if plants are treated with exogenous ABA (Singla & Grover, 1993). It is possible to derive information on importance of given gene / protein by studying its ABA-inducibility (Pareek *et al.*, 1997). Thus, ABA can be considered a common denominator of the stress response.

Since the first discoveries of the ABA-inducible LEA and RAB proteins in cotton (Baker, Steele & Dure, 1988), it has become evident that representatives of LEA protein families are produced in all plants and these are involved in the protection of cells against dehydration, whether environmentally induced or developmentally programmed (Rinne *et al.*, 1998). The tentative functions proposed for the induced proteins, according to the predicted protein structure (Dure, 1993; Lisse *et al.*, 1996) are, sequestration of ions or water, or preservation of membrane or protein structure (chaperone function). Many of the ABA-inducible proteins share a certain degree of structural similarity. In particular, they are typically hydrophilic and cysteine-and tryptophan-free, with a run of serines (Close *et al.*, 1989; Cohen *et al.*, 1991). They are also characterized by a conserved 15 amino acid, lysine-rich sequence near the carboxyl terminus. This consensus polypeptide forms an amphiphilic α-helix which may serve as an ion trap in dehydrated cells, sequestering ions that become concentrated (Close *et* al., 1993a, Dure, 1993). ABA-inducible proteins, including LEA proteins, remain soluble even after boiling, suggesting that these proteins have a high affinity for water (Close *et al.*, 1989; Ried & Walker-Simmons, 1990; Thomann *et al.*, 1992). Such proteins help maintain a minimal amount of water content during dehydration and in the process, stabilize cytoplasmic structures (Lane, 1991).

Bartels *et al.*, 1990, observed that the mRNAs expressed in dessicated leaves of the resurrection plant (*Craterostigma plantagineum*) and in the ABA-treated callus *in vitro* were similar. ABA has been found to play an important role in tomato in the regulation of protein accumulation during drought stress and recovery from stress (Bray, 1990). Following exposure of the plant to a water-related stress for a few days (or several hours depending on the plant or tissue), the elevated levels of endogenous ABA, that accompany the stress imposition, are thought to induce a group of proteins, that are presumed to play a protective role. This group of proteins, termed dehydrins are produced both in response to water deficit, ABA (RABs; Responsive to ABA) and during late embryogenesis). Thus, gene induction by osmotic stress in general, has been thought to be mediated by ABA in vegetative tissues and possibly also in embryonic tissues (Skriver & Mundy, 1990). Rao *et al.*, 1993, observed the induction of several water stress responsive proteins in rice seedlings and one such protein, namely 23kDa polypeptide, was found to be induced by ABA treatment and was immunologically similar to RAB16 family proteins. Covarrubias *et al* (1995) showed that the drought induced proteins in bean seedlings were also induced by ABA treatment. Zhang, Moloney and Chinnappa (1996) observed that osmotic stress-inducible protein of

Stellaria *longipes* was induced by ABA and PEG or drought and had structural similarity to dehydrins.

ABA has also been implicated to have a role in osmoregulation. It has been shown to regulate the level of some inorganic cations such as K^+ and Na^+ and of some osmotically active substances such as sucrose, proline and betaine (Hasson & Poljakof-Mayber, 1983; Stewart & Voetberg, 1985). Trotel-Aziz *et al* (2000) investigated the relationships between the changes induced in abscisic acid (ABA) and proline contents in canola (*Brassica napus* L.) leaf discs subjected to sequential hyper- and hypo-osmotic treatments. Changes in ABA content were found to precede that of proline, suggesting that ABA could exert regulatory effects on both osmo-induced proline accumulation and its subsequent mobilization during recovery.

Several genes have been shown to be induced by ABA during water deficit in a variety of plants (Bartels *et al.*, 1990; Mundy and Chua, 1988; Gurerrero *et al.*, 1990). However, there are also several genes induced by desiccation that apparently do not respond to ABA (Guerrero *et al.*, 1990). These observations indicate, that there are ABA-dependent as well as ABA-responsive signal transduction pathways between the water stress signal and gene expression (Yamaguchi-Shinozaki *et al.*, 1992).

1.1.1.2. Cell suspension cultures for *in vitro* water stress studies

Suspension cultured, undifferentiated plant cells offer a relatively homogeneous and experimentally controllable alternative for the study of cellular responses to different abiotic stresses and have been used extensively even to distinguish between short-term response and long-term adaptation involving physiological and biochemical changes (Fallon and Phillips, 1989a, b; Leone et al., 1994). Cell and tissue cultures are relevant to crop improvement strategies because they offer a means of rapid selection on a mass scale and the possibility of applying genetic engineering methods for the development of improved germplasm (Blits *et al.*, 1993). Use of *in vitro* techniques similar to the selection of variants in bacterial and fungal cultures have been advocated and employed in higher plants, which would have practical application in agriculture. Plant cell culture methods allow selection of variants resistant to water stress. Callus cultures have been used in the selection and isolation of stress-adapted cells (Kavi Kishor and Reddy, 1985) and subsequent plant regeneration (Liu *et al.*, 1985; Reddy & Vaidyanath, 1986; Siddeshwar & Kavi Kishor, 1989).

Molecular biology is being employed to detect gene products (proteins) and identification of genes for stress tolerance. Plant materials from highly tolerant species are used for this approach. The approach is to identify stress-induced proteins, to clone the respective genes and to determine their possible role in helping the cells cope with the induced stress. Cell suspension culture has been advocated as a convenient method for identifying and elucidating cellular traits contributing to stress tolerance in several crops since cell cultures offer the advantage of the ease of handling large populations in a small culturable area. They have been used extensively to study the effect of various stresses like water deficit, salinity and ABA in rice in terms of growth parameters and accumulation of certain metabolites (Kavi Kishor, 1988, 1989). Implicit in such studies is the assumption that the response of cell cultures to a given stress is similar to that of the whole plant.

In vitro tissue culture contributes a useful tool to study the cellular mechanisms of salt / drought resistance, since it allows a control on the homogeneity of the stress and its application at the cellular level, independently of regulatory mechanisms occurring at whole plant level. Reddy *et al* (1993) proved that cell cultures of rice, can be used as a rapid and reproducible experimental test system for evaluating osmotic stress responses and for studying genetics and molecular biology of stress responses in rice. They also showed that the stress responses *in vitro* (cell cultures) and *in vivo* (intact plants), atleast in terms of induction of a specific polypeptide, are similar, proving that cell cultures can be used in the study of certain stress responses, in place of intact organisms.

1.1.1.3. *In vitro* selection for water stress tolerance

There is an increasing awareness of the potential of tissue and cell culture for the production of new genotypes with valuable traits for agriculture, particularly in relation to stress tolerance. In addition, water stress tolerant cell lines also represent an ideal system for assessing the physiological effects of water stress at the cellular level (Handa *et al.,* 1983; Hasegawa *et al.,* 1984). Further, such tolerant lines, when compared with the normal sensitive callus, can provide a useful means of measuring the capacity and range of stress tolerance and may be used for elucidating tolerance mechanisms at the cellular level (Srivastava *et al.*, 1995). The genetic variability observed in plant cell cultures has allowed the isolation of variant and mutant cell lines that differ in their tolerance to either biotic or abiotic factors (Larkin & Scowcroft, 1981; Meredith, 1983; Salgado-Garciaglia *et al.*, 1985).

Two main strategies have been used to isolate drought tolerant plant cells:
(a) Single-step approach, in which the cells are exposed directly to high levels of the stress agent for selection (Bressan *et al.*, 1981).
(b) Step-wise selection, in which the cells are gradually exposed to increasing concentrations of the water stress agent (Gulati & Jaiwal, 1993; Santos-Díaz. & Ochoa-Alejo, 1994; Bressan *et al.*, 1982).

Polyethylene glycol and mannitol are the common osmotic agents used to simulate drought stress *in vitro* (Handa *et al.*, 1982; Gulati & Jaiwal, 1993). Plasmolyzing solutes such as sugar alcohols (eg. Mannitol), readily pass through the cell wall and cause temporary plasmolysis, until their movement into the cytosol leads to osmotic recovery (Attree & Fowke, 1993). In contrast, PEG molecules are too large to move through the cell wall and do not cause plasmolysis. PEG has been widely used as osmoticum to simulate drought stress in water stress experiments for plants (Appelgate, 1960; Jackson, 1962; Jensen, 1981; Steuter *et al.*, 1981; West *et al.*, 1980). When non-penetrating or less readily penetrating solutes are used, the more negative osmotic potential of the external medium due to these solutes can only be counter-balanced by uptake of other external solutes, tissue dehydration or the synthesis of organic osmotica. Kaufmann and Eckard (1971) reported that PEG 6000 produced changes in plant water relations similar to those caused by drying soil or dessication, at the same water potential since PEG does not penetrate the cell wall (Carpita *et al.*, 1979). Despite some claims for toxic effects of its contaminants, the purified PEG available commercially has been used without physiological impairments in various plant species such as tomato cell cultures (Bressan *et al.*, 1982; Bressan *et al.*, 1981; Handa *et al.*, 1983). *In vitro* selection for drought tolerance is most frequently carried out by including polyethylene glycol (PEG 4000/6000) in the medium. The addition of PEG to nutrient medium of cultured plant cells simulates water stress by acting as a non-penetrating osmotic agent which lowers the potential of the medium in which the cells are growing.

Heyser and Nabors (1979) were the first to report the selection of tobacco cell lines tolerant to stress induced by PEG. Bressan *et al* (1981) selected tomato cells tolerant to drought stress by exposing callus to nutrient medium containing PEG. The selected cells grew better than the control unselected cells on media containing the non-penetrating osmoticum. Sabbah & Tal (1990) reported development of callus cultures resistant to mannitol-induced water stress. Sumaryati *et al* (1992) reported regeneration of drought-tolerant plants from protoplast cultures of *Nicotiana plumbaginifolia*. Santos-Díaz & Ochoa-Alejo (1994) reported the isolation and characterization of PEG-tolerant cell clones using cell suspensions of the drought-sensitive chili pepper (*Capsicum annuum* L.) to improve drought tolerance. They observed that the tolerance of the cell lines adapted to 15% and 20% PEG was stable and not simple adaptation, as the adapted cells exhibited dependence for osmoticum for their growth. Srivastava *et al.*, 1995, reported the isolation of water stress tolerant clones of callus cultures of tomato, selected at 10% PEG. These clones were capable of growing when they were transferred back to the selective medium after a period of growth on the control medium, thus exhibiting stability for PEG tolerance selected. Geetha *et al.*, (1995) isolated a PEG-tolerant callus line of *Vigna mungo* (L.) Hepper as a spontaneous variant on 25% PEG-amended nutrient medium through step-wise selection method. The stability of PEG-tolerance was proved, by transferring the selected callus line back into PEG-selective medium. Barakat and Abdel-Latif (1995) reported that shoot regeneration was more in wheat embryogenic cells selected by direct exposure to high concentration of PEG (the direct method) than when the cells were selected after gradual passage to higher concentrations of PEG. .

1.1.1.3.1. Mechanisms of cell adaptation to water stress

In addition to the prospect of developing stress tolerant plants, *in vitro* selection studies also help to study the mechanisms by which cells adapt to the stress, enabling to get an insight into the possible mechanisms of the whole plants to tolerate the stress *in vivo*. The mechanisms by which these selected cells tolerate water stress have been investigated by many researchers (Singh *et al.*, 1985; Singh *et al.*, 1987; Iraki *et al.*, 1989a; Borkird *et al.*, 1991).

Osmotic adjustment through solute accumulation has been suggested as one of the possible means for overcoming osmotic stress in PEG-adapted cell suspensions of tobacco and tomato (Heyser & Nabors, 1981). A number of low molecular weight organic compounds and inorganic ions are known to be associated with osmotic adjustment in cultured cells under water stress (Handa *et al.*, 1983; Hasegawa *et al.*, 1984). Studies on intracellular solutes that contribute to osmotic adjustment in drought tolerant cell lines have been reported by Handa *et al* (1982), Sabbah & Tal (1990) and Gulati & Jaiwal (1993). Bressan *et al* (1982) and Handa *et al* (1982) reported a positive correlation between the ability for osmotic adjustment and tolerance to water stress in PEG-adapted cells in tomato. Similar observations were made in chili by Santos-Díaz. & Ochoa-Alejo (1994). Srivastava *et al* (1995) noted higher total soluble and reducing sugars in PEG-adapted cell clones of tomato, compared to that in unselected cells. Barakat and Abdel-Latif (1995) reported a decrease in Na^+, K^+, and Cl^- contents of the embryogenic calli with increasing concentration of PEG levels. Handa *et al* (1983) also observed a decrease in K^+ content with increasing PEG levels. However, Santos-Díaz and Ochoa-Alejo (1994) reported accumulation of 2-3 times more Na^+ content in tolerant cell clones of chili pepper than unselected controls. They also observed a positive correlation between K^+ accumulation and the degree of PEG tolerance. Srivastava *et al.*, 1995, noted that sugars, free amino acids and proline and K^+ in PEG-selected tomato cells was higher than in unselected cells and probably contributed to the osmotic adjustment, making the cells more tolerant. Proline provided maximum contribution, as its increase was more marked than any other solutes. Geetha *et al.*, (1995) found higher K^+ accumulation in PEG-selected callus line of *Vigna mungo*(L.) compared to that in the sensitive callus. Proline content was also higher in the tolerant callus line compared to the wild type cells, when subjected to PEG stress.

It has been widely observed that exposure to polyethylene glycol-induced water stress results in reduced growth. Bressan *et al* (1981) observed that the relative growth of tomato cells decreased markedly with increasing PEG concentration in the medium. Similar effects of high concentration of PEG on the relative growth weight of callus have been reported. Barakat and Abdel-Latif (1995) observed that increasing PEG concentration resulted in marked decrease in the relative growth weight of callus. However, Srivastava *et al* (1995) observed higher fresh and dry weights in PEG-adapted cell clones of tomato, compared to that of unselected clones.

A reduction in cell size has also often been noted in cell cultures that are grown under conditions of water stress (Hasegawa *et al.*, 1986). Iraki *et al* (1989a) observed a reduced rate of cell expansion in tobacco cells under water stress. In a later study, Iraki *et al* (1989b) observed that the reduction in cell size is in spite of the osmotic adjustment that results in development of turgor pressures that are several fold higher than in unadapted cells, suggesting that changes in extensibility or other mechanical properties of the cell wall are responsible for this self-imposed failure of adapted cells to expand. Binzel *et al* (1985, 1987) also observed that cells adapted to 30% PEG accumulate dry weight at rates comparable to unadapted cells but exhibit as much as a 5-fold decrease in average cell volume and a reduced rate of cell expansion.

1.1.2. Drought stress in black pepper

One of the main production constraints in black pepper productivity is water stress (Ramadasan, 1987) during the summer months of March and April. Prabhakaran (1997) developed a statistical model for estimation of yield loss in black pepper due to incidence of pests, diseases and drought in Kannur District of Kerala during 1989-1992 and predicted the average yield loss in black pepper due to these factors to be around 33%. The major contributor towards stand loss of the crop was foot rot caused by *Phytophthora capsici* which contributed about 9% loss followed by drought stress which amounted to 4% loss in the vines. Black pepper production in Kerala has been severely affected in the recent years also due to failure of monsoon. The drought during the years 2002 and 2003 affected the crop production severely (reports in www.peppertrade.com and www.spizes.com). Thus drought, has become a major production constraint, in addition to the disease, in black pepper production.

The most important developmental phase of black pepper that is highly sensitive to water stress is the flowering stage (Vasantha, 1996). Water availability during this period is very crucial for the crop, failure of which affects the flowering and fruit set and hence, productivity. Delayed monsoon postpones the flowering process. Soil moisture stress after the flower initiation is also detrimental to the crop as it results in shedding of young spikes, leading to lower yields. To overcome the hostile spell of dryness during the summer months of March to May, the pepper plantations need to be irrigated. Since pepper is a rain-fed crop and is grown over large acreage of land, irrigation is not commercially viable. It is therefore important to develop black pepper varieties tolerant to moisture stress.

The mechanism of drought resistance/tolerance differs with different agricultural crops (Vasantha, 1996). Those crops where the seed is the economically important part, mechanisms that maintain productivity and reproductive efficiency under drought, are important (Turner, 1979). On the other hand, in pasture systems, mechanisms that maintain leaf production and plant persistence through the periods of moisture stress are more important (Turner and Beggs, 1978). In contrast, in perennial and tree crops, like the plantation crops, persistence overrides production mechanisms (Jones, Turner & Osmand, 1981) and hence, those mechanisms which help these crops tide over the unfavourable drought conditions year after year for a long period of time, are of more significance. As black pepper is a perennial plantation crop, strategies for selection of drought-tolerant lines should be based on the persistence of the cultivar through intense drought conditions and not solely on yield performance during moisture stress.

Research on drought stress in black pepper, till recently have been scanty and sporadic. Geetha and Nair (1989) attributed low soil moisture as one of the reasons for spike shedding in black pepper. Stomatal resistance, transpiration rate and leaf water potentials are used to screen drought tolerant types in black pepper (Vasantha *et al.*, 1990). Vasantha *et al.* (1991) reported higher proline content in stressed Karimunda cultivar, followed by Kalluvally-4. Vasantha (1996) reported that total sugars and proline content in black pepper showed significant negative correlations with depleting moisture content. Vasantha (1996) also reported that seven physiological parameters have varietal significance at critical soil moisture content in black pepper and enable to classify the different cultivars of the crop for scoring purpose based on their response to drought. The characters identified are stomatal resistance, transpiration rate, leaf water potential, RWC, specific leaf weight, total *et al.,* sugars, proline content and nitrate reductase activity. Based on these characters Krishnamurthy *et al.* (1998) reported decreasing relative water content (RWC) with increasing severity in water stress in black pepper. It was also observed that in tolerant accessions of black pepper, membrane leakage was comparatively lower than that in susceptible accessions. A decreased transpiration was also noted in drought-tolerant accessions compared to control. In a recent study, Krishnamurthy *et al* (2000) reported the effect of water stress on membrane damage, relative water content and activities of different enzymes in black pepper and suggested the possibility of utilizing some of these parameters as a measure of water stress tolerance in the crop.

Further basic studies are necessary to gain a better insight into the changes taking place in black pepper during drought stress. These studies would help to understand the mechanism of drought response and tolerance in black pepper. The information from such work with the help of the available modern technologies would aid in improvement of the crop for survival under water-limiting conditions, which have become a recurring feature in Kerala in the recent years. The present study is an attempt to unravel the biochemical and physiological changes that take place in black pepper in response to induced water stress, using cell suspension cultures of black pepper as a model system.

1.2. Materials and Methods

1.2.1. Experimental material

(Fig. 3.1)

The experimental material used for all the studies on biotic and abiotic stress including *in vitro* selection for water stress-tolerant cell lines, was the embryogenic cell suspension culture obtained from the seed explant of *Piper nigrum* L. *var* Karimunda. The somatic embryogenesis from the seed explants of black pepper was initiated according to the method described by Biju *et al* (1996).

The initiation of somatic embryogenesis took 3 months and was seen with the emergence of a tuft of somatic embryos arising from the explant, accompanied by the spreading of a faintly visible granular layer of cells over the surface of the medium. The cells that spread over the medium were scooped out and sub-cultured into full or half strength SH- liquid medium (Schenk and Hilderbrandt, 1972; Appendix 1) for the multiplication of cells and for embryo development respectively. The suspension-cultures were incubated in dark on an orbital shaker at 100rpm at 25 ± 2°c. The cell suspension was then plated onto SH or ½ MS medium for the initial scaling up of callus production. The callus was later mass-multiplied by sub-culturing young embryogenic callus on to solid half-strength MS medium (Murashige & Skoog, 1962; Appendix 2) maintained as slants in culture tubes. After about 20-25 days, there was enough growth of callus in each culture tube for use in different experiments.

All the experiments including *in vitro* selection and studies on cell responses to stress were carried out with the embryogenic callus derived from a single seed of Karimunda variety of black pepper for the purpose of uniformity of the experimental material for all the studies.

1.2.1.1. Cell-suspension culture

Cell-suspension cultures of black pepper were initiated from the embryogenic callus that was multiplied and maintained on half-strength MS medium as described above. For experiments involving studies of cell responses to biotic and abiotic stress,

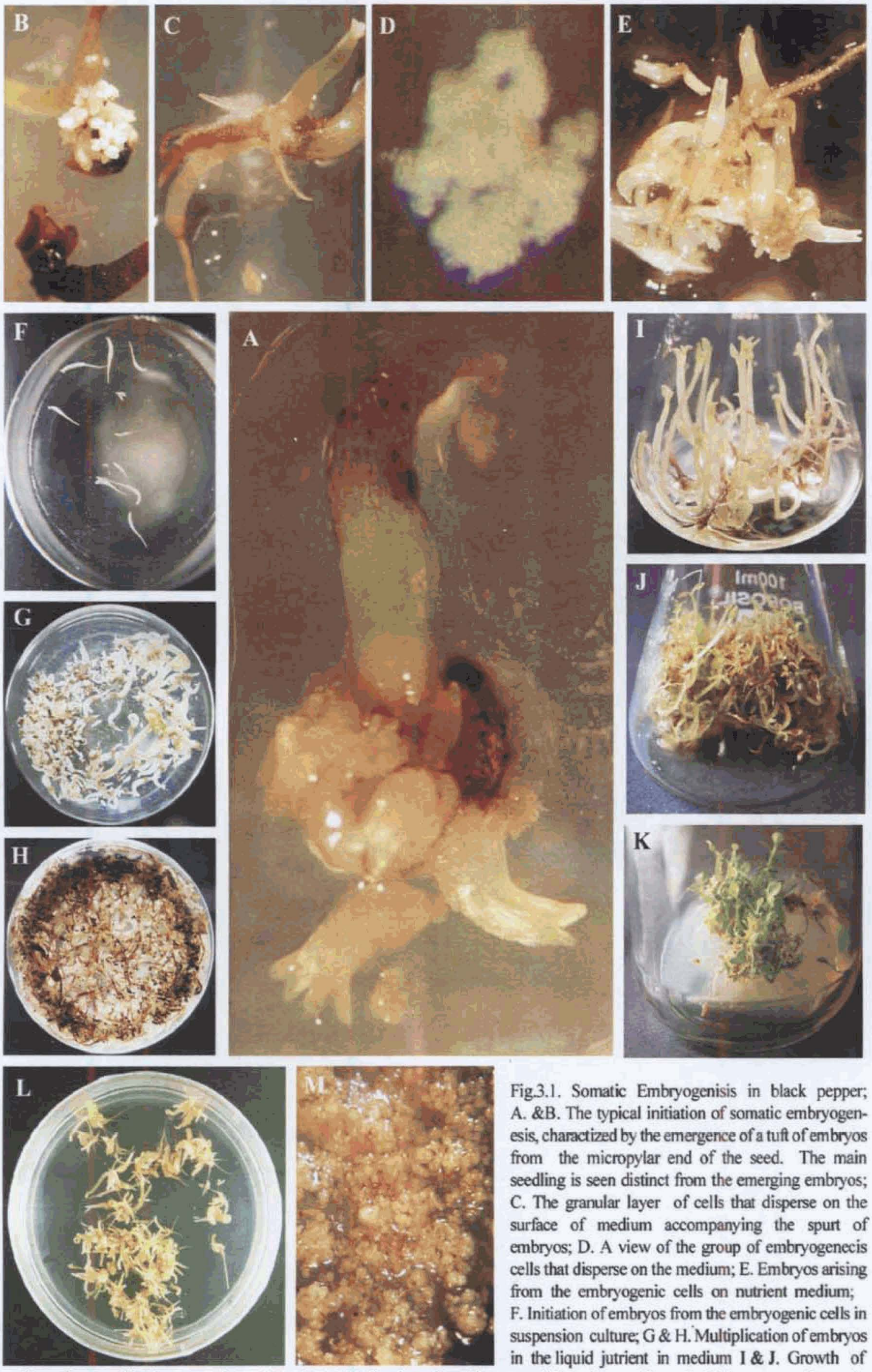


Fig.3.1. Somatic Embryogenisis in black pepper; A. &B. The typical initiation of somatic embryogenesis, charactized by the emergence of a tuft of embryos from the micropylar end of the seed. The main seedling is seen distinct from the emerging embryos; C. The granular layer of cells that disperse on the surface of medium accompanying the spurt of embryos; D. A view of the group of embryogenecis cells that disperse on the medium; E. Embryos arising from the embryogenic cells on nutrient medium; F. Initiation of embryos from the embryogenic cells in suspension culture; G & H. Multiplication of embryos in the liquid jutrient in medium **I & J.** Growth of embryos into seedling in suspension culture; **K.** Multiplication of seedlings on solid nutrient medium; **L.** Maintanance of somatic embryos; **M.** View of the embryogenic callus maintained on nutrient medium for use in different studies in the presenr work.

cell suspension cultures were established by inoculating 750mg of embryogenic callus in 30ml of full-strength SH medium. The cultures were incubated on an orbital shaker (Certomat) at 100 rpm in dark at 25±2°c. The callus was harvested at prescribed time intervals for different biochemical analyses.

1.2.2. Abiotic Stress - Water stress

Under abiotic stress, *in vitro* studies on the effects of water stress on black pepper cells were taken up by using polyethylene glycol to simulate water stress conditions. *In vitro* selection studies were also taken up for developing water stress-tolerant cell lines.

1.2.2.1. Induction of water stress

1.2.2.1.1. Preparation of PEG-amended medium

Polyethylene glycol (PEG 6000) was used to induce water stress *in vitro*. Since PEG interferes with solidification of culture medium, static culture conditions could not be used for water stress studies. Therefore suspension cultures were used, in which PEG was included in the liquid culture medium. For preparing PEG-amended medium, PEG-6000 was dissolved in double strength nutrient medium by heating if required, and the final volume was made up with distilled water to obtain a 1 X concentration. The pH of the 1 X medium was adjusted to 5.65 before autoclaving.

1.2.2.1.2. Dose response curve for PEG 6000

Before any experiments on the effect of PEG-mediated water stress on black pepper cells could be carried out, the inhibitory concentration of PEG 6000 on black pepper cells was to be determined. Hence, a dose-response curve for PEG 6000 was worked out using different concentrations of PEG. The growth of black pepper cells was determined at 0, 5, 10, 15, 20, 25, 30 and 40% PEG 6000. 25ml conical flasks containing liquid SH medium with different concentrations of PEG 6000 were inoculated with about750mg fresh weight of embryogenic callus. The flasks were incubated in dark on an orbital shaker. The callus from duplicate flasks of a treatment was harvested after 25days, by filtering through a nylon mesh and then under vacuum on a filter paper. The fresh weight of the callus was determined in each of the treatments and the net increase in fresh weight was calculated by substracting the weight of initial inoculum. The relative fresh weight increase compared to the control was determined by dividing this value with that for the control flasks, which is given by the following formula:

$$\text{Percentage increase in fresh weight} = \frac{X_n - X_o}{X_c = X_o} \text{ X } 100$$

The values were plotted on a graph to find the LD50 value of PEG 6000.

1.2.2.1.3. Induction of water stress and sampling of cells for biochemical analyses

Black pepper suspension-cultured cells were multiplied and maintained as described earlier. Callus from solid 1/2 MS medium (ca.0.750g) was transferred to 30ml liquid SH medium in 100ml flasks supplemented with 15, 25 and 30% PEG 6000. Control flasks were also maintained. The cells were harvested at 5, 10, 15, 20 and 25 day intervals by filtration. The PEG-treated cells were washed with 0.4M mannitol to remove the traces of polyethylene glycol sticking to the cells which interferes with various biochemical analyses. This also maintains the cells at the osmotic potential at which they are harvested, during the washing process. The cells were snap-frozen in liquid nitrogen and stored at -80°c until use.

1.2.2.2. Cell responses to PEG-mediated water stress

1.2.2.2.1. Osmolyte accumulation:

1.2.2.2.1.1. Inorganic solutes:

The inorganic ions were analyzed at different intervals of the growth cycle of the callus under different treatments. One gram fresh weight of the callus was taken in 25ml standard flasks and digested with 9:4 nitric acid : perchloric acid mix on a hot plate until the solution was clear. The solution was made up to 25ml and Na+, K+ and Ca2+ and Mg2+ concentrations in the solution were determined using an automated flame photometer (Black, 1965) using the same sample.

1.2.2.2.1.2. Organic solutes

1.2.2.2.1.2.1. Proline

Proline was extracted and determined colorimetrically by the method of Bates *et al* (1973). 0.1g d.wt. of callus harvested at different intervals, was homogenized in 10ml of 3% sulfosalicylic acid with acid-washed sand and filtered (Whatman#1 filter paper) to separate cell debris. 2ml of the filtrate of each sample was taken in test tubes and 2ml glacial acetic acid and 2ml acid-ninhydrin were added. The test tubes were then incubated in a boiling water bath for 1hr. The reaction was terminated by placing the test tubes in an ice bath. 4ml of toluene was then added to the cooled reaction mixture of the test tubes and vortexed. The toluene layer was pipetted out and proline was determined in the filtrate samples by measuring the absorbance of the red coloured proline-ninhydrin product at 520nm. Proline concentrations in the test samples were determined from a standard curve using quantities of authentic proline and calculated on a dry weight basis of the sample using the formula:

$$\text{nmoles per g tissue} = \frac{\mu\text{g proline/ml x 4ml toluene}}{115.5} \text{ X } \frac{5}{\text{g sample}} \text{ X } 1000$$

where, 115.5 is the molecular weight of proline.

1.2.2.2.1.2.2. Total free amino acids

Total free amino acids were extracted by homogenizing 0.1g of dry sample with 5ml of 80% ethanol with a small quantity of acid-washed sand in a mortar and pestle. The homogenate was centrifuged and the supernatant saved. The pellet was again extracted with 80% ethanol and centrifuged. The supernatants were pooled and used for the estimation of amino acids content by the ninhydrin method (Moore and Stein, 1948). Ninhydrin solution was prepared by dissolving 80mg stannous chloride in 50ml of 0.2M citrate buffer (pH5.0) and mixing this solution with 50ml of 2-methoxyethanol containing 2g of ninhydrin. To 0.1ml of the sample, 1ml of ninhydrin solution was added and the volume made up to 2ml with distilled water. The samples were heated in a boiling water bath for 20min and then brought to room temperature before adding 5ml of diluent solvent (1:1 water and n-propanol). The contents of the tubes were vortexed and the intensity of the purple colour developed was read at 570nm in a spectrophotometer. The reagent blank contained 0.1ml of 80% ethanol instead of the extract. The concentration of amino acids was determined from a standard graph of leucine taken in the range of 10μg -100μg. The values were expressed as μg equivalents of leucine per gram dry weight.

1.2.2.2.1.2.3. Reducing sugars

Reducing sugars were extracted from 0.1g dry weight with 5ml of hot 80% ethanol twice. The homogenate was centrifuged and the supernatants were pooled and evaporated to dryness by keeping the tubes in water bath at 80°c. The residue was dissolved in 5ml of water and aliquots of 0.1ml were taken for the estimation of reducing sugars present in the samples by Nelson-Somogyi method, as given by Sadasivam & Manickam (1996). The volume in each tube was made up to 2ml with distilled water after the addition of sample. 1ml of alkaline copper tartarate reagent was added to each tube. The tubes were placed in a boiling water bath for 10 minutes. The tubes were cooled and 1ml arsenomolybdic acid reagent was added to all tubes. The volume in each tube was made up to 10ml with water. The absorbance of the blue colour was read at 620nm after 10min. Glucose standard was processed similarly and a standard graph was plotted for calculating the amount of reducing sugars present in the sample. The values were expressed in terms of μg glucose per gram dry weight.

1.2.2.2.2. Estimation of phenolics

The ethanolic extract of cells used for estimating total amino acids was used to estimate the phenolics content of the cells under different treatments of water stress, according to the method described by Sadasivam and Manickam (1996). 0.5ml of the ethanolic extract was diluted to 3 ml with distilled water and 0.5ml of 1N Folin-Ciocalteau reagent was added. After 3min., 2ml of 20% Na_2CO_3 was added, the contents mixed thoroughly and placed in a boiling water bath for 1min., cooled and absorbance measured at 650nm with catechol as the standard. The phenolic content of the cells was expressed as of μg catechol per gram dry weight.

1.2.2.2.3. Measurement of cellular damage - Lipid peroxidation

The level of lipid peroxidation in the callus tissue was measured in terms of malondialdehyde (MDA) content, determined by a thiobarbituric acid reaction according to the method of Heath and Packer (1968). 200mg fresh weight of callus obtained at different intervals of time was homogenized in 5 ml 0.1% trichloroacetic acid and the mixture centrifuged at 10,000 rpm for 10min at 4^{o}c. To 1ml of the supernatant, 4ml of 20% TCA containing 0.5% thiobarbituric acid was added and vortexed. The mixture was heated at 95^{o}c for 30min, quickly cooled in ice bath for 15 min. and centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was read at 532nm and 600nm. The value for non-specific absorption at 600nm was subtracted from 532nm reading and the concentration of malondialdehyde (MDA) was calculated using MDA's extinction coefficient of $155 mM^{-1}cm^{-1}$, by the following equation and expressed as nmoles MDA per gram fresh weight.

$$\mathbf{D = \varepsilon.c.t}$$

Where, D = optical density or absorbance ($\lambda_{532} - \lambda_{600}$)

c = concentration (mmoles/l)

t = thickness of cell or path length (=1cm)

ε = extinction coeff.

1.2.2.2.4. Antioxidant enzymatic responses

1.2.2.2.4.1. Enzyme activity

Enzyme activities were expressed in terms of International Units (I.U.) Enzyme activity in International Units per ml of extract, known as the volume activity, was calculated by the equation,

$$\text{Volume activity (Units per ml)} = \frac{\text{V X (change in absorbance /min)}}{\varepsilon.\text{d.v}}$$

where, V = Total volume of assay mixture (in ml) v = volume of enzyme in assay mixture (in ml)

ε = extinction coefficient of substrate (that disappears) or the product (that appears) (expressed in l/micromoles/cm)

d = light path of cuvette in cm (1.0cm)

Therefore, Volume activity is the micromoles of product formed or substrate disappeared in 1 minute by the action of 1ml of enzyme and is expressed as units per ml of enzyme. Since one International Unit of enzyme transforms one micromole of substrate in 1min., one ml of enzyme solution would contain International Units equivalent to the volume activity.

Specific activity is calculated by dividing the volume activity (I.U./ml) with the protein concentration determined as mg/ml of the extract and is expressed as micromoles of transformed product per min. per mg protein. Protein was determined according by the method described by Lowry *et al* (1951). Thus,

$$\text{Specific activity} = \frac{\text{Volume activity (I.U./ml)}}{\text{mg protein/ml enz. extract}}$$

1.2.2.2.4.2. Enzyme extraction

2g f.wt. of the frozen suspension cultured cells stored at -80°c was ground to a fine powder with liquid nitrogen in a mortar and homogenized in ice-cold 50mM sodium phosphate buffer, pH 7.0. 1% (w/w) insoluble polyvinylpolypyrrolidone was added to the ground samples before addition of buffer, to decrease the oxidation of phenolics. The homogenized extracts were filtered through 2 layers of muslin cloth and the filtrate centrifuged at 10,000rpm for 30min. The supernatant was stored at -30°c until use for the assays of peroxidase, catalase and superoxide dismutase.

1.2.2.2.4.3. Enzyme assays

1.2.2.2.4.3.1. Superoxide dismutase (EC 1.15.1.1)

Superoxide dismutase was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The 3ml reaction mixture contained 50mM potassium phosphate buffer, pH 7.8, 0.1mM EDTA, 50mM Na_2CO_3, 13mM methionine, 63μM NBT, 1.3 μM riboflavin and 50μl enzyme extract. Tubes containing only the assay mixture without the cell extract served as controls. The samples and controls had two sets of tubes, each set with duplicate tubes. One set was illuminated under sunlight and the other set was maintained in dark. The test tubes containing the mixture were placed in the compartments of a dark box with a lid. The reaction was started by exposing the tubes to the sunlight by removing the lid of the dark box. The tubes were illuminated for 10min until the controls developed intense blue colour due to formazan formation. The illuminated control tubes served as absolute light controls. The formazan accumulation in different tubes was quantified using a Shimadzu UV-60A spectrophotometer by recording the absorbance of the developed blue colour at 560nm against the non-illuminated dark control that did not develop colour and served as blank to correct for background absorbance. The absorbance of the each of the illuminated samples was subtracted from that of similar samples maintained in dark to obtain the net absorbance value for each sample. The absolute control tubes served to determine the maximum A_{560}. The A_{560} value of each sample was subtracted from the maximum A_{560} ; the mathematical difference was then divided by the maximum A_{560} and multiplied by 100 to obtain the percentage of inhibition of NBT photoreduction. This value divided by 50 gave the value for 50% inhibition of NBT reduction.

The enzyme activity was determined using the formula:

$$\text{Activity} = \frac{100 - [(\text{OD}_s / \text{OD}_{lc}) \times 100]}{50}$$

where, OD_s = absorbance of sample
OD_{lc} = absorbance of absolute light control

The specific activity of the enzyme was obtained by dividing the above value with the amount of protein in mg present in the volume of enzyme used for the assay. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction as monitored at 560nm.

Activities of different forms of SOD were identified, by using KCN or H_2O_2 at final concentrations of 3mM and 5mM respectively, in the reaction mixture (Giannopolitis and Ries, 1977). KCN inhibits Cu/Zn SOD, but does not affect MnSOD or FeSOD, while H_2O_2 inactivates Cu/ZnSOD and FeSOD without affecting MnSOD. KCN at the final concentration of 3mM was added to the reaction mixture before the addition of H_2O_2 to inactivate peroxidases that interfere with the SOD assay (Yu & Rengel, 1999). MnSOD activity was determined in the presence of both 3mM KCN and 5mM H_2O_2 ; FeSOD activity was obtained by subtracting MnSOD activity from the activity yielded in the presence of 3mM KCN, and Cu/ZnSOD activity was calculated by the difference between total SOD activity and the sum total of MnSOD and FeSOD activity.

The SOD isozymes were separated by native polyacrylamide gel electrophoresis (PAGE) on 10% gels (Appendix 3) using a Hoefer Mighty Small vertical electrophoresis unit and were localized on the gels by the photochemical method of Weissiger and Fridovich (1973). The different isoforms (MnSOD, FeSOD and Cu/ZnSOD) were differentially stained by the selective inhibition of the various isoforms with NaCN or/and H_2O_2 (Hernandez *et al.*, 1999) (Appendix 4). The inhibitory action of H_2O_2 and NaCN of the different isoforms of SOD is described above.

1.2.2.2.4.3.2. Peroxidase (EC 1.11.1.7)

The peroxidase activity was determined by following the appearance of yellow colouration resulting from the oxidation of pyrogallol in the presence of hydrogen peroxide (Kar & Mishra, 1976). The reaction mixture consisted of 1ml of 50mM sodium phosphate buffer, pH 6.8, 0.5ml 10mM pyrogallol, 0.1ml of 100mM H_2O_2 and the volume made up to3ml by adding 1.4ml distilled water. The reaction was started by adding 25µl enzyme extract to the reaction mix and following the increase in absorbance at 420nm due to the oxidation of pyrogallol to purporogallin. The increase in absorbance at 420nm was recorded at an interval of 20sec for 3min. in a Shimadzu UV-160A, UV-

Visible spectrophotometer. The enzyme activity was expressed in Inernational Units and was calculated with the molar extinction coefficient of purpurogallin, $\varepsilon = 12\ mM^{-1}cm^{-1}$. One unit of enzyme was defined as micromoles of purpurogallin fomed per mg protein per minute.

1.2.2.2.4.3.3. Catalase (EC 1.11.1.6)

Catalase activity was assayed by following the disappearance of H_2O_2 at 230nm (Luck, 1965). The assay mix consisted of 2ml 50mM sodium phosphate buffer, pH6.8, and 1ml of 100mM H_2O_2. Reaction was started by adding 25µl of enzyme extract. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time and the enzyme activity is determined by following this decrease in absorbance at 230nm at 15sec intervals for 75sec. The enzyme activity in terms of the concentration of H_2O_2 was expressed in International Units (I.U.) and was calculated using the molar extinction coefficient of H_2O_2, $\varepsilon = 0.036\ \mu M^{-1}cm^{-1}$ (Sadasivam and Manickam, 1996). One unit of the enzyme was defined as µmoles H_2O_2 decomposed per min. per mg protein.

1.2.2.2.4.3.4. Enzymes of the AGC-Cycle

Enzymes of the AGC-cycle assayed in the present study include ascorbate-peroxidase, dehydroascorbate reductase and glutathione reductase.

1.2.2.2.4.3.4.1. Enzyme extraction

Crude fractions of cytosol and chloroplasts were prepared as described by Zhang and Kirkham (1995) and Miyake and Asada (1992). 2.0g f.wt. of the frozen cells stored at -80°c was ground to a fine powder in a mortar with liquid nitrogen and the mixture homogenized at 4°c with 10ml of extraction buffer (50mM sodium phosphate buffer, pH7.0 containing 0.33M sorbitol, 1mM $MgCl_2$, 2mM EDTA, 10mM NaCl, 0.5mM KH_2PO_4 and 1mM ascorbate, that formed an osmoticum). 1g polyvinylpolypyrrolidone was added to prevent oxidation of phenols that inhibit enzymes. The homogenate was filtered through 2 layers of cheesecloth and centrifuged at 4°c for 4min at 500 x g. The pellet, which consisted of debris was discarded and the supernatant was centrifuged again at 2000 x g for 15min. The supernatant was saved. The pellet that consisted of sedimented chloroplasts was gently washed with 10 ml of the extraction medium containing the osmoticum and centrifuged at 2,000 x g for 2min. The chloroplast pellet was finally resuspended in 2ml of the extraction buffer with the osmoticum and stored at -20^{0}C. 0.1%Triton X-100 was added to the chloroplast suspension to release the enzymes from chloroplasts when enzymatic activities were to be determined. The supernatant obtained at 2,000 x g was again centrifuged at 15,000 x g for 15min, and the resulting supernatant was used as the cytosolic fraction.

1.2.2.2.4.3.4.2. Enzyme assays

1.2.2.2.5.6.1. Ascorbate peroxidase (AP, EC 1.11.1.11)

AP activity was assayed by the method of Nakano and Asada (1981) by following the decrease in absorbance at 290nm due to AsA oxidation with slight modifications. The 3ml buffer mixture consisted of 0.5mM AsA, 0.1mM EDTA in 50mM sodium phosphate buffer, pH 7.0. 10μl of cytosolic enzyme extract or 50μl of chloroplastic extract was added to the buffer mix and the enzyme reaction was initiated by adding 10μl of 100mM H2O2 to reach a concentration of 0.1mM H_2O_2 in the final reaction mixture. H_2O_2 –dependent oxidation of AsA ($\varepsilon = 2.8 mM^{-1}cm^{-1}$) was followed by monitoring the decrease in absorbance at 290nm. One unit was defined as the amount of enzyme that oxidized 1μmol of ascorbate per min at room temperature under the above conditions.

1.2.2.2.5.6.2. Dehydroascorbate reductase (DR, EC 1.8.5.1)

DR activity was measured by following the formation of AsA at 265nm (Nakano and Asada, 1981; Cakmak *et al*, 1993). The 3ml buffer mixture for the enzyme reaction consisted of 2.5mM GSH and 0.1mM EDTA in 50mM sodium phosphate buffer (pH 7.0). 50μl of cytosolic extract or 80μl of chloroplast extract was added as the enzyme source. The enzyme reaction was initiated by adding 60μl of 10mM DAsA to reach a concentration of 0.2mM in the final reaction mixture. Formation of AsA ($\varepsilon = 14 mM^{-1}cm^{-1}$) from dehydroascorbate by reduction with GSH as electron donor, was followed by the increase in absorbance at 265nm for 75 sec at 15sec intervals. One unit was defined as the amount of enzyme that resulted in the formation of 1μM of ascorbate at room temperature under the conditions mentioned.

1.2.2.2.5.6.3. Glutathione reductase (GR, EC 1.6.4.2.)

GR activity was measured by following the oxidation of NADPH at 340nm (Scheadle and Bassham, 1977). The 3ml buffer mixture for the enzyme reaction consisted of 0.5mM GSSG and 3mM $MgCl_2$ in 50mM Tris-Cl, pH7.5. 50μl of the cytosolic extract was added as the enzyme source. The reaction was initiated by adding 30μl of 15mM NADPH to reach a concentration of 0.15mM NADPH in the final reaction mixture. The oxidation of NADPH ($\epsilon = 6.2 mM^{-1}cm^{-1}$) (Loggini *et al.*, 1999) was followed by measuring the decrease in absorbance at 340nm for 18sec at 20 sec intervals. One unit of GR was defined as the amount of enzyme that resulted in the oxidation of 1μmole of NADPH at room temperature under the conditions mentioned.

1.2.2.2.5. Water stress- and ABA-induced Proteins (WSP)

1.2.2.2.5.1. Water stress and ABA treatments

Water stress was induced as described above by incubating the cells in media containing different PEG regimes. ABA treatment was given by incubating the cells in liquid SH medium containing 200μM ABA for four days. ABA was dissolved in a small quantity of 0.4M $NaHCO_3$ and the volume made up to the desired volume to prepare a stock of 2000μM solution. The ABA containing medium was pipetted out and the cells washed with fresh SH medium. The cells were then resuspended in control SH medium or SH medium supplemented with different concentrations of PEG 6000. ABA controls were also maintained. The cultures were incubated for 10 days after which they were harvested and quick frozen in liquid nitrogen and stored at -80°c until use.

1.2.2.2.5.2. Total protein extraction

Total proteins were extracted according to Rao *et al.* (1993). 1.5g callus tissue was finely ground in 3ml of 0.2M Tris-HCl buffer, pH 8.1, containing 20mM EDTA, 2mM phenylmethyl sulfonyl fluoride 0.4%SDS and 2%β-mercaptoethanol at 4°c. The homogenate was centrifuged at 1,000 x g for 3 min, and the supernatant was collected and recentrifuged at 13,000 x g for 15 min. The resulting supernatant was used as the cytoplasmic extract. Protein was estimated by Lowry's method (1951).

1.2.2.2.5.2.1. SDS-PAGE of water-stress-induced proteins

The protein extracts were electrophoresed on a 12.0% SDS-polyacrylamide slab gel according to Laemmli (1970) (Appendix 5). 100μg protein was loaded uniformly in all the wells. The resolved proteins were visualized by coomassie blue R250 staining (Appendix 5.1) and the gel was documented using the gel documentation system, Alpha Imager (Alpha Innotech Inc.).

1.2.2.3. *In vitro* studies

1.2.2.3.1. Growth assay of cells in response to PEG

To determine the effect of different concentrations of PEG on the growth and differentiation of black pepper cells, 500mg of friable embryogenic callus was inoculated in 25ml SH liquid medium amended with required concentrations of PEG. For assessing the growth potential, the cultures were harvested after 25 days by collecting the cells on a filter paper in a Büchner funnel by aspiration followed by fresh weight determination.

The black pepper cells were also assessed for their regeneration ability in the different PEG-amended media by their prolonged exposure to the stress for 50d, until the cultures showed embryo formation. For determining the differentiation potential or regenerability of the cells after 25d exposure to the stress, uniform aliquots of 7 ml of the cells were plated on to control SH medium in petri-plates using a micropipette, the tip of which had a cut end. The regeneration ability was quantified in terms of the number of embryos produced per 5ml of the cell suspension plated on 25ml medium.

The growth and differentiation of cells on prolonged and step-wise exposure to higher intensities of stress was also studied.

1.2.2.3.2. *In vitro* selection

In vitro selection was carried out by both direct and step-wise selection methods. In the former, the cells were exposed directly to higher concentrations of PEG, while in the latter; the cells were initially subjected to lower levels of PEG followed by exposure to increasing concentrations. 500-750mg of the friable embryogenic callus was taken for *in vitro* selection experiments. Replicate flasks were maintained for selection for each of the PEG concentration. The cells were subjected to the PEG-mediated water stress for long durations of time by replenishing fresh medium at regular intervals of 25days. The cells were allowed to settle down and the supernatant spent medium was removed with a micropipette followed by addition of fresh medium. After the selection period, aliquots of the cells were plated on control SH solid medium in petri-plates for further growth and differentiation. The cell clumps and embryos which survived the rigorous selection regime were subjected to further stress for selecting truly water stress-tolerant lines.

1.2.2.3.3. Characterization of *in vitro* - selected cell lines

Selected cell lines were characterized biochemically after an exposure to stress for 45 days. Among the osmolytes, the cells were analyzed for inorganic ions, viz., K^+, Na^+ Mg^{2+} and Ca^{2+} and organic compounds, namely, reducing sugars, total free-amino acids and proline, as described in section 3.1.2.2.1. The phenolic content and lipid peroxidation in the cells was also determined as described in sections 3.1.2.2.2. & 3.1.2.2.3, respectively.

1.2.2.4. Statistical analysis

Results were analyzed by one-way ANOVA (Analysis of variance) with Post-Hoc tests. ANOVA tests the null hypothesis that several group means are equal in the population. Once it is determined that differences do exist among the means, the post-hoc range tests and pair-wise multiple comparisons determine which means differ. Range tests identify homogenous subsets of means that are not different from each other. Pair-wise multiple comparisons test the difference between each pair of means and yield a matrix that separate group means that are significantly different at an alpha significance level of 0.05. The ANOVA post-hoc test used for the analysis of results in the present study was the Duncan's multiple range test that makes pair-wise comparisons using a step-wise order of comparisons identical to the orders used by the student-Newman-Kent's test, but sets a protection level for the error state for the collection of tests, rather than an error rate for individual tests. It uses the standardized range statistic.

1.3 Results

Black pepper suspension cultures were used as model systems to study their biochemical responses to different regimes of water stress induced by polyethylene glycol.

1.3.1. Induction of water stress *in vitro*

1.3.1.1. Dose response curve for PEG 6000

Before any studies on water stress could be carried out, it was essential to determine the growth response of black pepper cells to different doses of PEG.

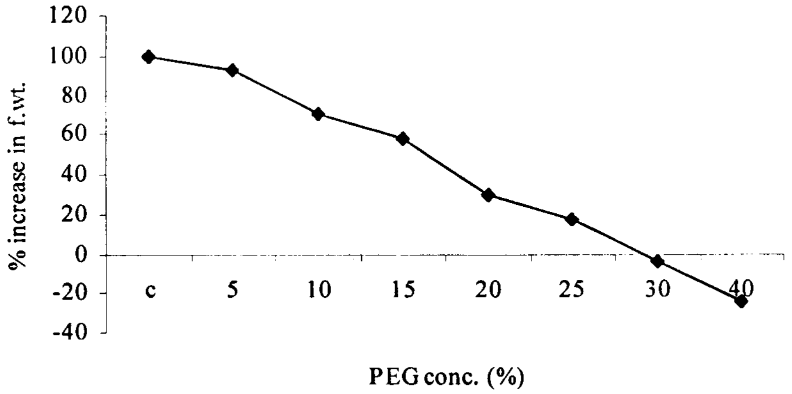


Fig. 1.1 Dose response curve of growth of black pepper suspension cultures in polyethylene glycol-amended medium

The effect of polyethylene glycol (PEG 6000) on the growth of black pepper suspension cultures was tested with different concentrations ranging from 5 to 40%. PEG at a concentration of 15% caused 50% inhibition of growth while 25% PEG resulted in 82.36% decrease in fresh weight. 30% PEG brought about complete inhibition of growth after 25 days of exposure to stress and resulted in negative fresh weight increase over the control (Fig. 1.1). Hence, in further studies, 15% PEG was used to apply mild water stress, while 25% PEG was used to induce severe water stress conditions while 30% PEG was used as a lethal dose. Fig.1.17 shows the growth of black pepper suspension cultures at these three PEG regimes.

1.3.2. Biochemical responses to PEG-induced water stress

Black pepper suspension cultures were used to study various biochemical responses to water stress induced by polyethylene glycol 6000 at the various intensities of stress mediated by 15 (mild), 25 (severe) and 30% (lethal) PEG.

1.3.2.1. Osmotic adjustment

1.3.2.1.1. Organic solutes

1.3.2.1.1.1. Amino acids

1.3.2.1.1.1.1. Total free amino acids

Total free amino acids were found to increase on imposition of water stress. While the cells exposed to mild water stress (15% PEG) showed a far greater increase in total amino acids during the initial stages of the stress period, which decreased over the period of time, the 25% PEG-treated cultures showed an increase which was not as massive as that seen in 15% PEG-treated cultures, but the levels were maintained more or less similar throughout the entire stress period. The 30% PEG-treated cultures however showed a marginal initial increase in the amino acid content compared to the control but declined after the 10th day and remained low till the end of the stress period **(Table 1.1).**

Table 1.1 Time-course accumulation of total free amino acids in black pepper suspension cultures subjected to varying degrees of PEG-induced water stress.

Tmt. No.	Tmt	Total amino acids (x 10^2 µg g^{-1} d.wt^{-1})					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
1	Control (0% PEG)	9.54 ± 0.1063	9.78 ± 0.0918	9.14 ± 0.0885	7.56 ± 0.1605	14.28 ± 0.1472	11.48 ± 0.2066
2	15% PEG		49.46 ± 0.9201	42.68 ± 0.8572	47.5 ± 0.5632	24.08 ± 0.2050	16.64 ± 0.1299
3	25% PEG		24.46 ± 0.3562	29.1 ± 0.2563	30.25 ± 0.6484	23.9 ± 0.1969	32.0 ± 0.5379
4	30% PEG		17.02 ± 0.0921	23.36 ± 0.1727	6.12 ± 0.2012	8.88 ± 0.2023	5.43 ± 0.1865

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

TMT	N	Subset for alpha = .05		
		1	2	3
1.00	24	10.3404		
4.00	24	11.7533		
3.00	24		24.7513	
2.00	24			31.1978
Sig.		.599	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.1.1.1.2. Amino acid profile

The amino acid profile showed a general increase in most of the amino acids in black pepper cultures subjected to PEG-induced water stress, compared to the control cultures. The increase in amino acids however showed a decline by the 25th day of exposure to the stress (Fig. 1.2). The control cultures showed a dominance of alanine and phenylalanine during the initial stages of growth while glutamine was found to be the predominant amino acid towards the end of the experiment. The prominent amino acids that increased during PEG-imposed water stress were alanine, glutamine, phenylalanine, serine and threonine. While alanine, phenylalanine glutamine and serine were abundant in 25% PEG-treated cultures, cultures exposed to 15% PEG had glutamine as the major amino acid. Glutamine levels were maintained at both mild and severe water stress even after prolonged exposure to stress. However, glutamine in 15% PEG-treated cultures was maintained higher than that in 25% PEG-treated cultures. Phenylalanine and alanine were abundant in cultures exposed to severe water stress (25%PEG). However, while they increased significantly during the initial stress period, they declined to minimal levels by the 20th day. Under mild stress, threonine increased gradually and maintained higher levels after 15th day of exposure till the conclusion of the experiment. Cultures exposed to 25% PEG, however, did not show an increase in threonine levels.

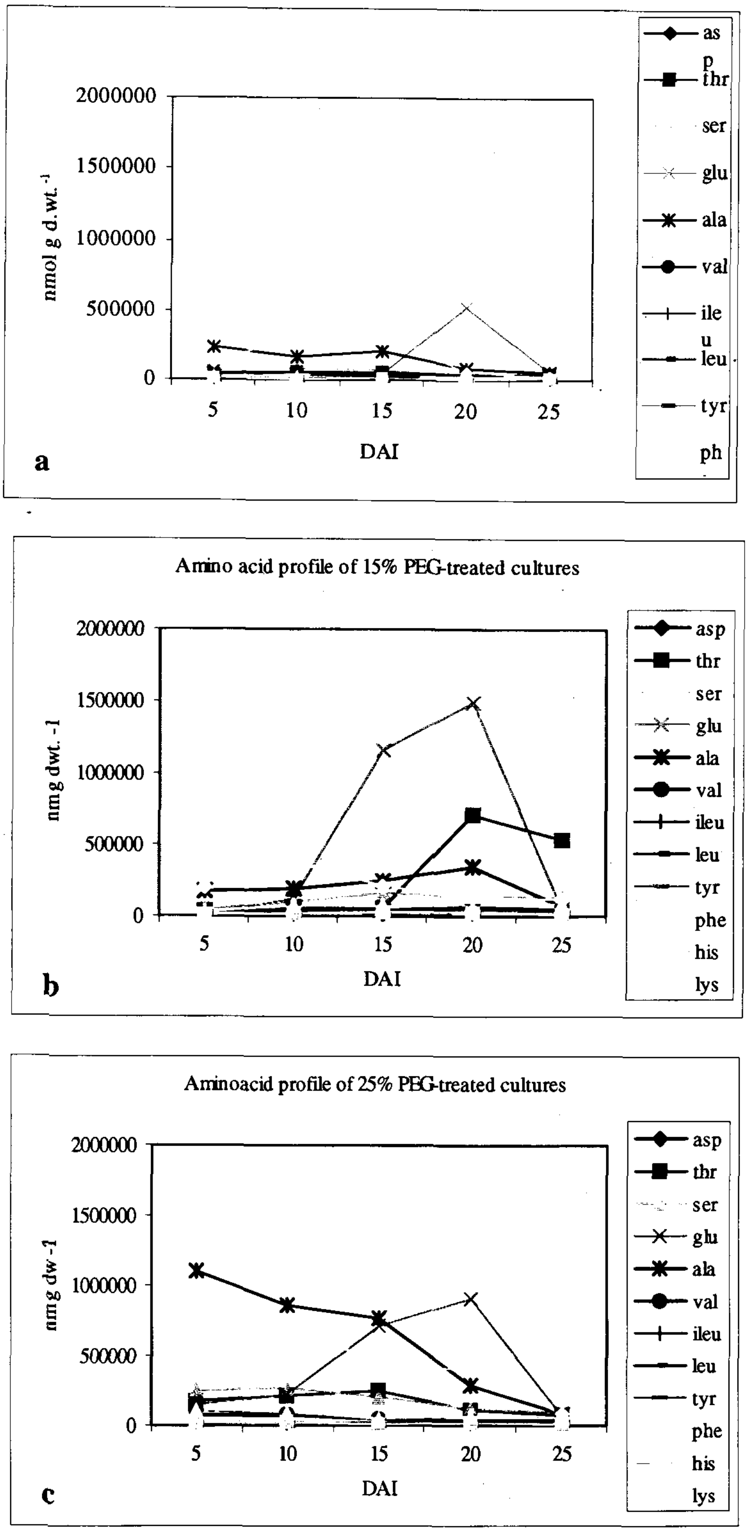


Fig. 1.2: Time-course of amino acid profile in control (**a**), 15% PEG-treated (**b**) and (**c**) 25% PEG-treated black pepper suspension cultures.

1.3.2.1.1.2. Proline

Proline was found to increase under mild water stress conditions. However, during the initial stages of the imposition of mild stress (15%), proline levels were maintained on par with that of the control cultures and the increase was significant only on the 15th day of exposure to stress. In cultures exposed to severe stress, namely, 25% PEG, a significant decrease in proline was noted initially, after which the levels regained but were however maintained lower than the levels seen in control cultures (Table 1.2).

Table 1.2. Time-course accumulation of total free amino acids in black pepper suspension cultures subjected to varying degrees of PEG-induced water stress.

Tmt. No.	**Tmt**	**Proline (nmol g^{-1} d.wt.$^{-1}$)**					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
1	Control (0% PEG)	0.54 ± 0.0403	0.61 ± 0.0387	0.665 ± 0.0162	0.67 ± 0.0462	0.443 ± 0.0158	0.667 ± 0.0113
2	15% PEG		0.625 ± 0.0071	0.659 ± 0.0191	0.973 ± 0.0091	1.085 ± 0.0272	1.273 ± 0.0631
3	25% PEG		0.188 ± 0.0124	0.508 ± 0.0111	0.458 ± 0.0202	0.437 ± 0.0086	0.368 ± 0.0196
4	30% PEG		0.14 ± 0.0092	0.444 ± 0.0106	0.455 ± 0.0164	0.373 ± 0.0059	0.742 ± 0.0212

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan

Tmt	N	Subset for alpha = .05		
		1	2	3
3.00	24	.4220		
4.00	24	.4572		
1.00	24		.6004	
2.00	24			.8751
Sig.		.516	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.1.1.3. Reducing sugars

There was a significant increase in reducing sugars on imposition of water stress, the increase being proportional to the severity of the stress (Table 1.3). However, though severe and lethal doses of PEG i.e., 25 and 30% PEG induced significant increase in reducing sugars, the levels in these cultures decreased progressively and fell below that seen in control cultures by the end of the experiment. On the other hand, though the levels of reducing sugars in cultures exposed to mild stress (15% PEG) were lower than that of 25 and 30% PEG-exposed cultures, the levels were maintained steady till the conclusion of the experiment.

Table 1.3. Time-course accumulation of reducing sugars in black pepper suspension cultures subjected to varying degrees of PEG-induced water stress.

Tmt. No.	**Tmt**	**Reducing sugars (x 10^4 µg glucose eq. g^{-1} d.wt.$^{-1}$)**					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
1	Control (0% PEG)	3.674 ± 0.0232	3.817 ± 0.0428	4.528 ± 0.0539	3.788 ± 0.0481	4.01 ± 0.14	4.467 ± 0.0453
2	15% PEG		5.030 ± 0.0941	5.9174 ± 0.036	5.249 ± 0.0628	6.496 ± 0.0196	6.113 ± 0.0255
3	25% PEG		4.938 ± 0.0764	5.291 ± 0.0688	4.962 ± 0.1022	4.388 ± 0.0570	3.222 ± 0.1380
4	30% PEG		8.8928 ± 0.076	7.659 ± 0.1867	3.248 ± 0.0653	5.656 ± 0.1412	3.077 ± 0.0187

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
1.00	24	4.0554	
3.00	24	4.4026	
4.00	24		5.3608
2.00	24		5.4212
Sig.		.354	.872

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

Harmonic Mean Sample Size = 24.000.

1.3.2.1.2.2. Na^+

The Na^+ content in black pepper cultures increased over the controls in all the water stressed treatments. However, 25 and 30% PEG-treated cultures showed higher values than the control cultures throughout the stress period. The 15% PEG-treated cultures showed an initial marginal increase but remained on par with the control cultures till the last day of exposure to stress (Table 1.5).

Table 1.5. Time-course accumulation of Na^+ in black pepper suspension cultures subjected to varying degrees of PEG-induced water stress.

Tmt. No.	Tmt	Na^+ (mg g^{-1}d.wt..^{1})					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
1	Control (0% PEG)	1.55 ± 0.1746	1.1 ± 0.1365	1.28 ± 0.0619	1.28 ± 0.0303	1.28 ± 0.0234	1.45 ± 0.1558
2	15% PEG		1.525 ± 0.0281	1.9 ± 0.0306	1.43 ± 0.0317	1.28 ± 0.7283	1.43 ± 0.1406
3	25% PEG		1.75 ± 0.0878	2.08 ± 0.1009	2.08 ± 0.1031	2.08 ± 0.1304	1.58 ± 0.0793
4	30% PEG		0.95 ± 0.1387	2.4 ± 0.1872	1.75 ± 0.1065	2.55 ± 0.1922	0.775 ± 0.0547

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Na^+

Duncan

Tmt	N	Subset for alpha = .05		
		1	2	3
1.00	24	1.3546		
2.00	24		1.6133	
4.00	24		1.6344	1.6344
3.00	24			1.8545
Sig.		1.000	.851	.053

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.1.2.3. Mg^{2+}

Black pepper cultures did not show much variation in Mg^{2+} content due to water stress imposition. The water-stressed cultures showed lower values of Mg^{2+} than the control. While 15 and 25% PEG-treated cultures had similar values and were on par with the control during the latter part of the stress period, 30% PEG- exposed cultures showed decreased Mg^{2+} content throughout the time-course experiment (Table 1.6).

Table 1.6. Time-course accumulation of Mg^{2+} in black pepper suspension cultures subjected to varying degrees of PEG-induced water stress.

Tmt. No.	Tmt	Mg^{2+} (mg g^{-1}d.wt..^{1})					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
1	Control (0% PEG)	2.055 ± 0.1503	2.05 ± 0.133	2.245 ± 0.0715	2.135 ± 0.0798	2.3 ± 0.1490	2.85 ± 0.1840
2	15% PEG		1.61 ± 0.0781	2.47 ± 0.1270	1.45 ± 0.0971	1.85 ± 0.1481	2.05 ± 0.1095
3	25% PEG		1.43 ± 0.1321	2.12 ± 0.1119	1.76 ± 0.1112	2.51 ± 0..4178	2.88 ± 0.0954
4	30% PEG		1.45 ± 0.1237	1.95 ± 0.1372	1.36 ± 0.1116	1.54 ± 0.1237	1.8 ± 0.058

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan^{a}

Tmt	N	Subset for alpha = .05		
		1	2	3
4.00	24	1.6535		
2.00	24		1.8619	
3.00	24		2.0630	
1.00	24			2.3105
Sig.		1.000	.052	1.000

Means for groups in homogeneous subsets are displayed.a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.1.2.4. Ca^{2+}

The Ca^{2+} content of all the water stressed treatments decreased compared to the control. The PEG-treated cultures were on par with each other (Table 1.7).

Table 1.7. Time-course accumulation of Ca^{2+} in black pepper suspension cultures subjected to varying degrees of PEG-induced water stress.

Tmt. No.	Tmt	Ca^{2+} (mg g^{-1}d.wt.$^{-1}$)					
		0d	5d	10d	15d	20d	25d
1	Control (0% PEG)	1.75 ± 0.1024	1.1 ± 0.0624	1.45 ± 0.0985	2.275 ± 0.0322	1.55 ± 0.1240	1.775 ± 0.0288
2	15% PEG		0.825 ± 0.0147	1.05 ± 0.0490	0.725 ± 0.0127	0.875 ± 0.0164	1.025 ± 0.0338
3	25% PEG		0.73 ± 0.0212	0.8 ± 0.0460	0.75 ± 0.0173	0.625 ± 0.0158	0.9 ± 0.0306
4	30% PEG		0.7 ± 0.027	1.375 ± 0.027	0.4 ± 0.0192	0.55 ± 0.0248	0.925 ± 0.0392

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
3.00	24	.9313	
4.00	24	.9490	
2.00	24	1.0370	
1.00	24		1.6465
Sig.		.389	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.2. Phenol accumulation

Total phenols increased in water-stressed cultures, with the increase being significant under severe stress conditions (Table 1.8). However the levels declined by the 15th and 10th day respectively in the 25 and 30% PEG-exposed cultures and decreased progressively thereafter. However, high phenolic content was maintained compared to the control, in cultures exposed to mild stress till the end of the stress period.

Table 1.8. Time-course accumulation of phenols in black pepper suspension cultures subjected to varying degrees of PEG-induced water stress.

Tmt. No.	**Tmt**	**Total phenols (x10^4 µg g^{-1} d.wt^{-1}.)**					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
1	Control (0% PEG)	4.012 ± 0.077	4.037 ± 0.1005	2.842 ± 0.0308	4.358 ± 0.0216	3.163 ± 0.0294	4.349 ± 0.1736
2	15% PEG		5.734 ± 0.0444	4.008 ± 0.0574	6.593 ± 0.1079	6.516 ± 0.0581	6.0242 ± 0.027
3	25% PEG		6.574 ± 0.0929	5.3962 ± 0.032	5.157 ± 0.0535	3.646 ± 0.1192	2.822 ± 0.0792
4	30% PEG		4.238 ± 0.0721	5.724 ± 0.0698	2.480 ± 0.0694	1.6959 ± 0.061	2.535 ± 0.0612

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan

Tmt	N	Subset for alpha = .05		
		1	2	3
4.00	24	3.4351		
1.00	24	3.7875		
3.00	24		4.5898	
2.00	24			5.4836
Sig.		.285	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.3. Lipid peroxidation

Lipid peroxidation, measured in terms of MDA content, increased in the water-stressed black pepper cultures over the control. However, while the MDA content increased with severity of stress, it was noted that the levels fell in 30% PEG-treated cultures after the 10th day and was significantly low from the 20th day of exposure to the stress. The cultures exposed to mild stress (15% PEG) registered a significant initial increase over the control cultures. However, though the MDA content was above the control throughout the stress period, the levels were maintained steady till the end of the stress period without any significant increase with time. In addition, the lipid peroxidation was seen to be lower in these cultures compared to the 25% PEG-treated cultures. In the 25% PEG-treated cultures, the MDA content was significantly higher than the control cultures over the entire period of water stress (Table 1.9).

Table 1.9. Time-course of lipid peroxidation in black pepper suspension cultures subjected to varying degrees of PEG-induced water stress.

Tmt. No.	Tmt	MDA content { $\times 10^2$ nm g^{-1} $f.wt^{-1}$ }				
		0d	10d	15d	20d	25d
1	Control (0% PEG)	3.373 ± 0.1791	3.677 ± 0.0831	6.5806 ± 0.069	11.42 ± 0.1083	10.26 ± 0.1577
2	15% PEG		8.323 ± 0.1721	11.81 ± 0.1766	13.742 ± 0.447	14.52 ± 0.1355
3	25% PEG		11.81 ± 0.1587	18.97 ± 0.2160	16.06 ± 0.0798	19.94 ± 0.1412
4	30% PEG		13.16 ± 0.0875	10.84 ± 0.1168	4.0645 ± 0.139	7.936 ± 0.1408

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan

Tmt	N	Subset for alpha = .05		
		1	2	3
1.00	20	7.1202		
4.00	20	7.9267	7.9267	
2.00	20		10.3155	
3.00	20			14.0866
Sig.		.570	.095	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 20.000.

1.3.2.4. Enzymatic antioxidant defenses

The effect of different regimes of PEG-induced water stress on the antioxidant enzymes of black pepper cells was studied. The enzymes included general antioxidant enzymes like superoxide dismutase, guaiacol peroxidase and catalase and the enzymes of the ascorbate-glutathione cycle.

1.3.2.4.1. Superoxide dismutase (SOD)

In general, total SOD activity was enhanced during water stress and increased with increase in the severity of the stress. However, while the activity rose substantially in 25 and 30% PEG-treated cultures during the initial 10 days, the levels declined thereafter. The total SOD activity in all the PEG-treated cultures reached levels below the control after the 15th day of exposure to stress, with cultures in 30% PEG showing maximum decrease. The increase in activity of total SOD in 15% PEG-treated cultures was gradual and did not fluctuate significantly unlike that shown by cultures at 25% and 30 % PEG, and remained at about the same levels throughout the course of the experiment (Fig.1.3).

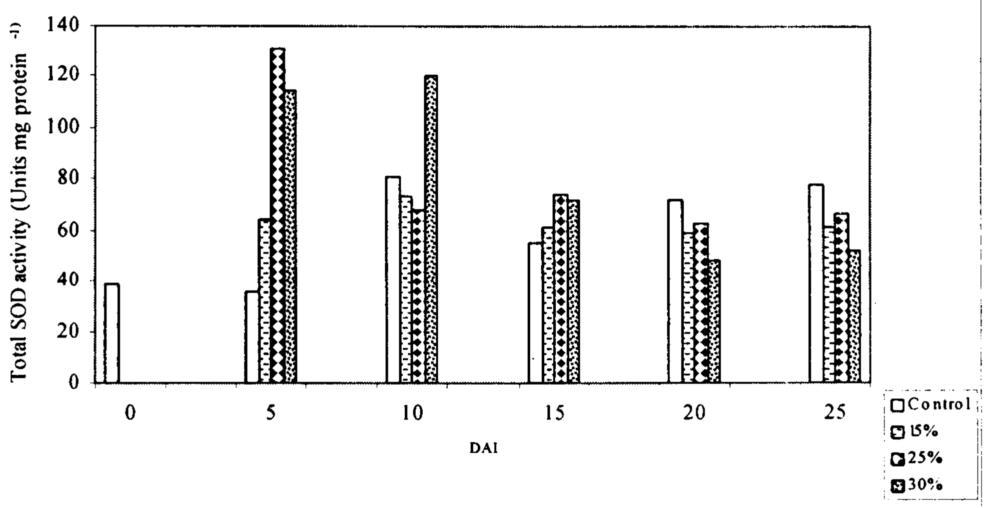


Fig. 1.3. Time-course change in total SOD activity in black pepper suspension cultures exposed to varying intensities of stress. Mean values of 4 replicates were used to plot the graph.

It was interesting to note that MnSOD was the dominant isoform of SOD in cultures exposed to mild stress throughout the experimental period and did not fluctuate significantly over a period of time. However, though cultures at 25% PEG showed an initial steep increase in activity of MnSOD, the levels decreased with progression of time. Similar was the case seen in cultures exposed to 30% PEG.

FeSOD maintained significantly low levels in 15% PEG-treated cultures. However, the cultures at 25 and 30% PEG showed a general increase in the activity of the enzyme in comparison with the control.

CuSOD increased significantly in 30% PEG-exposed cultures. The activity remained higher than the control till the 15th day. However, the activity decreased thereafter with time until the end of the experiment. Higher levels of activity were however compared to cultures exposed to 25 and 15% PEG. In cultures exposed to 15% PEG, the CuSOD activity was similar to that in control cultures during the initial period of the stress. However, the activity decreased by the 10th day, after which it again rose, and fluctuated till the end of the experiment and on the average; the levels of the enzyme were equal to or below that of the control cultures. In 25% PEG-treated cultures, a similar trend was observed. The activity of the isoform fluctuated during the initial phase of the stress period. With an initial decrease and rise in the activity, the levels dropped and were maintained below the control levels till the end of the control period.

It was observed that the most dominant form of SOD in control cultures throughout the experiment was CuSOD. MnSOD and FeSOD were also observed though MnSOD was comparatively higher.

In 15% PEG-treated cultures, the most dominant form was the MnSOD which was maintained high throughout their exposure to the stress. CuSOD was the next dominant form under these mild stress conditions. FeSOD however was significantly low.

In 25% PEG-treated cultures, the general trend was an increase in all the forms of SOD, with MnSOD showing greater increase during the initial phases of the stress, which later declined with concomitant increase in other forms of SOD with the duration of the

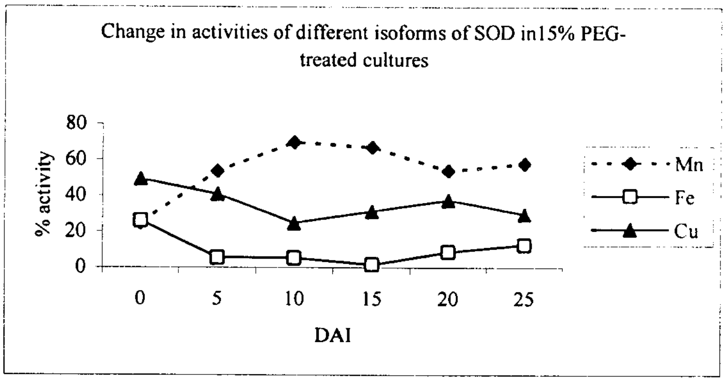


Fig. 1.4b

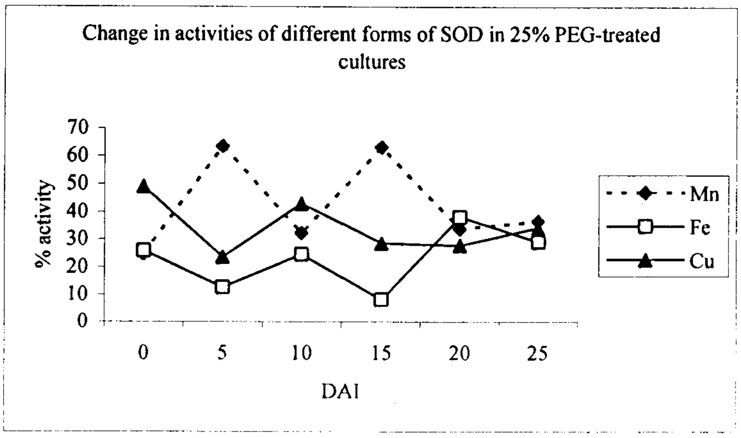


Fig. 1.4c

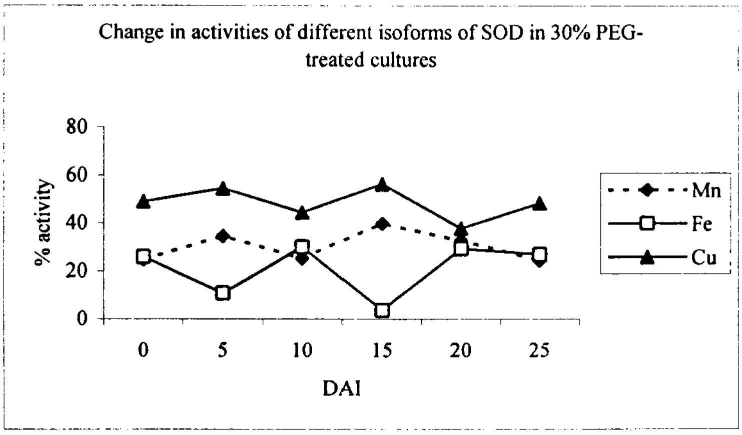
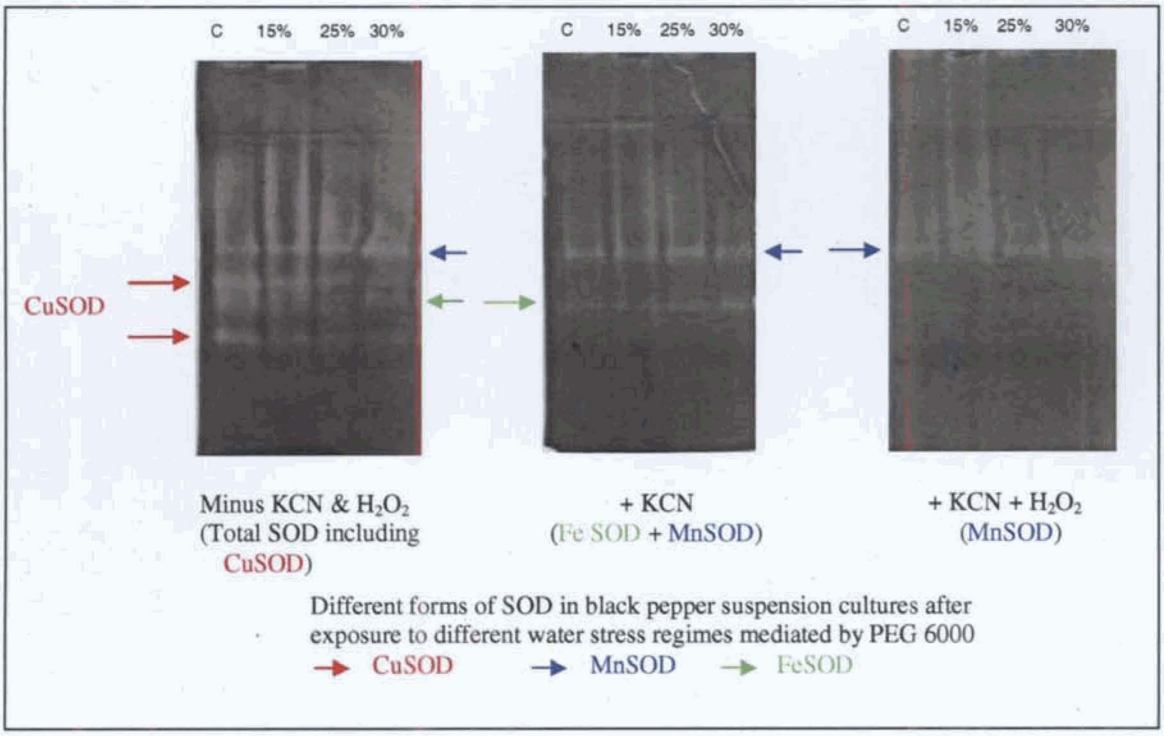


Fig. 1.4d

Fig.1. 4. Figures a,b,c and d show the time-course change in activities of Cu/Zn, Mn and Fe SOD isoforms in control, 15%, 25% and 30%- PEG-treated cultures respectively. Mean values of 4 replicates were used to plot the graphs.

Though there was a modulation in the activities of different isoforms of SOD, polyacrylamide gel electrophoresis showed no differences in the isozymes of these SOD isoforms during water stress (Fig. 1.5).



Minus KCN & H_2O_2 (Total SOD including CuSOD)

+ KCN (Fe SOD + MnSOD)

+ KCN + H_2O_2 (MnSOD)

Different forms of SOD in black pepper suspension cultures after exposure to different water stress regimes mediated by PEG 6000

Fig. 1.5: Native-PAGE showing isoforms of superoxide dismutase in black pepper suspension cultures on exposure to different regimes of PEG-induced water stress

1.3.2.4.2. Catalase (CAT)

Catalase activity was highly inhibited in cultures exposed to severe stress (25 and 30% PEG) and was far below the control levels. However, the cultures exposed to mild stress (15% PEG) showed increase in catalase activity over the control till the 10th day after which the levels fluctuated but remained marginally higher than the control levels (Fig.1.6 ; Table 1.10).

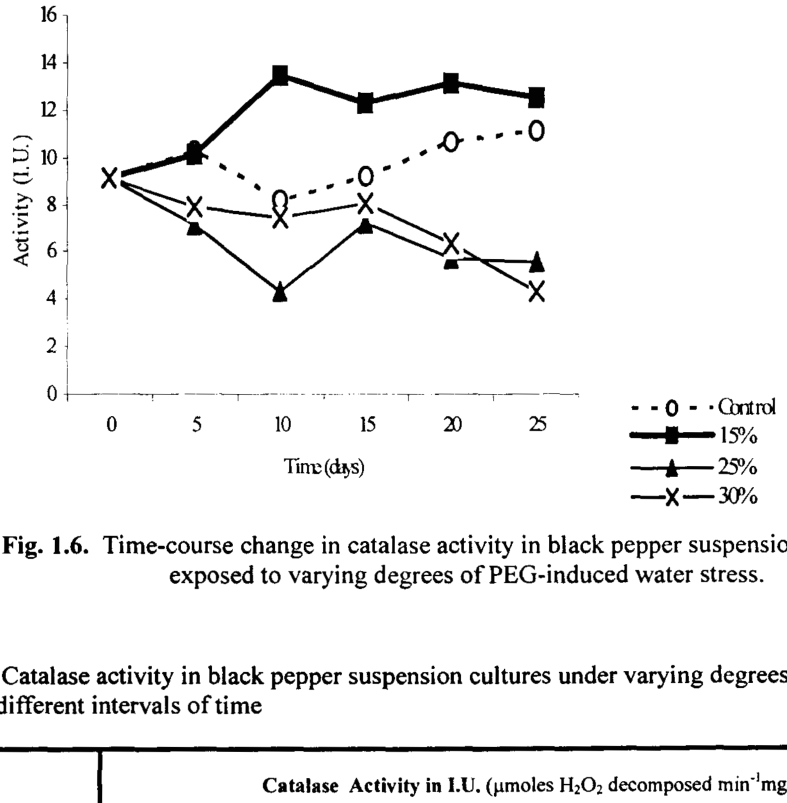


Fig. 1.6. Time-course change in catalase activity in black pepper suspension cultures exposed to varying degrees of PEG-induced water stress.

Table 1.10. Catalase activity in black pepper suspension cultures under varying degrees of water stress at different intervals of time

Tmt. No.	Tmt	Catalase Activity in I.U. (µmoles H_2O_2 decomposed $min^{-1}mg\ protein^{-1}$)					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
1	Control	9.12 ± 1.0826	10.28 ± 1.1825	8.21 ± 0.7718	9.16 ± 1.1328	10.66 ± 1.796	11.1 ± 0.3773
2	15% PEG		10.152 ± .4299	13.42 ± 0.3179	12.3 ± 0.3872	13.17 ± 0.3803	12.5 ± 0.4939
3	25% PEG		7.1 ± 0.5682	4.35 ± 0.6494	7.2 ± 0.5479	5.71 ± 0.5332	5.54 ± 0.6624
4	30% PEG		7.89 ± 0.7754	7.4 ± 0.6923	8.06 ± 0.3378	6.36 ± 0.3721	4.32 ± 0.6736

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan^{A}

Tmt	N	Subset for alpha = .05		
		1	2	3
3.00	24	6.8183		
4.00	24	7.3025		
1.00	24		10.0058	
2.00	24			11.8617
Sig.		.259	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.4.3. Peroxidase (PRX)

Peroxidase activity was higher in water-stressed samples compared to the control. While the activities rose sharply in 25 and 30% PEG-treated cultures by the 5th day after inoculation, the increase in 15% PEG-treated cultures was gradual. The increase in activity in 25 and 30% was short-lived and declined by the 15th day below the control levels. The gradual increase of the PRX activity in 15% PEG-treated cultures peaked on the 15th day whereafter it declined and maintained marginally higher levels above the controls till the last day of sampling (Fig.1.7; Table1.11).

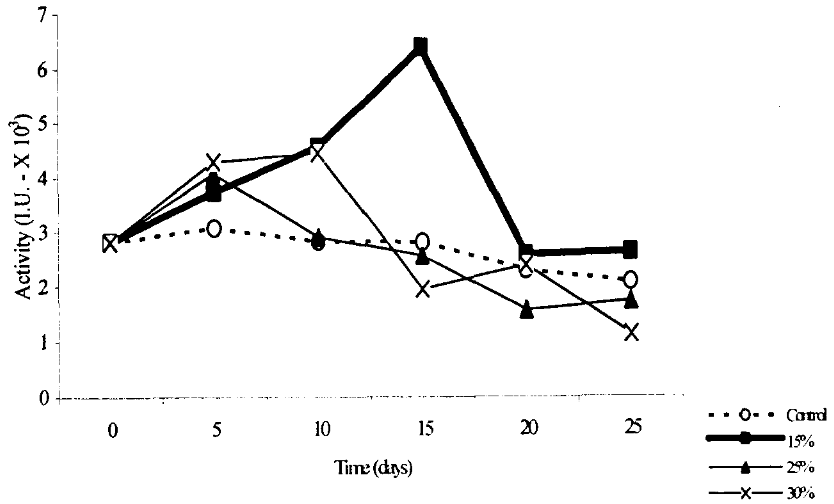


Fig. 1.7. Time-course change in peroxidase activity in black pepper suspension cultures exposed to varying degrees of PEG-induced water stress.

Table 1.11 Peroxidase activity in black pepper suspension cultures under varying degrees of water stress at different intervals of time

Tmt. No.	Tmt	Sp. Activity = x 10^3 I.U. (µmoles purpurogallin formed $min^{-1}mg\ protein^{-1}$)					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
1	**Control**	2.819 ± 0.1501	3.081 ± 0.1008	2.829 ± 0.1605	2.816 ± 0.2332	2.3 ± 0.2004	2.083 ± 0.0715
2	**15% PEG**		3.744 ± 0.3851	4.571 ± 0.3478	6.404 ± 0.2659	2.592 ± 0.3845	2.636 ± 0.2502
3	**25% PEG**		4.085 ± 0.3019	2.915 ± 0.1523	2.569 ± 0.1698	1.573 ± 0.1027	1.741 ± 0.1976
4	**30% PEG**		4.303 ± 0.2599	4.46 ± 0.2861	1.956 ± 0.1935	2.39 ± 0.1737	1.138 ± 0.2914

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
3.00	24	2.6657	
1.00	24	2.7258	
4.00	24	2.9048	
2.00	24		3.7345
Sig.		.479	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size

1.3.2.4.4. Enzymes of the AGC-cycle

1.3.2.4.4.1. Ascorbate peroxidase (AP)

Cytosolic ascorbate peroxidase activity was maintained significantly high than the controls till the 5th day in all the cultures exposed to PEG-induced water stress. The activity increased sharply in cultures at 15% PEG from the very first sampling on the 5th day, compared to the other water stressed samples. AP in 30% PEG-treated cultures (lethal water stress condition), activity declined sharply after the 5th day and was below the control levels after the 10th day of inoculation. The cultures exposed to 15 and 25% PEG remained significantly high over the control till the conclusion of the experiment.

Among these two treatments, however, the cultures exposed to 15% PEG showed higher activity than the cultures at 25% PEG during most of the experimental period (Fig.1.8; Table 1.12).

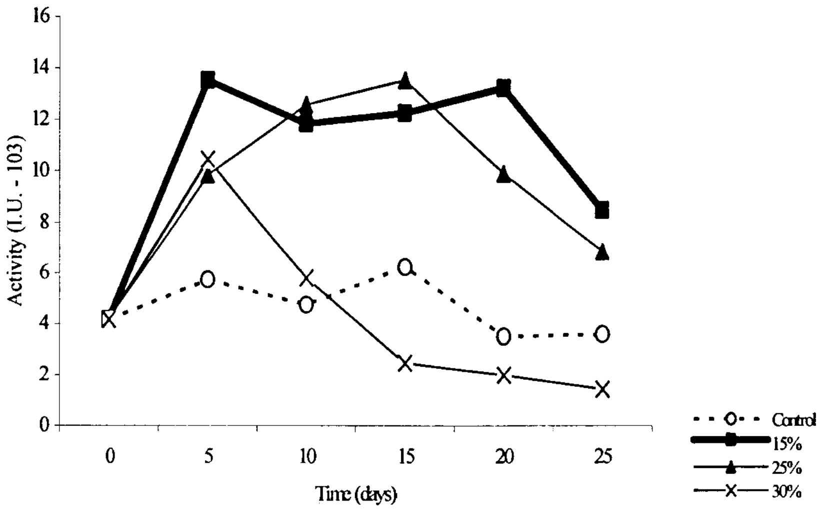


Fig.1. 8. Time-course change in activity of cytosolic AP in black pepper suspension cultures exposed to varying degrees of PEG-induced water stress.

Table 1.12. Cytosolic AP activity in black pepper suspension cultures under varying degrees of water stress at different intervals of time

Tmt. No.	Tmt	Sp. Activity= x 10^3 I.U. (µmoles AsA oxidized min^{-1}mg $protein^{-1}$)					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
	Control	4.163 ± 0.0483	5.727 ± 0.1824	4.754 ± 0.0747	6.244 ± 0.0886	3.51 ± 0.2373	3.595 ± 0.2159
	15% PEG		13.522 ± 0..2235	11.848 ± 0.2594	12.267 ± 0.3436	13.223 ± 0.1981	8.497 ± 0.2359
	25% PEG		9.837 ± 0.2795	12.577 ± 0.2950	13.535 ± 0.1294	9.901 ± 0.159	6.85 ± 0.2281
	30% PEG		10.448 ± 0.2754	.5.803 ± 0.2149	2.466 ± 0.2642	1.992 ± 0.1367	1.441 ± 0.134

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
4.00	24	4.3487	
1.00	24	4.6609	
3.00	24		9.4161
2.00	24		10.6226
Sig.		.708	.150

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

The activity of the chloroplastic AP was maintained high in the water-stressed cultures than in the control cultures throughout the stress period. The activity in cultures exposed to severe (25% PEG) and lethal (30% PEG) stress increased sharply over the control and 15% PEG-exposed cultures till the 10th day after inoculation, whereafter, the levels declined gradually. However, in cultures exposed to mild water stress (15% PEG), the activity increased gradually and was maintained at steady levels till the end of the experiment (Fig. 1.9; Table 1.13). Ascorbate peroxidase activity was lower in the chloroplastic fractions of the cultures than in the cytosol.

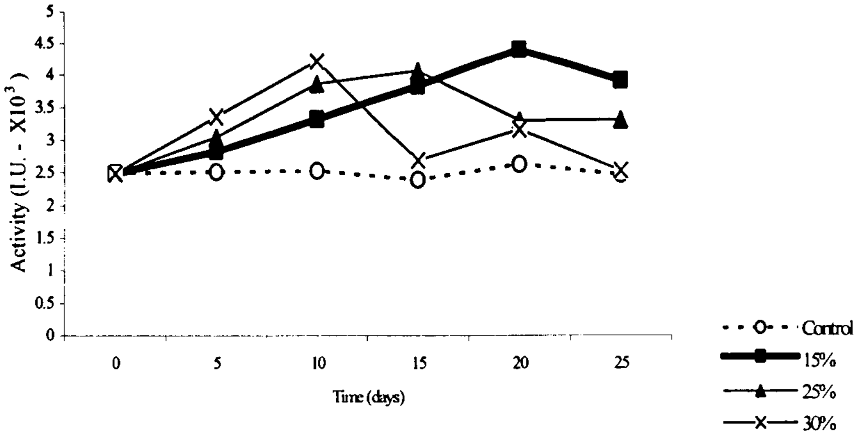


Fig. 1.9. Time-course change in the activity of chloroplastic AP in black pepper suspension cultures exposed to varying degrees of PEG-induced water stress.

Table 1.13. Chloroplastic AP activity in black pepper suspension cultures under varying degrees of water stress at different intervals of time

Tmt. No.	Tmt	Sp. Activity= x 10^3 I.U. μmoles AsA oxidized $min^{-1}mg\ protein^{-1}$)					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
1	Control	2.491 ± 0.1437	2.514 ± 0.0326	2.531 ± 0.0603	2.397 ± 0.0346	2.638 ± 0.1104	2.477 ± 0.0776
2	15% PEG		2.829 ± 0.1063	3.329 ± 0.1329	3.839 ± 0.1688	4.412 ± 0.0407	3.93 ± 0.0143
3	25% PEG		3.052 ± 0.0344	3.871 ± 0.0982	4.067 ± 0.0462	3.316 ± 0.0875	3.324 ± 0.0464
4	30% PEG		3.363 ± 0.0533	4.217 ± 0.0393	2.694 ± 0.0966	3.176 ± 0.2808	2.539 ± 0.0584

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05		
		1	2	3
1.00	24	2.5327		
4.00	24		3.0836	
3.00	24		3.3614	3.3614
2.00	24			3.4602
Sig.		1.000	.078	.528

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.4.4.2. Dehydroascorbate reductase

Dehydroascorbate reductase activity increased gradually in PEG treated cultures over the control. While there was an initial increase in activity in 30% PEG-treated cultures over all the other treatments, the levels fell to the control levels immediately and again rose to maintain marginally higher levels than the control. The increase in DR activity in cultures exposed to severe water stress conditions (25% PEG) peaked at 20th day after inoculation after which the levels declined but was maintained higher than the cultures exposed to mild water stress (15% PEG) and the control cultures. The cultures exposed to mild stress however showed gradual increase in enzymes and were maintained at higher levels above the control till the end of the experiment (Fig. 1.10; Table 1.14).

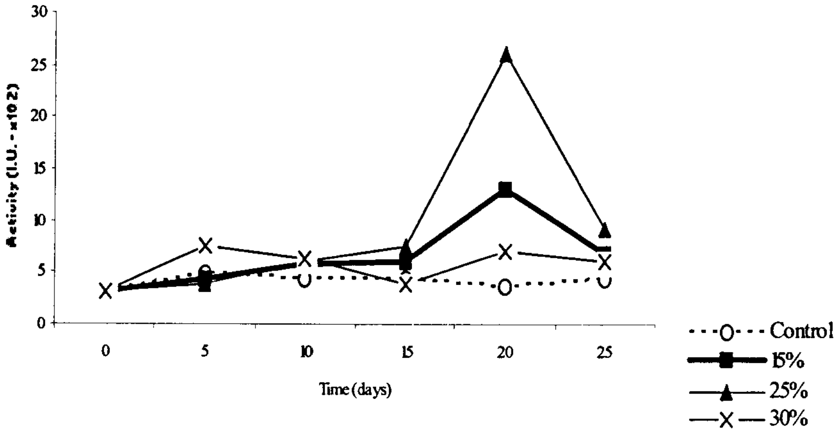


Fig. 1.10. Time-course change in the activity of cytosolic DR in black pepper suspension cultures exposed to varying degrees of PEG-induced water stress.

Table 1.14. Cytosolic DR activity in black pepper suspension cultures under varying degrees of water stress at different intervals of time

Tmt. No.	Tmt	Sp. Activity= I.U. (x 10^2 µmoles AsA min^{-1}mg $protein^{-1}$)					
		0d	5d	10d	15d	20d	25d
	Control	3.14 ± 0.3218	4.86 ± 0.2424	4.3 ± 0.1927	4.3 ± 0.2294	3.55 ± 0.2364	4.43 ± 0.1832
	15% PEG		4.33 ± 0.2268	5.75 ± 0.2028	5.96 ± 0.3411	13.07 ± 0.2115	6.85 ± 0.5075
	25% PEG		3.99 ± 0.2593	6.09 ± 0.1923	7.59 ± 0.2098	26.06 ± 1.489	9.26 ± 0.2365
	30% PEG		7.54 ± 0.1803	6.2 ± 0.1922	3.8 ±0.1828	6.95 ± 0.3066	5.95 ± 0.2076

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan

Tmt	N	Subset for alpha = .05	
		1	2
1.00	24	4.1597	
4.00	24	5.6362	
2.00	24	6.5391	
3.00	24		9.4735
Sig.		.081	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

Chloroplastic DR activity in cultures exposed to 30% PEG was maintained higher than other treatments till the 20th day after inoculation. However, cultures exposed to 15% PEG showed gradual increase in activity and sustained higher levels over all the other treatments by the end of the experiment on the 25th day after inoculation. 25% PEG-exposed cultures showed higher activity after 5th day of inoculation but declined below the control levels at 25th day after inoculation (Fig. 1.11; Table 1.15).

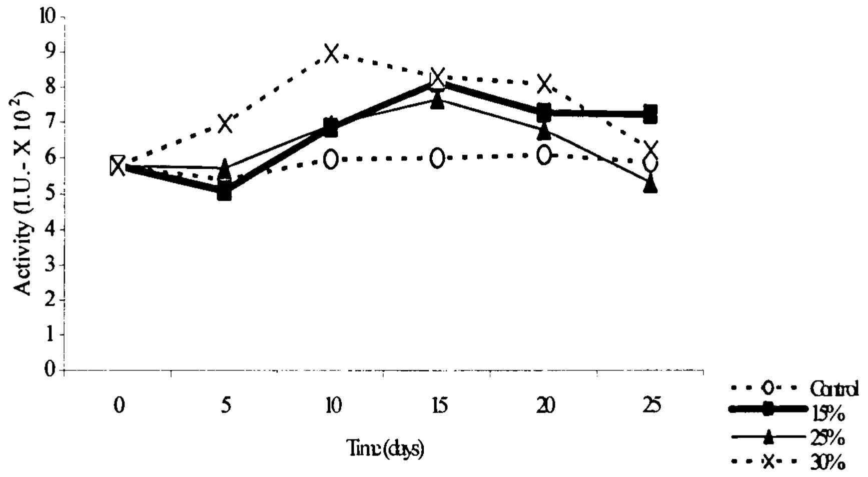


Fig. 1.11. Time-course change in the activity of chloroplastic DR in black pepper suspension cultures exposed to varying degrees of PEG-induced water stress.

Table 1.15. Chloroplastic DR activity in black pepper suspension cultures under varying degrees of water stress at different intervals of time

Tmt. No.	**Tmt**	**Sp. Activity = x 10^2 I.U. (µmoles AsA min^{-1}mg $protein^{-1}$)**					
		0d	**5d**	**10d**	**15d**	**20d**	**25d**
	Control	5.78 ± 0.0455	5.4 ± 0.0643	5.97 ± 0.1216	6 ± 0.2567	6.09 ± 0.023	5.89 ± 0.1023
	15% PEG		5.1 ± 0.1262	6.85 ± 0.0899	8.15 ± 0.1085	7.28 ± 0.2839	7.23 ± 0.0031
	25% PEG		5.72 ± 0.1246	6.93 ± 0.0325	7.68 ± 0.0309	6.79 ± 0.1321	5.33 ± 0.0141
	30% PEG		6.96 ± 0.1187	8.98 ± 0.1202	8.3 ± 0.0542	8.12 ± 0.0109	6.22 ± 0.1499

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets
Duncan^a

Tmt	N	Subset for alpha = .05		
		1	2	3
1.00	24	5.8319		
3.00	24		6.3857	
2.00	24		6.7971	
4.00	24			7.4027
Sig.		1.000	.117	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.4.4.3. Glutathione reductase

The GR activity increased sharply in cultures exposed to lethal water stress conditions (30% PEG) on the 5th day after inoculation, but decreased below control levels by the 10th day. However, the cultures exposed to 15% PEG showed gradual increase in activity and maintained higher levels than all the treatments till the end of the experiment.

While the AP and DR activities in the cytosolic fraction of all the stressed cultures remained high over the controls for the majority of the period of experimentation, the GR activity showed a sharp decline to levels below that of the control by the 10th day after inoculation in 30 and 25% PEG-treated cultures (Fig.1.12; Table 1.16).

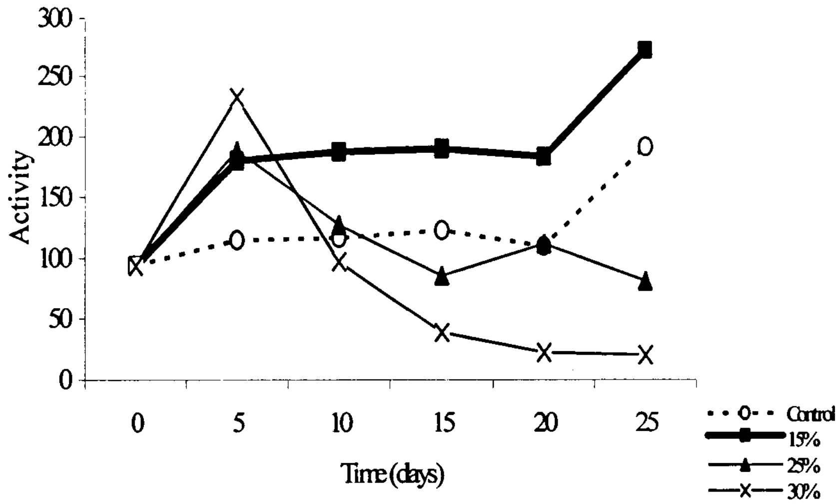


Fig. 1.12. Time-course change in the activity of cytosolic GR in black pepper suspension cultures exposed to varying degrees of PEG-induced water stress.

Table 1.16. Chloroplastic DR activity in black pepper suspension cultures under varying degrees of water stress at different intervals of time

Tmt. No.	**Tmt**	**Sp. activity = I.U. (μmoles NADPH oxidized min^{-1} mg $protein^{-1}$)**					
		0 DAI	**5 DAI**	**10 DAI**	**15 DAI**	**20 DAI**	**25d**
1	Control	93.54 ± 0.1098	115.17 ± 1.029	116.7 ± 0.4546	123.13 ± 0.569	110.1 ± 0.3164	192.2 ± 0.2858
2	15% PEG		180.3 ± 0.3512	187.89 ± 0.27	190.6 ± 0.237	184.11 ± 0.486	272.8 ± 1.6077
3	25% PEG		189 ± 0.2974	127.76 ± 0.256	85.9 ± 0.335	112.32 ± 0.227	81.3 ± 0.1454
4	30% PEG		233 ± 0.2496	97.4 ± 0.229	38.57 ± 0.1773	22.1 ± 0.1888	19.8 ± 0.0869

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan

Tmt	N	Subset for alpha = .05		
		1	2	3
4	24	8.3174		
3	24		11.5381	
1	24		12.7636	
2	24			18.5650
Sig.		1.000	.414	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

1.3.2.5. Water stress-induced proteins

Water stress induced accumulation of new proteins in black pepper suspension cultures that were absent in control cultures (Fig.1.13). SDS-PAGE of crude extracts of protein from callus cultures of different treatments revealed induction of three major polypeptides in all the PEG-treated cultures viz., 15, 25 and 30% PEG. These polypeptides had molecular weights, 39.94, 26.27 and 17.71 kD. It was interesting to note that these polypeptides were also induced by ABA treatment. Cultures pre-treated with ABA and then subjected to 15, 25 and 30% PEG also revealed the induction of these polypeptides. Some minor proteins were also induced in PEG-treated cultures that were not present in ABA-treated cultures. These included polypeptides with molecular weights 9.8 and 6.45 kD. It was further noted that two major proteins present in the control cultures were absent in the PEG- and ABA- treated cultures. These had molecular weights 32.8 and 16.45 kD.

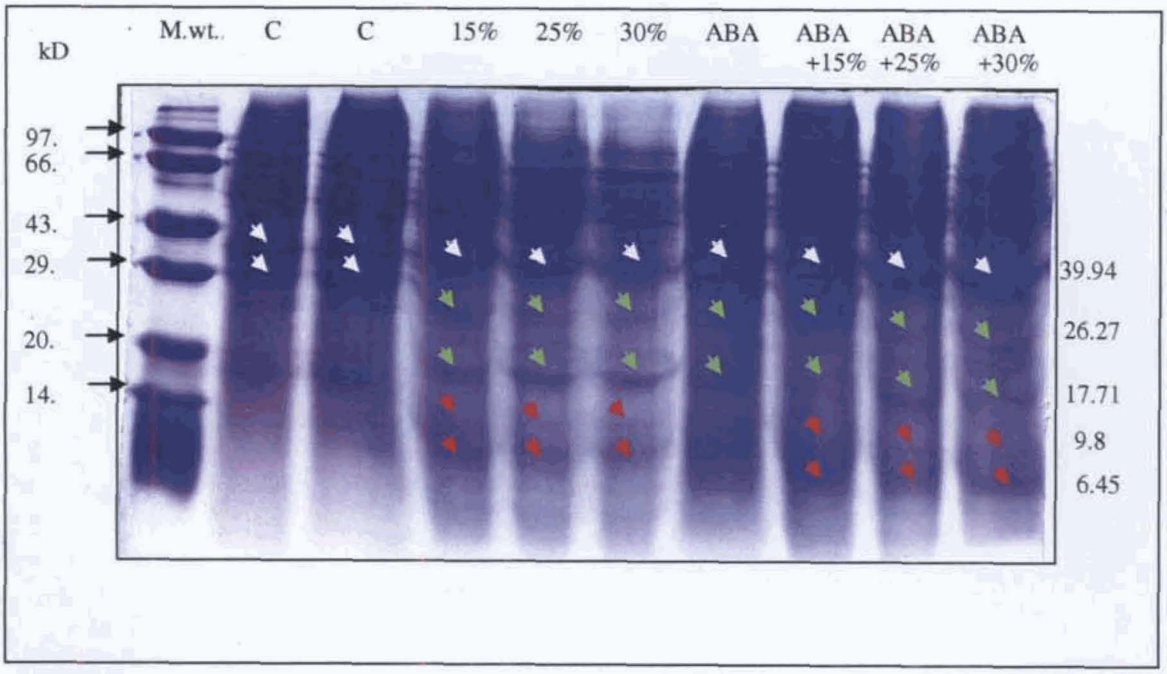


Fig. 1.13 SDS-PAGE of crude protein extracts of black pepper suspension cultures after exposure to PEG-induced water stress before and after treatment with ABA

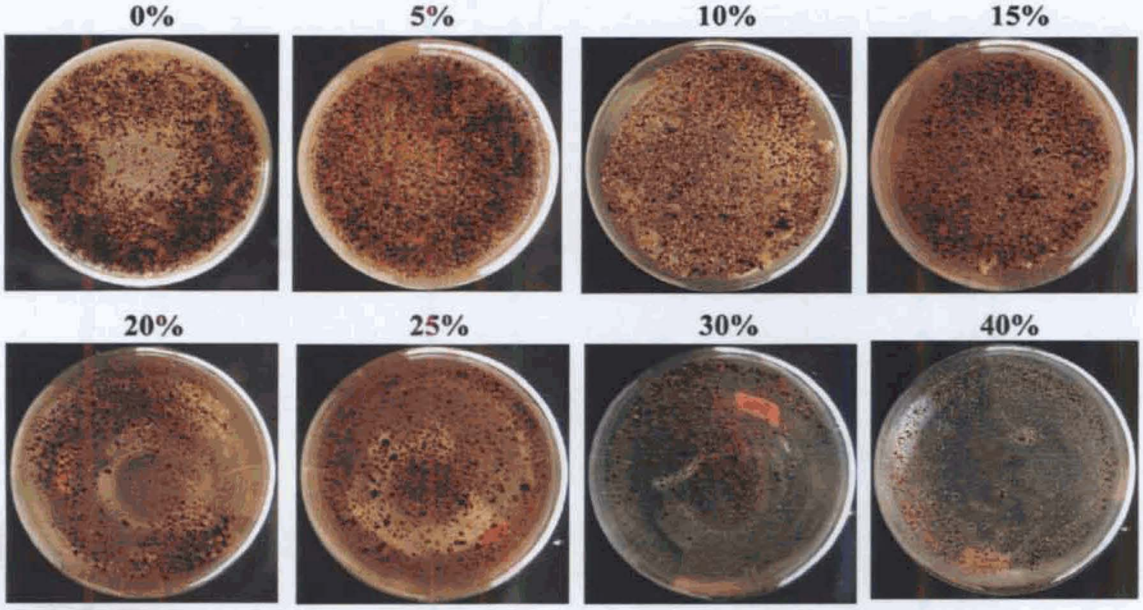


Fig.1.14. Effect of different concentrations of PEG 6000 on the growth of black pepper suspension cultures (after 25d of inoculation).

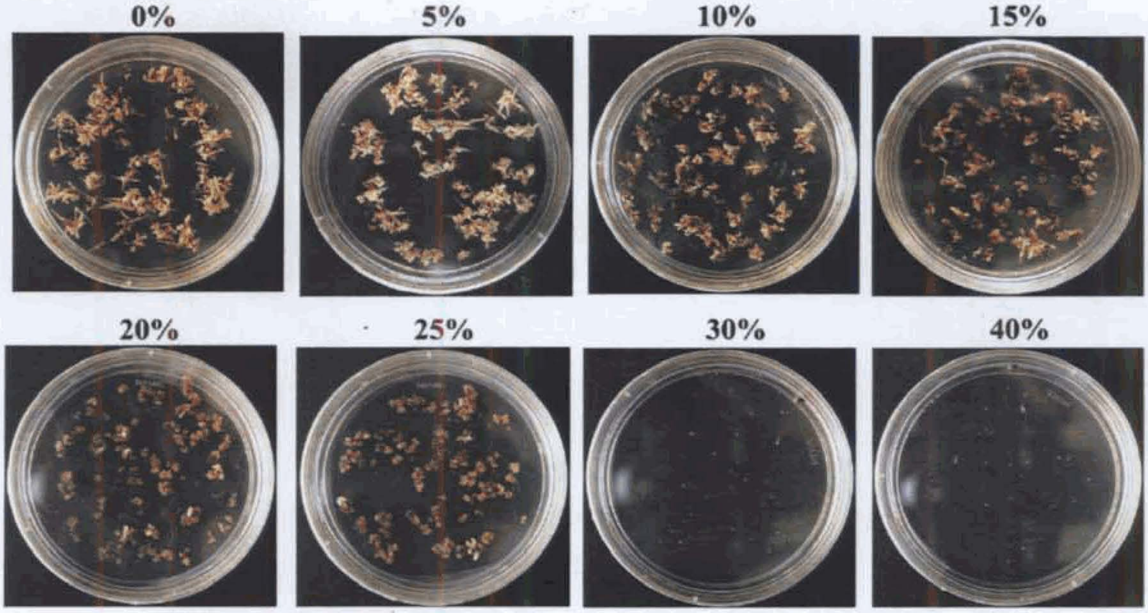


Fig. 1.15. Differentiation of black pepper suspension cultures on control medium after their exposure to different concentrations of PEG for 25 days.

1.3.2.6. *In vitro* selection studies

1.3.2.6.1. Growth characteristics

1.3.2.6.1.1. Effect of PEG on growth and regeneration of black pepper cells

Growth of black pepper cells was observed to decrease with increase in the intensity of the imposed water stress (Fig. 1.14; Fig.1.1 under section 4.1.1.1.). The cells exposed to 5% PEG exhibited growth on par with the control treatment, while it decreased progressively in cells exposed to increasing concentrations of PEG. The LD_{50} value of PEG that brought about 50% reduction in growth of black pepper cells was determined to be 15%. PEG at concentrations 20 and 25% significantly suppressed the growth. Cells subjected to 30 and 40% showed negative fresh weight readings, suggesting cell death.

Similar trends were observed for the regeneration ability of these PEG cultures when they were transferred to the control medium. On transfer back to the control medium after the water stress treatment for 25 days, the cultures exposed to 5% PEG differentiated into embryos on par with the control treatment (Fig. 1.15; Table 1.17). The number of embryos however decreased progressively in cultures that were exposed to increasing concentrations of PEG. The differentiation of embryos recorded a significant decrease in cultures exposed to 20 and 25% PEG, both the treatments being on par with each other. Cultures subjected to 30 and 40% PEG however did not show any signs of regeneration.

Table 1.17: Regeneration of black pepper cells on control medium after their exposure to different PEG regimes for 25d

Treatment	Average no. of embryos	%regeneration compared to control
Control	198.33 ± 8.5049	100.0 ± 0.0000
5%	180 ± 9.5393	90.73 ± 1.0693
10%	153.3 ± 11.5902	77.21 ± 2.6533
15%	105.87 ± 12.0139	53.17 ± 3.7669
20%	38.0 ± 2.6457	19.17 ± 1.4356
25%	34.3 ± 4.1633	17.27 ± 1.3687
30%	9.67 ± 1.5275	4.9 ± 0.9737
40%	0.0 ± 0.0000	0.0 ± 0.0000

The values shown are the average of 3 replicates ± standard deviation

On prolonged exposure to water stress, the rate of differentiation of cells in PEG-amended medium also recorded a similar trend. After 50d of incubation of black pepper cells in different PEG-amended media, the differentiation of cells decreased with increasing concentrations of PEG. However, PEG at 5% showed greater number of embryo-forming cell clumps which was higher than observed in the control (Fig.1.16; Table 1.18).

Table 1.18: Regeneration of black pepper cells in PEG-amended media on prolonged exposure

Treatment	Average no. of embryo forming clumps	Average % regeneration compared to control
Control	181.7 ± 7.6376	100.0 ± 0.0000
5%	217.0 ± 7.2111	119.5 ± 6.0144
10%	118.3 ± 7.5211	65.1 ± 2.0421
15%	75.0 ± 6.2450	41.2 ± 1.6929
20%	23.6 ± 5.5076	12.9 ± 2.4379
25%	13.3 ± 3.0550	7.4 ± 1.8339
30%	4.67 ± 2.0817	2.6 ± 1.2288
40%	0.0 ± 0.0000	0 ± 0.0000

The values shown are the average of 3 replicates ± standard deviation

From these results, it was concluded that 15% PEG imposed a mild water stress, while 25 and 30% PEG were severe and lethal stress levels (Fig.1.17).

It was interesting to note that the dry weight of cells subjected to prolonged water stress were maintained higher compared to the control, in spite of the fact that the fresh weight did not record a significant increase (Table 1.19).

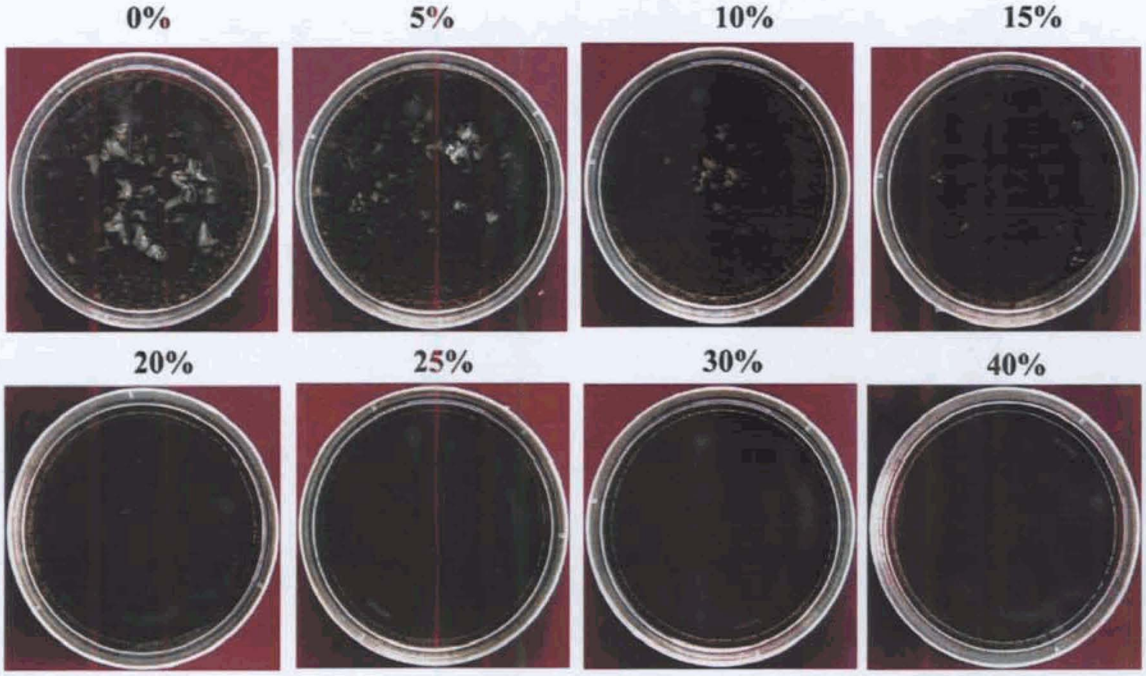


Fig. 1.16. Differentiation of black pepper suspension cultures in PEG-amended medium on prolonged exposure to the imposed water stress (50 d).

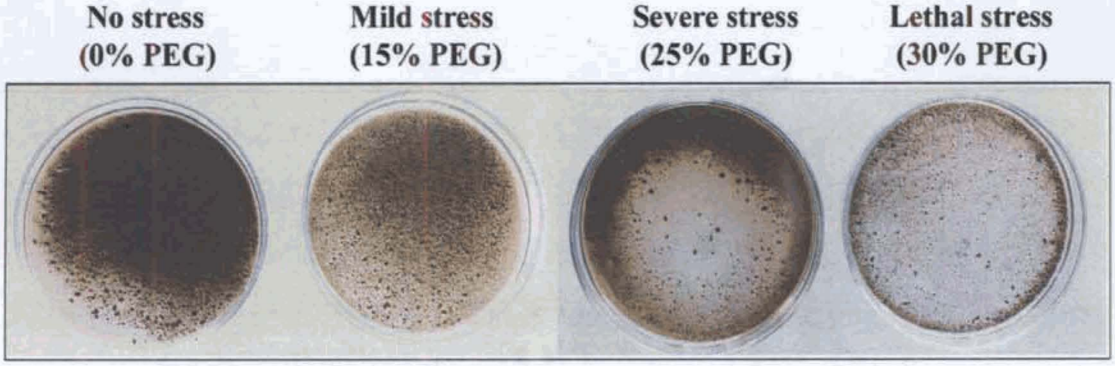


Fig. 1.17. Growth of black pepper suspension cultures under mild, severe and lethal water stress conditions, mediated by 15, 25 and 30% PEG respectively (at 25 days after inoculation).

Table 1.19: Time-course of change in fresh weight, dry weight and dry weight percentage of black pepper suspension cultures exposed to different regimes of PEG-induced water stress

Treatment	5d			10d			15d			20d			25d		
	Fwt.	D.wt.	D.wt. %	Fwt.	D.wt.	D.wt. %	Fwt.	D.wt.	D.wt. %	Fwt.	D.wt.	D.wt. %	Fwt.	D.wt.	D.wt. %
Control	1.9975 ± 0.133	0.215 ± 0.019	10.777 ± 1.01	2.5225 ± 0.077	0.273 ± 0.014	10.82 ± 0.439	2.16 ± 0.179	0.241 ± 0.026	11.148 ± 1.567	1.545 ± 0.120	0.2543 ± 0.120	16.45 ± 2.99	1.25 ± 0.086	0.204 ± 0.015	16.28 ± 1.5
15% PEG	1.4925 ± 0.869	0.1223 ± 0.032	8.1975 ± 1.15	1.4375 ± 0.08	0.3522 ± 0.031	24.503 ± 1.59	1.91 ± 0.184	0.383 ± 0.007	20.198 ± 2.27	1.505 ± 0.215	0.266 ± 0.215	17.641 ± 1.45	1.143 ± 0.127	0.204 ± 0.035	17.865 ± 2.41
25% PEG	1.31 ± 0.049	0.1556 ± 0.031	11.88 ± 1.371	1.4925 ± 0.084	0.2853 ± 0.053	19.02 ± 2.78	1.36 ± 0.081	0.287 ± 0.041	21.118 ± 1.81	1.513 ± 0.132	0.243 ± 0.132	16.03 ± 1.63	1.018 ± 0.142	0.144 ± 0.01	14.105 ± 2.18
30% PEG	1.25 ± 0.084	0.1435 ± 0.017	11.483 ± 0.846	2.3075 ± 0.092	0.5063 ± 0.024	21.94 ± 0.924	1.35 ± 0.088	0.248 ± 0.0	18.33 ± 2.16	1.265 ± 0.068	0.158 ± 0.068	12.474 ± 2.67	0.86 ± 0.074	0.093 ± 0.019	10.795 ± 1.973

The values shown are the average of 3 replicates with standard deviation

1.3.2.6.1.2. Regeneration efficiency of cells selected at different PEG regimes

I. Cells selected at different PEG concentrations were tested for their regeneration ability on control medium, after a period of 3 months of exposure to water stress. It was observed that 5% PEG exposed cultures showed maximum regeneration comparable to that of control cultures. The regeneration ability however decreased with the increase in PEG concentration used for selection of cells. While the cells exposed to 10% PEG regenerated into only 40% of embryos compared to the control, the regeneration percentage decreased further in cells exposed to 20 and 25% PEG (Table 1.20).

Table 1.20: Percent regeneration of black pepper callus selected at various PEG regimes, on control medium

Treatment	No. of embryos	% increase in the no. of embryos compared to control
Control (un-adapted)	163.0 ± 6.2449	100.0 ± 0.0000
5% PEG adapted	142.7 ± 10.2143	87.51 ± 5.2095
10% PEG adapted	60.0 ± 2.6457	36.84 ± 2.2185
20% PEG adapted	38.0 ± 3.0000	23.32 ± 1.8560
25% PEG adapted	20.7 ± 2.0816	12.69 ± 1.3836

The values shown are the average of 3 replicates ± standard deviation

II. Cells selected at different PEG regimes for 3 months were also subjected to higher concentrations of PEG for another period of 45 days before their transfer back to the control medium. In general, it was observed that the regeneration ability decreased with increase in duration and intensity of stress. The 5% PEG-selected cells when returned to control medium after the 3 months stress period, grew luxuriantly on the non-selective medium (Fig.1.18a: A1). However, when the 5% PEG-selected cells were passed to higher concentrations of PEG following their growth in 5% PEG, namely, 10, 15 and 25 % and then returned to control medium, the regeneration ability significantly reduced progressively with increasing concentrations of PEG. (Fig.1.18a: A2, A3 & A4 resp.). When the 5% PEG-selected cells were passed successively to higher concentrations, namely 15 and 20% with a subculture interval of 30 days each, the growth and regeneration ability of the callus was highly inhibited (Fig. 1.18a: A5).

Fig.1.18a

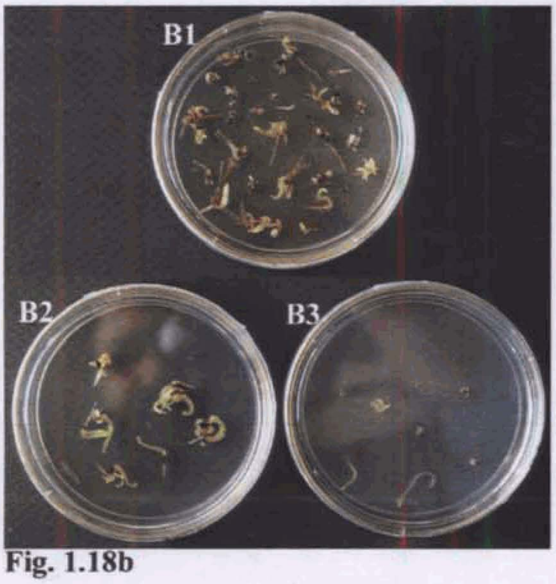


Fig. 1.18b

Fig. 1.18c

Fig. 1.18d

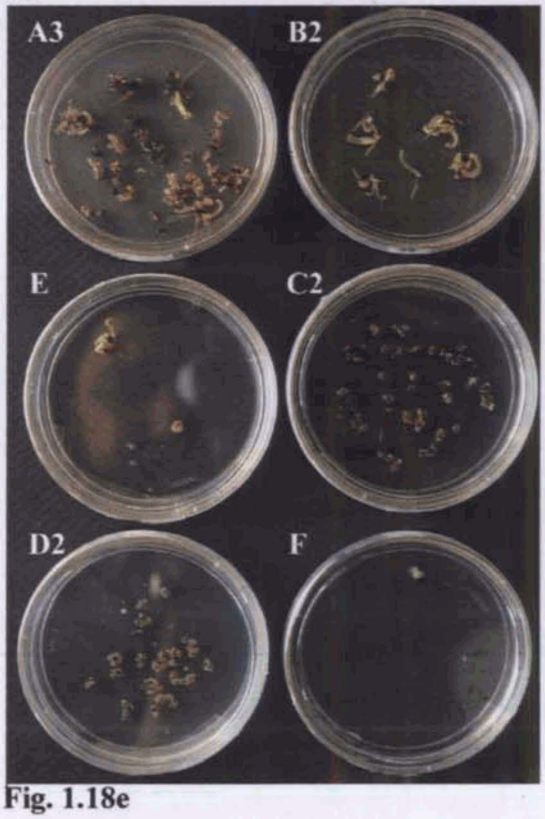


Fig. 1.18e

Fig. 1.18. Regeneration ability of black pepper suspension cultures selected at different PEG regimes for 3 months, on control medium after their further exposure to increasing intensity and duration of PEG-mediated water stress. Fig. 1.18a: Growth and regeneration ability of black pepper callus selected at 5% PEG for 3 months on return to control medium after exposure to varying intensities of stress. A1- 5% selected callus returned to control medium without further exposure to water stress; A2, A3 & A4- 5% selected callus exposed to a further stress period of 45d at 10, 15 and 25% PEG respectively, before its return to control medium; A5- 5% selected callus passed successively to higher conentrations of PEG, namely 15 and 20% PEG, each for a period of 30 days before its return to control medium. Fig. 1.18b: Growth and regeneration ability of 10% PEG-selected callus on control medium after its direct return to control medium (B1) or after further exposure to 15 or 20% PEG (B2 & B3 resp.). Fig. 1.18c: Behaviour of 20% PEG-selected callus after its immediate return to control medium (C1) or after a further exposure to 15% PEG (C2). Fig. 1.18d: Behaviour of 25% PEG-selected callus after its immediate return to control medium (D1) or after a further exposure to 15% PEG (D2). Fig. 18e shows the growth and regeneration of black pepper callus selected at different PEG regimes on control medium after their further exposure to 15% PEG. A3, B2, E, C2, D2 & F are 5, 10, 15, 20, 25 and 30% PEG-selected calli respectively on control medium after their further exposure to 15% PEG for a period of 45d.

The 10% PEG-selected cells when gradually exposed to increasing concentrations of 15 and 20% PEG before returning them to control medium, also resulted in significant reduction in the growth and differentiation of the cells. (Fig1.18b: B1, 2 & 3). 20 and 25% selected cells however failed to regenerate when they were further exposed to a milder stress, namely, 15% PEG, for a period of 45 days before their return to the control medium (Fig.1.18c: C1&2 ; Fig1.18d: D1&2).

Exposure of cells selected at different PEG regimes to milder water stress for a further period of 45 days before their transfer to control medium, resulted in different degrees of regenerability (Fig.1.18e: A3, B2, E, C2, D2 & F). It was observed that cells selected at 5% PEG showed better growth and differentiation than that selected at higher concentrations of PEG. While the callus selected at 5, 10 and 15% PEG differentiated into embryos, that selected at higher concentrations of PEG, namely, 20, 25 and 30% failed to differentiate into embryos when they were further exposed to 15% PEG, though the callus multiplied and showed growth. Cells subjected to 30% PEG did not show any visible growth, except for a chance regeneration of a small callus clump, which later did not grow.

1.3.2.6.2. Morphological characters

It was observed that in comparison with the control cultures, the regeneration of PEG-exposed cultures decreased (Fig. 1.19). Among the PEG-exposed cultures, the regenerability of black pepper calli exposed to milder water stress mediated by 15% PEG was higher than that of calli exposed to higher levels of stress, with the regenerating potential decreasing with increasing concentrations of PEG. Generally, the morphology of the calli showed a blackened appearance on exposure to stress (Fig. 1.19: B, C, D). However, while the blackened callus exposed to 15% PEG showed good number of embryogenic offshoots, the browned callus of 25% PEG-exposed cultures showed only limited number of embryo forming units (Fig. 1.19: C). On the other hand, the calli exposed to 30% PEG showed very little or no regeneration (Fig.1.19: D). Furthermore, black pepper cells exposed to PEG for a longer duration of time exhibited reduction in their cell size in comparison with the control cells (Fig. 1.19:E & F).

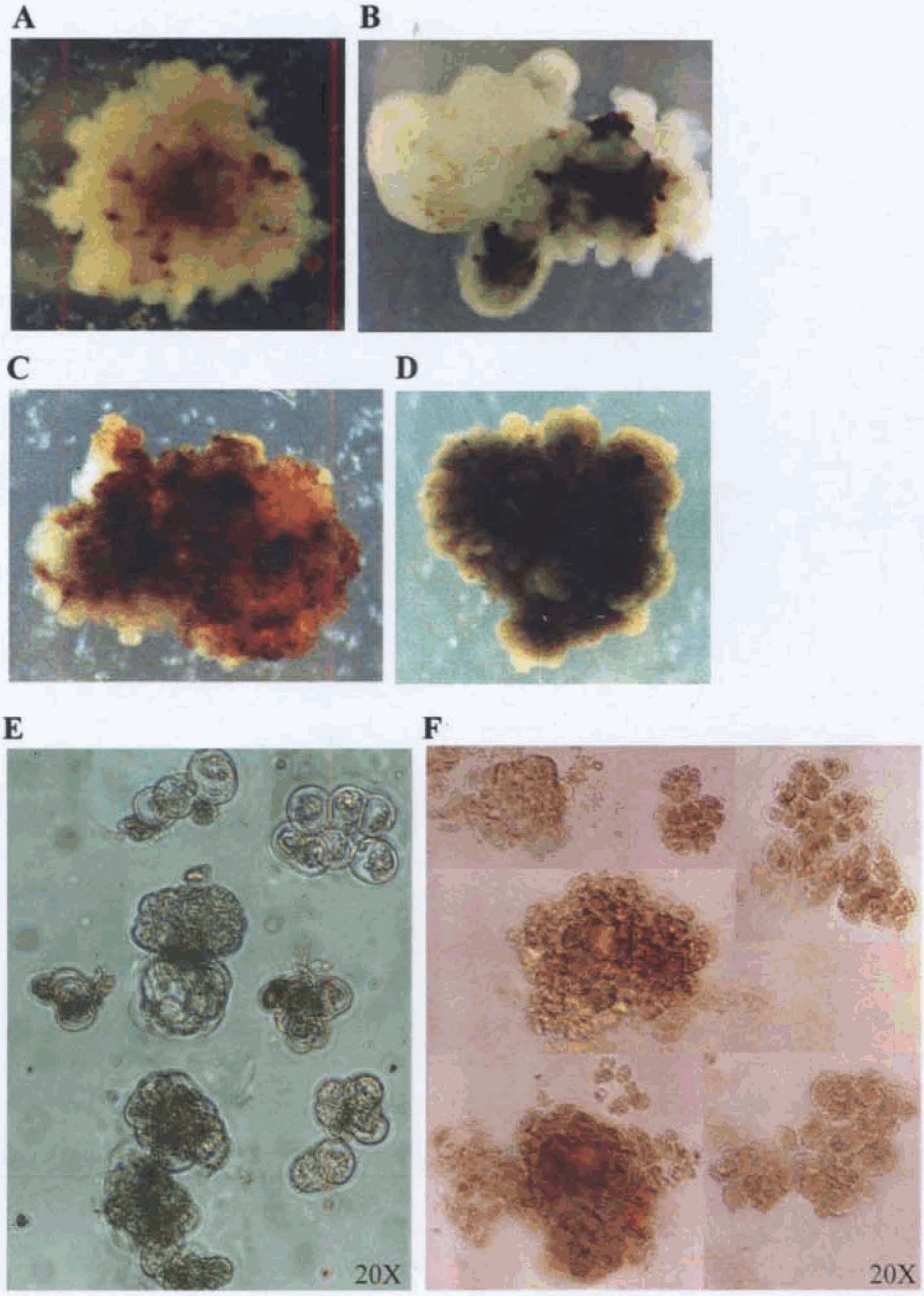


Fig. 1.19. Morphological characters of PEG-exposed black pepper callus and cells **A.** Differentiating callus of control cultures that shows numerous embryogenic off-shoots; **B, C & D** show callus of 15, 25 and 30% PEG respectively. The regeneration of PEG-exposed blackened callus clumps decreases with increase in the concentration of PEG. The 15% PEG-exposed calli (B) show good number of differentiating embryos. However, the differentiating ability of calli decreases significantly in 25% and 30% PEG-exposed cultures. The 25% PEG-exposed callus shows decreased number of embryogenic buds. The majority of the 30% PEG-exposed calli show very few or no embryogenic offshoots; **E. & F** show the cell size of black pepper cells of control and PEG-exposed cultures respectively at 20X magnification. On prolonged exposure to PEG-imposed water stress, the cell size decreases significantly.

1.3.2.6.3 *In vitro* selection

1.3.2.6.3.1. Step-wise selection

In the step-wise selection method, cells were first exposed to mild water stress induced by 15% PEG for 6 months followed by exposure to severe water stress mediated by 25% PEG, for another period of 6 months. The embryos and callus which survived in 15% PEG were grown briefly on control medium for a period of 20d before their transfer to 25% PEG. Transfer of the small growing cells and cell clumps of the 15% PEG-exposed cultures (Fig.1.20: A) to 25% PEG, after a period of 6 months resulted in the death of several cells and cell clumps (Fig1.20: B.). After a period of 6 months, small surviving cell clumps were transferred to the control medium for further multiplication. It was observed that these clumps blackened after a period of 5-6 days. However, these darkened clumps showed signs of fresh callus growth (Fig.1.20: C). These were allowed to regenerate on the same medium (Fig.1.20: D&E). There were two morphological types of calli - hard, compact and friable loose (Fig.1.20: F). While the former was not highly regenerable, the latter differentiated into embryogenic clumps that differentiated into embryos. The differentiating cell clumps and embryos were again returned to 25% PEG-amended medium to test the stability of their PEG-tolerance. However, the growth of these calli was highly inhibited which turned black on prolonged incubation (Fig.1.20: G). The cultures were incubated for a period of 2 months and the surviving embryos and calli were again plated back to the control medium for their recovery. All the embryos and calli again blackened. However, on further incubation for about 60 days, one of the blackened calli showed fresh growth of embryos (Fig.1.20: H). These regenerated embryos were sub-cultured again to control medium for multiplication and regeneration. The regenerated embryos were tested for their PEG tolerance by transferring them into medium amended with various concentrations of PEG, namely, 0, 10, 15 and 25% PEG. The embryos multiplied well in the control medium, while their growth and multiplication was inhibited in all the PEG-amended media (Fig.1.21: A, B). By the end of 2 months, the embryos of the selected cells in the control medium developed into seedlings, similar to the non-selected cells in the control medium (Fig.1.21: C), while those exposed to PEG showed inhibited growth. However, the selected cells growing in 10% PEG-amended medium showed better growth compared to those in 15 and 25% (Table 1.21).

Table 1.21: Behaviour of black pepper callus adapted to 25% PEG by step-wise selection, in PEG- amended medium

Treatment	No. of embryos	% increase in the no. of embryos
Non-adapted callus in control medium	153 ± 7.5498	100.0 ± 0.0000
Adapted callus in control medium	104 ± 4.5826	67.97 ± 8.0426
Adapted callus in 10% PEGmedium	39.7 ± 5.0332	25.95 ± 2.1617
Adapted callus in 15% PEGmedium	37 ± 6.0827	24.18 ± 3.4648
Adapted callus in 25% PEGmedium	11.3 ± 1.5275	7.38 ± 1.6747

The values shown are the average of 3 replicates with standard deviation

1.3.2.6.3.2. Direct selection

In the direct selection method, cells were directly exposed to higher concentrations of PEG. It was observed that cells exposed directly to 25% for longer duration (6 months), exhibited callus growth and embryo formation. However, the development of embryos into seedlings was suppressed (Fig.1.22: A). Transfer of the embryogenic clumps to fresh control medium also did not improve the regeneration ability and growth of the embryos (Fig.1.22: B). The callus however multiplied and was characteristically brown in colour and appeared to have lost regenerability Fig.1.22: C). This callus was tested for its tolerance by subjecting it to 15, 25 and 30% PEG for a period of 45 days. The growth of callus decreased with increasing concentration of PEG. Later these cells were transferred to control medium to assess their recovery. It was observed that the cells that were not exposed to PEG grew well when transferred to control medium, while the growth of cells exposed to 15 and 25% PEG was highly inhibited. The growth of the cells exposed to 30% PEG was minimal on the control medium.

Table 1.22: Behaviour of black pepper callus adapted to 25% PEG by direct selection method, in different PEG-amended media

Treatment	% increase in fresh wt. over control
Control (un-adapted)	100.0 ± 0.0000
Control (adapted)	86.37 ± 8.0011
Adapted callus in 15% PEG	46.27 ± 4.3851
Adapted callus in 25% PEG	11.53 ± 7.0249
Adapted callus in 30% PEG	-6.03 ± 4.9916

The values shown are the average of 3 replicates with standard deviation

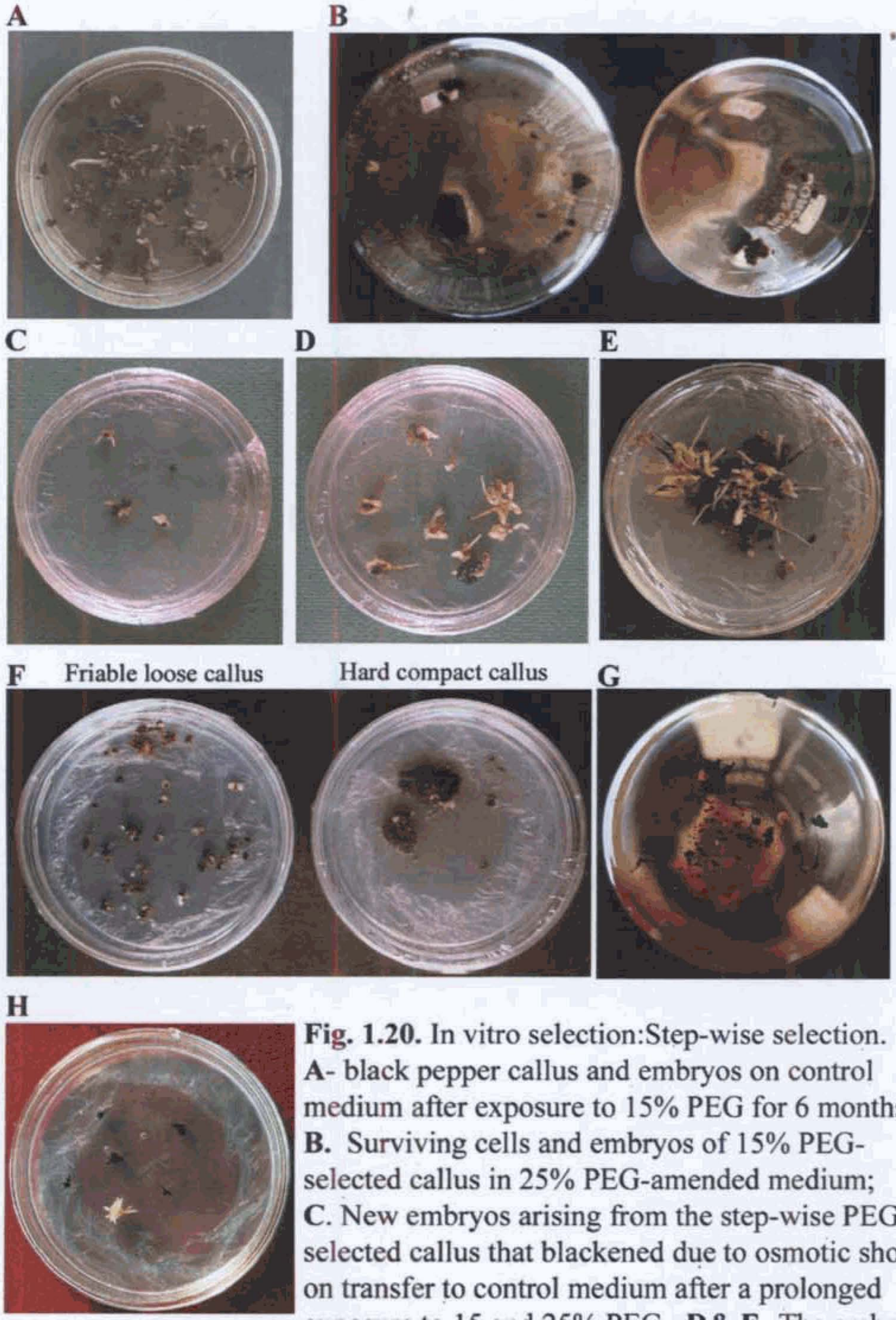


Fig. 1.20. In vitro selection:Step-wise selection. **A**- black pepper callus and embryos on control medium after exposure to 15% PEG for 6 months; **B.** Surviving cells and embryos of 15% PEG-selected callus in 25% PEG-amended medium; **C**. New embryos arising from the step-wise PEG-selected callus that blackened due to osmotic shock on transfer to control medium after a prolonged exposure to 15 and 25% PEG. **D& E**- The embryos being multiplied on control medium. **F.** The two morphological types of calli that grown from the selected embryos -loose-friable and hard compact; **G**- Blackening and death of step-wise selected friable callus and embryos on exposure to 25% PEG for a period of 2 months; **H**- Osmotic shock experienced by callus and embryos from G, on transfer to control medium. One of the blackened callus shows fresh sprouts of embryos after a period of about 60days- embryos from step-wise PEG-selected callus.

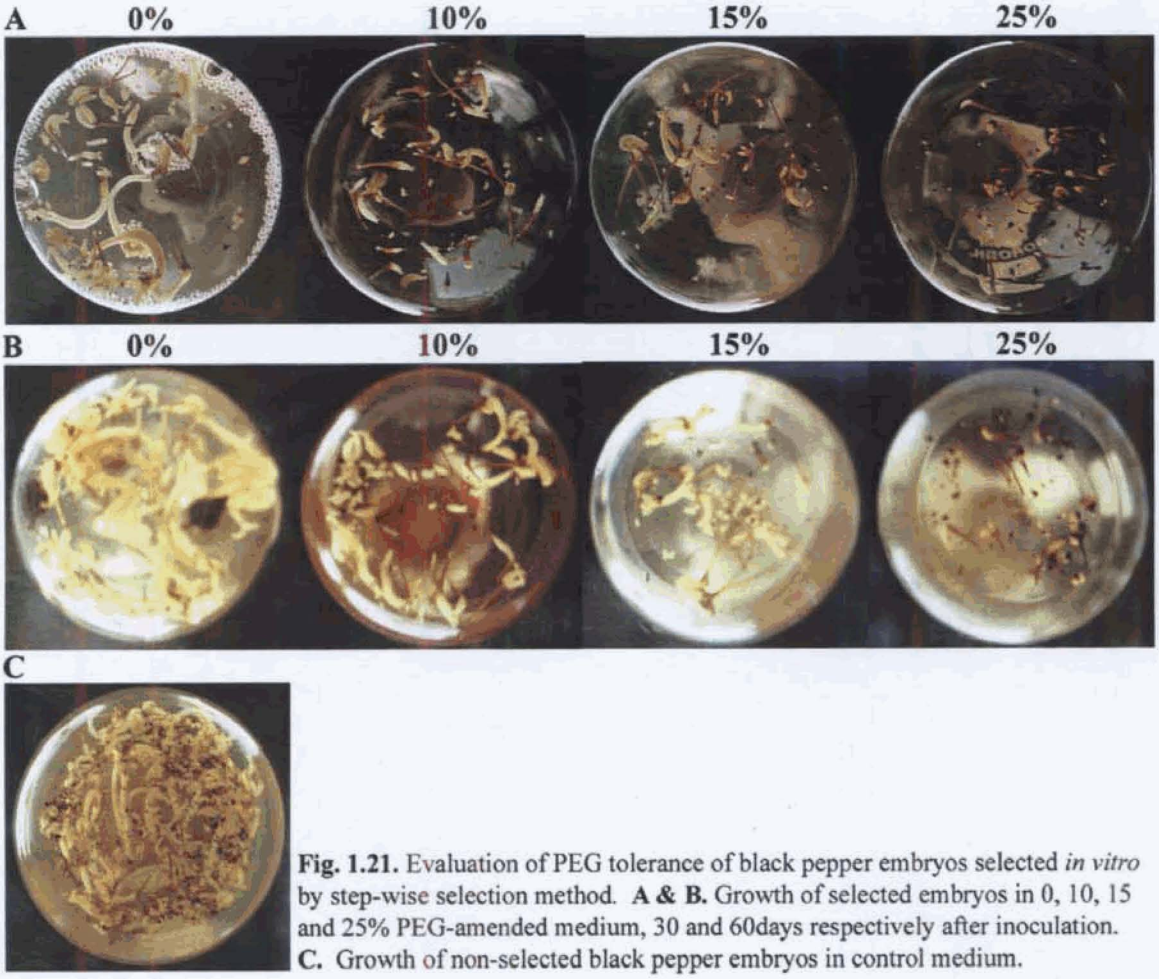


Fig. 1.21. Evaluation of PEG tolerance of black pepper embryos selected *in vitro* by step-wise selection method. **A & B.** Growth of selected embryos in 0, 10, 15 and 25% PEG-amended medium, 30 and 60days respectively after inoculation. **C.** Growth of non-selected black pepper embryos in control medium.

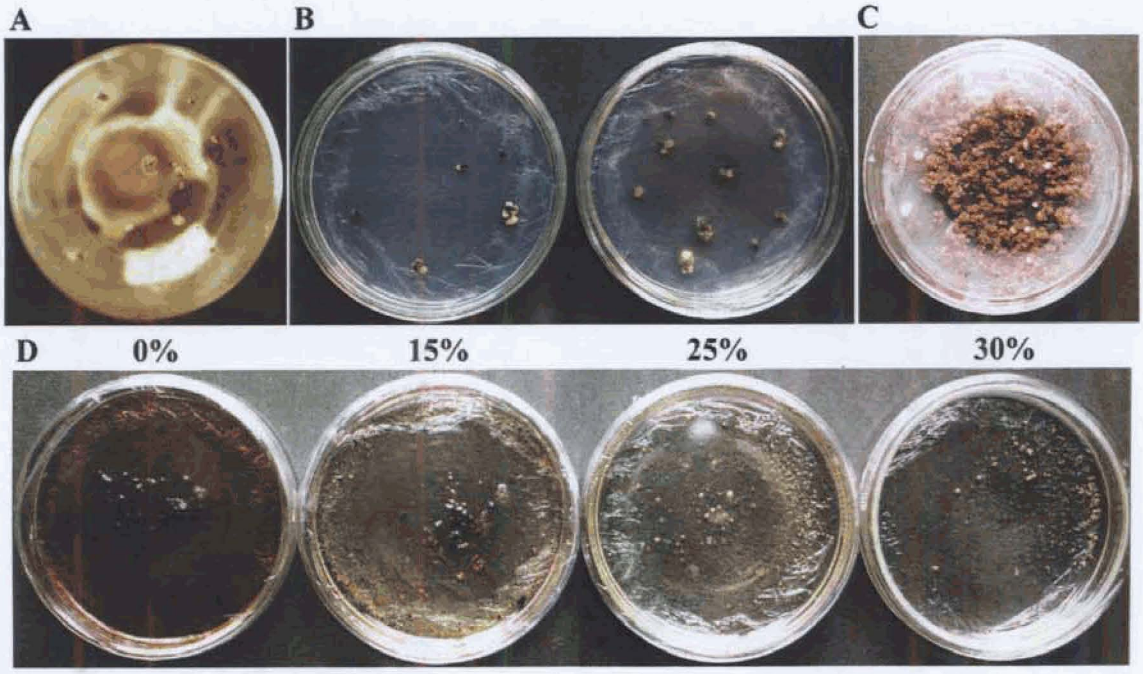


Fig. 1.22. *In vitro* selection of black pepper cells for PEG-tolerance: Direct Selection. **A.** Suppressed growth of callus clumps/embryos in 25% PEG after an exposure period of 6 months; **B.** Transfer of the surviving embryos to control medium does not help them regenerate; **C.** Growth and multiplication of the selected callus on control medium- characteristically brown in colour; **D.** Evaluation of the multiplied selected callus from C for PEG tolerance. The panel shows the decrease in growth of the selected callus in different PEG-amended media- callus does not have the ability to tolerate higher concentrations of PEG.

Cells exposed to 30% PEG for a period of 6 months showed very feeble growth (Fig.1.23:A). On transfer to solid control medium, these cells exhibited minimal growth (Fig.1.23: B). These cells, when exposed to 15 and 25% PEG, showed better growth in the former than in the latter (Fig.1.23: C). The majority of the 30% PEG-selected cells did not show any signs of regenerability. However, there were a few sporadic cells exhibiting embryo formation, when transferred to 15% PEG-amended medium, after a period of 7 months (Fig.1.23:D).

Cells exposed to 40 and 50% for 6 months did not show any signs of growth. The cells appeared bleached and sustained at these high concentrations for a long period of time without any increase in biomass (Fig.1.23: E &F resp.).

1.3.2.6.3.3. Characteristics of cells exposed to PEG-mediated water stress

1.3.2.6.3.3.1. Biochemical characters

1.3.2.6.3.3.1.1. Osmolyte accumulation

The cells adapted to 25% PEG by both step-wise and direct selection methods were characterized biochemically for osmolyte accumulation, lipid peroxidation and phenol accumulation after 45 days of exposure to stress. The cells were analyzed for inorganic ions, K^+, Na^+, Mg^{2+} and Ca^{2+} and organic osmolytes, namely, reducing sugars, total free amino acids and proline. Concentrations of these osmolytes in the PEG-adapted cell lines, selected by the step-wise (ST_{15-25}) and by the direct method (ST_{25}) are summarized in Table 1.23.

Table 1.23: Comparison of inorganic ion accumulation in water stress-adapted and non-adapted black pepper cells in control and PEG-amended medium

Tmt No.	Treatment	Inorganic ions {mg/g dwt}			
		K	Na	Mg	Ca
1	Non-adapted cells in Control medium	14.63 ± 1.4525	0.97 ± 0.0959	2.38 ± 0.2790	2.48 ± 0.1134
2	Non-adapted cells in 25% PEG-amended medium	18.56 ± 2.3619	1.31 ± 0.0934	2.21 ± 0.8322	1.63 ± 0.1476
3	ST_{15-25} in control medium	13.8 ± 2.560	1.05 ± 0.2422	2.49 ± 0.2076	2.41 ± 0.4425
4	ST_{15-25} in 25% PEG-amended medium	28.81 ± 4.5568	1.47 ± 0.1023	1.63 ± 0.1862	0.87 ± 0.2890
5	ST_{25} in control medium	14.3 ± 1.6581	0.92 ± 0.1273	2.57 ± 0.2254	2.3 ± 0.4174
6	ST_{25} in 25% PEG-amended medium	22.65 ± 1.9125	1.39 ± 0 2450	1.04 ± 0.4574	0.72 ± 0.3775

Four replicates were used for each treatment. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

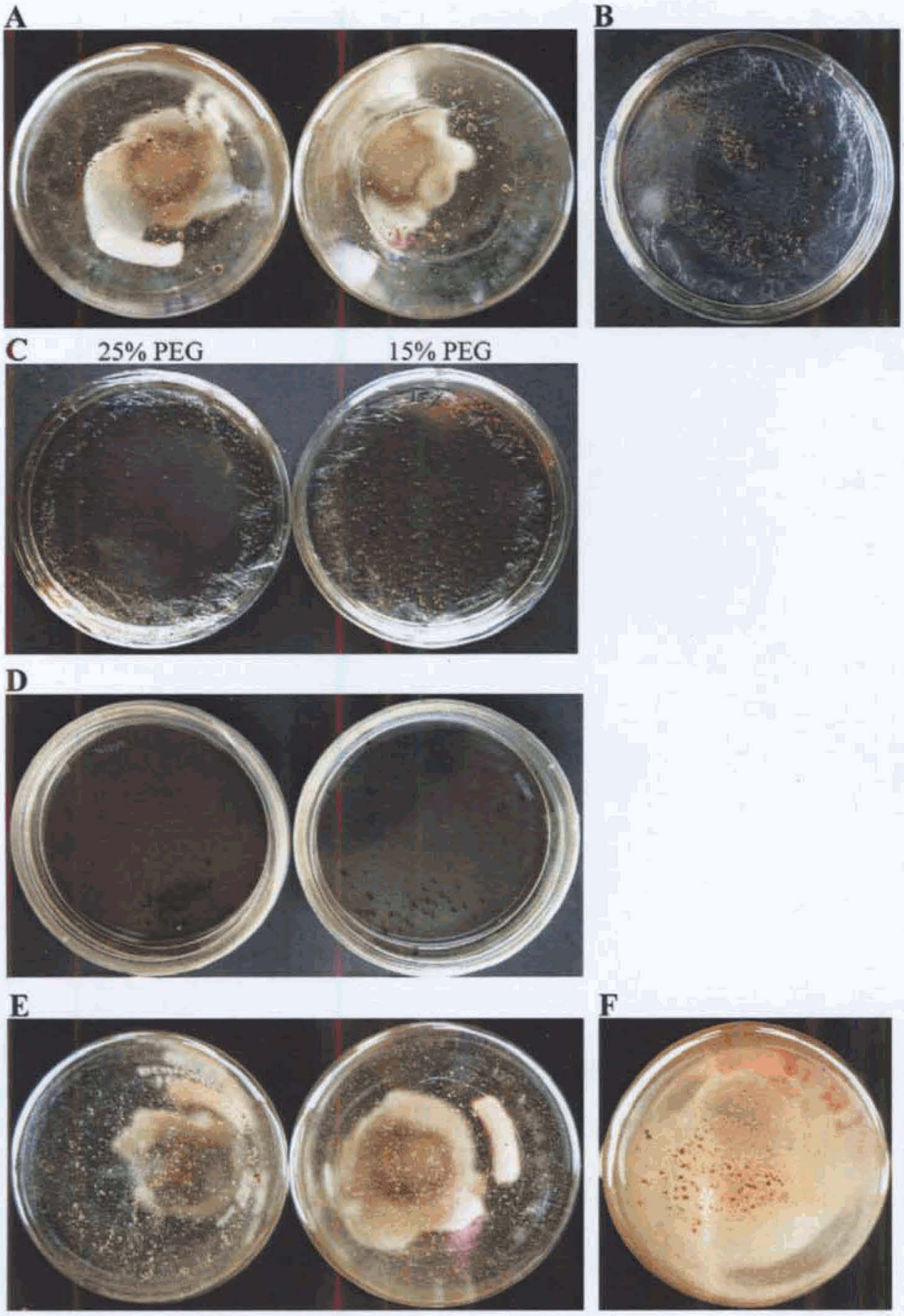


Fig. 1.23. Behaviour of black pepper cells exposed to higher concentrations of PEG. **A.** Cells exposed to 30% PEG for a period of 6 months show very feeble growth; **B.** 30% PEG-selected cells when transferred to control medium show only minimal growth; **C.** The 30% PEG-selected cells evaluated in 15 and 25% PEG for their tolerance. The biomass is more in 15% PEG - amended medium than in 25% PEG-amended medium; **D.** Transfer of 30% PEG-selected cells to a milder stress mediated by 15% PEG resulted in sporadic appearance of a few embryos after a period of 7 months, which however did not show any further development; **E.& F.** Feeble growth of black pepper cells in 30 and 40% PEG-amended media respectively, after a prolonged exposure of 6 months.

Post-Hoc tests

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 4.000.

K^+

Duncan[a]

Tmt	N	Subset for alpha = .05			
		1	2	3	4
3	4	13.8000			
5	4	14.3000			
1	4	14.6300			
2	4		18.5600		
6	4			22.6500	
4	4				28.8100
Sig.		.675	1.000	1.000	1.000

Na^+

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
5	4	.9200	
1	4	.9700	
3	4	1.0500	
2	4		1.3100
6	4		1.3900
4	4		1.4700
Sig.		.306	.210

Mg^{2+}

Duncan[a]

TMT	N	Subset for alpha = .05		
		1	2	3
6	4	1.0400		
4	4	1.6300	1.6300	
2	4		2.2100	2.2100
1	4			2.3800
3	4			2.4900
5	4			2.5700
Sig.		.068	.072	.291

Ca^{2+}

Duncan[a]

TMT	N	Subset for alpha = .05		
		1	2	3
6	4	.720		
4	4	.870		
2	4		1.630	
5	4			2.300
3	4			2.410
1	4			2.480
Sig.		.521	1.000	.468

In general, among the inorganic ions, K^+ and Na^+ ions increased while Mg^{2+} and Ca^{2+} decreased significantly in black pepper cells exposed to water stress compared to the cells in the control medium. This trend was seen in all cell lines irrespective of whether they were control cells that were not previously subjected to selection or those that were selected for water stress adaptation. It was observed that on exposure to water stress, the increase in Na^+ content in the control non-selected cultures was on par with that of the selected cell line cultures. However, the increase in K^+ accumulation was significantly higher in the selected cell lines compared to the control, under the imposed water stress.

Among the organic osmolytes, total free amino acids, proline and reducing sugars increased upon imposition of water stress in both the selected cell lines as well as the control cells. It was however observed that the selected lines accumulated higher amounts of total free amino acids than the control non-selected cell lines in 25% PEG-amended medium. Among the selected clones, the cell line selected by the step-wise method recorded higher concentrations of total free amino acids than the cell line selected by the direct method, in the PEG-amended medium. Reducing sugars increased only marginally in all the cell cultures, on imposition of water stress. The two selected cell lines also did not vary much in their reducing sugar content. In the case of proline, there was a marginal increase in the levels in the adapted cell lines over the control, on imposition of water stress. However, while the cell line obtained by the step-wise selection method recorded only a marginal increase over the control non-selected cells under water stress, the cells selected by the direct method seemed to accumulate rather higher levels (Table 1.24).

Table 1.24: Comparison of organic osmolyte accumulation in water stress-adapted and non-adapted black pepper cells in control and PEG- amended medium

Tmt. No.	Treatment	Organic osmolytes		
		Proline (μmol g^{-1} dwt^{-1})	**Total a.a.** (x 10^2 μg g^{-1} dwt^{-1})	**Red. Sugars** (x 10^4 μg glu.eq. g^{-1} dwt^{-1})
1	Unadapted cells in Control medium	0.581 ± 0.0648	9.49 ± 0.4135	4.1109 ± 0.1395
2	Unadapted cells in 25% PEG-amended medium	0.663 ± 0.0933	11.83 ± 0.1995	5.8269 ± 0.0599
3	$ST_{15\text{-}25}$ in control medium	0.567 ± 0.0373	9.62 ± 0.2652	4.2379 ± 0.2748
4	$ST_{15\text{-}25}$ in 25% PEG-amended medium	0.71 ± 0.0703	15.61 ± 1.1335	6.2351 ± 0.2310
5	ST_{25} in control medium	0.48 ± 0.0918	9.31 ± 0.4444	4.416 ± 0.0573
6	ST_{25} in 25% PEG-amended medium	0.88 ± 0.2155	13.86 ± 0.8082	6.0146 ± 0.0668

Four replicates were used for each treatment. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

Post-Hoc tests

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 4.000

Proline

Duncan[a]

Tmt	N	Subset for alpha = .05		
		1	2	3
5	4	.48000		
3	4	.56700	.56700	
1	4	.58100	.58100	
2	4		.66300	
4	4		.71000	
6	4			.88700
Sig.		.239	.111	1.000

Total free amino acids

Duncan[a]

Tmt	N	Subset for alpha = .05			
		1	2	3	4
5	4	9.3100			
1	4	9.4900			
3	4	9.6200			
2	4		11.8300		
6	4			13.8600	
4	4				15.6100
Sig.		.522	1.000	1.000	1.000

Reducing Sugars
Duncan

Tmt	N	Subset for alpha = .05			
		1	2	3	4
1	4	4.110900			
3	4	4.237900	4.237900		
5	4		4.416000		
2	4			5.826900	
6	4			6.014600	6.014600
4	4				6.235100
Sig.		.285	.140	.121	.072

1.3.2.6.2.3.2.2. Lipid peroxidation and phenol accumulation

Exposure to 25% PEG resulted in increase in phenolics and lipid peroxidation in both adapted and non-adapted cell cultures. However, while the adapted and non-adapted cells reacted similarly in terms of lipid peroxidation, the phenolic content of the adapted cell lines were higher than that of the non-adapted control cells under water stress. Among the adapted cell lines, the cell line selected by the step-wise selection method showed greater phenolics accumulation than the cell line selected by the direct method (Table 1.25).

Table 1.25: Comparison of phenolics accumulation and lipid peroxidation in water stress-adapted and non-adapted black pepper cells in control and PEG- amended medium

Tmt. No.	Treatment	Phenols ($x10^4$ µg g^{-1} d.wt.$^{-1}$)	MDA content ($x10^2$ nm g^{-1} fwt^{-1})
1	Unadapted cells in Control medium	4.2641 ± 0.1310	10.0121 ± 1.4763
2	Unadapted cells in 25% PEG-amended medium	5.3236 ± 0.1374	12.8161 ± 0.8677
3	ST_{15-25} in control medium	4.1362 ± 0.0286	10.6145 ± 2.7455
4	ST_{15-25} in 25% PEG-amended medium	5.9673 ± 0.1506	13.32 ± 1.2827
5	ST_{25} in control medium	4.2747 ± 0.0811	10.4923 ± 1.4843
6	ST_{25} in 25% PEG-amended medium	5.721 ± 0.0264	13.86 ± 2.0715

Post-Hoc tests
Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 4.000

Phenols

Duncan[a]

Tmt	N	Subset for alpha = .05			
		1	2	3	4
3	4	4.136200			
1	4	4.264100			
5	4	4.274700			
2	4		5.323600		
6	4			5.721000	
4	4				5.967300
Sig.		.095	1.000	1.000	1.000

Lipid peroxidation

Duncan[a]

Tmt	N	Subset for alpha = .05		
		1	2	3
1	4	10.012100		
5	4	10.492300		
3	4	10.614500	10.614500	
2	4	12.820000	12.820000	12.816100
4	4		13.329400	13.329400
6	4			13.861200
Sig.		.051	.052	.440

1.4 Discussion

Limited water availability affects plant growth and development. Due to the vagaries of monsoon that have become a regular feature in recent years, it has become increasingly important to develop plants to yield under unfavourable conditions. Therefore, a better understanding of the mechanisms that enable plants to adapt to water deficit conditions and maintain growth, development and productivity during stress periods would help in breeding for drought tolerance/resistance.

Black pepper being a rain fed crop is highly affected by water stress conditions. Kerala witnessed drying up of thousands of black pepper vines in Kannur district due to drought (Sadanandan, 1993) during the years 1973, 1977, 1983, 1987, 1989. Prabhakaran (1997) reported that the average yield loss in black pepper in Kannur District of Kerala during 1989-1992 was partly due to drought stress which amounted to 4% loss in the pepper vines. Delayed monsoon has become a regular feature in Kerala during the recent years. Hence research into the development of drought tolerance/resistance in this crop is gaining importance.

In the present study, we used a model system of cell suspension cultures of black pepper to study the various biochemical responses of black pepper cells to water stress. Water deficit conditions were simulated by inclusion of polyethylene glycol 6000 in the culture medium. Polyethylene glycol at different concentrations was used to induce different degrees of water stress.

The study on the effect of different concentrations of polyethylene glycol revealed that PEG at a concentration of 15% brought about 50% of growth inhibition, while 25 and 30% PEG resulted in 80 and >100% inhibition of growth respectively. If different concentrations of PEG are responsible for different rates of growth, it can be assumed that these different levels of stress would also have different degrees of effect on the biochemical parameters. The present study was therefore undertaken to investigate the modulations of different biochemical parameters like active oxygen scavenging enzymes, osmoregulation and changes in protein profile in black pepper cells subjected to different degrees of water stress. Based on the preliminary results on the effect of polyethylene glycol on black pepper suspension cultures, PEG at concentrations of 15, 25 and 30% PEG were used to simulate mild, severe and lethal water stress conditions to study the effect of these different degrees of water stress on the biochemical responses of the black pepper cells.

1.4.1. Biochemical responses to water stress

1.4.1.1. Osmotic adjustment

A general increase in the levels of different solutes was observed on imposition of water stress in black pepper suspension cultures. Plant cells that experience dessication to the point of turgor loss must regain turgor through osmotic adjustment to resume growth. Therefore, under water stress conditions, during which the plant cells generally tend to lose water to the outside, accumulation of solutes facilitates the uptake of water from the surroundings, which is otherwise not possible in environments with depleting moisture. Therefore, the accumulation of organic and inorganic solutes in black pepper cells subjected to PEG-induced water stress indicates the attempt by the cells to counteract the loss of water by osmotic adjustment.

1.4.1.1.1. Organic osmolytes

1.4.1.1.1.1. Amino acids

Among the organic osmolytes, reducing sugars and free amino acids contributed significantly to the solute concentration in the cells. Total free amino acids were found to increase significantly in black pepper suspension cultures under water stress conditions, the increase being more in cells exposed to mild stress during the initial phase of the stress period. In control cultures, alanine and phenylalanine were the predominant amino acids during the early stages of the experiment, which later showed a decline. Glutamine was however the major amino acid during the latter half of the experiment. Glutamine was also found to be the predominant amino acid under mild as well as severe water stress. However, while the levels of glutamine was maintained steady over a period of time under mild stress conditions, the levels dropped in cells exposed to severe stress. Phenylalanine and alanine levels showed a significant increase under severe stress conditions that was not observed under mild stress. However, the levels declined very soon on prolonged exposure which is consistent with the high phenolic content recorded in the 25% PEG-treated cultures during the initial stages of stress exposure. Handa *et al* (1983) also observed increases in alanine and phenylalanine in addition to increased glycine, serine, valine, methionine, isoleucine, leucine, lysine and hystidine content in tomato cells adapted to polyethylene glycol-induced water stress. However, they observed decrease in concentrations of glutamate and glutamine in the adapted cells. It was suggested from this observation that glutamate being the precursor of proline, might have decreased due to increased synthesis of proline. However, the observed levels of glutamate or glutamine in unadapted tomato cells were not high enough to account for the levels of proline observed in the adapted cells. Hence, it can be suggested that the increase in and maintenance of sufficient levels of glutamine observed in black pepper cells exposed to mild stress in the present study may be due to its increased synthesis to ensure sufficient pools of precursor for the synthesis of proline under mild stress. Similar to observations made in the present study, Mapelli *et al* (2001) also observed that glutamine which remained one of the major component of the total free amino acids in the xylem of walnut trees during the non-stress period, continued to remain as one of the major components under water stress. In addition, the % increase in aspartate and glutamate were also noted. The authors suggested that the increase in amino acids in the leaf sap during the stress may be a consequence of different causes, namely, protein hydrolysis, synthesis or conversion.

Yang *et al.,* (2000) studied the contents of proline, ornithine, arginine and glutamic acid in detached rice leaves on imposition of water stress and observed that the proline content consistently increased with the progression of stress. While ornithine and arginine were significantly higher in stressed leaves compared to the control, the glutamic acid levels recorded an initial increase but declined below the controls by the end of the stress period. Barathi *et al* (2001) reported increased amino acid content in mulberry leaves on imposition of drought stress. The stressed leaves showed a 2.5 fold increase in proline.

Proline, considered to play a major role in osmoregulation in plants under water deficit conditions, did not accumulate to significantly high levels in black pepper cells under water stress over the control, in the present study. Cells exposed to mild stress showed significant increase in proline over the control only on the 15th day of exposure to stress. The proline levels however were maintained on par with the control cells during the initial phases of the stress. On the other hand, cells exposed to severe and lethal stress conditions showed a considerable decline in proline content compared to the controls. Though the cells attempted to increase their proline content during the course of time, the levels were far below that of the control cells throughout the experiment.

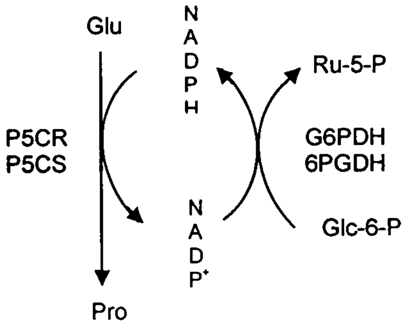
In a recent review on the roles of osmolyte accumulation during water stress, Hare *et al* (1998) suggested that to understand the physiological significance of differences in the types of osmotic solute accumulated by various species is to consider the preferential accumulation of any osmolyte as a reflection of the availability of its precursors under adverse conditions. Thus, the increase in proline observed in the present study, especially in the cells exposed to mild water stress may be due to the abundant increase in glutamine which forms the precursor for proline synthesis. Black pepper cells exposed to 25% PEG in the present study also recorded higher levels of glutamine, but the levels were far below than that found in the cells at 15% PEG. This is consistent with the observation that while the cells at 15% PEG maintained proline levels on par with the control cells during the initial phases of the stress period or higher levels after the 10th day of exposure to stress, the 25% PEG-exposed cells attempted to regain the proline levels that fell below the control levels during the initial phases of the stress period.

The importance of increase in free proline content during water stress could be attributed to its role in reducing the various adverse effects of water deficit in plant cells. Proline accumulation has been implicated to have an osmoregulatory role during water stress conditions (Aspinall and Paleg, 1981). Santos Diaz and Ochoa Alejo (1994) observed a positive correlation between proline accumulation and the capacity of cell cultures of drought-sensitive *Capsicum annuum* and *Larrea tridentate*, a plant species highly tolerant to drought, to grown in conditions of PEG-induced water stress. Desmukh *et al* (2001) noted positive correlation between proline accumulation and intensity of PEG-induced water stress in *Sorghum bicolor* cultivars. The authors suggested that proline, in addition to other parameters like phenols, reducing sugars, starch and protein, could be considered as a reliable marker for assessment of water stress tolerance in different genotypes of sorghum. Madhusudhan *et al.,* (2002) noted a better maintenance of proline content and free amino acid content in a drought tolerant cultivar of groundnut. Han *et al* (2003) also observed increased proline alleviated osmotic potential in sea buckthorn, indicating that the plant had characteristics of drought tolerance at low water potential.

Proline is also known to protect cell membranes and enzymes (Paleg *et al.*, 1981; 1984) and as a reservoir of energy and amino groups for post-stress growth (Fukutaku and Yamada, 1984). Wu *et al* (2001) reported increase in proline content and activity of protective enzymes in reed callus in response to PEG-induced osmotic stress. The authors observed that the sand reed callus recorded a greater increase in proline content and enzyme activity than in water reed callus.

In addition to any biophysical protective effects of free proline, it has been proposed that it plays an important role in ameliorating shifts in cellular redox potential which accompany water stress. Under *in vivo* conditions, plants under water stress are exposed to light intensities that exceed those that can be used for carbon assimilation. When the continued exposure to photons is not matched with the regeneration of $NADP^+$ under water stress conditions due to decrease in CO_2 uptake by light-induced stomatal closure, resulting in reduced photosynthesis, a redox imbalance is likely to result in photoinhibition. This would enhance the use of O_2 instead of $NADP^+$ as the electron acceptor in photosynthesis. Hare *et al* (1998) proposed that a stress-induced increase in the transfer of reducing equivalents (NADPH) into proline by Δ^1- pyrroline-5-carboxylate (P5C) synthetase and P5C reductase may be a protective mechanism to ameliorate shifts in cellular redox potential that occur during water stress. The observation of Kiyosue *et al.,* (1996) in *Arabidopsis* that though severe dehydration induced the enzymes of proline synthesis, there was no net accumulation of free proline, strengthens the suggestion that cycling between proline and its precursors may be an important homeostatic mechanism to forestall redox imbalance associated with smaller water deficits. This supports the observation made in the present study, wherein a significant increase in glutamine, the precursor for proline synthesis, was observed in the water-stressed cells. Hare *et al* (1998) suggest that enhanced flux through the substrate cycle may confer a yield advantage at least under modest stress, although uncoupling of the process under severe stress might overwhelm the benefit. The glutamine levels in cells exposed to mild water stress in the present study remained on par with the control until the 10th day and increased significantly only from the 15th day, which is similar to the trend observed for proline accumulation, signifying that the proline levels increased due to increase in the glutamine levels in cells exposed to mild water stress. In 25% PEG-treated cultures, though glutamine recorded an increase from the 15th day, the levels were far below than that observed in cells at 15% PEG. Moreover, this increase was not favourable for proline synthesis, as can be seen from the decline in proline levels in cells exposed to severe water stress. This suggests that under severe water stress conditions, the recycling between glutamine and proline is severely affected or that the enzymes Δ^1- pyrroline-5-carboxylate (P5C) synthetase and P5C reductase, catalyzing the conversion of the precursor, glutamine to proline, are greatly inhibited under severe water stress.

The maintenance of low proline levels during the initial phase of the mild water stress period observed in the present study may not necessarily imply that proline does not play a major role in drought stress tolerance in black pepper. It is possible that the cells do not require the accumulation of this solute for maintenance of their growth during the initial phase of water stress period. The absolute concentrations of the solute may not be important for attributing a role for the solute in osmotic adjustment. This is supported by the observations made by Hare *et al* (1998) that the absolute osmolyte concentrations in plants during water stress are unlikely to mediate osmotic adjustment. Consistent with this suggestion, Rascio *et al* (1994) reported that proline, despite its low concentration in the leaf, seems to be an important osmotic component because its content was highly correlated with the osmotic potential. Metabolic benefits of osmolyte accumulation may augment the classically accepted roles of these compounds. Hence, it is important to reconsider the significance of accumulation of these solutes during water stress. Hare *et al* (1998) also suggest that there is no reason to assume that any end product of metabolic adjustment resulting in osmolyte accumulation should be the primary effector of stress tolerance. The metabolic implications of an increase in osmolyte synthesis and/or decline in osmolyte degradation warrant at least as much attention as the osmotic consequences of these processes, since maintenance of turgor or protection of subcellular structure alone is likely to be inadequate for continued growth under adverse conditions. Thus, it can be suggested from the present study that in addition to aiding in osmoregulation, the accumulation of proline on prolonged exposure to mild stress might have an adaptive value, due to its possible role in maintaining the cellular redox potential, or as a reserve of reducing power, as has been frequently suggested by others that proline degradation upon relief from stress might provide carbon, nitrogen and energy for recovery (Hare and Cress, 1997). Another important role attributed to proline accumulation is that the replenishment of NADP$^+$ supply by proline synthesis from glutatamine catalyzed by P5C synthetase and P5C reductase, supports redox cycling and this is considered to be important in plant antioxidant defence mechanisms during stress (Babiychuk *et al*, 1995). Since NADPH is required to maintain glutathione (GSH) and ascorbate in the reduced state for use in the ascorbate glutathione cycle of the antioxidant system, it is known that the oxidative pentose phosphate pathway is an important component of antioxidative defence mechanisms (Juhnke *et al.*, 1996). Pentose phosphate pathway provides the NADPH required for this by converting the NADP$^+$ formed during proline synthesis to NADPH during the conversion of ribulose -5-phosphate to glucose-6-phosphate catalyzed by the enzymes of the pathway as shown in the equation below:



Thus proline also plays an important role in potentiating the pentose phosphate pathway activity, thus helping in the antioxidative defence (Hare and Cress, 1997). Hence, in addition to osmoregulation, the significance of accumulation of proline observed in the present study after prolonged exposure to mild water stress, needs to be explored in black pepper. However, it can be suggested from the present study that proline accumulation may have a role in ameliorating the adverse effects of water stress under mild stress conditions and that it does not seem to play a significant role under severe water stress, as is seen from the heavy decline in its levels from the beginning of the stress period. However, the implication of increase in proline content to water stress tolerance should be done cautiously since there are also reports that show that proline could just be a symptom of water deficit and may not be directly involved in water deficit tolerance, as has been shown by Hanson (1980). Hanson and Hitz (1982) suggested that proline accumulation under stress is a primitive response of living organisms to increasing osmotic pressure in the cellular mileu and is, probably, a metabolic response of little advantage. Kameli and Lösel (1993) found that proline was not correlated with tolerance of water stress in their studies on Triticum durum since it increased only later and at lower water potentials.

1.4.1.1.1.2. Sugars

Sugars have also been found to contribute to osmotic adjustment in many plants under water stress conditions. Increased levels of sucrose and/or reducing sugars have frequently been reported and have been proposed to contribute towards turgor maintenance in water-stressed tissues of plants (Kellar and Ludlow, 1993; Pilon-Smits *et al.*, 1995; Pelleschi *et al.*, 1997). In the present study, similar to trends observed in the accumulation of other osmolytes, reducing sugars were also noted to increase significantly in water-stressed black pepper cells. The increase was markedly higher in 30% PEG-exposed cultures during the initial stages of the water stress period. However, the levels declined by the 15th day to the control levels after which they fluctuated and remained low by the end of the experiment. Though the increase in reducing sugars was proportional to the severity of the stress, the levels in cells exposed to higher concentrations of PEG (25 and 30%) decreased progressively with the prolongation of the stress period and declined below the control levels by the end of the experiment. However, under mild stress conditions, the increased levels of reducing sugars were maintained steady till the end of the stress period, suggesting that under mild stress, though the increase in reducing sugars was not as high as shown initially by cells exposed to severe and lethal stress, the cells were able to maintain steady levels of reducing sugars for a longer period of the imposed water stress.

Sugar accumulation has long been demonstrated to be an important determinant of dehydration tolerance (Ingram and Bartels, 1996). It has been suggested by Hare *et al* (1998) that cells of stressed plants are likely to have an increased demand for readily metabolizable carbohydrate since tolerance depends on the energy status of the cells in which appropriate responses are induced. Sucrose provides carbon for cellular metabolism only after its conversion to hexose phosphates. Sucrose and hexoses have been implicated to have signaling capabilities during stress responses (Hare *et al*, 1998). Sugars may also play important regulatory functions in addition to their osmotic role. Sugars are known to not only sustain the growth of sink tissues in plants, but also affect sugar-sensing systems that regulate the expression, either positively or negatively, of a variety of genes involved in photosynthesis, respiration, starch and sucrose synthesis and degradation and nitrogen metabolism (Koch, 1996). Bai and Rajagopal (2000) reported increase in osmotically-active soluble sugars in coconut leaves on imposition of drought stress and also observed that they increased to a greater extent in the tolerant genotypes compared to the susceptible ones. Kameli and Lösel (1995) reported that sugars, particularly glucose, made the largest contribution to osmotic adjustment in wheat leaves under water stress and that it was more efficient in younger leaves. The authors suggested that changes in monosaccharide content may regulate osmotic activity more efficiently than changes in sucrose, since the osmotic value can be doubled by splitting the disaccharide molecules, without changing the water content. Hexoses are reported to be required for cell walls and other structural components in developing tissues (ap Rees, 1984). The increase in reducing sugars in black pepper cells exposed to mild stress observed in the present study may thus have one or many of these roles and may be of an adaptive value since the growth of cells is not affected significantly due to the imposed stress. However, the roles of these sugars need to be explored. The decrease in reducing sugars however, in 25% PEG-exposed cultures, could be explained to be due to the severe inhibition of enzymes responsible for the hydrolysis of starch and sucrose. Moreover, the low amount of reducing sugars in these cultures could also be due to the fact that during severe water stress, the main reducing sugar, glucose, is highly detrimental to the survival of the cells, since, glucose can participate in the Maillard reaction, that can lead to many end products that are known to cause protein inactivation and DNA damage (Fujimaki *et al.*, 1986). Thus, the decrease in reducing sugars at severe water stress regime observed in the present study seems to be a protective measure to prevent cellular damage. However, the increase in levels of reducing sugars under mild stress may be an adaptive response, by helping in osmoregulation. Pelah *et al* (1996) observed increase in sucrose and decrease in glucose levels in the aspen leaves during water stress.

Contrary to some reports that implicate detrimental effect of accumulation of reducing sugars during water stress, there are several reports that indicate that reducing sugars contribute significantly to osmotic potential in water stressed plant tissues. Garg *et al* (2001) noted increase in reducing sugars in addition to proline and free amino acids in all genotypes of moth bean with increase in water stress. Iannucci *et al* (2002) observed increase in reducing sugars and a decrease in non-reducing sugars in leaves of water-stressed annual clover plants. Gebre and Tschaplinski (2002) reported that mono-saccharides, particularly glucose and fructose, accounted for most of the osmotic adjustment in chestnut oak and understorey dogwood during severe drought.

1.4.1.1.2. Inorganic solutes

Among the inorganic solutes analyzed in the present study, black pepper cells appeared to accumulate higher levels of K^+ on imposition of water stress, and the accumulation was proportional to the severity of stress. However, the K^+ levels were observed to be significantly low in cells exposed to lethal stress imposed by 30% PEG, compared to the controls. Na^+ content also increased in all the water stressed cultures but the increase was more significant in cells exposed to severe and lethal stress, while the cells exposed to mild water stress had Na^+ levels that were in general, comparable to the control cells. While Mg^{2+} content did not show any significant increase in cells exposed to 15 and 25% PEG during water stress, cells exposed to 30% PEG showed a decreasing trend. Ca^{2+} content showed a heavy decline in all the water stress treatments.

Thus, in the present study, it was observed that black pepper cells predominantly accumulated potassium ions, reducing sugars and free amino acids in higher levels under water stress and it can be suggested that these solutes may contribute significantly to osmotic adjustment under PEG-induced water deficit conditions. Potassium ions, sugars, free amino acids and proline have long been known to be important components of osmotic adjustment. Premachandra *et al* (1995) reported that sugars and K^+ were the major contributors to solute concentration in sorghum under water stress. The authors noted that concentrations of most of the solutes like sugars, K^+, proline, amino acids, Mg^{2+}, Ca^{2+} and glycine betaine tended to be higher in the tolerant sorghum cultivar than the susceptible plants under water stress conditions. However, the most notable differences between the tolerant and susceptible genotypes reported by these authors were with respect to the contributions of sugars and K^+ ions, with sugars contributing more to the osmotic adjustment during the early development of stress, while K^+ ions accounting for an increasingly larger fraction of adjustment as the water deficit intensified. In the present study also, the levels of K^+ ions in black pepper cells remained high under both mild (15% PEG) as well as severe (25% PEG) water stress conditions, compared to the control, till the end of the experiment, pointing to the possibility that these ions play a significant role in osmotic adjustment during PEG-induced water stress. Jones *et al* (1980) also showed that K^+ was the major cation contributing to osmotic adjustment in sorghum. Morgan (1992) reported K^+ accumulation that contributed about 78% towards osmotic adjustment in certain wheat lines during drought stress. An increase in K^+ ions in leaves of plants under water stress could increase stomatal responsiveness to leaf water deficit. The increase in K^+ ion concentration observed in the black pepper suspension cultures could point to the possibility that a similar increase *in planta* could be the reason for the increased stomatal resistance observed by Krishnamurthy *et al* (1997) in rooted cuttings of black pepper accessions with increasing intensity of drought stress.

Santos Diaz and Ochoa Alejo (1994) observed an important contribution of K^+ to osmotic potential in calluses of drought-sensitive *Capsicum annuum* cells and highly tolerant *Larrea tridentata* on exposure to PEG-induced water stress *in vitro*, the levels being higher in the former than the latter.

He *et al* (1993) reported increased K^+ accumulation and H^+ extrusion by sorghum roots during PEG-induced osmotic stress. They further observed that the K^+ accumulation was inhibited by cycloheximide, indicating that the accumulation may be related to the synthesis of stress-induced proteins. Zhou *et al* (1999) also reported that increased K^+ content in root tissues of poplar induced by osmotic stress could be inhibited completely by cycloheximide. Liu *et al* (2000) observed that among the ramie germplasms, the drought resistant varieties showed greater increases in K^+ concentrations compared to the susceptible varieties during drought stress.

Appropriate supply of crop plants with potassium is known to improve their water management and consequently results in higher resistance to stresses, including droughts. The anti-stress potassium action is a consequence of higher K^+ ion concentration in the soil solution either due to natural fertility or K fertilization. K^+ has been reported by many researchers to improve water status in plants during water stress. Deka and Baruah (1998) reported increased K^+ accumulation in two water stress-resistant cultivars of rice under water stress conditions. Yadav *et al* (1999) reported that soil application of K mitigated the adverse effects of water stress in finger millet and thereby increased grain yield under water stress conditions. Soil application of K^+ was also observed to have a beneficial effect in overcoming soil moisture stress and increasing physiological parameters and carbon partitioning in the tropical legumes, mungbean and cowpea (Sangakkara *et al,* 2000).

Huang (2001) reported that higher potassium and lower Fe accumulations in shoots could contribute to better drought tolerance of tall fescue cultivars. Vyas *et al*

(2001) reported that the detrimental effects of water stress on yield of clusterbean were markedly less at higher K levels. The authors suggested that K application helped plants in maintaining favourable internal tissue water and metabolic activities under water stress. The activities of nitrate reductase and glutamine synthetase generally increased while glutamate dehydrogenase activity decreased with increasing K levels. Ashraf *et al* (2001) observed that considerable osmotic adjustment occurred in pearl millet plants experiencing water deficit under high K supply. Contents of total free amino acids in the leaves of two pearl millet lines increased significantly with increase in K supply under water stress. Using experimental data Grzebisz *et al* (2002) proved that appropriate nutrition of sugarbeet plants with potassium allowed them to survive during the critical growth stages and, consequently, reduced the risk of yield losses caused by drought.

Hence, from the available literature, it can be suggested that the significant increase in K^+ observed in black pepper cells under mild stress conditions in the present study could have an adaptive value by maintaining favourable water status inside the cells and also other metabolic activities.

Among the other inorganic ions analyzed in the present study, Na^{2+} recorded an increase in black pepper cells exposed to severe and lethal stress compared to the control, while the cells under mild stress recorded an initial increase after which they declined with time and were on par with that of the control cells. While the Mg^{2+} content remained on par with the control in cells exposed to 15 and 25% PEG, the levels declined in cells exposed to lethal stress imposed by 30% PEG. The Ca^{2+} levels however decreased in all the water stress treatments over the control. Similar observations have been made by Santos Diaz and Ochoa Alejo (1994) who observed that Na^{2+} and Ca^{2+} contents in the cell cultures of *Capsicum annuum* and *Larrea tridentata* did not contribute significantly to the osmotic potential. On the other hand, K^+ ions made a major contribution to the osmotic potential of the *Capsicum annuum* cells.

Thus, in summary, black pepper cells in general accumulated significant concentrations of reducing sugars, free amino acids and K^+ ions during PEG-induced water stress. In addition, the black pepper cells also recorded moderate increase in proline levels in response to water stress. The results obtained in the present study are consistent with those of Krishnamurthy *et al* (1998) who reported increased total free amino acids and reducing sugars in addition to increased stomatal resistance in rooted cuttings of black pepper accessions on imposition of water stress. Similar observations have been made by other researchers in different plants. Handa *et al* (1983b) also observed increase in intracellular concentrations of reducing sugars, total free amino acids, proline, K^+, NO_3^-, Na^+ and Cl^- in addition to malate, citrate and quarternary ammonium compounds with decreasing external water potential. Rascio *et al* (1994) observed reducing sugars, proline, K^+ and Cl^- to be major contributors to osmotic adjustment in durum wheat under water stress. The authors also observed that the accumulation of ions (Cl-, K+) and proline was concurrent with an increase in the binding-strength of tightly and weakly bound water, respectively, indicating that these solutes had a role in maintaining the internal water status of the cells. Srivastava *et al* (1995) also reported increased levels of soluble proteins, total and reducing sugars, free amino acids, L-proline and K+ in water stress tolerant cells of tomato during water stress, compared to the control. Bai and Rajagopal (2000) also observed increased accumulation of osmotically active sugar and amino acids in coconut leaves on imposition of drought stress. Drought tolerant genotypes were observed to accumulate more solutes than the susceptible genotypes during severe stress period. Iannucci *et al* (2002) observed increase in potassium, reducing sugars and proline concentrations and a decrease in non-reducing sugars in leaves of water-stressed annual clover plants. Garg *et al* (2001) noted increase in proline, free amino acids and reducing sugars in all genotypes of moth bean with increase in water stress.

Thus, from the observations made by other researchers in different plants under water stress, it can be concluded from the present study that the increase in the concentration of these osmolytes in black pepper suspension cultures during water stress is indicative of their role in osmotic adjustment. In general, it could be concluded from the present study that while cells under mild water stress showed far greater ability for osmoregulation, the cells exposed to 25 and 30% PEG underwent severe physiological damage with the result that the physiological function of osmoregulation was highly affected, especially on prolongation of the stress period. The fact that the adapted cells remain small and do not expand as much as the unadapted cells (as mentioned under section 1.3.2.2.1.1), observed in the present study points to the possibility that the process of synthesis of osmotic solutes such as sugars and free amino acids from the carbon reserves limits the synthesis of macromolecules like proteins and cell wall material, which are required for growth processes as suggested by Handa *et al* (1983b). According to these authors, a decrease in elasticity (and/or extensibility) could account for the restricted growth of cells observed under water stress.

1.4.1.2. Total Phenols

Phenols were found to increase significantly in water-stressed black pepper cells compared to the controls, the increase being steady in the cells exposed to moderate water stress, than in those exposed to severe stress. While the cells under mild water stress maintained high phenolic content till the end of the experiment, those exposed to 25 and 30% PEG showed an initial increase in phenolics, but the levels declined as the stress period progressed. Chakraborty *et al* (2002) also reported increase in phenol content and activities of polyphenol oxidase, phenylalanine ammonia lyase and peroxidase in two drought-tolerant cultivars of tea on imposition of drought stress but decreased on extended drought period. This observation could be extrapolated into the results obtained in the present study, that the responses elicited during the mild stress could be taken as tolerant responses of black pepper during water stress which decline on prolonged water stress as is seen in the cells exposed to severe water stress (25 and 30% PEG).

Phenolic compounds have been implicated to have a role in inhibiting stomatal opening in plants. Plumbe and Willmer (1986) observed that the phenolics, p-coumaric acid, caffeic acid, chlorogenic acid, salicylic acid and particularly sinapic acid, which accumulate in leaf tissues during water stress, were strong inhibitors of stomatal opening in *Commelina communis* epidermal strips. The authors suggested that all water stress compounds except proline and glutamine, which had no effect on stomatal behaviour, inhibited opening and caused some closure of initially open stomata. Krishnamurthy *et al*

(1998) observed increased stomatal resistance in black pepper accessions with increasing water stress intensity. This could be interpreted to be due to the possible increase in phenols during the stress, similar to the increased phenolic content observed in the black pepper cell suspension cultures in the present study, which may play a role in the inhibition of stomatal opening.

Polyphenols are also known to possess anti-oxidant properties (Blokhina, 2000). This property lies in their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron that has a chain-breaking function. They also chelate transition metal ions, thus helping in the termination of Fenton reaction (Rice-Evans *et al.*, 1997). Flavonoids, one of the members of phenolic compounds, modify the lipid packing order and decrease membrane fluidity, thus sterically hindering the diffusion of free radicals and restricting peroxidative reactions (Arora *et al.*, 2000). Thus, it can be suggested from the results obtained in the present study that the increase in phenolic content under mild stress conditions could have a protective role in inhibiting to a great extent, the lipid peroxidative reactions that occur during water stress. This is supported by the observation that the lipid peroxidation in cells exposed to 15% PEG is limited to lower levels than that observed in cells exposed to 25% PEG. Phenols and flavonoids are also known to act as protective filter in plants against the damage by UV-B radiation. Al Wakeel (1999) reported increase in phenols and flavonoids in hydroponically grown plants of *Cucurbita pepo* on exposure to normal visible light after a period of water stress and UV-B irradiation. The authors suggested that the plants exposed to water stress are sensitive to UV-B irradiation and that these substances could act as a protective filter against the UV-B rays. Thus, the response of increased phenolic compounds observed in black pepper cells during PEG-imposed water stress in the present study, could suggest that such an increase *in planta* could mitigate the effects of the natural UV-B radiations in black pepper vines during drought conditions.

Desmukh *et al* (2001) observed increase in total phenols in a cultivar of Sorghum bicolor that exhibited drought tolerance. The authors suggest that phenols, along with proline, reducing sugars, starch and proteins, could be additional reliable characters of drought tolerance in Sorghum bicolor. Kouki and Manetas (2002) also observed increase in total leaf phenolics and tannins under water stress in the Mediterranean evergreen sclerophyll, *Ceratonia siliqua.*

1.4.1.3. Lipid peroxidation

Lipid peroxidation was found to increase significantly in all the water-stressed black pepper suspension-cultures, irrespective of the intensity of stress. However, the amount of lipid peroxidation in cells under moderate water stress was found to be lower compared to that in cells exposed to severe stress. The significantly higher MDA content in 25% PEG-treated cultures could be due to the increased lipid peroxidation on prolongation of the stress period. It was interesting to note that though the MDA content of cells exposed to severe stress (25% PEG) was much higher than in the control and in cells under mild stress, the cells under lethal stress conditions (30% PEG) recorded a significant decline in MDA content after an initial rise. The MDA content fell even below the control levels after the 15th day. Such a decrease has not been reported in the literature. However, the decrease in MDA content observed in the present study on prolonged exposure to lethal water stress condition could be attributed to the heavy decline in growth and viability of cells exposed to 30% PEG, as a result of which the metabolic processes could have been severely inhibited with the consequent reduction in the processes leading to lipid peroxidation.

The observations made by Jiang and Zhang (2002c) on responses of maize leaves to different degrees of PEG-induced water stress are similar to the results obtained in the present study. The authors observed that lipid peroxidation was far higher under moderate stress compared to that under mild stress and it was suggested that the content of catalytic Fe which is critical for H_2O_2-dependent hydroxyl radical production (Fenton reaction) and the oxidized forms of ascorbate and glutathione pools, namely dehydroascorbate (DHA) and oxidized glutathione (GSSG) markedly increased resulting in significant oxidative damage to lipids and proteins under moderate water stress. Gogorcena *et al* (1995) also suggested that the 2-fold increase in the content of "catalytic Fe" could explain the augmented levels of lipid peroxides and oxidatively modified proteins found in water-stressed nodules of pea because of the known requirement of lipid and protein oxidation for a transition catalytic metal. Thus, the increased lipid peroxidation observed in black pepper suspension cultures under severe water stress observed in the present study can be interpreted to be due to a possible increase in the Fe^{2+} content which could have triggered the chain of processes leading to production of ROS (Fenton reaction) and lipid damage. This hypothesis also gains support from the studies of Iturbe-Ormaetxe *et al* (1998) who noticed consistent decreases in antioxidants and accumulation of catalytic Fe, malondialdehyde and oxidized proteins in pea plants exposed to water deficit. The authors suggested that the increase in catalytic Fe and the lowering of antioxidant protection may be involved in the oxidative damage caused by severe water deficit but not necessarily in the incipient stress induced by moderate water deficit. Since the antioxidant system in the black pepper cells under severe water stress in the present study was largely inhibited, unlike that observed in cells under mild water stress, it can be postulated that the weak antioxidant defenses together with a possible Fe^{2+} increase could have resulted in uncontrolled lipid peroxidation in the former. Aziz and Larher (1998) observed increase in the MDA content in excised leaf discs of rape plants when subjected to moderate osmotic stress *in vitro* and suggested that the concurrent increase in lipoxygenase, superoxide dismutase and ascorbate peroxidase enzymes were biochemical adjustments induced by the osmotic stress which could partly rely on changes in membrane lipid composition, which in turn are related to the shift in balance between the production and scavenging of the reactive oxygen species. In an earlier study, Sairam and Srivastava (2000) reported that the drought-tolerance in a particular wheat genotype, as represented by higher membrane stability and chlorophyll and carotenoid contents and lower lipid peroxidation, is related to its higher antioxidant enzyme activity. Sairam and Srivastava (2001) observed increased hydrogen peroxide and consequent lipid peroxidation and membrane injury in wheat cultivars under water stress. However, the tolerant cultivars showed lower MDA content compared to the susceptible genotypes. The authors suggested that the degree of oxidative stress and antioxidant enzyme activity were closely associated with the tolerance or susceptibility of a genotype to water stress.

1.4.1.4. Antioxidative enzymes

Molecular oxygen is essential for all aerobic organisms including plants, but reactive oxygen species (ROS) produced from it destroy cellular components. Plants therefore have developed an efficient ROS scavenging system consisting of both enzymatic and non-enzymatic components to protect themselves against the deleterious effects of these oxygen free radicals. The major enzymes involved are superoxide dismutase, catalase and peroxidase and the enzymes of the ascorbate-glutathione cycle. Smirnoff (1993) suggested that in addition to the direct measurements of free radical production or the consequence of oxidative damage (lipid peroxidation), measurements of the activity of protective enzymes can provide information about the extent to which tissue is exposed to reactive oxygen species because an increase in the capacity of antioxidative defense may occur in response to increased levels of ROS. Thus, we examined the changes in the levels of these enzymes in black pepper cells exposed to varying intensities of water stress.

1.4.1.4.1. Superoxide dismutase

Superoxide dismutase recorded a general increase in all the cultures exposed to water stress during the initial phase of the water stress, signifying increased production of superoxide radicals due to water deficit. The greater increase in the enzyme activity in cultures exposed to severe and lethal stress shows the intensity of superoxide generation and the consequent cellular damage and is an effort by the cells exposed to intense water stress to contain the damage caused by these radicals. Though there was an initial increase in SOD activity in all the water-stressed cultures, the enzyme levels declined on prolonged exposure to the stress. However, while the enzyme activity decreased sharply in cells exposed to severe stress conditions, mediated by 25 and 30% PEG after the initial steep increase, the activity in cells exposed to mild water stress remained at almost similar levels throughout the stress period. Decrease in SOD activities was also observed by Chowdhury and Choudhuri (1985) in jute and Quartacci and Navari-Izzo (1992) in sunflower under water stress. Zhang and Kirkham (1994) also observed an initial increase followed by a decline in SOD activities in different wheat species on exposure to drought stress. The authors explained the decrease in SOD activity to be either due to reduced synthesis or enhanced degradation of the enzyme. Hence, it can be supposed from the results obtained in the present study that while the steep rise in the enzyme activity in cells exposed to severe and lethal water stress (25 and 30% PEG) suggested increased ROS generation and was an attempt by the cells to scavenge the increasing amounts of superoxide radicals produced by the severe stress, the scavenging ability of the cells was depressed very soon, denoting that the enzymes could have been inactivated by the prolonged exposure to intense stress. Morever, the degradation of the enzyme also could have taken place due to increased accumulation of H_2O_2 formed by the significantly higher increase in SOD activity during the initial phases, as suggested by Zhang and Kirkham (1994), without the back-up of a concominant and sustained increase in the defense against these more toxic oxygen species evolved from superoxide radicals. It can also be suggested from the results obtained, that the maintenance of SOD activity at about the same levels in cells exposed to mild water stress throughout the stress period could be due to the similar rates of synthesis and degradation of the enzyme, since the measured enzyme activity is the net result of both synthesis and degradation of the enzyme. The decreased enzyme activities in all the water-stressed cultures compared to the control towards the end of the stress period suggests that the superoxide radical accumulation in the water-stressed cells could be more compared to the control cultures. This assumption is supported by the observation that lipid peroxidation in water stressed cultures was more compared to that in control cultures, at all stages of the stress period. The increased rate of lipid peroxidation in the water-stressed cultures till the end of the stress period could have resulted in a greater rate of inactivation of the enzyme during the latter part of the stress period, more so in the case of cells exposed to 25 and 30%. In contrast to the reports on decrease in SOD activity under water stress conditions mentioned above, an increased activity of the enzyme was observed in the tolerant line of maize, LIZA, compared to the susceptible line, LG11 (Del Longo *et al.*, 1993). Van Rensburg and Krüger (1994) also observed increased activity of SOD in tobacco on imposition of drought stress. Baisak *et al* (1994) observed increase in SOD in wheat leaves subjected to water stress by incubation in different concentrations of polyethylene glycol solutions.

Study of the different isoforms of SOD is also important in addition to the studies on total SOD activity during water stress imposition, since the isoforms have different subcellular locations (Yu and Rengel, 1999) and different metabolic roles (Bowler *et al.*, 1992). It is known that Cu/Zn SOD is commonly found in the cytosol, Mn SOD in the mitochondria and Fe SOD in the chloroplasts. Moreover, measuring the total SOD activity alone may not reveal some of the important interactions that exist between the different isoforms.

Thus, in addition to studying the total SOD activity, the expression of the activities of the different isoforms of SOD under water stress was also examined in the present study. It was observed that Cu/Zn SOD is the major isoform of SOD in control cultures. The isoform was also maintained high in cells under higher water stress conditions, especially during intense and prolonged stress. Under mild water stress, Mn SOD was observed to be the most dominant form of superoxide dismutase and after prolonged exposure to these mild stress conditions, Cu/Zn SOD also gained activity signifying that Mn SOD and Cu/Zn SOD played an important role in containing the adverse effects of the imposed water stress. However, with intensity and severity of stress (25 and 30% PEG), Fe SOD also was induced in addition to Cu/Zn SOD to control the severe damage caused by high intensity water stress. Under mild water stress, however, Fe SOD did not play a major role and was maintained at low levels compared to the activity in all other treatments. Similar to results obtained in this study, Yu and Rengel (1999) observed that total SOD activity markedly increased in narrow-leafed lupins, due to an increase in the activities of Cu/Zn SOD and Fe SOD with increasing magnitude of drought stress. However, when drought stress was relieved, the activity of Cu/Zn SOD returned to the control level while the Fe SOD activity was maintained high over the control. Mn SOD was found to be unaffected by drought stress. Thus, it was suggested that Cu/Zn SOD and Fe SOD and not Mn SOD were involved in the defense against drought-induced oxidative stress in lupins. The authors also noted that Cu/Zn SOD consistently increased with the duration of water stress while FeSOD increased significantly during the initial stages and was maintained at high levels without further increase on further prolongation of the stress period. Thus, it was suggested that Cu/Zn

SOD was primarily related to the degree of water deficit, while other mechanisms could have been involved in regulating the Fe SOD activity. Jansen *et al* (1990) also noted higher levels of Cu/Zn SOD in drought-tolerant maize inbreds.

Though there were differences in the activities of the different isoforms of SOD in black pepper suspension cultures on imposition of water stress, there were no differences in the isozyme pattern of these SOD isoforms between the control and the water-stressed cultures. Our results are similar to those reported by Ruiz Lozano *et al* (1996) who observed no changes in the SOD iso-enzyme pattern as a consequence of water stress treatment, in spite of increase in the activity of SOD.

In the present study it can be suggested that there was an increase in the different isoforms with the degreee and duration of the stress. While MnSOD alone was sufficient under mild stress conditions during the initial stages, CuSOD also gained role in containing the adverse effects of water deficit during the later stages of the mild stress. It could be suggested that Fe SOD did not play a major role under mild stress and that its induction may not be essential under mild stress conditions even after prolonged duration since the damage could be controlled with Cu/Zn SOD and Mn SOD. However, at higher intensities of water stress, Cu/Zn SOD was the major isoform from the early stages, which was followed by the increase in Mn SOD and Fe SOD after prolonged exposure to severe stress conditions. Fe SOD was active only under severe stress conditions. All the isoforms of SOD were equally active during the latter part of the severe stress, thus indicating that all the three isoforms were necessary to counter the very adverse effects of severe stress on prolonged duration. The increased activity of total SOD in 25% PEG cultures over the 15% PEG-treated cultures observed in the present study may be due to the additional higher activities of Fe SOD in the former compared to its lower activity in the latter. Malan *et al*, (1990) reported a positive correlation between drought resistance and Cu/Zn SOD activity in maize varieties. Zhu and Scandalios (1994) showed differential accumulation of the different genes of Mn SOD in response to ABA and high osmoticum. Their studies revealed that the mitochondrial Mn SODs induced during these treatments were mediated through ABA and not induced by osmoticum. By the use of ABA-deficient mutants, the authors observed that high osmoticum did not induce certain

Mn SODs induced by ABA. These isoforms of Mn SOD were thus thought to be induced through the synthesis of ABA under high osmoticum. Thus, the authors concluded that the Mn SODs are ABA-induced proteins required for plant cells to survive under environmental stress. The increase in Mn SOD in black pepper cells under mild water stress conditions can be thought to be an adaptive response. However, further studies are required to support this hypothesis. Kaminaka *et al* (1999) also reported the expression of Mn-SOD gene (sodA1) and one of the cytosolic Cu/Zn SOD gene (sodCc2) during drought stress in rice seedlings that were also inducible by ABA. They also reported that while Fe SOD gene (sodB) and another cytosolic Cu/Zn SOD gene (sodCc1) were also induced by ABA, the mRNA level of sodB was decreased and sodCc1 gene was not induced by the drought treatment. The authors suggested that the phytohormone, ABA and active oxygen species are associated with the regulation of SOD genes under environmental stresses. In the present study, it was also observed that some water-stress proteins are induced by ABA. It is tempting to presume that some of the antioxidant enzymes induced under water stress in black pepper suspension cultures could also be mediated by ABA. However, this possibility needs to be investigated further.

1.4.1.4.2. Enzymes involved in scavenging H_2O_2

Superoxide activity merely transforms one form of reactive oxygen species to a more toxic form, namely, hydrogen peroxide (H_2O_2). Removal of this more reactive oxygen species is essential, since the absence of such a mechanism would result in a chain of reactions that would affect the very survival of the cells. The various enzymes, which scavenge this highly toxic reactive oxygen species, are catalase, peroxidase and the enzymes of the ascorbate-glutathione cycle.

1.4.1.4.2.1. Catalase

In the present study, it was observed that catalase activity was severely inhibited in cultures exposed to severe and lethal water stress conditions while in the cultures exposed to mild stress, the activity showed an initial increase after which the levels were maintained only at marginally high levels than the control. Catalase scavenges H_2O_2 by

1.4.1.4.2.2. Peroxidase

Another enzyme involved in the removal of H_2O_2 is peroxidase, usually known as guaiacol peroxidase to distinguish it from ascorbate peroxidase. In contrast to ascorbate peroxidase which has a high degree of specificity for ascorbic acid as the electron donor, guaiacol peroxidases are characterized by their broad specificity with respect to electron donors. Peroxidases remove H_2O_2 by utilizing it for the oxidation of different substrates (RH_2). These substrates act as electron donors for the reduction of hydrogen peroxide, to water, thus getting oxidized in the process. Thus, peroxidases participate in a number of physiological processes, such as the biosythesis of lignin, plant development and organogenesis via the degradation of indole acetic acid (IAA) (Schneider and Wightman, 1974) and the production of ethylene, which include such oxidation reactions.

In the present study, there was an initial increase in peroxidase activity in all the water-stressed cultures over the control. However, though the increase was higher under severe water stress conditions compared to the cultures exposed to milder stress, the levels dropped in the former by the 15th day of exposure to PEG. The cultures exposed to milder stress showed gradual increase in activities and peaked on the 15th day but dropped by the 20th day of exposure to stress. Similar increase in activity of peroxidase was observed by Zhang and Kirkham (1994) in wheat species. Increase in peroxidase activity was also observed by Chakraborty *et al* (2001) in tea on imposition of drought stress.

Increased peroxidase activity is an attempt by the cells to counteract the adverse effects of the production of hydrogen peroxide. Increased peroxidase activity during water stress has been suggested by Zhang and Kirkham (1994) to be due to the release of H_2O_2 from the membranes with which peroxidase is usually associated. Other explanations given by the authors for the increase in peroxidase are that it could be due to the synthesis of the enzyme *de novo* during water stress or it could also be due to the possibility that water stress could increase the substrates for peroxidase, such as glutathione, ascorbate and phenolics that act as scavengers of active oxygen species. The increase of these metabolites during water stress to scavenge the increasing amounts of

H_2O_2 would necessitate increase in the activity of peroxidase since peroxidase is required to catalyze the oxidation of these electron donors to facilitate the transfer of electrons from these substrates to H_2O_2, thus helping them in the ROS scavenging ability of these substrates.

In the present study, however, higher activities of peroxidase in the water stress treatments could not be sustained till the last day of the stress period, though the levels were marginally high in the cultures under mild water stress. Hence, it could be inferred that peroxidases were not active enough to scavenge the increasing levels of hydrogen peroxide in black pepper cells.

1.4.1.4.2.3. Enzymes of the Ascorbate-Glutathione cycle

A more efficient way of scavenging hydrogen peroxide that is primarily produced in the chloroplasts due to the disruption of the electron transport chain present in these organelles, is by the concerted action of a set of enzymes of the ascorbate-glutathione cycle (AGC), which is present both in the cytosol and the chloroplasts.

1.4.1.4.2.3.1. Ascorbate peroxidase

It was noticed in the present study that the enzymes of the AGC were more active than the catalases and peroxidases in all the water stress treatments. Ascorbate peroxidase activity increased significantly over the control in all the water stress treatments, the initial increase being more in cultures exposed to increasing severity of the stress. However, the activity in cultures exposed to lethal water stress conditions (30% PEG) declined by the 5th day and were below the control levels after the 10th day. The activities remained higher in cultures exposed to severe and mild water stress conditions, the activity being more in the latter than in the former. Similar results were reported by Baisak *et al* (1994) in wheat. AP activity increased in wheat leaf segments exposed to mild water stress induced by polyethylene glycol. However, severe water stress resulted in decline in the AP activity below the control levels. The authors suggested that the chloroplast-generated H_2O_2 can be scavenged only at low levels of water stress and the efficiency of the H_2O_2-scavenging system declines under severe water stress conditions in wheat. Ascorbate peroxidase is the first enzyme of the AGC-pathway that detoxifies H_2O_2 using ascorbate as the reductant (Nakano and Asada, 1981). The severe decline in AP activity in cultures exposed to lethal water stress conditions observed in the present study may be due to the inactivation of the enzyme by the high accumulation of H_2O_2 in these cultures exposed to extreme water stress. Thus, though ascorbate peroxidase (AP) scavenges H_2O_2 more efficiently than catalase, its activity is inhibited by the rising H_2O_2 levels.

The chloroplastic fraction of cells had lower AP activity compared to that of the cytosolic fraction but all the water stressed cultures maintained higher activity than the control. Though the chloroplastic AP activity of the cultures exposed to severe and lethal stress showed an initial sharp increase, the levels declined by the 10th day. However, the enzyme activity in cultures exposed to mild stress increased gradually and remained higher than all the other treatments after the 10th day. This again shows that the severity of water stress triggers higher enzymatic activity to control the damage caused by the reactive oxygen species but the activity declines soon which may be due to the inactivation of the enzyme by the high levels of ROS on prolongation of the stress period. The decline in the antioxidant capacity under severe and lethal stress may also result from a restricted supply of NAD(P)H for the ascorbate-glutathione pathway and from the Fe-catalyzed Fenton reactions of ascorbate and glutathione with activated oxygen, as suggested by Gogorcena *et al* (1995) from his studies on the antioxidant defenses against activated oxygen in pea nodules subjected to water stress. This hypothesis for the reason of decline in the antioxidant enzymes in black pepper suspension cultures under severe and lethal water stress however needs to be confirmed. The gradual increase and maintenance of activity at higher levels in cultures exposed to mild water stress conditions suggests that the cultures are able to maintain a balance between the production and removal of ROS by an active AGC-pathway, thereby maintaining growth, as was observed in the present study.

Del Longo *et al* (1993) reported higher ascorbate peroxidase activity in drought resistant maize, LIZA than in the susceptible line, LG11. Zhang and Kirkham (1996) observed decreased activity of AP and MR, but increased activity of DR in the cytosolic fraction of sorghum under drought stress. In sunflower however, the authors noted that the cytosolic acitivities of AP and MR in the drought-stressed plants remained on par with the control, while the DR activity increased. The enzymatic activities of chloroplastic fractions in sorghum and sunflower were not affected by drought, except for the AP activity in sunflower, which showed an increase under drought conditions. It was observed that activities of the enzymes involved in AGC were generally higher in the cytosolic fraction than in chloroplasts of sorghum, whereas in sunflower, the activities were usually higher in chloroplasts than in the cytosolic fraction.

In the present study, it was observed that catalase and peroxidase activity increased only marginally in the water stressed treatments over the control, suggesting that these enzymes could have only a very limited role in the antioxidant machinery of the cells during water stress. The ascorbate peroxidase activity was however shown to increase significantly over controls in water-stressed cultures, showing that AP has a major role in scavenging hydrogen peroxide formed under water stress conditions. Studies of Sairam *et al* (1998a) however revealed that in wheat, catalase also was important in addition to ascorbate peroxidase in imparting tolerance against drought-induced oxidative stress. The authors observed that superoxide dismutase activity, however, did not show significant differences among the genotypes under irrigated as well as water stress conditions, similar to results obtained in the present study.

AGC is considered a more efficient enzymatic way to break down H_2O_2 than CAT and POD (Cakmak *et al.*, 1993). The main difference between the two H_2O_2-scavenging enzymes, AP and CAT, is the consumption of NADPH (the reducing power) in the former, so as to make available more $NADP^+$ for further reduction by electrons from ferredoxin of the electron transport chain. Since oxidation of NADPH by the ascorbate-glutathione cycle provides PS I with additional electron acceptors, i.e., $NADP^+$, AP is considered to be superior to catalase in the ability to remove excess electrons from the over-reduced electron transport chain that results during water stress conditions or by the exposure of the chloroplasts to excess excitation energy during drought stress. Thus, it can be suggested that the higher activity of AP than catalase and peroxidase helps the cells, especially those exposed to milder stress, to sustain the normal functioning of the electron transport chain and maintain the ROS at permitted levels, thus containing to a considerable extent, the damage caused by them.

1.4.1.4.2.3.2. Dehydroascorbate reductase

Cytosolic dehydroascorbate reductase (DHAR) increased in all water-stressed cultures above the controls, the increase being more in cultures exposed to severe and lethal water stress. However the cultures exposed to lethal water stress declined immediately after an initial sharp increase though the levels were maintained over the control till the end of the experiment. The 25% PEG-exposed cultures showed higher activity than the cultures exposed to mild stress (15% PEG).

The chloroplastic DHAR activity was lower than the cytosolic activity but the activity was maintained at higher levels in all the water stressed-cultures compared to the control. The activity was maintained at steady levels in cultures exposed to mild stress than in cultures exposed to severe water stress, in which the activity showed a significant increase from the beginning of the stress period, but immediately declined.

The increased activity of DHAR in water-stressed cultures signifies the effort by the cells to replenish ascorbate used up by the ascorbate peroxidase activity. Higher DHAR levels sustain higher levels of ascorbate, which keeps the AGC-pathway live under water stress conditions. Zhang and Kirkham (1996) reported higher cytosolic DHAR activity in sorghum and sunflower during drought stress. The authors suggested that the higher enzymatic activities of the AGC in sorghum than in sunflower could explain the relatively low lipid peroxidation in the former than the latter. In the present study it was observed that the lipid peroxidation was more in 25% PEG-treated cultures than the cultures exposed to mild water stress, which showed maintenance of steady levels of all the AGC enzymes studied, thus keeping the reactive oxygen species under controllable amounts. Kubo *et al* (1999) studied the effects of various environmental stresses on the activities of several antioxidant enzymes in *Arabidopsis thaliana* and observed that water deficiency enhanced the activities of DHAR and guaiacol peroxidase.

1.4.1.4.2.3.3. Glutathione reductase

Glutathione reductase increased in all the water-stressed black pepper suspension cultures, with the cells under mild stress maintaining higher levels than the controls throughout the stress period. The activity however declined below control levels by the 10th day in cultures exposed to severe and lethal stress, showing that the enzyme activity could not be maintained on prolongation of the severe water stress conditions. GR catalyzes the rate-limiting step of the ascorbate-glutathione pathway. The increase in GR activity results in an increase in the ratio of $NADP^+$ / NADPH, as suggested by Baisak *et al* (1994), thereby, ensuring the availability of $NADP^+$ to accept electrons from the photosynthetic electron transport chain. This would help minimize the flow of electrons to O_2, consequently minimizing the formation of $O_2^{\cdot -}$. Elevated levels of GR also maintain a high ratio of GSH/GSSG, which is required not only for the regeneration of ascorbate but also for the activation of several chloroplastic CO_2-fixing ezymes. Baisak *et al* (1994), while studying the active-oxygen scavenging enzymes of wheat leaves subjected tow water stress induced by different concentrations of polyethylene glycol, observed that the GR activity increased in all the water-stressed leaves compared to the control but showed decline on increasing severity of the stress. Farrant, (2000) observed increase in glutathione reductase in angiospermic resurrection plants, *Myrothamnus flabellifolius* and *Xerophyta humilis* on dehydration. Jiang and Zhang (2002 a) observed increased glutathione reductase activity in maize leaves exposed to PEG-induced water stress. It was also noticed that the activity was higher in leaves exposed to milder water stress and that the leaves exposed to moderate stress failed to sustain the activities after a long exposure to stress, similar to results seen in the present study.

There are several reports that support the view that a concerted action of several antioxidant enzymes help in controlling the adverse effects of ROS in plants under water stress. Del Longo *et al* (1993) suggested that the high-level activity not only of SOD, but also of catalase, ascorbate peroxidase and glutathione reductase in the drought-tolerant line of wheat, LIZA, could be responsible for lower levels of $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$, which could in turn be the reason for the tolerance of the plant.

Loggini *et al* (1999) observed that the drought-sensitive cultivar of wheat, Adamello showed increase in GR activity on imposition of drought, which returned to normal control levels once the stress was relieved. However, the drought-tolerant cultivar, Ofanto, did not show any increase in the enzyme activity, showing that the levels of GR and hydrogen peroxide-glutathione peroxidase (GP) activities sufficient to maintain the balance of cellular components were present in Ofanto. They also suggested that AP and monodehydroascorbate reductase and other antioxidant enzymes may also play a role in maintaining low levels of hydrogen peroxide in the cells. In the sensitive cultivar, all the defense mechanisms returned to control levels rapidly upon rehydration suggesting that the defense mechanisms in this cultivar prevent plants from suffering irreversible damages during drought, while the more drought-tolerant cultivar, Ofanto avoids drought stress by maintaining high photosynthetic activity and does not suffer an oxidative stress high enough to trigger the defense mechanisms active in cv Adamello.

Yu and Rengel (1999) suggested that an increase in enzyme activities under mild drought stress could be indicative of increased production of ROS and/or build-up of a protective mechanism aimed at reducing oxidative damage triggered by water deficit experienced by plants. Thus, the enzyme activities induced by the mild stress conditions in the present study can be assumed to be of adaptive value and that the initial high increase of the enzyme activities induced by the severe stress may be the attempt to overcome the high intensity damage initiated in the cells from the initial stages of the stress period. Some of the responses elicited could also be due to a consequence of stress after an initial effort by the cells to adapt to the stress. The responses of the cells to mild stress were gradual and were sustained at balanced levels throughout the stress period, indicating that they may be adaptive mechanisms to the stress. However, further studies may be needed to confirm this.

Farrant (2000) compared the mechanisms of protection against oxidative stress induced by dehydration in the angiosperm resurrection plants and found that the activity of antioxidant enzymes increased during drying and remained high at low water contents in all species, ameliorating free radical damage from both photosynthesis and respiration. However, the nature and extent of antioxidant up-regulation varied among the species.

Sairam and Srivastava (2001) also noticed increased antioxidant activity in water stress tolerant cultivars of wheat compared to the susceptible cultivars and suggested that the degree of oxidative stress and antioxidant activity are closely associated with the tolerance or susceptibility of a genotype to water stress.

Jiang and Zhang (2002a&b) based on their studies on the inter-relationship among water stress-induced abscisic acid accumulation, the generation of reactive oxygen species and the activities of several antioxidant enzymes, namely, SOD, catalase, AP, GR in leaves of detached maize plants exposed to PEG-induced water stress, observed that water stress-induced ABA accumulation triggers the increased generation of ROS, which in turn, leads to the up-regulation of the antioxidant defense system. Thus, the increase in the antioxidant enzymes observed in the present study could be assumed to due to the increase in ROS. A possible role for ABA in mediating the water stress responses was observed in black pepper suspension cultures in the present study (section 4.1.1.5., below) and therefore, it can be postulated that ABA may have a role in increasing the activities of the antioxidant defense enzymes in black pepper throught the production of ROS. However, this hypothesis needs to be confirmed by further studies. In another study, Jiang and Zhang (2002c) noted that mild water stress mediated by PEG increased the generation of superoxide radical and hydrogen peroxide and consequently the activities of superoxide dismutase, catalase, ascorbate peroxidase (AP) and glutathione reductase (GR) and antioxidant metabolites like ascorbate and reduced glutathione. However, the moderate water stress failed to further enhance the capacity of antioxidant defense systems compared to the mild stress, which together with increased hydroxyl radical production due to increase in catalytic Fe resulted in severe damage to lipids and proteins. This explanation could support the observation obtained in the present study in which the activities of antioxidant enzymes were severely inhibited in black pepper suspension cultures exposed to severe stress and caused higher lipid peroxidation and damage, resulting in highly inhibited growth of the black pepper cells under these stress conditions. In the cells under mild stress however, the antioxidant enzymes remained active for a long period of time during the water stress imposition.

1.4.1.5. Water stress proteins

Polyethylene glycol-induced water stress resulted in the induction of three major polypeptides in black pepper suspension cultures with molecular weights, 39.94, 26.27 and 17.71.

The results obtained in the present study are similar to those reported by Rao *et al* (1993) in rice seedlings in response to PEG-induced water stress. The authors reported the induction of three polypeptides with apparent molecular weights of 15, 23 and 46kD. In the present study too, the apparent molecular weights of the proteins induced in black pepper suspension-cultured cells during PEG-imposed water stress were in this range, viz., 17.71, 26.27 and 39.94. Bray (1990) also noticed differential expression of several proteins in tomato leaves on imposition of drought stress. While a few proteins increased during the stress period, the others decreased on prolonged exposure to stress. Recovery from water deficit resulted in the loss of the drought-induced polypeptides and the increase in several others that are characteristic of the non-stress condition. Drought-induced poloypeptides have been found in many plants (Perez-Molphe-Balch *et al.*, 1996; Riccardi 1998). The dehydrin family of proteins accumulates in a wide range of plant species under dehydration stress [late embryogenesis abundant (LEA) D11 family], which range in size from 9 to 200 kDa (Close, 1996). There are several reports on induction of a number of dehydrin-like proteins during dehydration or drought stress (Han and Kermode, 1996; Pelah 1997). Wechsberg *et al* (1994) found that the accumulation of 18-, 28-, 31-kDa dehydrin-like proteins in the seeds of crowfoot (*Ranunculus scleratus*) depended on different stages of water stress. Jiang and Huang (2002) also observed induction of dehydrin proteins with polypeptides in the range of 23-60kDa, especially, 23 and 27 kD, in tall fescue cultivars on imposition of drought stress and the intensities of these proteins increased with the increase in intensity of drought stress. Dehydrins are hydrophilic, and heat stable and may protect other proteins and help maintain the physiological integrity of cells (Bray, 1993; Close 1993). Accumulation of dehydrin proteins could protect plant cells from further dehydration during drought stress (Cellier *et al.*, 1998; Han & Kermode, 1996).

In the present work, treatment of cultures with ABA also resulted in the induction of the water stress-induced polypeptides indicating that the induction of these proteins to PEG-induced water stress may be mediated by ABA. The induction of the same set of proteins induced during water stress and by ABA treatment points to the possibility that ABA may have a role in mediating the water stress response. Similar observations were made by Rao *et al* (1993) who observed that the proteins induced during PEG-induced water stress in the rice seedlings were also induced by ABA treatment. Dehydrin synthesis in response to ABA has also been observed by Cellier *et al* (1998) and Giordani *et al* (1999).

ABA has been known to increase in response to drought stress in many plants (Zeevaart and Creelman, 1988; Popoya *et al*, 2000). ABA has been implicated a role in modulating specific gene products (Bray, 1988; Guerrero and Mullet, 1988; Alves and Setter, 2000) and coordination of the stress response (Hanson and Hitz, 1982; Jiang and Zhang, 2002a, b, c). Bray (1990) noticed that there was a correlation between the increase in the majority of the polypeptides during drought stress and ABA accumulation. It was also reported that ABA returned to non-stress levels after prolonged exposure to stress, which again correlated with the decrease in the polypeptide levels indicating that ABA plays a role in the regulation of protein accumulation during drought stress. Mao *et al* (1995) observed a correlation between dehydrin gene transcript level and endogenous ABA content in maize.

It was also observed in the present study that small polypeptides with molecular weights, 9.8 and 6.45 were induced in cultures exposed to PEG as well as ABA-pre-treated cultures exposed to PEG but were missing in cultures treated with ABA alone. The presence of certain polypeptides in black pepper cultures treated with PEG or ABA-pre-treated PEG-exposed cultures and not in the cultures treated with ABA alone points to the possibility that these polypeptides are induced on PEG treatment and were independent of pre-treatment with ABA. This indicates that these responses of cells to water stress may not be mediated by ABA. It can thus be presumed from these results that there are both ABA-dependent as well as ABA-independent pathways that mediate the responses of black pepper cells to water stress.

The pathways of expression of dehydrins have been found to be either ABA-independent or only dehydration-dependent (Espelund 1995; Whitsitt et al., 1997). Yamaguchi-Shinozaki and Shinozaki (1994) also reported that ABA-independent and the ABA-dependent signal transduction pathways might exist between stress and dehydrin gene expression. Giordani et al. (1999) suggested that these two pathways of regulating dehydrin transcript accumulation might have cumulative effects. Jiang and Huang (2002), based on their experiments on tall fescue, suggested that the enhancement of drought tolerance by ABA application, as manifested by higher turf quality during drought stress, was not related to the induction of dehydrins in tall fescue. ABA-treated plants maintained higher RWC and lower electrolyte leakage. The ability to distinguish different sets of polypeptides during drought stress may provide some clues about the mechanism of induction of drought-induced polypeptides.

It was also observed in the present study that two major polypeptides of molecular weight 40.94 and 32.8, present in the control cultures disappeared on treatment with PEG or ABA, indicating that water stress severely inhibited the two major proteins present in the black pepper cells. The fact that ABA treatment also results in the disappearance of these major proteins also indicates the possibility that ABA simulates certain water stress responses in black pepper.

Basic studies on the changes and modulations of different drought stress-induced proteins would help identify physiological traits that could be incorporated into breeding programmes to improve drought tolerance of black pepper. Since not many studies have been undertaken in this direction, the objective of this study was to determine the alteration in soluble proteins of black pepper cells subjected to water stress.

Since rehydration studies were not taken up in the present work, the status of these polypeptide changes after recovery from water stress is unknown. Hence, further work needs to be done to implicate a role for these proteins during water stress. Implication of any adaptive value to the induced proteins during water stress also needs further studies. Further, whether ABA does accumulate in the cells during the PEG-induced water stress was also not assessed. However, it can be postulated based on the induction of similar proteins by both ABA and water stress that the levels of ABA may be indeed modulated during water stress. It can also be supposed from the results obtained in the present study that there are different mechanisms that regulate the synthesis of different polypeptides induced during water stress in black pepper suspension cultures. The hypothesis gains support from the suggestion made by Jiang and Zhang (2002b &c) that ABA plays an important role in water stress responses in maize leaves. Based on their extensive studies on responses of maize leaves to ABA treatment and PEG-induced water stress, the authors suggested that water stress induces accumulation of ABA which in turn triggers the production of active oxygen species (AOS) mediated by membrane-bound NADPH-oxidase, resulting in the induction of antioxidant defense systems against oxidative damage in plants. The authors observed increase in the activity of plasma membrane associated NADPH-oxidase, production of ROS and the activities of several antioxidant enzymes and antioxidant metabolite content on treatment of exogenous ABA and water stress in maize leaves.

Cell cultures have been used to study the qualitative and quantitative changes in polypeptides under stress conditions (Reddy *et al.*, 1993; Leone *et al.*, 1994) Reddy *et al* (1993) reported induction of a 23 kD polypeptide in rice suspension cultures on imposition of PEG-induced water stress which was the same as the one induced in intact rice seedlings, proving that synchronized cell populations of rice could be used as a rapid and reproducible experimental test system for evaluating osmotic stress responses and for studying genetics and molecular biology of stress responses in rice.

The present study was an attempt to observe the changes in protein expression taking place in black pepper cells under *in vitro* water stress conditions to understand the metabolic changes taking place during water stress. Implicit in this study is the assumption that black pepper cells react similarly to water stress under both *in vitro* and *in vivo* conditions. However, the results obtained in the present study need to be confirmed by *in vivo* studies in intact plants to demonstrate that the cell suspension cultures used in the present study can serve as a model system that can be exploited for studying various *in vivo* stress responses under *in vitro* conditions. Studies on polypeptide changes in black pepper during water stress have not been studied in detail except for a report by Thankamani (2001). Since there are many reports that ABA- and water stress-induced proteins are also induced during late embryogenesis in the seeds of different plants (Espelund *et al.*, 1992; Curry *et al.*, 1991; Curry *et al.*, 1993; Kiyosue *et al.*, 1994a) it would be interesting to study if the proteins induced in PEG-treated cultures observed in the present work are similar to the late embryogenesis abundant (LEA) proteins that may be induced during the latter part of the developmental and maturing phase of the seeds of black pepper and also to study and compare the functions of these proteins.

In recent years, the list of genes whose transcription is up-regulated in response to water stress is increasing and the functions for some of the polypeptides that these genes encode are being studied and their likely role in stress physiology is being determined (Kiyosue *et al.*, 1994b; Urao *et al.*, 1994; Koizumi *et al.*, 1993; Velasco *et al.*, 1994; Ishitani *et al.*, 1995; Bray, 2002). In a recent review, Bartels and Nelson (1994) state that the understanding of the mechanisms which regulate gene expression and the ability to transfer genes from other organisms into plants will expand the ways in which plants can be utilized. The identification of induced-proteins is one of the first steps to study the differential gene expression during water stress, which in due course would aid in isolating genes correlated with water stress tolerance. Since studies on the metabolic processes that occur during water stress in black pepper are meagre, the present study on induction of water stress proteins is an initial step towards the goal of improvement of black pepper for drought tolerance. The characterization of these proteins would be a beginning to determine if these polypeptides have any adaptive value under water stress conditions in black pepper.

To summarize the studies on responses to abiotic stress, it can be said from the the present study that the black pepper cells exposed to mild water stress exhibited steady maintenance of certain physiological parameters during the entire stress period though quantitatively, some of these parameters did not match those exhibited by cells under severe and lethal water stress during the initial stages of the stress period. It was further observed that in general, the cells exposed to stringent stress conditions initially responded in a manner that attempted to overcome the deleterious effects of the imposed water stress, but failed to sustain these responses as the stress period progressed. The heightened physiological responses in these cells during the initial stages of the stress period were the same as that exhibited by cells under mild stress, but did not keep pace with the prolongation of the stress unlike the cells under mild stress, in which the physiological responses to water stress were maintained steady. This therefore, could possibly be the reason for the inhibition of important metabolic processes with the resultant physiological damage and inhibition of growth in cells under severe and lethal water stress. Thus, it can be assumed that the responses elicited and maintained steady by cells under mild water stress conditions but are severely inhibited in cells under severe water stress on prolongation of the stress period may possibly be those that help adapt the cells to water stress and therefore can be considered as adaptive responses or traits of tolerance.

In studies involving the identification of tolerant responses of plants to water stress, responses of a drought tolerant cultivar are compared with that of a drought-sensitive cultivar. However, in the absence of a truly drought tolerant cultivar in black pepper, this study was taken up with a view to analyze the differential responses of black pepper cells to varying intensities of water stress, with a postulation that if the black pepper cells could tolerate mild water stress conditions to a great extent and maintain sufficient growth, the responses elicited by the cells under these mild stress conditions *in vitro* could simulate the putative tolerant responses. It was observed in the present study that the cells under mild stress conditions exhibited sustained metabolic responses that are known to be adaptive and impart protection under depleting moisture conditions in many plants. The responses of these cells exposed to mild water stress could thus be postulated to simulate the putative tolerant responses as these cells are capable of maintenance of sufficient growth and metabolic processes even under the stressed conditions.

Thus the results obtained in this work, satisfy the objectives proposed before the study was taken up, by providing leads in identifying and implicating certain responses of black pepper cells as those that may be adaptive to water stress. Further detailed studies based on this basic information could in future lead to improvement of the crop for drought tolerance.

1.4.2. *In vitro* selection studies

1.4.2.1. Response of black pepper cells to polyethylene glycol

In studies involving *in vitro* selection of stress tolerant plants, it is essential to regenerate plantlets from the adapted cell lines after their exposure to the selection regimes, so as to have a fruitful meaning for the selection experiments. Hence, the effect of PEG on the growth and differentiation of black pepper cells was determined in the present study.

1.4.2.1.1. Effect of PEG on callus growth

It was observed that the growth and differentiation of black pepper cells was greatly inhibited at increasing concentrations of PEG 6000. While PEG at lower concentrations, viz., 5 and 10% did not affect these parameters significantly, higher concentrations significantly affected them. Barakat and Abdel-Latif (1995) also reported decrease in relative growth of callus of different wheat cultivars with increasing concentrations of PEG. Geetha *et al* (1995) observed complete inhibition of callus growth of *Vigna mungo* at 30% PEG, similar to observations made in the present study. Bressan *et al* (1981) reported similar decrease in the relative growth of tomato callus with increasing concentrations of PEG in the nutrient medium. Bressan *et al* (1982) explained the reduction in fresh weight of callus in tomato on exposure to 30% PEG to be due to the reversal of the gradient favoring uptake of water by cells from the surroundings, thus causing water to leave the cells.

It was interesting to observe in the present study that though there was a decrease in fresh weight gain by cells exposed to higher intensity of stress compared to the control, the dry weight was not much affected, suggesting that the black pepper cells on exposure to higher concentrations of PEG maintain dry weight accumulation even during water stress that did not favour increase in their fresh weight. Similar increase in dry weight in

PEG-adapted cells was observed by Handa *et al* (1982) and Geetha *et al* (1995). Dry weight increase could be due to the increase in solutes in cells exposed to water stress as observed in the present study.

1.4.2.1.2. Effect of PEG on regeneratbility of callus

The study on regeneration ability of cells subjected to different PEG concentrations showed that the differentiation of embryos from the embryogenic cells decreased with increasing PEG concentration.

It was interesting to observe that 5% PEG promoted growth and differentiation of the cells and was on par with the control treatment. The fresh weight increase in 5% PEG-amended medium was comparable with that observed in the control cells. On transfer back to the control medium, the 5% PEG-exposed cultures exhibited embryo development which was also on par with the control treatment. The higher concentrations of PEG however resulted in progressive decrease in the growth and regenerability of the cells. 20 and 25% PEG highly inhibited the differentiation, while 30% caused complete inhibition of regenerability, showing that increasing intensity of stress severely affected differentiation of embryos, while lower concentrations did not significantly affect the regenerability. It was further interesting to note that on prolonged exposure to PEG, the differentiation of 5% PEG-exposed cultures in terms of embryo forming units in the PEG-amended medium was higher than that observed in the control cultures. The differentiation of cultures in PEG medium however decreased with increasing concentrations of PEG, similar to the trend observed on return of the PEG-exposed cultures to the control medium.

Promotion of growth and differentiation of embryos by low levels of polyethylene glycol has been reported by many groups. Geetha *et al* (1995) reported stimulation of growth in *Vigna* callus on exposure to low concentrations of PEG, namely, 5-10%. Walker and Parrott (2001) also reported improved germination frequencies in soybean somatic embryos by supplementing the nutrient medium with 5% polyethylene glycol, similar to the observations made in the present study with black pepper cells. It is also well known that partial dessication of mature somatic embryos improves conversion frequencies (conversion of embryos to plants) (Hammatt and Davey, 1987). Plasmolyzing (D-mannitol and d-sorbitol) as well as non-plasmolyzing (polyethylene glycol) osmotica have been shown to enhance germination of embryos from a variety of different plant species (Finkelstein and Crouch, 1986; Attree *et al.*, 1991). Non-penetrating osmotica, like polyethylene glycol, that cannot pass through the cell wall and therefore do not cause plasmolysis, are known to be more effective in promoting somatic embryo maturation in some conifer species. Finkelstein and Crouch (1986), Egli (1990) and Xu *et al* (1990) based on their studies in rapeseed, soybean and alfalfa showed that embryo maturation is frequently associated with a low osmotic potential in tissues or medium surrounding the embryo. A short dessication period is known to be an important phase during the later stages of embryo development. Hence, the increase in the regeneration of black pepper embryos in the 5% PEG treatment over the control on prolonged exposure observed in the present study could be attributed to the supplementation of this dessication phase by polyethylene glycol, resulting in a better differentiation of embryos comparable to the control.

It was also observed in the present study that concentrations of PEG beyond 15%, namely, 20 and 25% failed to promote differentiation of embryos. Walker and Parrott (2001) also observed that 5% PEG was the only concentration that significantly improved embryo germination and conversion in soybean compared to the control. Further, they also observed that globular-stage embryos transferred to a medium containing 20% PEG failed to histo-differentiate and turned necrotic. This was similar to the observations made in the present study in which the black pepper embryos which did manage to differentiate into embryos from the callus subjected to 20 and 25% PEG, failed to develop further on prolonged exposure to the stress, showing that these high concentrations of PEG were highly inhibitory for the growth and development of embryos.

Regenerability of callus was further inhibited when cells exposed to different PEG concentrations, were further subjected to higher PEG levels. This suggested that the severity and length of the stress period highly influenced the regenerability of the callus. However, it was seen that 5% PEG-exposed cultures performed better than cultures exposed to higher concentrations, when they were further subjected to a mild water stress mediated by 15% PEG. It could be suggested that 5% PEG induced adaptability in cells to tolerate greater intensity of stress. It was also observed that cells subjected to 20 and 25% PEG did not regenerate into embryos when they were further exposed to 15% PEG, suggesting that these high levels of PEG caused irreversible damage to the regenerating ability of these cells. However, these cells could maintain cell multiplication during the imposition of the stress, on the control medium. The 30% PEG-exposed cultures on the other hand showed no signs of growth, which may be due to the lethal level of stress imposed.

In short, it was observed in the present study that while 5-15% PEG did not affect the growth and differentiation of the embryogenic callus, concentrations 20 and 25% affected both these parameters to a great extent. However, callus growth was maintained, though differentiation of embryos was highly inhibited. Concentrations 30 and 40% severely inhibited both growth of callus and differentiation of embryos. Hence, in the present study, 15%, 25 and 30% were considered to induce mild, severe and lethal water stress respectively.

1.4.2.2. *In vitro* selection

In vitro selection of black pepper cells for water stress tolerance was carried out by both direct as well as step-wise selection methods. It was observed that cells selected by the step-wise method could adapt better to higher severity of PEG-induced water stress than when selected by the direct method. Exposure of cells to milder water stress mediated by 15% PEG aided better growth and differentiation of these cells in 25% PEG-amended medium.

1.4.2.2.1. Step-wise selection

From the growth and differentiation assays taken up in the present study, it was observed that 15% PEG induced moderate water stress that supported both callus multiplication as well as differentiation. Hence, cells were first adapted to the moderate stress induced by 15% PEG, before subjecting them to severe stress mediated by 25%

PEG. This helped a better adaptation of the cells to 25% PEG, a concentration that severely inhibits embryo development. However, though callus selected on 15% PEG produced embryos when transferred to 25% PEG-amended medium, it was observed that both the callus and embryos could maintain only minimal growth in this medium. Hence, they were returned back to the control medium to develop further. It was interesting to note that these calli and embryos blackened immediately on transfer to the control medium suggesting an osmotic shock due to the sudden transfer from a high water stress regime to a hypotonic medium. Handa *et al* (1982) also reported similar osmotic shock experienced by 25% PEG-adapted tomato cells, on transfer to control medium. Santoz-Diaz and Ochloa-Alejo (1994) also observed the dramatic decrease in survival of chilli pepper cells selected at 25% PEG on the control medium. Handa *et al* (1982) suggested that the 25% PEG-adapted tomato cells that experienced osmotic shock on the control medium grew better in 25% PEG medium than in the PEG-free medium, suggesting stability of the adaptation to water stress conditions. However, in the present study, it was noted that though the cells selected on 25% PEG by the step-wise method experienced osmotic shock on their transfer back to the control medium, the tolerance to 25% PEG was not stable. These cells and embryos had the ability to adapt to 25% PEG-induced stress, on prolonged exposure to PEG and maintained limited growth. But, on transfer to the control medium, the cells resumed multiplication after the initial osmotic shock. The embryos developed from these cells could however establish better in the control medium than in medium amended with 25% PEG. This was proved by testing the growth of the embryos obtained by the stepwise selection method from the 25% PEG-adapted callus by transferring them to media amended with different concentrations of PEG, viz., 10, 15 and 25%. It was observed that the embryos grew better in the control medium and the number and growth of seedlings decreased with increase in the PEG concentration. The decrease in 10 and 15% PEG-amended medium was similar and moderate. However, the growth of seedlings in 25% PEG-amended medium was severely inhibited. These seedlings however sustained in this medium without significant growth for 2 months, when the experiment was concluded. Hence it can be concluded from the present study that step-wise selection method resulted only in the adaptation of the cells to higher intensity of water stress (25% PEG) and could not induce tolerance.

Acquisition of tolerance to water stress should enable the cells to survive, multiply and grow under water stress conditions as reported by Handa *et al* (1982). However, since the black pepper cells that were found to be putatively tolerant to 25% PEG medium in the present study did not multiply or differentiate significantly in 25% PEG medium after a transient passage through the control medium, it can be suggested that the putative tolerance observed is only an adaptation to the stress and not a stable tolerant trait. Bresssan *et al* (1981) also reported adaptation as a likely mechanism in tomato cells by which resistance was increased at 25% PEG after selection.

1.4.2.2.2. Direct selection

In the direct selection method, it was observed that cells exposed to longer duration of intense stress, mediated by 25% PEG, lost the ability to regenerate into embryos, unlike the cells selected by the step-wise method. However, the cells maintained growth in the stress medium though the biomass accumulation by these cells was not as significant as that of control, non-selected cells. These cells were therefore transferred to the control medium for increasing their biomass. However, when these cells were returned back to the 25% PEG medium, the growth was significantly inhibited compared to their growth in the PEG-free medium. However, these cells grew better in 15% PEG- mediated moderate water stress. These results again suggest that the cells were not truly tolerant to 25% PEG-mediated severe water stress, the conditions under which they were selected over a period of 6 months, but were adapted to the stress conditions due to their prolonged exposure to the imposed stress.

Hence it could be concluded from the present study that the tolerance of embryos and cells selected by the step-wise selection method or of the cells selected by the direct method, was not stable. However, these could adapt to the severe stress conditions by maintenance of minimal growth on the stressed media.

Reports of successful development of water stress-tolerant plants from cells selected for PEG- tolerance *in vitro* are meagre. Sumaryati *et al* (1992) reported the regeneration of tolerant plants from cells selected for water stress tolerance. Barakat and Abdel-Latif (1995) reported shoot regeneration in certain wheat varieties from embryogenic calli subjected to direct selection method by exposing these calli to higher concentrations of PEG whereas, the calli subjected to step-wise selection method did not regenerate into shoots. Siddeswar and Kavi Kishor (1989) reported the regeneration of plants from polyethylene glycol adapted callus of rice. However, the concentrations of PEG to which the calli were exposed, were very low, the highest concentration tried being 5%, which is the lowest concentration tried in the present study. Moreover, it can be argued that since it has been reported that PEG at lower concentrations promotes and supports growth and differentiation similar to control cultures in many plants (Walker and Parrott, 2001, Geetha *et al*, 1995), the plantlets regenerated at 5% PEG reported by Siddeshwar and Kavi Kishor (1989) may be due to the promotive effects of the low concentrations of PEG used in the culture medium. These however may not be tolerant to higher concentrations of PEG at which plant cells are usually selected *in vitro* for water stress tolerance.

Though the reports of successful development of plants tolerant to water stress by *in vitro* selection are few, there are several reports of selection of cell lines tolerant to PEG-induced water stress in different plants. Bressan *et al* (1982) reported the isolation of 25% PEG-adapted tomato cell lines that failed to develop into plants. Santos-diaz and Ochoa-Alejo (1994) reported isolation of PEG-tolerant cell lines of chilli pepper by step-wise selection method, capable of growing in the presence of high concentrations of PEG, namely 20 and 25% PEG. These cells were exposed to 15% PEG, for several months (6-8 months) prior to their exposure to higher concentrations, viz., 20 and 25%. The cells selected at 20% grew better in the presence of 5-10% PEG. The results obtained in the present study with black pepper cells are similar to these observations. It was observed in the present study that exposure of black pepper cells to moderate water stress mediated by 15% PEG for 6 months, was helpful in adaptation of cells to a higher concentration of PEG, namely, 25%, that was highly inhibitory to wild-type cells. In addition, 25% PEG-selected cells grew better in low concentrations of PEG than in 25% PEG. Handa *et al* (1982) also reported selection of tomato cells tolerant to PEG-induced water stress by transferring the cells to successively higher concentrations of PEG after allowing them to initially adapt to a lower concentration. Geetha *et al* (1995) also reported the isolation of 25% PEG-adapted cell lines in *Vigna mungo.*

1.4.2.2.1. Characterization of PEG-adapted cells

1.4.2.2.1.1. Morphological characters

The black pepper cells exposed to higher intensities of water stress for a prolonged period of time exhibited reduced cell size, suggesting limited cell expansion. Similar reduction in cell size was observed by Handa *et al* (1982) in tomato cells that were adapted to PEG-induced water stress. The authors suggested that the decrease in the amount of expansion/turgor (elasticity and extensibility) during the adaptation of the cells was responsible for the increased turgor pressures observed in these cells and for their restricted growth. Hence, it can be assumed that the limited cell expansion could be an adaptive mechanism by cells to maintain high turgor in conditions of limited water availability. Carpita *et al* (1990b) also reported reduction in cell size of tobacco cells under water stress. The authors observed that cell volume in tobacco cells adapted to dessication or salt stress is reduced to as little as 1/8 that of unadapted cells. The authors suggested that cells adapted to either NaCl or desiccation accumulate substantial amounts of sugars, proline, and other amino acids as additional osmolites, but do this at the expense of synthesis of cell wall polymers. The reduced cell volume associated with adaptation occurs despite maintenance of more than adequate turgor needed to drive cell expansion under normal conditions. The authors deduced from their work that a lesion in xyloglucan metabolism is responsible for reduced cell expansion.

1.4.2.2.1.2. Biochemical characters

The cells adapted to 25% PEG were characterized biochemically for osmolyte accumulation, lipid peroxidation and phenol accumulation.

1.4.2.2.1.2.1. Osmolyte accumulation

Osmolyte accumulation in non-adapted as well as adapted cell lines was observed to be higher under water stress conditions compared to the cells in control medium.

Among the inorganic osmolytes, it was observed that K^+ and Na^+ ions increased while Mg^{2+} and Ca^{2+} decreased significantly in the selected lines as well as non-selected control cells, when exposed to 25% PEG compared to the accumulation of these ions when grown under non-stressed conditions. The increase in Na^+ ions was only marginal. However, the increase in K^+ ions was highly significant. Among the organic osmolytes, total free amino acids and reducing sugars were more prominent under water stress conditions in both selected as well as non-selected cell cultures. However, while amino acids accumulation was significantly high, the reducing sugars were only marginally higher than the control non-selected cells under water stress conditions. Among the selected cell lines, the cell line selected by the step-wise method seemed to accumulate higher concentrations of amino acids than that selected by the direct method. However, the two selected cell lines did not differ significantly in their reducing sugar content. Though an increase in proline content was observed in the adapted cell lines over the non-adapted control cells under water stress, the increase was not very significant. Moreover, the cells selected by the direct method accumulated marginally higher proline than the cells selected by the step-wise method. In general, it was observed that the accumulation of osmolytes in the non-selected as well as selected cell lines was similar in the control medium and was lesser than the osmolyte content seen during water stress conditions.

Handa *et al* (1982) also observed increase in solute levels in adapted cell lines of tomato compared to the unadapted cells. The authors observed that the maximum contribution to the osmotic potential of the cells was made by reducing sugars and by K^+ ions, similar to the results obtained in the present study with black pepper cells. Handa *et al* (1982) suggested that the process of conservation and/or generation of osmotic solutes such as sugars and free amino acids itself limits the synthesis of macromolecules which are likely required for growth processes, which is the main reason for the limited expansion of the adapted cells, as is also observed in the present study. Santos-Diaz and Ochoa-Alejo (1994) also reported an increase in K^+ levels in chili pepper cell cultures adapted to higher levels of polyethylene glycol. The authors observed a positive correlation between PEG-tolerance and the K^+ levels in chili pepper cell cultures, which accounted for about 40-70% of the osmotic potential in the cell clones, thus, making a major contribution to osmotic potentials in the PEG-tolerant cell clones. Geetha *et al* (1995) also reported significant increase in K^+ levels in PEG-adapted cell lines of *Vigna mungo*.

Proline has often been found to increase during water stress in many plants (Chandler and Thorpe, 1986, Iriyogen *et al.*, 1992). In the present study however , it was observed that though the adapted cells showed increase in proline content compared to the non-selected control under water stress, the increase was only marginal.

From the results obtained in the present study, it can be suggested that the selected cell lines behaved similar to the control, non-selected cells under water stress conditions. Water stress induced the accumulation of K^+, Na^+, total free amino acids and reducing sugars and proline in both adapted and non-adapted cells, with the concentrations of most of these osmolytes being marginally higher in the former than in the latter. Accumulation of K^+ and total free amino acids was significantly higher in the adapted cell lines compared to the control non-selected cells, under water stress conditions. Though reducing sugars and proline increased, their concentrations were only marginally higher than the control non-selected cells, during water stress. Increase in K^+, Na^+, total free amino acids and reducing sugars and proline is a response of black pepper cells to water stress, as mentioned previously in a previous section of this chapter (Section 4.1.1.1.2., under Biochemical responses to water stress). The accumulation of osmolytes, especially, K^+ and total amino acids, by the adapted cell lines was similar to that shown by the 15% PEG-exposed black pepper cells. It was observed that the osmolyte accumulation in black pepper cells exposed to severe stress mediated by 25% PEG declined with the duration of the stress period. However, the adapted cell lines maintained higher levels of these osmolytes under 25% PEG, even after a prolonged exposure of 45 days. This is similar to the trend shown by the wild type black pepper cells exposed to 15% PEG, which maintained steady amounts of osmolytes for a longer period of time, suggesting that the responses of black pepper to mild stress mediated by 15% PEG can be assumed to be adaptive. However, it should be said here, that a significant increase in reducing sugars was noticed in black pepper cells exposed to 15%

PEG, a trend which was not exhibited by the adapted cell lines. Proline accumulation in the adapted cell lines was however similar to that shown by wild-type black pepper cells exposed to 15% PEG, with the levels increasing only marginally on imposition of water stress.

1.4.2.2.1.2.2. Lipid peroxidation and phenol accumulation

The MDA content of the adapted as well as the non-adapted cells increased when they were subjected to water stress mediated by 25% PEG. However the lipid peroxidation was higher in the adapted cell lines compared to the non-adapted cells. Lipid peroxidation is a common occurrence under water stress conditions. Since the lipid peroxidation in the non-adapted cells and the selected cell lines increased under water stress compared to that under non-stressed conditions, it could be assumed that the selected cell lines are not highly tolerant to the imposed water stress and behaved on par with the non-adapted cells. Since the lipid peroxidation in selected cell lines was lower when they were incubated in the non-stressed media it can be assumed that the imposed water stress induced lipid peroxidation to a similar extent as that in the non-adapted cells, again pointing to the possibility that the selected cell lines exhibited only adaptation to the water stress rather than being tolerant to the imposed stress.

Phenol accumulation was observed to be higher in cells exposed to water stress. However, the selected cell lines showed greater accumulation of phenolics than the non-adapted cells. Phenolics accumulation, as discussed in section 4.1.1.2. of this chapter, could be a protective mechanism in the adapted cell lines to mitigate the adverse effects of oxidative stress ensuing water deficit. Hence, the cells exposed to prolonged water stress accumulated more phenolics that could have helped to protect themselves against the severe oxidative stress accompanied with water stress.

From the observations made in the present study, it can be concluded that the attempt of selecting a truly water stress-tolerant cell line of black pepper was unsuccessful. The black pepper cells selected by the step-wise or the direct method were however adapted to the imposed water stress due to prolonged exposure, possibly by physiological adaptation. The fact that embryos regenerated from cells selected at 25%

PEG by the step-wise selection procedure did not exhibit tolerance to 25% PEG suggests that a true genetic selection for water stress tolerance did not take place. This was also true for black pepper cells selected by the direct selection method. However, the studies on biochemical characterization of the selected cell lines suggest that some of the characters exhibited by them are similar to the general responses of the wild-type black pepper cells to PEG-mediated water stress implicating these characters for possible adaptation.

BIOTIC STRESS

CHAPTER TWO

BIOTIC STRESS

2.1. Review of literature

Fungal disease of crop plants is one of the main biotic stresses that result in considerable yield and economic losses. Plants serve as food not only for animals and insects, but also for a variety of microbes like bacteria, nematodes and fungi. Though plants are constantly challenged by diverse possibly pathogenic microorganisms, and cannot move from one place to another to evade the potential pathogens, they are successful in defending themselves against most of these microbes. This is because plants are equipped sufficiently well with innate defense mechanisms, which protect them from being devoured by microbial attacks. In general, plants are resistant to most potential pathogens and only few plant - microbial interactions exist where the plant is considered as a susceptible host and the micro-organism a virulent pathogen, able to establish an infection process followed by plant pathogenesis (Ebel and Scheel, 1997; Lebeda *et al.*, 1999a). Thus, resistance against microbes seems to be a rule and susceptibility (i.e., disease), an exception when microorganisms and plants meet.

In recent years, considerable information has accumulated on the mechanisms by which plant cells respond to microbial attack. Research in plant physiology, pathology and molecular biology has focused on plant defense reactions, mainly for determining the genetic and molecular mechanisms responsible for plant resistance to infection. Moreover, with the advent of modern technologies like genetic engineering, it has become possible to transfer genes of interest into crop plants that confer on them, tolerance / resistance to their pathogens. The inheritance of resistance to disease is generally dependent on multiple genes. To demonstrate the role of genes in plant resistance will require a concerted application of biochemical and molecular approaches.

Several species of *Phytophthora* have been known to be pathogenic to various crop plants, incurring heavy crop losses around the world. Research into the biochemistry, physiology and molecular biology of plant defense reactions has made it possible to dissect and analyze the defense mechanisms, which the plants have evolved against this group of fungi. Such fundamental studies might in the future, lead to the identification of useful genes for the management of *Phytophthora* diseases.

Research on different aspects of interaction specificity and defense mechanisms of plants against potential fungal pathogens has received great attention in the last few years (Parbery, 1996; Kuć, 1997; Bolwell and Wojtaszek, 1997; van Loon and van Strein, 1999; Hammond-Kosack Jones & Parker, 2003; Matsumura *et al.*, 2003; Chu *et al.*, 2004).

Plants defend themselves from attempted infection by elaborating a number of diverse defense responses (Hahlbrock and Scheel, 1987; Aziz *et al.*, 2003; Baldwin, 2003), including localized cell death known as a hypersensitive response (HR), oxidative burst involving free radical production, cell wall fortification by deposition of callose, lignins and hydroxyproline-rich glycoproteins, production of protease inhibitors (Dixon and Harrison, 1990; Ryan and Farmer, 1991; Côté and Hahn, 1994), accumulation of hydrolytic enzymes such as β-1,3-glucanase and chitinase and synthesis of anti-microbial compounds called phytoalexins (Kombrink *et al.*, 1986; Collinge and Slusarenko, 1987) and pathogenesis-related (PR) proteins (Kuć, 1995; Cordelier *et al.*, 2003). Studies by a number of researchers using biochemical and molecular biological approaches have shown that the activation of these presumptive defense responses is often correlated with disease resistance in specific plant-microbial interactions (Hahn *et al.*, 1985; Bell *et al.*, 1986; Jahnen and Hahlbrock, 1988; Habereder *et al.*, 1989; Von Roepenack-Lahaye *et al.*, 2003).

A key event of plant responses to pathogen attack lies in the plant's ability to recognize the pathogen and quickly mount the battery of inducible-defensive measures. Whether a plant is susceptible or resistant to infection depends upon many subtle interactions between molecules produced by the plant and those produced by the pathogen. In a susceptible reaction, a microbe which is pathogenic, manages to overcome the defense mechanisms of the plant and invades and establishes itself in the host, thus taking control over the host metabolism. The speed of a plant's response to infection affects the level of resistance expressed.

The induction of the defense responses in plant tissues is presumed to be mediated by initial recognition process between plant and pathogen, involving the detection of signal molecules or elicitors by the plant cells (Bowles, 1990). Disease results either from failure of this recognition event or the ability of the pathogen to avoid or overcome the resistance response.

2.1.1. Elicitors – signals of pathogens

To identify microbial threats, plants must be able to perceive signals from a large variety of potential pathogens to trigger the general "non-host" resistance. In recent years, a set of biological signal molecules (referred to as elicitors) from different pathogens, have been identified and characterized with respect to the defense responses induced in plant cells. Resistance-inducing factors, called "inducers" (Hayami *et al.*, 1982) or "elicitors" (Keen, 1975; DeWit, 1986) have been found in the culture filtrate, hyphal cell walls or spore germination fluid of pathogenic fungi and have been variously characterized as polysaccharide, glucan, chitin, chitosan, glycoprotein, peptide and lipid (Darvill and Albersheim, 1984, DeWit, 1986, Ralton *et al.*, 1986). Through research on the biochemistry of plant defenses, it has been shown that "elicitor" fragments from fungal cell walls can act as powerful signaling agents to activate plant defenses during plant – pathogen interactions (Ryan, 1987; Yoshioka *et al.*, 2001; Fliegmann *et al.*, 2003). Biochemical analysis of the induction of plant defense responses has been facilitated by these elicitors, which are capable of inducing defense responses, when applied to plant tissues. The significance of elicitors in plant-parasite interactions and their modes of action in the induction of active resistance have been reviewed by Lamb *et al.*, (1989) and Yoshikawa *et al* (1993). No pathogenic fungus exists that does not produce an elicitor. For eg., β-glucans, chitin and /or chitosan, which are commonly present in the hyphal cell walls of many pathogenic fungi, can act as non-specific elicitors (Shiraishi *et al.*, 1994).

Fungal elicitors have been used to achieve new insights into disease responses of plants induced by transcriptional activation (Dixon and Lamb, 1990; Takemoto *et al.*, 2000). It has been proposed that the specific recognition of such molecules would be a basis for pathogen-host specificity (Keen, 1982). Studies have shown that treatment of potato (Takemoto *et al.,* 1999a) and tobacco (Takemoto *et al.,*1999b) with HWC elicitor, induced resistance responses such as cell death and accumulation of PR-proteins. Most of the defense-responsive genes cloned till date, are transcriptionally activated in response to the microbial elicitor molecules (Dixon and Harrison, 1990). Fellbrich (2000) reported the accumulation of defense-associated gene transcripts in cultured *Petroselinum crispum* cells by Pep-25, an oligopeptide fragment of a Phytophthora sojae 42-kDa cell wall protein, and a cell wall elicitor preparation derived from *Phytophthora nicotianae* var. *parasitica* (Pp-elicitor).

The promoters of genes induced by the elicitors are ideal for expression of the transgenes used for the disease resistance. The information of defense responses induced during elicitor treatment helps in engineering new defense barriers in plants, prior to pathogen ingress.

2.1.1.1. Elicitors of *Phytophthora*

It has been found that *Phytophthora* possess two causal factors of compatible and incompatible reactions (Doke and Tomiyama, 1980), namely, (1) the hyphal wall components (HWC) which elicit a non-specific reaction in host tissues, similar to that elicited by infection with an incompatible race, and (2) the water soluble glucans (WSG), which prevent the hypersensitive response elicited by infection or by HWCs (Kuc *et al.*, 1976; Garas *et al.*, 1979; Doke *et al.*, 1980).

Elicitor molecules of *Phytophthora* species are chemically diverse. They include isolated hyphal cell walls (Doke &Tomiyama, 1980) or oligosaccharides released from them, lipids (eg. Arachidonic acid and eicosapentaenoic acid in *P. infestans*- Creamer and Bostock, 1986; inositol sphingophospholipid in *P. capsici* (Lhomme *et al.*, 1990) ; and proteins (Billard *et al.*, 1988). The best-characterized elicitor of fungal origin is a β-linked heptaglucoside from race 3 of *P. megasperma* f.sp. *glycineae*, which causes root rot of soybean.

Proteinaceous elicitors can be either glycoproteins or holoproteins. Endoxylanase, a 46 kDa glycoprotein, isolated from *P. parasitica* var. *nicotianae* induces the synthesis of phytoalexins in tobacco (Farmer and Helgesson, 1987). Parker *et al* (1991) isolated a 42kDa glycoprotein from the culture filtrate of *P. megasperma* f. sp. *glycinea*, which elicits the production of coumarin phytoalexins in parsley cells and protoplasts. Ricci *et al.*, (1989) isolated small peptides from *P. cryptogea* and related species and showed that they elicited both local and systemic defense responses in tobacco. In an effort to explore the antigenic potential of surface structures of different *Phytophthora* spp to elicit non-cultivar specific defense responses in parsley, Fellbrich (2000) identified proteinaceous cell wall fraction in *P. parasitica* that contained active elicitors. Brunner *et al* (2002) identified a 13 amino acid peptide fragment (Pep-13) within a 42-kDa cell wall transglutaminase from various *Phytophthora* species that triggers a multifaceted defense response in parsley cells. Fellbrich *et al.*, (2002) identified another cell wall protein, a 24 kDa necrosis-inducing *Phytophthora* protein 1 (NPP1) in *Phytophthora parasitica* that evoked the same pattern of defense responses in parsley as pep-13.

Many pathogenic *Phytophthora* species produce extracellular holoproteins of 98 amino acids with a molecular weight of about 10,300 Da, and are grouped under the name 'elicitins' (Huet and Pernollet, 1989; Ricci *et al.*, 1989). Elicitins are produced by most of the *Phytophthora* species. *P. cryptogea, P. cinnamomi, P. dreschleri, P. parasitica* and *P. capsici,* secrete elicitins named cryptogein (cry), cinnamomin(cin), Dre elicitin(Dre), parasiticein (para) and capsicein (cap) respectively in their culture filtrates(Billard *et al.*, 1988). They are considered by some researchers as toxins that enable *Phytophthora* species to kill the host tissue before using it as a growth substratum (Pernollet *et al.*, 1993). They are not only toxins but also act as fungal signals in the plant–*Phytophthora* interactions. These elicitins were shown to produce the incompatible reaction when treated on tobacco (a non-host) plants and cell cultures (Yu, 1995).

Hyphal wall components of several *Phytophthora* species cause the hypersensitive reaction of plant cells of both host and non-hosts. These elicitor molecules activate various defense mechanisms in plants, which include lignification (Graham & Graham, 1991), accumulation of phytoalexins (Castoria *et al.*, 1992), oxidation of phenolics (Maniara *et al.*, 1984), generation of active oxygen species (Sanchez *et al.*, 1992; Doke & Miura, 1995; Yoshioka *et al.*, 2001) and activation of defense enzymes like phenylalanine ammonia lyase (Yoshioka *et al.*, 1995). Crude cell wall extracts of the fungal pathogen *Phytophthora sojae* (Pmg-elicitor) were found to induce various defense responses like H_2O_2 accumulation, peroxidative cross-linking of cell wall proteins, activation of phenylpropanoid metabolism, accumulation of phenolic compounds and induction of PR-proteins in soybean suspension cultures (Groten and Barz, 2000).

Activation of defense mechanisms has been observed in both susceptible and resistant interactions, following the release of elicitors. Both virulent and avirulent races have elicitors in their cell walls, and are released from the fungal cell wall upon contact with the host cell surface. The release of elicitors, which has a very important role in pathogenesis, is brought about by the action of enzymes of host-origin. β-1, 3 glucanase of soybean releases β-1,3 glucan, an elicitor in the mycelial cell wall (Ham *et al.*, 1991) of *P. megasperma*. Arachidonic acid, the elicitor of *P. infestans* is released by potato lipoxygenase (Vaughn & Lulai, 1992; Ricker & Bostock, 1994).

The timing and amount of release of elicitors from the fungal cell wall is important in determining the susceptibility or resistance of the host to *Phytophthora*. In incompatible interaction, the release of fungal elicitors is quicker due to the speed with which the host enzymes act on the fungal cell wall to release elicitors, thereby triggering the defense mechanisms of the plant earlier, than is observed in a susceptible interaction. The early activation of host defense responses in the resistant host restricts the spread of the pathogen.

2.1.2. Suppressors

Once the chemical and physical barriers have been established in plant tissues, the penetration, growth and / or reproduction of the pathogens are crucially inhibited.

Therefore, the ability to overcome the host's resistance is essential for the successful proliferation of pathogens (Oku, 1980). In other words, the specificity cannot be explained solely by the production of elicitors, but is, rather, determined by the substances that are able to circumvent or negate the active resistance of host plants (Heath, 1981). Metabolites from several fungi can delay or suppress the active resistance of host plants such as hypersensitive cell death (Doke, 1975; Storti *et al.*, 1981), the NADPH-dependent generation of superoxides (Doke, 1983) and the accumulation of phytoalexins (Doke *et al.*, 1979; Kessman and Barz, 1986), with resultant conditioning of host cells such that they become susceptible even to avirulent or non-pathogenic micro organisms (Kodama *et al.*, 1989). Such compounds are known as **suppressors** (Oku *et al.*, 1977). In Oku's review (1980) and in a previous early report (Oku *et al.*, 1980), suppressors were defined as 'determinants for pathogenicity without apparent phytotoxicity'. In other words, (i) they are produced by pathogens at the site of infection; (ii) they participate in suppression of general resistance and in the induction of local susceptibility in host plants; (iii) they are host-specific; and (iv) they are non-toxic to plants.

Suppressors have been characterized as glyco-proteins, glyco-peptides, peptides and anionic and non-anionic glucans. Suppressors by themselves are non-toxic to plant cells and do not cause any visible damage to or necrosis in host tissues or protoplasts, and thus should be distinguished from host-specific toxins (HSTs). However, the suppressors appear to function in a similar manner to HSTs, suppressing the defense responses of their own hosts (Kohmoto *et al.*, 1987; Yamamoto *et al.*, 1984) and conditioning host cells, may be by disturbing fundamental functions of host plasma membranes, such that they become accessible to pathogens (Comstock and Scheffer, 1973; Otani *et al.*, 1975).

Suppressors of *Phytophthora* have been characterized as glucans of 17-23 glucose units containing β-1,3 and β-1,6 linkages and are found in the mycelial cell walls of *Phytophthora* species. They are also released during germination of cytospores (Doke *et al.*, 1980; Doke & Tomiyama, 1980b). Cytospores of *Phytophthora infestans* secrete small anionic and non-anionic water-soluble glucans into the germination fluid, and the amounts of both types of glucan increase during incubation. These glucans have been known to suppress, in a race-cultivar specific manner, both hypersensitive cell death and production of phytoalexin by potato tubers that is induced by an incompatible race of the fungus (Doke *et al.*, 1979, 1980).

Suppressors are usually secreted by and into the spore germination fluid of pathogens. This is because, the majority of phytopathogenic fungi commonly infest and infect through their conidiospores and hence, the initial interaction between plants and pathogens is mediated by the substances that are secreted into spore germination fluids.

2.1.3. Inter-play of elicitors and suppressors of *Phytophthora* in determining host-specificity

Phytophthora species are aggressive plant pathogens, despite the fact that they contain numerous elicitors that can potentially be recognized by plants, including the glucan elicitors and the proteinaceous elicitins. One possibility is that *Phytophthora* produces suppressors, i.e. compounds that prevent recognition of elicitors by the host, as described above. Another is that the fungus down-regulates the production of elicitor-active components upon contact with its host plant. In potato, compatible races of the late blight fungus prevent the hypersensitive death of infected cells, which is usually seen during the interaction with incompatible races. This is possibly through water-soluble inhibitor glucans (Doke *et al.*, 1979; Garas *et al.*, 1979; Doke & Tomiyama, 1980; Currier, 1981) which enable them to colonize the host.

Investigations on the interaction between HWCs and membrane-rich fractions (Doke *et al.*, 1975; Doke *et al.*, 1979; Blume *et al.*, 2000) show that a reactive site (a receptor) for HWCs may be located on host cell membranes and that binding of HWCs to the receptor may cause an induction of the hypersensitive response of the cells. Glucans from compatible races, unlike those from incompatible ones prevent the interaction between HWCs and the membrane-rich fractions of host tissues (Doke *et al.*, 1975). Thus, it has been suggested that glucans possibly occupy the binding site for HWCs on the protoplast membrane or cause a configurational change of the binding site, resulting in no effective binding of the HWCs to the receptor. Thus, specific suppression of the reactivity of host cell to hyphal wall components by glucans from compatible races may contribute to the establishment of compatible interactions (or aggressisveness of the fungi) as well as host-pathogen specifity (Doke & Tomiyama, 1980).

The suppressive action of glucans is more characteristic of a compatible reaction between races and cultivars rather than incompatible ones. It has been shown that the hypersensitive reactivity elicited by HWC from *P. infestans* in potato protoplasts was suppressed by treating the protoplasts with glucans from a compatible race (Doke & Tomiyama, 1980). Suppressors of *Phytophthora* block active defenses in host plants. Infection of potato by incompatible races of *Phytophthora infestans* causes rapid death of the infected cells (Hypersensitive reaction) (Coffey & Wilson, 1983; Doke, 1982; Ferris, 1955). Compatible races of the late blight fungus prevent this active response by the potato cells, possibly through water-soluble inhibitor glucans. Hence, compatible races are able to go on to colonize and destroy their host. The hypersensitive reaction in potato tissues or protoplasts that is induced by an incompatible race or by hyphal cell walls of *P. infestans* was prevented or delayed by water-soluble glucans from zoospores of the compatible race (Doke, 1975; Doke and Tomiyama, 1977; Doke *et al,* 1980;). Similarly, the NADPH-dependent generation of superoxides, which was induced by the hyphal cell-wall fraction (a non-specific elicitor), prior to hypersensitive cell death and production of rishitin, was suppressed by the water-soluble glucans in a race- and cultivar-specific manner (Doke, 1983, 1985). It has also been shown that water-soluble glucans from compatible races of *P. infestans* could inhibit the hypersensitive response elicited by HWC from incompatible and compatible races, in potato protoplasts (Doke and Tomiyama, 1980) as well as in tuber tissue (Doke *et al.*, 1979).

The role of WSGs in host selectivity has also been demonstrated in *P. capsici*-tomato and *P. capsici*-sweet pepper compatible interactions (Sanchez *et al.*, 1991a; Sanchez *et al.*, 1991b). The detection of water-soluble glucans from zoospore germination fluid of virulent *P. capsici* (Sanchez *et al.*, 1994) and suppression of elicitor-induced death of suspension-cultured cells of susceptible sweet pepper and tomato and not that of resistant pepper and tobacco by these glucans suggests that the fungus may release suppressor glucans for establishment of susceptibility in host plants. Moreover, during the growth of fungus in the inter-cellular spaces of leaf tissue, polygalacturonase plays an important role as a virulence factor (Sanchez *et al.*, 1994). It breaks down pectic substances in host intercellular spaces for symptom development and also produces initial nutrients (possibly galacturonic acid) for fungal growth in the intercellular spaces of host tissue.

2.1.4. Cell suspension cultures - model systems for studying plant defenses

Plants defend themselves against pathogens in a largely cell autonomous manner, greatly distinct from the vertebrate immune system. Each plant cell is capable of defense against pathogens by both pre-formed and induced mechanisms. Crude or purified elicitor preparations released from pathogen cell walls have been used with plant cell suspension cultures to induce and study the biochemistry, enzymology and molecular biology of inducible plant defenses (Campbell and Ellis, 1992a; Dixon *et al.*, 1994; Hahlbrock *et al.*, 1995; Binet *et al.*, 2001; Mithofer *et al.*, 2001). The infection of plants by pathogens and the resulting defense responses occur initially at single cells and are experimentally difficult to synchronize, since higher plant tissues consist of various types of cells at different stages of growth and differentiation. Tissue culture is a simple experimental system of homogenous cells, where control of environment and genetic uniformity are easily realized. For experiments on the biochemical and molecular biological mechanisms involved in defense responses, suspension-cultured cells at the same stage of growth and responsive to fungal elicitors have been used to reduce the complexity of plant-pathogen interactions.

Plant cells exposed to elicitors, whether crude fungal cell wall fragments or defined molecules such as purified proteins and avirulence gene products, respond with a battery of cellular changes (Yu, 1995; Hammond-Kosack and Jones, 1997; Yang et al., 1997). Some of these responses, such as changes in ion fluxes and the generation of reactive oxygen species, occur very rapidly and may involve events that occur primarily at the post-translational level. Other responses, such as the accumulation of phytoalexins and synthesis of chitinases, glucanases and other pathogenesis-related proteins, involve induction of gene expression (Dixon and Lamb, 1990; Yang *et al.,* 1997).

Cell suspension cultures have been shown by many researchers (Schmidt *et al.*, 1998; Garcia-Pérez, 1998; Szabo *et al.*, 1999) to respond to elicitors in a manner similar to the response of the plants to the pathogen. Use of cultured cells instead of whole plants and replacing the live pathogen by elicitor-active components of the pathogen, can reduce the high complexity involved in the study of the plant-pathogen interactions. A number of systems have been developed in which the resistance or susceptibility expressed in intact plants is also expressed in callus cultures derived from them (Miller and Maxwell, 1983). The defense reactions in fungus-infected leaves have been shown to resemble those in elicitor-treated cell cultures, as extensively demonstrated in parsley (Hahlbrock *et al.*, 1995).

Elicitors from both pathogens as well as non-pathogens have been used for studying the defense responses. Cell suspension cultures of parsley (*Petroselinum crispum*) have been used as a system for studying the non-host resistance responses to *Phytophthora sojae* and *Phytophthora infestans*, two important fungal pathogens. Incubation of parsley cells with the glycoprotein elicitor from *P. sojae* resulted in dramatic biochemical changes in the affected plant cells, e.g., the generation of reactive oxygen intermediates (ROI), increased ion fluxes across the plasma membrane, and changes in gene activity. The same processes were observed upon infecting parsley cells with *P. infestans.* Cell suspension cultures were used by Garcia-Pérez *et al* (1998) to investigate the hypersensitive reaction in resistant and susceptible cultivars of chilli pepper, in an attempt to explain the factors that trigger the defense response in the host. Kombrink and Hahlbrock (1986) used cell cultures of tobacco and elicitor-active fragments of *P. nicotianae* (pathogen of tobacco) to facilitate the biochemical studies of induced defense reactions of tobacco to the pathogen. Oelofse and Dubery (1996) used cell cultures of tobacco to study the relative importance of various inducible mechanisms operative in tobacco-*P. nicotianae* interactions. Davis and Ausubel (1989) established a model system of cell suspension culture in *Arabidopsis* for studying plant-pathogen interactions with the bacterial elicitor α-1,4-endopolyagalacturonic acid lyase (PGA lyase). The responses of suspension-cultured cells of non-host, parsley, treated with the cell wall elicitor preparation from soybean pathogen, *P.megasperma* (a non-pathogen of parsley) were similar to those induced *in vivo* in Parsley plants (Dietrich *et al.*, 1990). The defense responses induced in parsley plants, namely, the transcriptional activation of phenylpropanoid pathways, the production of furanocoumarin phytoalexins (Hahlbrock and Scheel, 1987, 1989) and cell wall-bound phenolics, as well as the synthesis of several PR (pathogenesis-related) proteins (Somssich *et al.*, 1986, 1988), were mimicked in suspension-cultured parsley cells and protoplasts treated with an elicitor preparation from the *P.megasperma* cell wall (Scheel *et al.*, 1986; Somssich *et al.*,1989). This and several other studies have shown that the response of plant cells to elicitor treatment is very similar to that observed during microbial infection (Fritzemeier *et al.*, 1987; Habereder *et al.*, 1989).

Plant tissue cultures have been used to provide an environment in which certain aspects of defense responses can be examined more readily than in the case of the whole plant (Matthyse, 1983). The detailed study of individual steps of some of these defense responses, for eg., the hypersensitive cell death in whole-plant tissue, has many limitations, since, with the infection sites occurring asynchronously, only very few cells react, which are spatially separated by the bulk of non-affected tissue. David and Currier (1986) established a system in which potato cells in suspension culture responded to HWC, as a model of fungus-plant interactions and examined in detail, the various steps of cytoplasmic aggregation leading to cell death in this system and found that this cytoplasmic aggregation phenomenon might also be associated with actin filaments in potato cells in suspension. Water-soluble glucans from incompatible races of *P. infestans* have been shown to inhibit the hypersensitive response in protoplasts (Doke and Tomiyama, 1980) as well as in tuber tissue of potato (Doke *et al.*, 1979). Because of these similarities in the response of potato tuber cells and protoplasts, it appears that the protoplast assay could be used to study the biochemical aspects of the hypersensitive response. This could help determine the biochemical mechanism of disease resistance in this host-pathogen interaction.

Some researchers have used a system of reduced complexity that consisted callus and live pathogen as a tool to study the infective process of the pathogen, as they found that the ultra structural changes in compatible and incompatible interactions in calli were similar to those occurring in intact plants infected by a fungus (De Zoeten *et al.*, 1982; Trigiano *et al.*, 1984; Beech and Gessler, 1986). Naton *et* al. (1996) used a system of immobilized fungal germlings of *P. infestans* infecting cultured parsley (*Petroselinum crispum*) cells that reflected a non-host relationship that exhibited major features of the early plant defense reactions. It proved to be very useful for investigating the cell-death-related metabolic changes in individual infected cells. Casares *et al.*, (1994) have studied the infection process of *P. cambivora* in callus from susceptible and resistant species of chestnut. Vieitez (1961) proposed the use of callus formed spontaneously on cuttings and inoculated with *P. cinnamomi as* a quick test for detecting susceptible or resistant plants.

Model systems of callus and cell suspension cultures in combination with elicitor preparations have proven to be good tools in the identification and isolation of defense-related genes in many plant species (Hahlbrock *et al.*, 1995). Putative defense-related genes, such as genes encoding for chalcone synthase and chalcone isomerase (Hahlbrock and Scheel, 1989) and phenylalanine ammonia lyase (PAL) (Habereder *et al.*, 1989) and still unidentified genes (Schmelzer *et al.*, 1989) have been isolated with such model systems. Rompf and Kahl (1999) observed that a defense related gene, PRP-4, was similarly regulated in elicited cell cultures and leaves. Suzuki *et al.*, (1995) used a homogeneous suspension culture of tobacco cells, which respond to a fungal elicitor derived from the cell wall fraction of *Phytophthora infestans*, to study the signal transduction pathway that leads to activation of defense genes. Several responses previously associated with disease resistance in other plants were induced in suspension-cultured *Arabidopsis* cells treated with the bacterial elicitor α-1,4-endopolyagalacturonic acid lyase (PGA lyase) (Davis & Ausebel, 1989).

2.1.5. Plant defense responses

It has been proposed that most of the plants have a battery of constitutive and inducible features that can protect them against infection by most of the fungal and other pathogens. Some of the constitutive defense factors include structural and developmental features, toxic chemicals, enzymes degrading fungal pathogenicity factors (e.g. phytotoxins) and lack of receptors, signals or nutrients, required for the pathogen. The induced defense mechanisms include active oxygen species, phytoalexins and other toxic, non-proteinaceous molecules, cell wall fortifications, defense-related enzymes and other proteins and cell death. These defenses are not specific for particular species of a pathogen. If a microorganism has sufficient pathogenicity factors for successful parasitism or pathogenism of a plant species, it has established a basic compatibility with the plant (Heath, 1997).

From a physiological perspective, plant defense mechanisms in plants can be classified as passive and active. Static (passive) or structural defenses involve constitutive properties such as the thickness or hardness of cell walls and the presence of anti-microbial substances, which restrict the ingress of the pathogen by the structural barriers. Active resistance involves the biochemical responses induced in the host after the pathogen has gained entry into the host. These include biochemical reactions, which result in the formation of chemical and physical barriers, such as phytoalexins, infection inhibitors, superoxides, PR proteins, lignin, callose and hydroxyproline-rich glycoprotein (Lamb *et al.*, 1989; Ouchi, 1991). The defense responses produced locally around the site of infection are designed to strengthen barriers against invasion and the defenses, induced both locally and systemically, i.e. the active resistance, are meant to weaken and destroy the invading pathogen. Resistance is due to a combination of physical and chemical barriers, which are either preformed or induced only after infection. Lebeda *et al* (2001) have reviewed extensively on the various defense mechanisms elaborated in plants against biotic stress.

2.1.5.1. Structural (passive) defenses

To restrict the ingress of fungal pathogens, plants possess mechanical barriers like lignin, suberin, and callose and produce abundant low molecular weight anti-microbial compounds like phenols, quinines, alkaloids and others. Plant cell wall is the first barrier and penetration of the cell wall appears to be the first requirement for pathogenesis of fungal pathogens. There are at least two types of structural defenses that act as physical barriers in plants. Pre-formed mechanisms exist constitutively before infection and induced mechanisms are formed only after infection. The aerial surfaces of plants are covered with a wax layer. Epi-cuticular waxes represent an important interface between plant and the pathogen. In fungus-plant interactions, the thickness of cuticle and the amount of waxes have a positive correlation with host resistance. The cell wall is the basic structure, which protects cells against mechanical and chemical injury. If a pathogen breaks through these barriers, the plant responds by enhanced synthesis and insertion of structural compounds in the cell wall (Kohomoto *et al.*, 1995).

The main structural barriers include lignin, suberin and callose. Modification of cell walls by lignification (Kohle *et al.*, 1984), suberization (Espelie, K.E., 1986), hydroxyproline-rich glycoproteins (HRGPs) (Showalter and Varner, 1989), callose deposition (Kohle *et al.*, 1985), contribute to strengthening the cell wall architecture and are aimed at obstructing the pathogen's access to plant cell nutrients. Deposition of lignin, a polymeric cell wall phenolic that is incorporated in standard conditions to the xylem cell walls, is synthesized during pathogen attack. It is resistant to degradation by microorganisms and can serve as a barrier to wall off pathogen-infected tissue (Dixon and Paiva, 1995). Lignin is synthesized via the phenylpropanoid biosynthetic pathway. Lignified hyphae, which result during the lignification process that takes place during the plant defense response, lose their plasticity and cannot continue growth. Deposition of lignin and other cell wall-bound phenolics has been described for many plant species in response to microbial attack. Egea *et al.*, (2001) reported accumulation of lignin-like polymers in cell suspension cultures of *Capsicum annuum* in response to elicitation by both lyophilized mycelium and culture filtrate of *P. capsici*. Suberin is a polyester of hydroxy acids and dicarboxyl acids and is another polymer involved in the formation of structural barriers. The defensive lignification and suberization makes the cell wall more fast, restricts the cleavage of its compounds, reduces the nutrient flow between plant and pathogen and also the transport of mycotoxins (Nicholson and Hammerschmidt, 1992). Another post-infectional defense is the deposition of callose and formation of tyloses in the xylem vessels (Smart, 1991). Mithofer (2002) reported callose deposition in root cell walls of soybean as a defense response to attempted infection by the incompatible pathogen, *Phytophhtora sojae*. The enzyme callose-synthetase (E.C. 2.4.1.34) catalyzes the formation of β-1, 3-glucans, which are the components of extracellular matrix (Lebeda *et al.*, 1999).

2.1.5.2. Biochemical (active) defenses

Active plant defense mechanisms, thought to debilitate or injure the pathogen are very complex and complicated and they include various responses that are activated after the entry of the pathogen. These responses act synergistically to control the establishment of the pathogen, once the pathogen overcomes the structural barriers put forth by the host plant. These include death of the plant cells, namely the hypersensitive reaction (HR), the induction of the phenylpropanoid pathway and synthesis of lignin (lignification), accumulation of anti-microbial phytoalexins, synthesis of hydroxyproline-rich glycoproteins (HRGP) and hydrolytic enzymes such as chitinase and β-1,3-glucanase (Boller, 1985), capable of attacking the surface polymers of pathogen, tannins and o-quinones (Bell, 1981), PR-proteins, active oxygen species and proteinase inhibitors (Ryan *et al.*, 1985) and elicitation of the production of volatile compounds (e.g. ethylene) (Vidyasekharan, 1997).

2.1.5.2.1. Hypersensitive reaction

Cells are programmed to die if their continued existence is detrimental. The incompatible interaction between plants and pathogens is characterized by the induction of various defense mechanisms. One of the most effective induced defense responses in plants (Keen, 1990; Goodman and Novacky, 1994) is the **hypersensitive response (HR)**, in which the cells around the infection site rapidly necrose. This response is associated with a coordinated and integrated set of metabolic alterations that are instrumental in impeding further pathogen ingress, as well as in enhancing the capacity of the host to limit subsequent infection by different types of pathogens (Goodman & Novacky, 1994; Van Loon, 1997). At the infection site immediately after invasion by a pathogen, the affected plant cell undergoes highly dynamic metabolic and structural changes and suddenly dies. This process of induced cell death is extremely rapid and is considered to be a major means of halting growth of the pathogen.

The hypersensitive reaction is a result of the interaction of a dominant resistance gene in the plant with a pathogen race-specific avirulence gene product. The term hypersensitivity is credited to Stakman (1915) and was originally described as a rapid, localized necrosis of plant cells at the infection site. A number of morphological, physiological and molecular changes have been identified that appear coincident with the rapid cell death component of HR. Altered ion fluxes across the plant cell membrane, generation of active oxygen species, changes in the phosphorylation state of regulatory proteins and transcriptional activation of plant defense systems culminate in cell death at the site of infection, synthesis of lytic enzymes (Boller, 1987; Linthorst *et al.*, 1990), the production of pathogenesis-related proteins (Takahashi, 1975), protease inhibitors (Ryan, 1990), local accumulation of phytoalexins (Dixon, R.A., 1986; Paxton and Groth, 1994) and cell wall rigidification as a result of callose, lignin and suberin deposition (Hammond-Kossack & Jones, 1996; Yang *et al.*, 1997). These changes have been proposed to contribute alone or together to disease resistance. The multitude of biochemical processes associated with HR make the dead cells, and adjacent living cells, inhospitable for microbes (Kombrink and Somssich, 1995) and as a result, the pathogen is contained within the immediate vicinity of the infection site, thus preventing the spread to the non-infected parts of the plant. Plants that have undergone an HR show increased resistance against subsequent microbial attacks. This non-localized and long-lasting induced protection, which is active against a broad spectrum of pathogens, is called systemic acquired resistance (SAR) (Ross, 1961).

Defense by hypersensitive reaction (HR) occurs frequently in specific incompatible host plant-fungus interactions and is mostly challenged by avirulent isolate

(Hammond-Kosack & Jones, 1996). Infection of potato by incompatible races of *Phytophthora infestans* causes rapid death of the infected cells (Hypersensitive reaction) (Ferris, 1955; Doke, 1982; Coffey & Wilson, 1983). Naton *et al.*, (1996) reported that rapid cell death was the major defense response in parsley cell cultures infected with germlings of *P. infestans*. Graham and Graham (1999), reviewed data that suggests that hypersensitive cell death is necessary to trigger the competency of surrounding living cells to respond to elicitors of defense responses. They also present the interesting hypothesis that these competent surrounding cells enter a hypersensitive cell death programme that is "rescued" by scavengers of active oxygen species and this is necessary for the cell to perform the metabolic functions associated with localized tissue defense. HR-linked death in plants requires active plant metabolism and depends on the activity of host transcriptional machinery (He *et al.*, 1994) in contrast to cell death by necrosis, which does not require the active participation of the cell.

2.1.5.2.2. Hypersensitive reaction and the oxidative burst

Associated with triggering of defense responsive genes, there is a rapid production of active oxygen species such as superoxide anion radical, hydroxyl radical and hydrogen peroxide following pathogen infection. The accumulation of H_2O_2 is one of the earliest responses of plant-pathogen recognition. This process is referred to as 'oxidative burst' and is known to play an important role in plant defense (Low and Merida, 1996). The oxidative burst involving production of free radicals and causing cross-linking of proteins in plant cell walls is a very early response to infection. Gozzo (2003) has reviewed the role of oxidative burst in systemic acquired resistance in plants against pathogen attack.

It has been observed that the pathogen-infected tissue generally exhibits higher rate of respiration (Uritani and Asahi, 1980; Hutcheson and Buchanan, 1983). Generation of reactive oxygen species has also been frequently found in plant-pathogen interactions (Doke, 1983; Sutherland, 1991; Mehdy, 1994) as well as elicitor-treated cultured cells (Apostol *et al.*, 1989a), including parsley (Nürenberger *et al.*, 1994). Defense reactions that precede rapid cell death, such as the synthesis of cell-wall material, translocation of cytoplasm and nucleus, and expression of defense-related genes (Freytag *et al.*, 1994) are all energy-demanding processes. Thus, it is conceivable that the infected cells need to be supplied with sufficient energy to fulfill all the requirements to complete the hypersensitive reaction, including rapid cell death. Naton *et al.*, (1996) demonstrated that the activation of energy metabolism and the accumulation of intracellular peroxides are correlated with induced rapid cell death, and that this rapid cell death terminates growth of the fungal pathogen.

The origin of reactive oxygen species in plant cells is still a matter of debate (Sutherland, 1991). Oxygen radicals are generated by the major electron transport processes, mitochondrial respiration, photosynthesis, and microsomal activity (Richter *et al.*, 1977; Rich and Bonner, 1978; Michalski and Kaniuga, 1981). Studies by Doke (1985) and Doke and Chai (1985) suggest the existence of a membrane-located superoxide-generating NADPH oxidase that may be involved in the oxidative burst in association with the hypersensitive reaction.

Induction of oxidative burst in elicited cells is rapid and does not require transcription, unlike most other plant defense responses, which involve induction of batteries of defense-related genes. In contrast to the elicitor-induced oxidative burst, these defense responses require at least several hours for cumulative transcription and translation before maximal response is observed, although initial transcriptional activation may be apparent within one hour (Dixon and Paiva, 1995).

Several functional roles for the oxidative burst in plant defense have been described. Higher concentration of hydrogen peroxide in plant tissues increases the resistance. At the concentration known to be produced in plants, it could be directly toxic to pathogens. Currently, it is considered that the generation of reactive oxygen species (the oxidative burst) and hydrogen peroxide are substantially responsible for plant cell death (Bolwell & Wojtaszek, 1997). The oxidants may function directly, in cell wall cross-linking of structural proteins or as part of signaling mechanisms (Kuc, 1997). In general, it has been suggested that H_2O_2 could be involved in: (1) direct killing of the pathogen; (2) act as a second messenger in defense reactions (HR or synthesis of phytoalexins); (3) as a factor involved in plant cell reinforcement, a mechanism restricting pathogen development (Mehdy *et al.*, 1996).

At a higher level of H_2O_2, hydroxyproline and proline-rich cell wall glycoproteins are rapidly oxidatively cross-linked in cell walls after fungal elicitor treatment (Hammond-Kosack & Jones, 1996). The H_2O_2-mediated cross-linking of cell wall structural proteins makes the cell wall more refractory to digestion by microbial cell wall-degrading enzymes (Bradley *et al.*, 1992; Levine *et al.*, 1994). Wu *et al.*, (1995) produced transgenic potato plants with a fungal glucose oxidase driven by figwort mosaic virus 35S promoter. The transgenic plants showed 2-3 fold increase in H_2O_2 production and enhanced protection to potato late blight caused by *Phytophthora infestans*. This also substantiates the application of this system to a broad-spectrum of diseases. Similar report of enhanced resistance to fungal and bacterial pathogens by induced H_2O_2 production has been reported by Kachroo *et al* (2003) in transgenic rice with a fungal glucose oxidase gene.

Reactive oxygen species (ROS), mainly highly aggressive oxygen radicals, are thought to cause lipid peroxidation, which may represent a self-propagating process and a source of new radical species (Sutherland, 1991; Tzeng and De Vay, 1993). Unsaturated fatty acids may become more and more oxidized and may be removed from the lipid bilayer. H_2O_2 has been proposed as being the trigger of the activation of lipoxygenases (Kulkarni *et al.*, 1990). Thus, enhanced degradation of fatty acids that have been released by lipases may occur via the lipoxygenase pathway. This may cause increased membrane deterioration, finally resulting in the collapse of the protoplast. Rusteruci *et al.*, (1999) reported increased lipid peroxidation in relation with the hypersensitive reaction in cryptogein (a purified protein from *Phytophthora cryptogea*)-elicited tobacco leaves. The lipoxygenase-dependent production of free polyunsaturated fatty acid (PUFA) hydroperoxides, was suggested to be responsible for tissue necrosis and to be one of the features of hypersensitive programmed cell death.

2.1.5.2.3. Cytoplasmic aggregation

The hypersensitive response at the microscopic level is characterized by aggregation of cytoplasm at the point of infection, rapid cytoplasmic streaming, quickly followed by loss of cytoplasmic streaming and cell death. This response is rapid, usually occurring within 30 min. of penetration by the fungus (Tomiyama *et al.*, 1979). This structural change, called cytoplasmic aggregation, is observed when a fungus invades a plant cell, and is one of the first visible events observed in the defense response of plants. It is defined as the rapid translocation of cytoplasm and the nucleus to the site of fungal penetration of a cell, especially in case of a resistant reaction.

Takemoto *et al* (1997) observed that cytoplasmic aggregation is the early resistance-associated event that is observed in potato tissues either after penetration of an incompatible race of *Phytophthora infestans*, the potato late blight fungus, or after treatment with hyphal wall components (HWC) prepared from *P. infestans.* Kitazawa *et al* (1973) and Doke and Tomiyama (1980) also reported the induction of cytoplasmic aggregation in potato tissues and protoplasts upon penetration by *P. infestans* or stimulation by HWC. Davis and Currier (1986) observed that most of the potato protoplasts treated with the elicitor, arachidonic acid, reacted immediately, showing cytoplasmic aggregation to one site in the protoplast followed by loss of cytoplasmic contents. Tomiyama *et al* (1982) reported that cytoplasmic aggregation is indispensable to the induction of defense responses. In studies of several plants, it was demonstrated that the motility associated with cytoplasmic aggregation is dependent on the rearrangement of actin filament (Gross *et al.*, 1993; Kobayashi *et al.*, 1994).

2.1.5.2.4. Phytoalexins

Genes activated in plant disease resistance include those which encode enzymes that are involved in secondary metabolic pathways. These enzymes catalyze biosynthesis of wall appositions and phytoalexins, which are accumulated in cells that are in direct contact with the pathogen and in the surrounding cells. Low molecular weight, non-proteinaceous compounds produced, formed as products of the secondary metabolism of plants as a response to pathogen infection, are known as phytoalexins (Kuć, 1997). The presence of elicitors also stimulates the plant to produce great amounts of phytoalexins. The phenolic compounds, terpenoids and isoflavonoids have received great attention. Where resistance is associated with cell necrosis, e.g. in a hypersensitive response, phytoalexin biosynthesis and accumulation occur within the infected cell and also, possibly, the neighbouring cells. Park *et al* (2002) reported phenylpropanoid defense responses in the form of accumulation of glyceollin and other conjugates of isoflavones, daidzein and genistein in soybean cells immediately proximal to the point of treatment of wall glucan elicitor from *Phytophthora sojae*. Fellbrich (2000) reported accumulation of furanocoumarin phytoalexins in cultured parsley cells treated with cell wall elicitor of *Phytophthora parasitica*. Phytoalexin accumulation is a local and not a systemic response to infection.

2.1.5.2.5. Proteins and enzymes in plant defense

Protein-protein interactions play a crucial role in plant-pathogen recognition and defense responses (Hutcheson, 1998). Depending on their function during the defense response, proteins can be grouped into three classes: (1) The structural proteins in the first class, participate in strengthening and repairing of the cell wall or modification of the properties of the extra-cellular matrix; (2) The second class of proteins exhibits direct anti-microbial activities or catalyzes the synthesis of anti-microbial compounds. (3) The third class comprises of proteins that are activated during pathogenesis (Schoeltens-Toma *et al.*, 1991).

2.1.5.2.5.1. Structural proteins

These are proteins that function in strengthening the physical barriers to prevent pathogen invasion (Lebeda *et al.*, 2001). These include extensins namely, hydroxyproline-rich glycoproteins (HPRGs) and glycine-rich proteins (GRPs). Extensins are basic, highly glycosylated proteins. They are synthesized as soluble monomer polypeptides inside the cells and secreted to the apopolast and incorporated into the cell walls.

Enzymes like phenylalanine-ammonia lyase and peroxidase also participate in strengthening the cell walls through lignin synthesis. Deposition of callose in response to pathogen attack is also involved in structural defenses (Smart, 1991). The enzyme involved in this is callose-synthetase that catalyzes the formation of β-glucans, which are components of the extra-cellular matrix.

2.1.5.2.5.2. Proteins involved in anti-microbial activities

This class of proteins has either direct anti-microbial activity or catalyzes the synthesis of anti-microbial compounds. These include proteases that act on the pathogen and inhibit their growth; lipoxygenases which form free radicals that react on fatty acids to produce volatile and non-volatile fatty acid-derived secondary metabolites that could directly attack invading pathogens; peroxidases that participate in the synthesis of ethylene and phenolic compounds like quinones, tannins and melanins that are toxic to pathogens (Sitbon *et al.*, 1999); phenylalanine ammonia lyase, sesquiterpene cyclase 4-coumarate-CoA ligase, cinnamic acid-4-hydroxylase, cinnamyl alcohol dehydrogenase, chalcone synthase, and chalcone isomerase (Vidhyasekaran, 1993a) that are involved in synthesis of low-molecular-weight substances such as phytoalexins, phenols, lignins, tannins, and melanins, which are inhibitory to fungal pathogens (Bell, 1981; Vidhyasekaran, 1988a, b, 1990, 1993a). Some proteins form a complex with fungi and insect proteases and inhibit their activities and the regulation of the synthesis of these proteins is mediated by signaling molecules like ethylene, salicylic acid and jasmonic acid through the signal transduction pathway which is initiated by the binding of the pathogens with the receptors of the plant cells.

2.1.5.2.5.2.1. Phenylalanine ammonia lyase (PAL, E.C. 4.3.1.5)

PAL is a very important enzyme in secondary metabolism (Henstrand *et al.*, 1992) and catalyzes the first step in the phenylpropanoid pathway that is responsible for the synthesis of various phytoalexins, induced during a pathogen attack. Phenylalanine, the substrate of PAL is the first metabolite in the phenylpropanoid pathway that is involved in the synthesis of phytoalexins. Lignin, a polymeric cell wall phenolic, is synthesized by the phenylpropanoid pathway. The phenylpropanoids are derived from cinnamic acid, which is synthesized from phenylalanine by the action of phenylalanine ammonia-lyase (PAL), the rate-limiting entry point into phenylpropanoid metabolism. While PAL is the entry point into phenylpropanoid metabolism, cinnamyl alcohol dehydrogenase (CAD) is specifically involved in the biosynthesis of lignin and related cell wall-bound polyphenolics (Hotter, 1997). Induction of the activities of phenylpropanoid biosynthetic enzymes such as PAL by addition of fungal elicitor to cell suspension cultures have served as useful markers for studying the activation and kinetics of defense-related phenylpropanoid metabolism (Dixon and Paiva, 1995). Sasabe *et al* (2000) observed expression of defense genes such as phenylalanine ammonia lyase gene in tobacco BY-2 cells following treatments with Escherichia coli preparations of INF1, the major elicitin of *P. infestans*. Zhao *et al* (2000) reported activation of phenylalanine ammonia lyase, peroxidase and polyphenoloxidase in the leaves of tobacco seedlings treated with chitosan that induced resistance in tobacco seedlings against *Phytophora parasitica.*

2.1.5.2.5.3. Proteins induced during pathogen attack: PR-proteins

Proteins participating in defense mechanisms after pathogen attack are generally called pathogenesis-related proteins or PR-proteins (Antoniw and White, 1987; Van Loon, 1989; Van Loon & Van Strein, 1999). While the first two classes of proteins accumulate around the site of infection and restrict the pathogen ingress, PR- proteins appear to act directly on the invading pathogen (Verburg and Huynh, 1991). Gene expression is considerably altered when plants respond to any stress stimuli, including pathogen attack or elicitor treatment. Some such genes triggered in defense against the pathogen, include those coding for pathogenesis-related (PR) proteins. Numerous plant species have been reported to accumulate PR proteins upon attack by micro-organisms. The most widely used operational definition of PR proteins is that of polypeptides with relatively low molecular weights (M_r, 10,000-40,000), that accumulate extra-cellularly in infected plant tissue, exhibit basic nature with low pH optima for their catalytic activity, high resistance to proteolytic degradation, high temperature stability, and often, but not always, possess extreme isoelectric points (Van Loon, 1985). Among PR proteins, plant hydrolases such as 1,3-β-glucanases and chitinases have been suggested to be involved in plant resistance against fungal pathogens (Schlumbaum *et al.*, 1986; Legrand *et al.*, 1988; Pan *et al.*, 1991). .

A class of defense genes, induced at a late stage in the defense responses, not only in the invaded region, but also systemically, includes genes for PR-proteins. PR proteins have been thought to play an important role in the defense mechanisms of plants, because most of them show anti-fungal action. These pathogenesis-related proteins, defined as proteins coded for by the host plant but induced specifically in pathological or related situations (Antoniw & Pierpoint, 1978; Van Loon *et al.*, 1994), are low molecular weight, soluble proteins which are over-expressed in the infected plant tissues and not only accumulate locally in the infected leaf, but are also induced systemically, associated with the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria and viruses. They have unique properties such as high solubility at low pH (Van Loon, 1976) and resistance to many kinds of proteases (Pierpoint, 1983). Both acidic and basic isoforms of PR proteins have been reported in plants. The acidic forms accumulate in apoplast while the basic forms accumulate in vacuoles of the host cells. Exceptions have also been reported; a few acidic PR proteins accumulate in vacuoles. Among the PR proteins, basic forms alone have been shown to have fungitoxic action. Pathogens develop in the apoplast in the initial stages and only in the necrotrophic phase do they disrupt the vacuole, releasing the PR proteins into the extracellular space. It suggests that the PR proteins may act only in the later stages of pathogenesis, resulting in symptom suppression. Virulent pathogens delay the accumulation of PR proteins. The delay may be due to delay in signal induction and transcription. It is also possible that delay in elicitor release from the fungal cell wall, degradation of elicitor, action of host specific elicitor, and presence of suppressor would have contributed to the delay in signal induction.

Induction of PRs has been found in many plant species belonging to various families (Van Loon, 1999). The criteria used for the inclusion of new families of PRs are, : (i) protein(s) must be induced by a pathogen in tissues that do not normally express the protein(s), and (ii) induced expression must have been shown to occur in at least two different plant-pathogen combinations, or expression in a single plant-pathogen combinations, or expression in a single plant-pathogen combination must have been confirmed independently in different laboratories (Van Loon & Van Strein, 1999).

PR proteins are divided into 5 major groups according to their solubility, relative molecular mass Mr, biochemical activity and other chemical properties (Dixon *et al.*, 1995). The function of the majority of the PR-proteins is known or can be inferred. PR-2 family of proteins are endo-β-1, 3-glucanases and PR-3, -4, -8 and –11, are classified as endochitinases. PR-6 proteins are proteinase inhibitors, implicated in defense against insects, herbivores, microorganisms, and nematodes (Ryan, 1990; Koiwa *et al.*, 1997). PR-7 has so far been characterized only in tomato, where it is a major PR and acts as an endoproteinase. PR-9 family of peroxidases is likely to function in strengthening plant cell walls by catalyzing lignin deposition in reaction to microbial attack. PR-5 family belongs to the thaumatin-like proteins with homology to permatins that permeabilize fungal membranes. Some of these proteins have been found to possess anti-fungal activity against oomycetous fungi. Recently, a 22kD PR-5 protein from potato was shown to bind to actin, along with a 32kDa basic chitinase, and the actin-binding complex formed was suggested to be involved in cytoplasmic aggregation, thereby participating in the potato cell's defense against *Phytophthora infestans* (Takemoto *et al.*, 1997). Hoegen *et al.*, (2002) reported massive accumulation of pathogenesis-related (PR) proteins in the extracellular leaf space in potato leaves in response to with the late blight pathogen *Phytophthora infestans*, or treatment with fungal elicitor. The most abundant of these proteins was purified to apparent homogeneity and identified as a new. Edreva *et al.,* (2002) reported accumulation of PR-proteins in beta-cryptogein (a proteinaceous elicitor from *P. cryptogea*) treated tobacco leaves as part of the acquired resistance.

Most of the PR proteins can be considered as direct antimicrobial proteins or enzymes (Ponstein *et al.*, 1993; Niderman *et al.*, 1995). The two major PR-proteins with enzymatic activity are β-1, 3-glucanases and chitinases (Daugrois *et al.*, 1990).

These enzymes play an important role in cleavage of fungal cell wall components. Enhanced production of them was noted in many plant-pathogen interactions, in connection with both hypersensitive reaction and systemic acquired resistance (SAR) (Sticher *et al.,* 1997). They occur in healthy plants mainly in the apoplast. β-1, 3-glucanases together with chitinases release glucan and/or chitin fragments from the cell walls of the fungal pathogen (De Lorenzo *et al.*, 1997). Glucans and N-acetyl glucosamines, the monomeric components of cellulosic and chitinaceous walls respectively, thus released, have resistance-elicitor activity and increase the plant's induced response to infection (Repka, 1993).

2.1.5.2.5.3.1. Glucanases

Enhanced production of some enzymes and their increasing activity is one of the most important processes in plant defense. These enzymes occur frequently in many isoforms and are involved in the synthesis of defense substances or have a direct antimicrobial activity. In some specific plant-fungal pathogen interactions, the presence or activities of enzymes can be used as biochemical markers of the degree of resistance and / or susceptibility

Among PR proteins, plant hydrolases such as 1,3-β-glucanases and chitinases have been suggested to be involved in plant resistance against fungal pathogens (Schlumbaum *et al.*, 1986; Legrand *et al.*, 1988; Pan *et al.*, 1991). Glucanases belong to group PR-2 of PR proteins and breakdown β-1, 3-glucans which are components of fungal cell wall (Yoshikawa *et al.*, 1993). β- 1, 3- glucanase solubilizes elicitor-active glucan molecules from the cell walls of invading fungal pathogens (Mauch and Staehelin, 1989), in turn inducing its own production and that of other defense enzymes involved in the synthesis of antimicrobial phytoalexins and cell wall barriers. It was proposed that the degraded glucans act as elicitors to trigger the defense responses of the host system, thereby arresting the pathogen ingress. Yoshikawa *et al* (1990) reported that treatments which led to higher β-1,3 glucanase activity in soybean hypocotyls resulted in greater general resistance to the fungus. Plant glucanases have been known to play a role in the general resistance of plants by the indirect method of releasing elicitor-active molecules from pathogens (Keen, 1993).

Extra-cellular and intracellular forms of β- 1, 3- glucanases are known. The extracellular forms, which are wall-bound, are acidic in nature, while the intracellular (vacuolar) are basic. While the vacuolar forms act as a last line of defense, the extracellular enzyme is involved in the recognition and release of elicitors (Ramachandra Kini *et al.*, 2000). In bean, pea and pearl millet and tomato, basic β- 1, 3- glucanases have been found to be highly inhibitory against their respective pathogens (Much *et al.*, 1988; Mauch and Staehlin 1989; Pozo *et al.*, 1999; Ramachandra Kini, K *et al.*, 2000). It has been observed that the anti-fungal activity of plant protein extracts against different *Phytophthora* species lies in basic glucanase forms (Pozo *et al.*, 1999). The β- 1, 3-glucanases isolated from pepper stems that were highly inhibitory against *Phytophthora capsici* was also basic in nature (Kim and Hwang, 1997).

β- 1, 3- glucanases show a complex isozyme pattern with isoforms differing in their biochemical characteristics, primary structure, antigenicity, enzyme activity, subcellular localization and antifungal properties (Simmons, 1994).

As β-1, 3- glucanase is a direct defense enzyme. In contrast to the products of complex biosynthetic pathways such as phytoalexins, they may be valuable targets for engineering defense in transgenic plants (Kim and Huang, 1997). Transgenic tobacco plants have been developed that express the cloned soybean glucanase gene and it was observed that the transformed plants were resistant to several pathogens, including *Phytophthora parasitica* var. *nicotianae* (Yoshikawa *et al.*, 1993). Yoshikawa *et al* (1993) have produced transgenic tobacco plants with glucanase driven by 35S promoter showing increased resistance to *R. solani.* Li *et al* (2002) reported transformation of tobacco cultivars with the antifungal protein chitinase and beta-1, 3-glucanase genes via Agrobacterium mediated transformation and the primary results indicated that the offsprings of transgenic plants have good resistance to *Colletotrichum* and *Phytophthora.*

2.1.5.2.5.4. Antioxidant defenses

2.1.5.2.5.4.1. Anti-oxidant enzymes

2.1.5.2.5.4.1.1. Superoxide scavenging enzyme

2.1.5.2.5.4.1.1.1. Superoxide dismutase (SOD) (EC 1.15.1.1)

Superoxide dismutase is an antioxidant enzyme, which is involved in plant defense reactions. SODs are detoxification metallo-enzymes that catalyze the dismutation of superoxide radicals to molecular oxygen and a more toxic reactive oxygen species, namely, hydrogen peroxide.

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Enzymes with SOD activity are classified into three classes according to the metal co-factors: copper/zinc, manganese, and iron form (McCord and Fridovich, 1969; Bowler *et al.*, 1994; Kanematsu and Asada, 1994). Plants generally contain Mn-SOD in the mitochondrial matrix and peroxisomes, Fe-SOD in chloroplasts, Cu/Zn-SOD, that is inhibited by KCN, mainly in cytosol and in chloroplasts (Asada, 1992; Bowler *et al.*, 1994; Palma *et al.*, 1986; Salin, 1988). The superoxide radicals, which form the substrate for this enzyme, are more often produced in incompatible interactions, for example between potato and incompatible race of *P. infestans*. Superoxide dismutase scavenges the superoxide radicals, in the process producing hydrogen peroxide. Higher concentration of hydrogen peroxide in plant tissues increases resistance. At the concentration known to be produced in plants, it could be directly toxic to pathogens. At a higher level of H_2O_2, hydroxyproline and proline-rich cell wall glycoproteins are rapidly oxidatively cross-linked in cell walls after fungal elicitor treatment (Hammond-Kosack & Jones, 1996). Fodor *et al.,* (1997) observed increased activity of superoxide dismutase among the enzymatic changes taking place during the development of systemic acquired resistance in non-inoculated leaves of tobacco after inoculation of lower leaves with tobacco mosaic virus. Barna and Pogany (2001) suggested that elevated activity of superoxide dismutase in tomato plants regenerated from tumors induced by

Agrobacterium tumefaciens could play a significant role in their tolerance to *Phytophthora infestans.*

2.1.5.2.5.4.1.2. H_2O_2-scavenging enzymes

2.1.5.2.5.4.1.2.1. Peroxidase (POD) (EC 1.11.1.7)

The hydrogen peroxide formed by the action of superoxide dismutases on superoxide radicals is scavenged by peroxidases and catalases. Peroxidases are frequently associated with plant defense against pathogens. These are heme-containing enzymes that are usually glycosylated and catalyse large variety of reactions (Siegel, 1993) by the following mechanism:

$$XH_2 + H_2O_2 \rightarrow X + 2H_2O$$

Peroxidases catalyse the oxidation of substrates like phenol and its derivatives by utilizing hydrogen peroxide. They are responsible for the dehydrogenation of sinapylalcohol and coniferyl alcohol during the lignin synthesis. Peroxidases participate in the synthesis of flavones, stilbenes and other phenolic secondary metabolites. They are represented by many isoenzymes. Peroxidase polymorphism could also be used as a biochemical marker related to the different levels of field resistance (Lebeda *et al.*, 1999). Peroxidases are associated with the cell wall and participate in processes that occur in the extracellular matrix (Buonario & Montalbini, 1993). They also remove the toxic hydrogen peroxide from tissues; participate in synthesis of phenolic compounds and in the building of intermolecular bonds during the organization of the cell wall at the sites of infection by pathogens (Repka & Slovakova, 1994). The production of phenolic compounds includes synthesis of quinones, tannins, melanins and also the polymerization of lignin and suberin composing monomers. Tannins and melanins are dihydroxyphenol and quinone oligomers and are toxic to pathogens. Lignin and suberin are involved in structural defense. Peroxidase also participates in the synthesis of ethylene, the concentration of which increases frequently in pathogenesis process (Tudzynski, 1997). The activity of peroxidases is generally enhanced after a pathogen attack, because they participate in defensive lignification and synthesis of phenolic compounds that are effective against pathogens (Nicholson & Hammerschmidt, 1992). The increase in their activity coincides with increased H_2O_2 levels (Kuzniak *et al.*, 1999). Egea (2001) reported increase in peroxidases with an associated increase in lignification in resistant *Capsicum annuum* suspension cultures on elicitation with *P. capsici* elicitor. Sen *et al* (2002) reported increase in specific activity of peroxidase in infected leaves of tolerant cultivars of Taro (*Colocasia esculenta*) compared to the susceptible cultivars, on attempted infection by *Phytophthora* leaf blight disease. Edreva *et al.*, (2002) also reported an increase in peroxidase activity in tobacco leaves treated with beta cryptogein , an elicitin of *Phytophthora cryptogea*, over controls. Das *et al.*, (2002) reported elevated activity of peroxidase in Gamma rays-induced mutant of Indian cultivar of potato, resistant to *Phytophthora infestans.*

2.1.5.2.5.4.1.2.2. Catalase (CAT) (EC 1.11.1.6)

Catalase is included in plant defense reactions and is one of the main enzymes playing a role in catabolism of H_2O_2. It is a tetrameric heme protein, occurring in almost all aerobic organisms. This enzyme decomposes the hydrogen peroxide to water and oxygen:

$$2\,H_2O_2 \longrightarrow 2\,H_2O + O_2$$

Plant catalases are predominantly peroxisomal enzymes and most of them contain a carboxy terminal consensus sequence for peroxisomal import. The catalases play the role of a specific peroxidase and their function is protection of cells from the toxic effects of hydrogen peroxide. This enzyme is the competitor of peroxidase, as both the enzymes use the same substrate. A considerable decrease in catalase activity was noted in correlation with very high peroxidase activity in tobacco plants infected with *Erysiphe cichoracearum* (Buonario & Motalbini, 1993). Catalase activity is usually associated with compatible plant-pathogen interactions. Vanacker *et al* (1998) reported large increases in foliar catalase activity in barley cultivar susceptible to *Blumeria graminis*, while the resistant cultivar did not show any increase. Molinari (2001) also reported significant inhibition of catalase activity in roots of resistant tomato inoculated with

Meloidogyne incognita, while such inhibition was not observed in the roots of susceptible line. The inhibition of catalase was also observed with salicylic acid treatment, which is known to induce systemic resistance in plants.

2.1.5.2.5.4.1.2.3. Enzymes of the Ascorbate-glutathione cycle

Enzymes of the ascorbate glutathione cycle (AGC) are known to be differentially induced in different plant-pathogen interactions and serve to scavenge the hydrogen peroxide formed during the dismutation of superoxide radicals by superoxide dismutase. Reactive oxygen intermediates (ROI) that are produced during the oxidative burst are known to activate programmed cell death and induce anti- microbial defenses and thus play a critical role in the defense of plants against invading pathogens. The main player in the ascorbate-glutathione cycle is the ascorbate peroxidase (AP), which has increased ability to scavenge hydrogen peroxide than catalase. Many reports suggest that suppression of active oxygen species-detoxifying enzymes favour increased resistance in plant-pathogen interactions. Mittler *et al.,* (1999) reported that transgenic antisense tobacco plants with reduced capability to detoxify ROI (i.e., antisense APX or CAT) were found to be hyper-responsive to attack by *Pseudomonas syringae.* They activated PCD in response to low amounts of pathogens in comparison to control plants, which did not show activation of PCD in response to the pathogen.

Vanacker *et al* (1999) studied the differential responses of three oat lines differing in their resistance to attack by biotrophic fungal pathogen *Blumeria graminis* and noted that resistance correlated with increased total foliar glutathione, an increase in the ratio of reduced to oxidized glutathione and with decreased total activities of foliar ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase and monodehydroascorbate reductase.

2.1.5.2.5.5. Lipoxygenase (LOX) [EC 1.13.11.12]

Lipoxygenase (LOX) activity has been identified consistently during pathogen-induced defence responses. Lipoxygenase is a non-heme iron-containing dioxygenase, which deoxygenates unsaturated fatty acids to yield hydroperoxides and forms free radicals. Its activity rapidly increases after pathogen attack (Maccarone *et al.*, 1997). Reactions of free radicals can result in the production of toxic volatile and non-volatile fatty acid-derived secondary metabolites that could directly attack invading pathogens. Alternatively, lipoxygenase can cause irreversible membrane damage and cell death (Hammon- Kosack & Jones, 1996). Lipoxygenase-dependent peroxidative reactions were observed in cryptogein-elicited tobacco leaves that was suggested to be responsible for the programmed cell death associated with the hypersensitive response (Rusterucci *et al.*, 1999) occurring on elicitation. Kolomietes *et al* (2000) reported the involvement of a specific leaf LOX gene of potato (*Solanum tuberosum*), designated POTLX-3, in defense responses against pathogens. POTLX-3 mRNA accumulation was induced in potato leaves treated with ethylene or methyl jasmonate or infected with either virulent or avirulent strains of *Phytophthora infestans*, the causal agent of late blight. LOX activity assayed during an incompatible interaction in leaves peaked 3 days earlier than during a compatible interaction. During the resistance response, POTLX-3 was induced within 6 hours, increased steadily through 24 hours, and its mRNA continued to accumulate for a week after inoculation. In contrast, when a plant was susceptible to P. infestans, induction of mRNA accumulation in response to inoculation was inconsistent and delayed. Noehringer *et al.*, (2000) reported that treatment of parsley cell cultures with a fungal elicitor from *Phytophthora sojae* triggered the induction of a lipoxygenase isoform, which may be involved in the de novo synthesis of defense-response inducers, such as jasmonic acid or 12-oxo-phytodienoic acid.

2.1.7. Black pepper – *Phytophthora* pathosystem – *Rationale of the present study*

The main production constraint in black pepper cultivation in addition to abiotic stress is the massive destruction of vines by *Phytophthora* foot rot or Quick wilt disease, caused by *Phytophthora capsici*. *Phytophthora* foot rot was first reported in Indonesia (Muller, 1936) in 1936. The devastation caused by this disease was studied in detail in Sarawak, Malaysia (Holliday and Mowat, 1963). Though the vine death was reported in India as early as 1902 in Wayanad region of Kerala (Butler, 1902), the causal organism was identified as *Phytophthora* only during 1966 (Samraj and Jose, 1966). Crop loss of

905 and 119 tonnes of black pepper was reported from Kannur and Calicut districts respectively of Kerala, India due to vine death of 9.4 and 3.7% respectively Balakrishnan *et al.*, 1986).

Integrated disease management involving cultural, chemical, biocontrol methods coupled with host resistance has been thought to provide a viable solution to combat the disease (Sarma *et al.*, 1994). Chemical control involves use of copper fungicides like Bordeaux mixture and systemic fungicides like metalaxyl, while biological control uses biocontrol agents like *Trichoderma*, *Gliocladium* and vescicular arbuscular mycorrhiza (VAM) to control the pathogen. Venugopal,

A high degree of host resistance in black pepper to the pathogen is lacking at present (Sarma, Anandaraj and Venugopal,. Hence, development of resistance for *P. capsici* would be of high relevance. Reports on successful induction of resistance in black pepper are scanty. Shylaja *et al* (2000) attempted tissue culture techniques to induce tolerance to *Phytophthora capsici* using somaclonal variations in black pepper. Basic understanding of the host-pathogen interactions is a pre-requisite for any programme on attempting induction of host resistance in black pepper. Jebakumar *et al* (2001) reported induction of defense enzymes in a greater magnitude in a tolerant line of black pepper, compared to the susceptible lines, and induction of PR-proteins, on attempted infection with *P. capsici.* Significant work needs to be done to unravel the various defenses induced in the host on infection with the pathogen for improving the crop for resistance against *P. capsici.* Studies on the finer details of the host-pathogen interactions at the cellular level would be more rewarding. Hence the present work was taken up to study some of the defense responses that take place at the cellular level using a model system consisting of cell suspension cultures of black pepper and hyphal wall components of *Phytophthora*, to minimize the complexities involved in the studies under *in vivo* conditions.

2.2 Materials and Methods

Studies on host-pathogen interactions in black pepper-*Phytophthora* pathosystem were taken up in the present programme using a simplified model system in which cell suspension cultures of black pepper were used in the place of whole plants and the live pathogen, replaced with hyphal wall-elicitors from *Phytophthora* spp.

2.2.1. Reaction of black pepper leaves to different species of *Phytophthora*

A preliminary study was undertaken to study the pathogenicity of different species of *Phytophthora* on black pepper with a view to select a species which could be used as a non-pathogen to study the incompatible-interactions between black pepper and the non-pathogen and compare it with the host responses taking place in a compatible-interaction. Three species of *Phytophthora* maintained in the National Repository of Phytophthora at Indian Institute of Spices Research, Calicut, Kerala, were taken for the study. The *Phytophthora* species used were isolates from four different hosts and included *P. capsici* isolate, 98-176, a known virulent isolate from black pepper, *P. parasitica* isolates, 00-47 and 00-49 from *Vinca* and tomato respectively, *P. meadii* isolate, 99-183a, from cardamom.

Ten-millimeter discs from the advancing margins of young growing mycelium of 3-day old culture of each of the isolates were placed on the ventral side of detached black pepper leaves, both with and without pinprick. The leaves were incubated for 4 days in a moist chamber consisting of a closed box with moistened filter paper lining. The mycelial discs were kept moist by regularly wetting the absorbent cotton plugs placed over the discs. The reaction of the leaves to the different *Phytophthora* isolates was recorded by observing the spread of the infection lesion over 96 hours.

2.2.2. Model system for studying host-pathogen interactions

The black pepper-*Phytophthora* interactions were studied using a model system consisting of suspension-cultures of black pepper and hyphal wall elicitors from *Phytophthora* spp.

2.2.2.1. Hyphal wall-elicitors

2.2.2.1.1. Fungal culture and maintenance:

The black pepper isolate of *Phytophthora capsici,* namely, 98-176 and the cardamom isolate of *Phytophthora meadii*, 99-183a, tested in the above study to be pathogenic and non-pathogenic respectively, were used for all the experiments in the present study. The isolates were purified on a *Phytophthora*-selective antibiotic PVPH (Tsao, 1970) (Appendix 6) -supplemented carrot agar medium. The pure cultures of the fungi were maintained on carrot agar medium (Appendix 7) in petri-plates with periodic sub-culture to fresh medium at 3 d intervals.

2.2.2.1.2. Mass multiplication of *Phytophthora*

The *Phytophthora* species were mass multiplied in GYP broth as modified by Hall *et al* (1969) (Appendix 8). Briefly, ten mycelial plugs of 10mm diameter taken from the margins of a 3-day old pure culture of the fungus were inoculated in 150ml of modified GYP broth in 1l Roux bottles. The cultures were incubated in diffuse light at 28°c. After an incubation of 10-12 days, the mycelial mats were harvested, washed twice in distilled water, filtered under vacuum to remove excess moisture and stored at -80°c until use.

2.2.2.1.3. Isolation of hyphal wall components

Hyphal wall components (HWC) were isolated from the harvested mycelium of *Phytophthora* according to the method followed by Doke and Tomiyama (1980). The frozen mycelium was ground in a mortar with pestle in liquid nitrogen to a fine powder and then homogenized with 50mM acetate buffer, pH 4.5 in a 1:5 ratio (w:v) using a polytron homogenizer, under cold conditions. The slurry was then sonicated (Sonics sonicator) at 60% amplitude for 30min in cold. The sonicate was centrifuged at 10,000 rpm for 20min and the resultant pellet was used to isolate the hyphal wall components.

The pellet obtained above was washed twice with the acetate buffer, and re-suspended in 0.1M borate buffer pH 8.8 (in a 1:5 ratio), with a brief homogenization and then autoclaved at 120°c for 15min. The slurry was filtered and the filtrate was concentrated in a flash evaporator to a minimum volume of about 25-50ml. The concentrated filtrate was dialyzed overnight against distilled water in dialysis tubing with a cut-off limit of 2kD (Sigma). The non-dialyzable material was centrifuged to remove the lipid contents. Alternatively, lipid was removed by treating the extracts with an equal volume of diethyl ether in a separating funnel by shaking to form a gel in the ether phase. The ether phase was evaporated and the water residue obtained was used as the hyphal wall components (HWC). The HWC solution was lyophilized and suspended in 10mM Tris-HCl buffer, pH 7.4, sonicated briefly for 3 minutes to obtain a clear solution, which was then filter-sterilized through a membrane filter (0.22μm), for use in all the experiments with cell- suspension cultures.

2.2.2.1.4. Quantification of the elicitor

The carbohydrate content of the isolated hyphal wall components was determined by phenol-sulphuric acid method (Dubois *et al.,* 1956) to quantify the elicitor fraction in terms of glucose equivalent units. One ml of the hyphal wall elicitor was hydrolyzed in 5ml of 2.5 N HCl by incubating in a boiling water bath for 3 h. The solution was cooled and neutralized with solid sodium carbonate until the effervescence stopped. The volume was made up to 10ml and 0.2ml aliquots of this solution were used for the estimation of glucose content using the phenol-sulphuric acid method (Dubois *et al.*, 1956). To each sample, 1ml of 5% redistilled phenol and 5ml of 96% H_2SO_4 were added and the mixture allowed to stand for 20 min. The yellow-orange product formed by the reaction of hydroxymethyl furfural (the dehydrated form of glucose obtained during acid hydrolysis), with phenol was read at 420nm to estimate the glucose content of the sample. The cabohydrate content of the sample was determined from a standard graph of glucose and was expressed in terms of μg glucose equivalents per ml. The glucose content obtained after hydrolysis of the HWC solution was observed to be similar to that determined directly on the HWC solution without hydrolysis. Hence, thereafter, the HWC solution was used directly to quantify the elicitor in terms of glucose equivalents by phenol-sulfuric acid method. The HWC solution was lyophilized and suspended in a required volume of 10mM Tris-HCl buffer, pH 7.4, as described above to obtain a final elicitor concentration of 2μg glucose equivalents per microlitre of the elicitor solution.

2.2.2.2. Suspension-culture

Black pepper suspension cultures were prepared by inoculating embryogenic callus in liquid SH medium, as described before under section 3.1.1.1. of Materials & Methods of the chapter, Abiotic Stress.

2.2.3. Determination of optimal elicitor concentration

The optimal concentration of HWC of *P. capsici* and *P. meadii* to be used with black pepper cells was determined using 1ml cell suspension cultures taken in sterile glass vials. The cultures were inoculated with aliquots of sterile stock solution of hyphal wall elicitor to obtain different concentrations of HWC, viz., 0, 50, 100, 150, 200 and 400μg ml^{-1} cell suspension. After an incubation of 24 hours, the viability of cells was determined by the MTT -colorimetric assay and FDA staining.

2.2.4. Viability of black pepper cells after elicitor treatment

2.2.4.1. MTT colorimetric assay

The viability of black pepper cells after incubation with HWC was determined by MTT (3-(4,5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide)-colorimetric assay as described by Sanchez *et al* (1992) with a slight modification in that the formazan formed was extracted in ethanol instead of iso-propanol. The assay is based on the reduction of the tetrazolium salt to an insolube formazan by the viable cells. Aliquots of 400μl of the suspension cultures of the different treatments were taken in eppendorf tubes. 80μl of 5mg/ml MTT solution was added to the mixture and incubated for 2hrs. After the incubation, the contents of the tubes were centrifuged briefly and the supernatant discarded. 1ml of absolute alcohol was added to the pellet and incubated for about 30 minutes to extract the accumulated formazan from the cells. The mixture was centrifuged at 2,000 rpm for 10min to collect the supernatant. The absorbance of the purple colored formazan in the supernatant was measured at 570nm. The absorbance of mock-inoculated control was taken as 100% viability. The percentage cell death was calculated by the equation: $(1-A_t/A_c)$ X 100, where A_t and A_c are the absorbance values of formazan of the treated cells and HWC non-treated cells, respectively.

2.2.4.2. FDA- viability staining

The vital dye fluorescein diacetate (FDA) was used to distinguish living from dead cells/clumps according to the procedure described by Widholm (1972). A stock solution of 5mgml^{-1} FDA was prepared in acetone and diluted with culture medium to a final concentration of 50μgml^{-1}. 200μl of the cell suspension of different treatments were taken in eppendorf tubes and 10μl of the diluted FDA stock was added. The cells were incubated in the stain solution for 15min. The stain solution was subsequently replaced with fresh culture medium and the cells were examined under epi-fluorescence light using UV filter sets: (a) Excitation filter, 450-490nm; dichroic mirror, 510nm; barrier filter, 520nm; (b) Excitation filter, 330-380nm; dichroic mirror, 400nm; barrier filter, 420nm. The staining is based on the principle that the colorless fluorescein diacetate (FDA) diffuses easily into cells and is acted upon by the esterases present in viable cells, releasing the fluorochrome, fluorescein, which fluoresces green under UV. The dead cells do not exhibit the green fluorescence at 450-490nm.

2.2.4.3. Determination of cell death in terms of change in fresh weight

Cell death was measured in terms of change in fresh weight at different intervals of time after elicitation, namely 6, 12, 24, 48 and 72h after elicitor treatment. An initial fresh weight of 2.0g was inoculated in 100ml flasks containing 30ml liquid SH medium. The % change in fresh weight was calculated by the equation:

$$\%\ \text{change in f.wt.} = \frac{\text{Fin.wt.} - \text{Ini. wt.}}{\text{Ini. wt}} \times 100$$

2.2.5. Preparation of hypersensitivity inhibitory factor (Water soluble glucan)

An inhibitor of hypersensitive reaction of black pepper cells was prepared from the germination fluid of *P. capsici* obtained from zoospore suspensions of the fungus as described by Doke and Tomiyama (1977). For zoospore suspensions, mycelial plugs of 3d old cultures were placed in sterile petri-plates, with the mycelial side facing upward. Sterile distilled water was poured into the plates such that the water level just reached the mycelial end of the mycelial plugs. Care was taken to ensure that the discs were not submerged in water. The plates were incubated under fluorescent tube lights at 25-28°c. After an incubation of 48h, the plates were examined for zoosporangia formation. The plates were chilled in the freezer for 10 min. and incubated in dark at room temperature for 1hr to liberate the zoospores from the sporangia. The zoosporangia liberated zoospores into the surrounding distilled water. This zoospore suspension was aseptically transferred to sterile flasks and the concentration of zoospores was determined by a haemocytometer. The zoospore concentration was adjusted to $1x10^5$ zoospores ml^{-1} by dilution with sterile distilled water. The zoospores were encysted by adding $CaCl_2$ to the zoospore suspension to give a final concentration of 0.1mM. The flasks were incubated overnight on an orbital shaker at 100rpm at 25°c. The zoospores encysted and germinated luxuriantly by 24hours. The germinating zoospores were filtered out from the liquid to obtain the germination fluid. The fluid was concentrated 10-fold and dialyzed in dialysis tubings with a 2kD cut-off limit against distilled water. The concentrated germination fluid was freeze-dried and suspended in 0.01M Tris-Cl, pH 7.4. The sample was quantified in terms of glucose content by phenol-sulfuric acid method of Dubois *et al* (1956).

2.2.5.1. Effect of water-soluble glucan on hypersensitive reaction

An experiment was set up to study the effect of water-soluble glucan isolated from the germination fluid of *P. capsici* on the hypersensitive reaction elicited in black pepper suspension cultures after elicitor treatment. 1ml aliquots of fine suspensions of black pepper cells were taken in sterile vials. The cultures were incubated for 12 hrs with water-soluble glucans at a concentration of 1mg ml^{-1}. The hyphal wall elicitors of *P. capsici* or *P. meadii* were then added to a final concentration of 150μgml^{-1}. The cultures were incubated for 24hrs. Controls that lacked WSG/HWC or both WSG and HWC were maintained. The cultures were examined for their viability using FDA and the percentage of green / blue fluorescent cells were calculated.

2.2.6. Preparation of cell suspension cultures for studies with elicitor

750mg fresh weight of cells was inoculated in 15 ml of SH medium in 100 ml flasks, and incubated in dark on an orbital shaker. After a period of 48 h, the suspension cultures were inoculated with filter-sterilized solutions of hyphal wall components of either *P. capsici* or *P. meadii*, at a final concentration of 150μgml^{-1}. Four replicates were maintained for each treatment. Controls were mock inoculated with Tris-Cl buffer, pH 7.4, for a final concentration of 10mM. The cultures were incubated in dark and harvested at 6, 12, 24, 48, and 72h after addition of the elicitor, in all experiments, except where mentioned otherwise. The suspension cultures were harvested under vacuum on a büchner funnel with a filter paper. The cells were snap frozen in liquid nitrogen soon after harvest and stored at -80^{o}c until use.

2.2.7. Cell responses to elicitor treatment

2.2.7.1. Phenolics production

Cellular browning due to elicitor treatment was quantified in terms of phenolics accumulation in the cells by the method described by Malick and Singh (1980). 200mg f.wt. of cells were extracted in 80% ethanol and the ethanolic extracts were used for the estimation of phenolics as described under section 3.1.2.2.2. of materials and methods of the chapter, Abiotic Stress. The phenolic content of the cells was expressed as of μg catechol per 200mg fresh weight.

For the measurement of extra-cellular phenolics, the suspension cultures at different intervals after elicitor treatment, namely 0, 6, 12, 24, 48 and 72hrs, were centrifuged at 10,000 rpm for 10min at 4^{o}c and the supernatant obtained, served as the sample. The phenolic content in the supernatant was measured as described above and expressed in terms of μg catechol per ml of the medium. Comparisons were made between treatments, by taking care to inoculate equal amount of callus in known volume of the culture medium while setting up the experiment.

2.2.7.2. Superoxide radical generation

The superoxide anion generation was detected according to the method of Beleid El-Moshaty *et al* (1993) as described by Velazhahan and Vidyasekaran (1999). 250mg elicitor-treated and untreated cells were collected at 10min, 30min, 45min, 3h, 6h and 12h. on a muslin cloth and rinsed with deionized water, vacuum infiltrated with 3ml of 10mM phosphate buffer (pH7.8) containing 0.05% nitroblue tetrazolium (NBT)in 25ml flasks. The flasks were placed on a rotary shaker at 140rpm at 25°c for 1h. 2ml samples were drawn from each flask, filtered and heated for 15min at 85°c. The mixture was allowed to cool to room temperature and the A_{580} was measured to detect formazan accumulation using a spectrophotometer.

2.2.7.3. Lipid peroxidation

200mg fresh weight of callus obtained at different intervals of time was used to estimate lipid peroxidation. The MDA content, a measure of lipid peroxiation, was measured by the method of Heath and Packer (1968) as described under section 1.2.2.2.3. of Materials & Methods of the chapter, Abiotic Stress.

2.2.7.4. pH measurement

Suspension-cultures were initiated by inoculating 1.0g f.wt. of cells in 20ml liquid SH medium. The elicitors were added after 48h of inoculation to a concentration of 150μg ml^{-1}. The initial pH of the medium was noted and the change in extra-cellular pH over a period of time was recorded by an Elico pH meter.

2.2.7.5. Cytological studies

The changes taking place in the cells on elicitation was observed using a Nikon microscope by light as well as epifluorescence microscopy. The cells were stained *en masse* in eppendorf tubes. For staining callose, an aliquot of cell suspension was taken in an eppendorf tube. The cells were allowed to settle down and the medium was decanted. The cells were incubated in aniline blue solution for 10min. The stain was prepared fresh by dissolving 0.005% (w/v) aniline blue in 50mM sodium phosphate buffer, pH9.0 and stirring until the colour turned dull. The stain solution was replaced with water and the cells examined under a Nikon microscope equipped with UV epi-fluorescence. Callose was detected using appropriate filter set with excitation filter 330-380nm; dichroic mirror, 400nm and barrier filter, 420nm.

Lignin was stained by phloroglucinol-HCl method (Jensen, 1962). Prior to staining with the stain solution, the cells were extracted with ethanol, to effect a parial removal of soluble (non-wall-bound) phenolics. The stain consisted of a saturated solution of phloroglucinol in 20% of 2N HCl and was prepared by dissolving 2.0g of phloroglucinol in 80ml of 20% ethanol followed by addition of 20ml of conc. HCl.

2.2.7.6. Lignification

Lignification in elicited cells was quantified by homogenizing the cells in methanol and determining lignin in the alcohol insoluble residue (AIR) as thioglycollic acid-derivatives following alkali hydrolysis as described by Campbell and Ellis (1992).

2g of suspension-cultured cells were homogenized in buffer as mentioned below, for the extraction of protein/enzyme. The residue obtained in the muslin cloth while filtering the crude enzyme extracts of cells before centrifugation, was used for the analysis of lignin. The residue, which is mainly the wall residue, was suspended in 4ml of methanol and ground again in two changes of methanol and incubated at 80°c for 2hrs in tight screw-capped glass vials. The mixture was centrifuged at 10,000 x g for 10 min. The pellet was dried in an oven at 37°c for 48 hours and their dry weights determined. 25mg dry weight of each sample was ground in 5ml of 0.5M NaOH and extracted for 24h to hydrolyze wall-bound phenolics. The incubation mixture was neutralized with 1.0ml of 2M HCl and centrifuged at 10,000 rpm for 10min. The pellet was washed twice with distilled water and suspended in 5ml of methanol, centrifuged at 10,000rpm for 10min. The pellet was air-dried and re-suspended in 2M HCl and 0.5ml thioglycollic acid (TGA). The contents were taken in screw cap tubes and the tubes sealed tightly. The contents were incubated at 80°c for 2h and cooled on ice. The mixture was centrifuged at 10,000rpm for 10min. and the pellet re-suspended in 5ml of distilled water, vortexed and centrifuged again for 10min. The pellet was re-suspended in 5ml of 0.5M NaOH in screw-capped tubes and gently agitated on a rotary shaker at 100rpm for 12h at room temperature. The tubes were later incubated overnight at 4°c. The contents of the tubes were centrifuged and the supernatant collected. The pellet was washed twice with 2ml distilled water and the supernatants obtained after centrifugation were pooled with the NaOH extract obtained above. The combined supernatant was acidified by adding 1ml of conc. HCl and the tubes capped and the mixture incubated at 4°c for 4h to precipitate the lignin-thioglycollic acid (LTGA) derivatives. The precipitate was collected by centrifugation at 10,000rpm for 10min. The pellet obtained was washed twice with 0.1M

HCl and centrifuged. The pellet was then dissolved in 3ml of 0.5M NaOH and centrifuged. The colour of the supernatant ranged from light yellow to dark yellow to brown. Since absorbance of these samples at 280nm was above the readable range of the spectrophotometer, 200µl of each sample was made up to 5ml with 0.5M NaOH (25 X dilution) and the absorbance taken at 280nm. Results were expressed as the increase in absorbance at A_{280} nm per 250mg alcohol insoluble residue (AIR).

2.2.7.7. Enzymatic changes induced by elicitor treatment

2.2.7.7.1. Enzyme activity

Enzyme activity was calculated as described under section 3.1.2.2.4.1. of Materials & Methods described in the chapter, Abiotic Stress. In addition to International Units, enzyme activity was also expressed in terms of katals/mg protein. One katal is the amount of enzyme that catalyzes the conversion of one mole of substrate to product per second under the conditions of the assay.

2.2.7.7.2. Antioxidant enzymes

The anti-oxidant enzymes, namely, superoxide dismutase, catalase, peroxidase and the enzymes of the ascorbate-glutathione cycle were extracted and assayed as mentioned under section 3.1.2.2.4. of Materials & Methods in Chapter 1 on Abiotic Stress.

2.2.7.7.3. β-1, 3 Glucanase (EC 3.2.1.6)

Enzyme extraction

2g f.wt. of cells stored at -80°c was ground to a fine powder in a mortar with liquid nitrogen and homogenized in 0.5M sodium acetate buffer, pH 5.2 containing 15mM 2-mercaptoethanol and 2mM PMSF, a protease inhibitor. The crude extracts were passed through two layers of muslin and centrifuged at 10,000xg for 30min at 4°c.

Enzyme assay

β-1,3 glucanase activity in the crude extracts was measured colorimetrically by the method of Pan *et al* (1991) with some modifications. The principle of the assay is based on the measurement of the rate of production of reducing sugars from a substrate, laminarin, by the β-1, 3 glucanase contained in the enzyme extracts. 62.5μl of the crude enzyme extract was incubated with 62.5μl of 4% laminarin that was dissolved in 0.1M sodium acetate buffer, pH5.2, and the reaction mixture incubated at 37°c for 20min. Enzyme and substrate blanks were also maintained and treated similarly with the test samples. The enzyme blank consisted of 62.5μl 0.1M sodium acetate buffer, pH 5.2 and 62.5μl enzyme. The substrate blank consisted of 62.5μl 4%laminarin and 62.5μl buffer. The reaction was stopped by boiling at 100°c for 5 min. The volume of the mixture was made up to 1ml with distilled water and 0.1ml aliquots were taken from this to determine the reducing sugars in terms of glucose by the method of Nelson (1994). The formation of glucose was taken as a linear function of the enzyme concentration in the extract. β-1,3 Glucanase activity was measured in μkatal. One microkatal was defined as the quantity of enzyme catalyzing the formation of 1μmole of glucose equivalents s^{-1}mg protein $^{-1}$.

2.2.7.7.4. Phenylalanine ammonia lyase (PAL, EC 4.3.1.5)

Enzyme extraction

The enzyme extract prepared for assaying peroxidase, catalase and SOD was used for the PAL assay.

Enzyme assay

PAL activity was determined by measuring the production of trans-cinnamic acid from L-phenylalanine spectrophotometrically at 290nm. The reaction mixture consisted of 0.1ml of extract, 0.1ml of 0.01M borate buffer and 0.1ml of 0.15M phenylalanine dissolved in borate buffer. Enzyme and buffer blanks were maintained. The samples were incubated at 38°c for 1h. The reaction was stopped by adding 0.5ml of 1M TCA. The resultant mixture was diluted 10 times before measuring the absorbance at 270nm. Enzyme activity was expressed in nkatal. One nanokatal was defined as the quantity of enzyme catalyzing the formation of 1 nmole trans-cinnamic acid s^{-1}mg protein^{-1}.

2.2.8. Bioassays to determine the effect of elicitor treatment of black pepper tissues on *P. capsici* growth

2.2.8.1. Cell-suspension bioassay

Cell suspension cultures were inoculated with elicitors of either *P. capsici* or *P. meadii* at a final concentration of 150μg ml^{-1}. After a period of 24hrs, the cultures were inoculated with zoospore suspension of *P. capsici* with a concentration of 1 x 10^5 cells ml^{-1}. The cultures were examined under the microscope after 36 hrs of incubation for the effect of elicitor treatment of cells on the growth of *P. capsici.*

2.2.8.2. Cut-leaf bioassay

Excised black pepper leaves of uniform age were used in the leaf bioassay. The cut ends of the petioles were dipped in sterile distilled water containing 150μg ml^{-1} elicitors of either *P. capsici* or *P. meadii* in sterilized glass vials. Controls were also maintained by mock inoculating the sterile water with Tris-Cl buffer, pH 7.4. After incubation with the elicitors for 48 hours, the cut-leaves were transferred to sterile vials containing zoospore suspension of *P. capsici* with a concentration of 1 x 10^5 zoospores ml^{-1}. The growth of *P. capsici* hyphae around the petiole was followed over a period of time.

2. 3 Results

The black pepper – *Phytophthora* interactions were studied with a model system consisting of cell suspension cultures of black pepper and hyphal wall components of *Phytophthora* spp.

2.3.1. Preliminary studies

2.3.1.1. Reaction of different *Phytophthora* spp. on black pepper leaves

P. capsici isolate from black pepper caused infection on black pepper leaves within 24hrs of inoculation while the other two species of *Phytophthora* used in the study, namely *P. parasitica* isolates from tomato and vinca and *P. meadii* isolate from cardamom caused a hypersensitive reaction, generally observed in incompatible interactions. The HR consisted of a small necrotic lesion limited to the vicinity of the site of inoculation. In contrast, *P. capsici* produced spreading water-soaked lesions with the characteristic fimbriate margins and brought about the necrosis of the entire leaf lamina, by 96hrs after inoculation (HAI) (Fig. 2.1: B1, B2, B3 & B4). The H on the dorsal surface, the HR was visible to the naked eye as a group of red spots (Fig.2.1: C). Under the low resolution of a stereomicroscope, the HR on the ventral surface of the leaf appeared as small deep red to brown spots with interspersed green tissue, (Fig.2.1: D).

2.3.1.2. Cell suspension culture-elicitor model system to study black pepper-*Phytophthora* interactions

HWC of *P. capsici* and *P. meadii* was used in cell suspension cultures of black pepper, to study the defense responses elicited in the cells. The defenses induced on elicitation include hypersensitive browning of cells, superoxide radical production, cellular changes at the microscopic level, exudation of phenolics, formation of physical barriers and changes in enzyme levels.

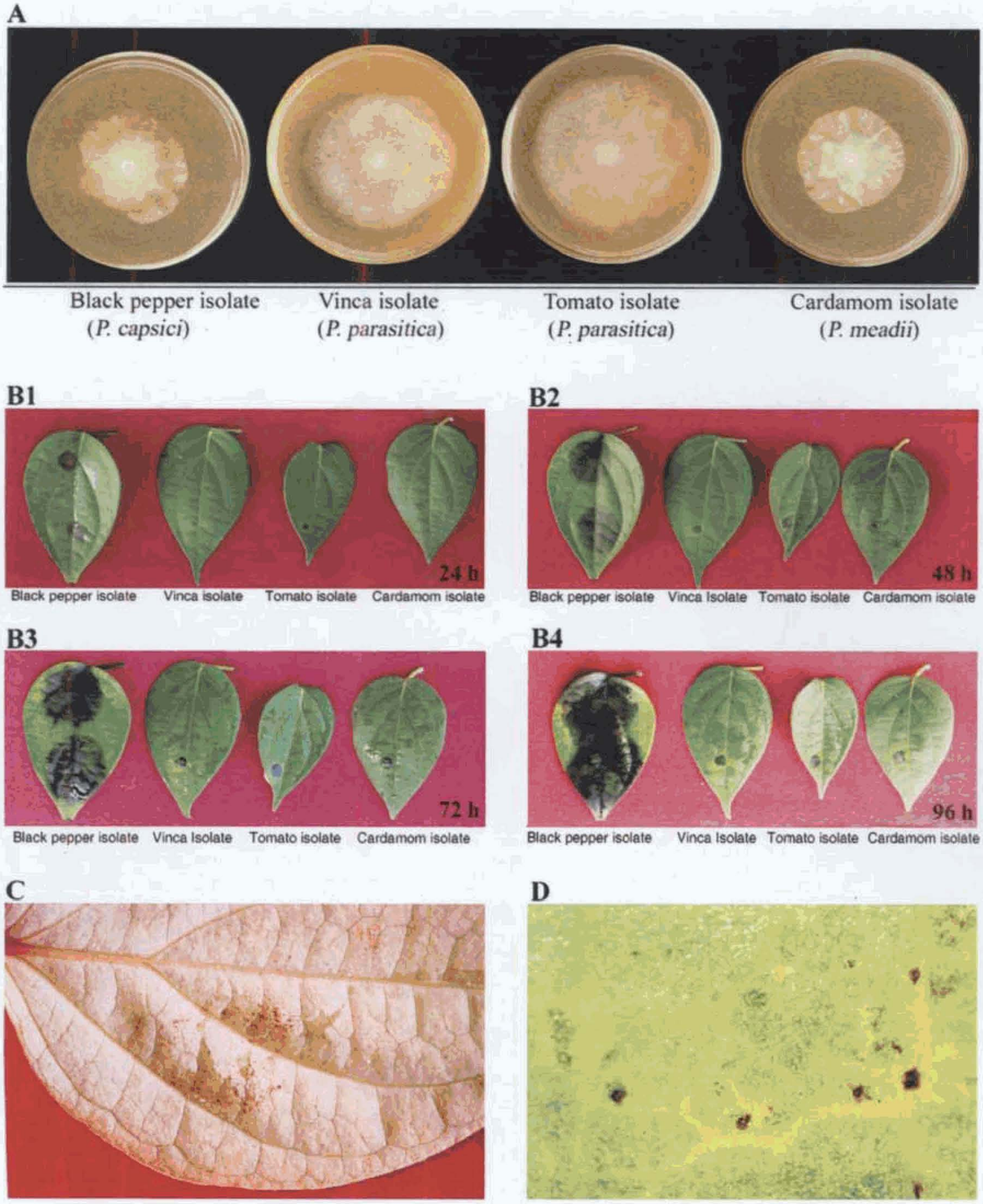


Fig. 2.1 Reaction of Blackpepper to *Phytophthora* species from different hosts. **A**. *Phytophthora* isolates from different hosts; **B1, B2, B3** and **B4**. Responses of black pepper leaves to inoculation with different *Phytophthora* isolates at 24, 48, 72 and 96 hrs after inoculation; **C.** A portion of dorsal surface of the leaf showing HR (deep red spots) on inoculation with incompatible *Phytophthora* isolates (*P. parasitica* / *P. meadii*).; **D.** Stereomicroscopic view of HR on the ventral side of the leaf.

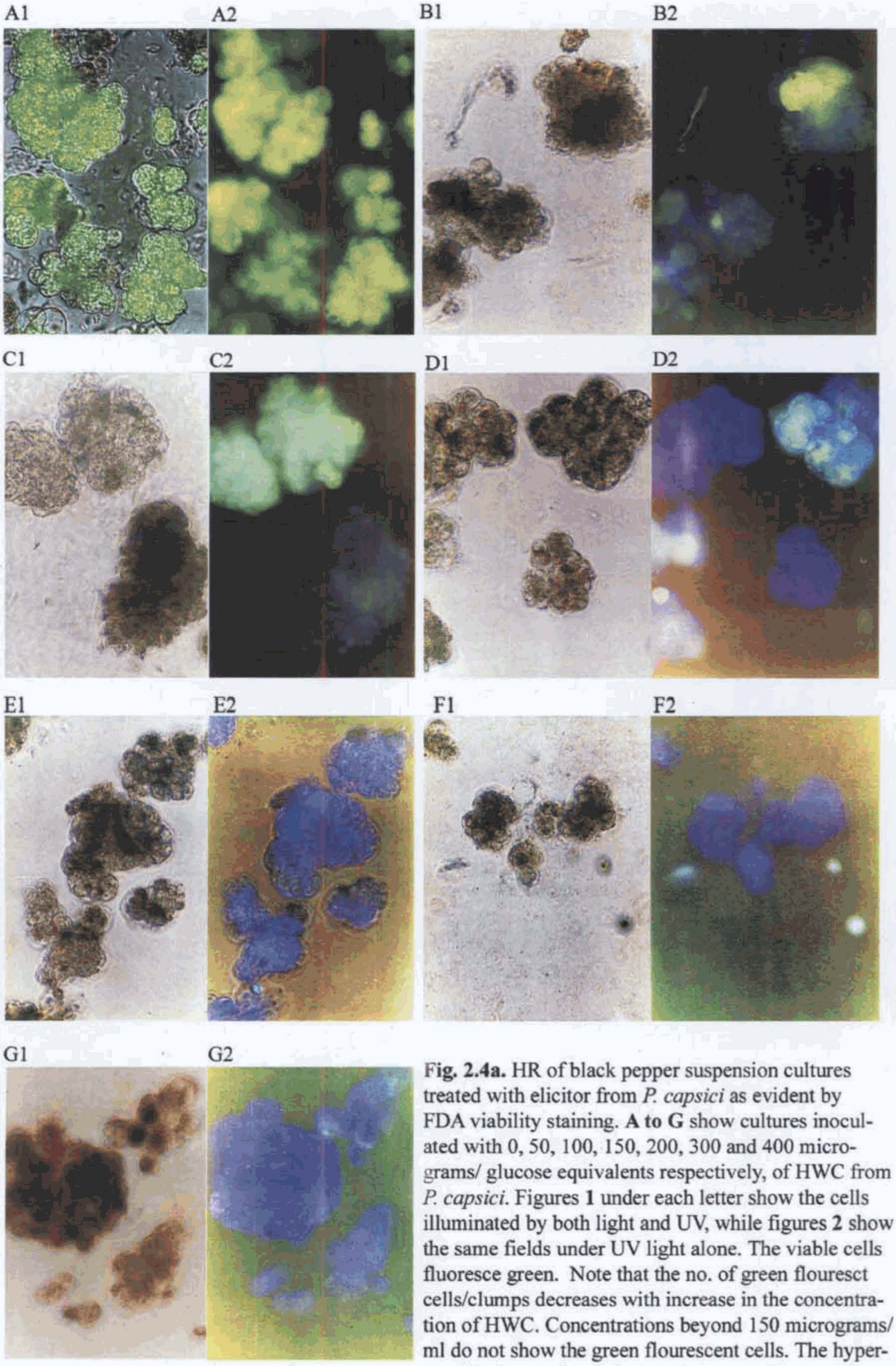


Fig. 2.4a. HR of black pepper suspension cultures treated with elicitor from *P. capsici* as evident by FDA viability staining. **A to G** show cultures inoculated with 0, 50, 100, 150, 200, 300 and 400 micrograms/ glucose equivalents respectively, of HWC from *P. capsici*. Figures **1** under each letter show the cells illuminated by both light and UV, while figures **2** show the same fields under UV light alone. The viable cells fluoresce green. Note that the no. of green flouresct cells/clumps decreases with increase in the concentration of HWC. Concentrations beyond 150 micrograms/ ml do not show the green flourescent cells. The hypersensitively reacting cells autofluouresce blue.

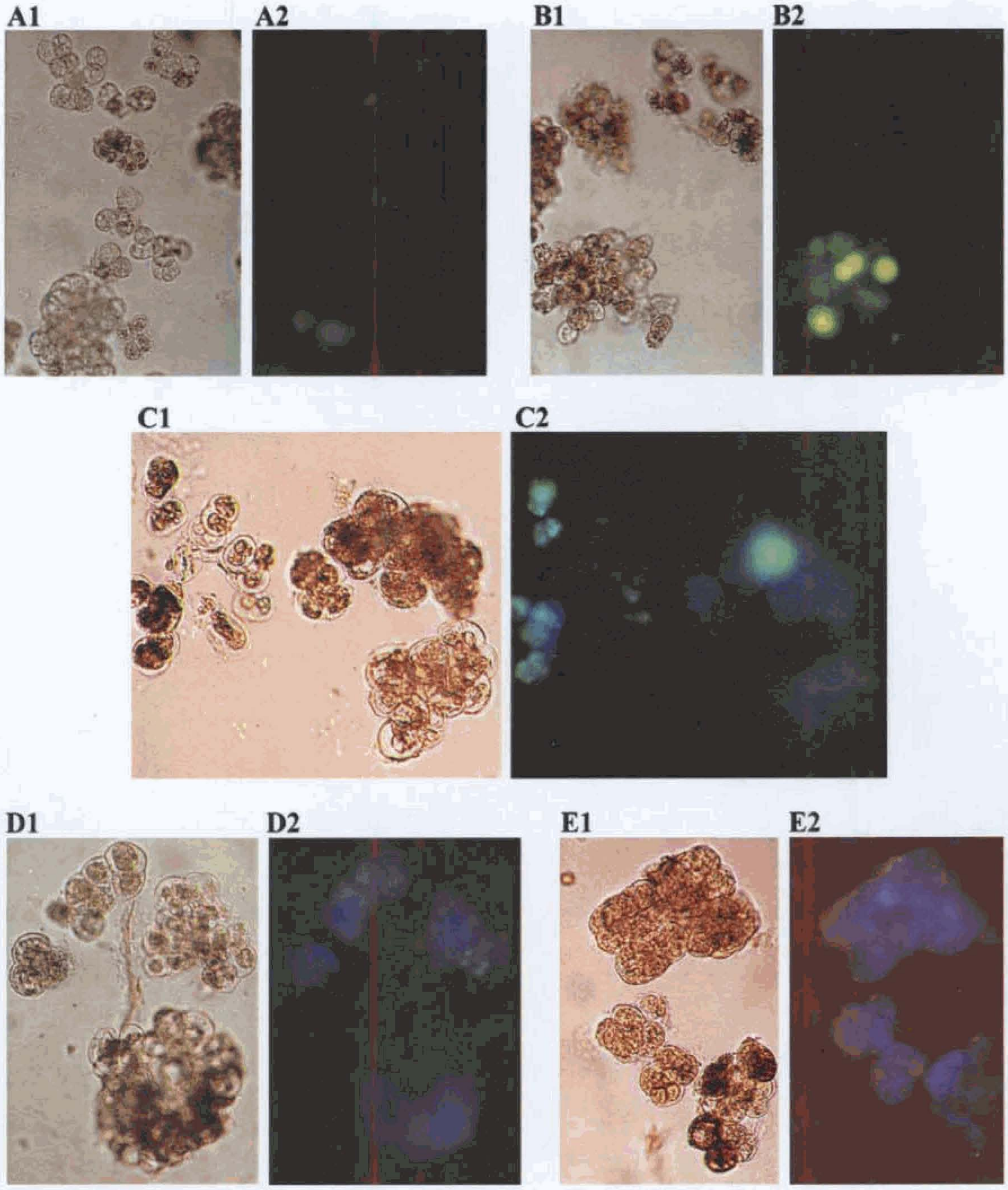


Fig. 2.4b. HR of black pepper suspension cultures inoculated with HWC from P. meadii as evident by FDA viability staining. A-E show cell clumps of suspension cultures inoculated with 50, 100, 150, 200 and 300 micrograms glucose equivalants of HWC respectively. A1, B1, C1, D1 and E1 show cell clumps illuminated by light fluorescent and UV-epifluorescent light, while A2, B2, C2, D2 and E2 show the same clumps under UV-epifluorescent light alone. Note that in contrast to cultures inoculated with PcF. the PmE-tretaed cultures show cell clumps with reduced viabiltiy at low concentrations of the elicitor.

2.3.1.2.1. Determination of optimal concentration of HWC for induction of defenses

The hyphal wall components were mainly gulucan and were quantified in terms of glucose equivalents. The hypersensitive cell death induced by various concentrations of HWC of *P. capsici* and *P. meadii* in black pepper cell suspension cultures was assessed after 24hrs of elicitor treatment by MTT-colorimetry assay and FDA viability assay to determine the optimal concentration of HWC to be used in cell suspension cultures to study the defense responses.

2.3.1.2.1.1. MTT-colorimetric assay

Cell death in elicitor-treated cells was assessed by the MTT-colorimetric assay after 72 days of elicitation. The cell death was quantified by recording the decrease in the blue MTT-formazan product, over the control. Cell death percentage increased with increasing concentration of the elicitors (Figs.2.2 & 2.3) in both *P. capsici* and *P. meadii* HWC-treated cultures at 24hrs after elicitation. The cell death percentage was higher in *P. meadii* HWC-treated cells compared to that in cells treated with HWC from *P. capsici* at different concentrations. Elicitor at 150μg ml^{-1} concentration resulted in 55 and 60% cell death in PcE- and PmE- treated cultures respectively. Since HWC of *P. meadii* at concentrations beyond 150 μg ml^{-1} resulted in significant reduction in the viability of cells, 150μg ml^{-1} was used as the optimal concentration of both *P. capsici* (PcE-) and *P. meadii* (PmE-) elicitors in further studies, for eliciting the defense responses in black pepper suspension-cultured cells.

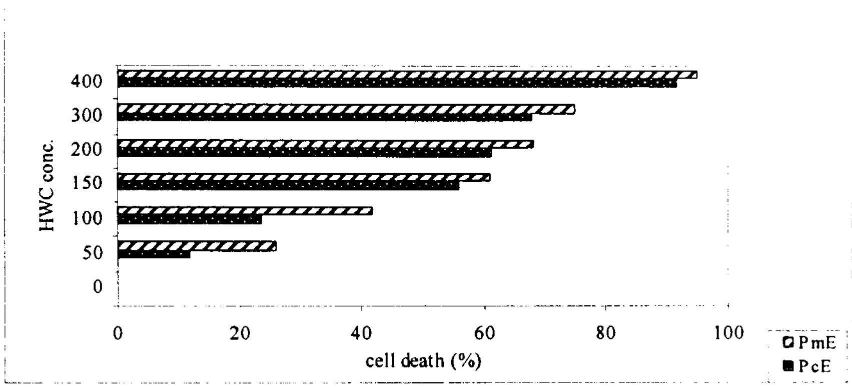


Fig. 2.2: Cell death in black pepper suspension cultures on treatment with different doses of HWC based on MTT-colorimetric assay. The plotted values are the average of 4 replicates.

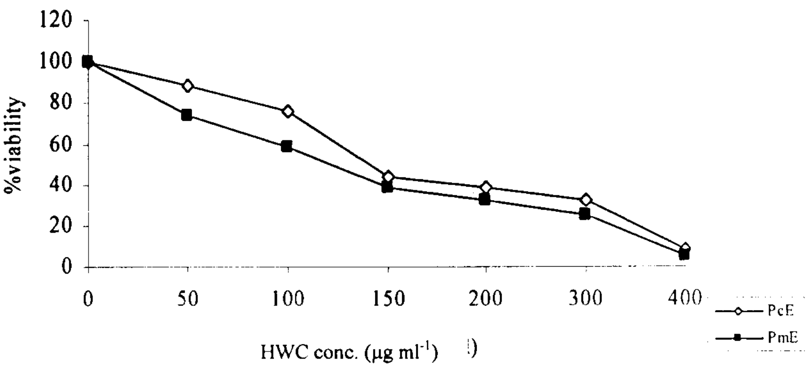


Fig. 2.3: Dose effect of HWC from *P. capsici* and *P. meadii* on the viability of black pepper suspension-cultured cells, based on MTT-colorimetric assay. The plotted values are the mean of 4 replicates.

2.3.1.2.1.2. Viability test by vital staining with FDA

Staining with vital dye, FDA was used to distinguish living from dead/ hypersensitively reacting cells (Fig.2.4a&b). Control cultures, which received mock inoculations showed live cells that fluoresced green under UV epifluorescent light (excitation filter, 330-380nm; dichroic mirror, 400nm; barrier, 420nm). The number of green fluorescent cells however decreased in elicitor treated cells (Fig. 2.5). Elicitor-treated cells consistently showed the presence of cells/cell clumps, which did not stain with FDA and auto-fluoresced blue. The number of auto-fluorescing cells increased with increasing concentration of elicitor. *P. capsici* elicitor at a concentration of 150μg ml^{-1} resulted in 75% of the cells/clumps showing blue auto-fluorescent cells. The percentage of cells showing blue auto-fluorescent cells was more in *P. meadii* elicitor-treated cells at different concentrations compared to that in *P. capsici* elicitor-treated cells. A concentration of 100 μg ml^{-1} of *P. meadii* elicitor was sufficient to cause 75% of cells to auto-fluoresce blue. *P. capsici* elicitor at concentrations only above 200μg ml^{-1}, resulted in 100% blue auto-fluorescent clumps. However, in the case of *P. meadii* elicitor, concentrations as low as 150 μg ml^{-1} resulted in 100% blue auto-fluorescent cells.

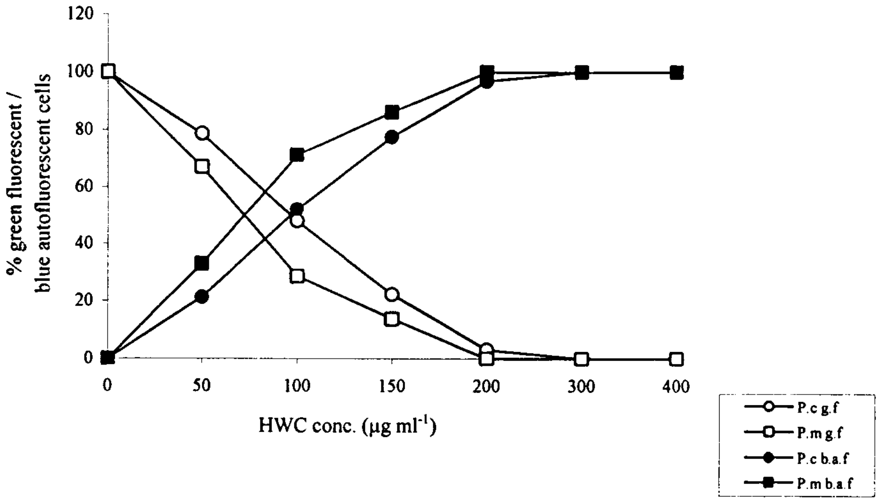


Fig. 2.5: Effect of different doses of HWC from *P. capsici* and *P. meadii* on the proportion of FDA stained and blue auto-fluorescent cells in black pepper suspension. The plotted values are the average of 4 replicates. P.c and P.m. denote PcE-treated and PmE-treated cells respectively. G.f denotes green fluorescent cells, while a.f. denotes blue auto-fluorescent cells.

The MTT-colorimetric assay and FDA viability assay revealed decrease in cell viability with increasing concentration of the elicitor. The decrease in viability as revealed by the MTT-colorimetric assay however was gradual with increasing concentrations, while FDA staining showed a sharp decrease in viability with increasing concentrations of HWC. According to the MTT-colorimetric assay, viability was evident even in cultures treated with a high dose of HWC (300µg ml^{-1}). However, the FDA test showed decreased viability as revealed by decreasing number of green fluorescent cells, at low concentrations of HWC. HWC at a concentration of 100µg ml^{-1} showed only 50% viability, by FDA assay, while the MTT assay showed 77% viability. The difference between the two assays was marked at higher concentrations, with FDA staining recording only 3.1 and 0% viability at 200 and 300 µg ml^{-1} respectively, while the MTT assay revealed approximately 38 and 32% viability respectively at the same concentrations of HWC. The FDA test however resulted in differential staining of the control and hypersensitively reacting cells, the latter being stained blue in contrast to the green fluorescence exhibited by viable cells, as expected by the FDA assay.

Cell death observed in elicitor-treated cultures was also reflected in fresh weight decrease over a period of time after elicitation. The % decrease in fresh weight in PmE-treated cultures was more compared to that in PcE-treated cultures at different intervals of time (Fig. 2.6).

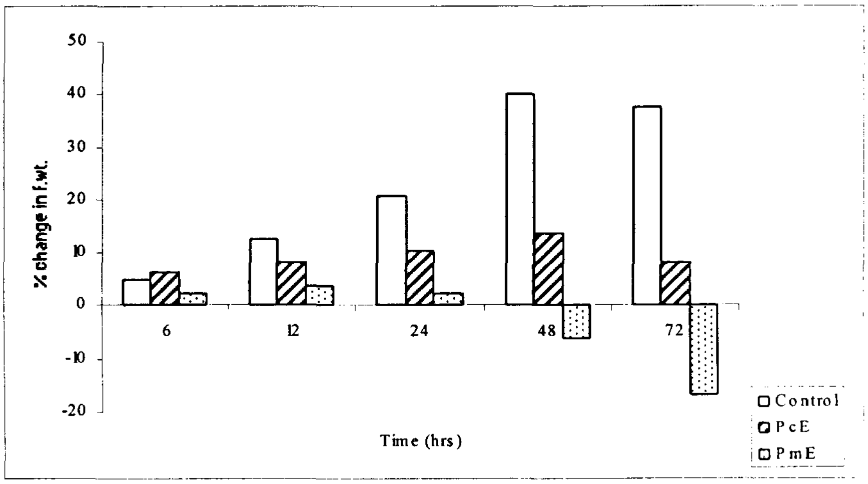


Fig.2.6: Percentage change in fresh weight in black pepper suspension cultures after elicitation. The plotted values are the average of 4 replicates.

2.3.1.2.2. Isolation of water soluble glucans

Water-soluble glucans were isolated from the germination fluids of *P. capsici*. The dialyzed concentrated germination fluid was used as the crude water-soluble glucan at a concentration of 1mg glucose equivalents ml^{-1}.

2.3.2. Defense responses:

The various defenses observed in black pepper cells on elicitation were:

2.3.2.1. Hypersensitive browning and phenolics

The characteristic feature of black pepper suspension cultures on elicitation with hyphal wall components of *Phytophthora species* was the hypersensitive browning of the cells, accompanied by marked reduction in fresh weight with increasing incubation time. The hypersensitive reaction was visually evident after 24hrs of inoculation of the hyphal wall elicitor. The cell browning was more marked in *P. meadii* elicitor-treated cells than in *P. capsici* elicitor-treated cells (Fig.2.7A). The cellular phenolics increased due to hypersensitive browning (Fig. 2.8). In addition to cellular browning and accumulation of phenolics in the cells, the culture medium also showed darkening due to exudation of phenolics by cells into the medium. Phenolics exudation in *P. meadii* elicitor (PmE)-treated cells was visible as early as 12hrs. The intensity of extra-cellular phenolics exudation increased with incubation time and was higher in PmE-treated cutures than in the *P. capsici* elicitor (PcE)-treated cultures (Fig. 2.7B; Fig.2.9).

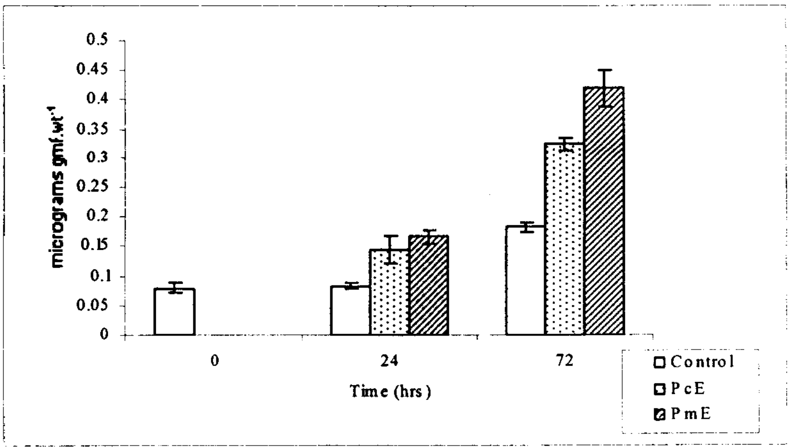


Fig.2.8: Phenolics accumulation in black pepper cells on elicitation. The plotted values are the average of 4 replicates.

Control PcE-treated PmE-treated

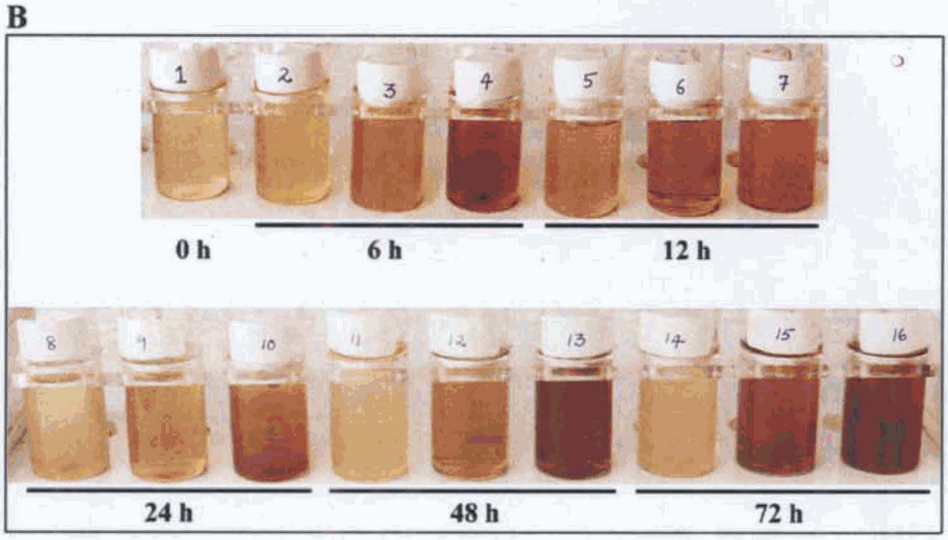


Fig. 2.7. Hypersensitive reaction in response to elicitor treatment; **A**. Hypersensitive browning of black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*. Note the cell browning in the elicitor treated cultures compared to the control. The intensity of cell browning is higher in PmE-treated cultures compared to PcE-treated cultures; **B**. Time-course of phenolics exudation in black pepper suspension cultures on elicitation. The vials show extra-cellular phenolics exudated into the culture filtrates of suspension cultures. Vials 1,2,5,8,11 & 14 - control; vials, 3,6,9,12 & 15 - PcE-treated; and vials 4,7,10,,13,16 - PmE-treated.

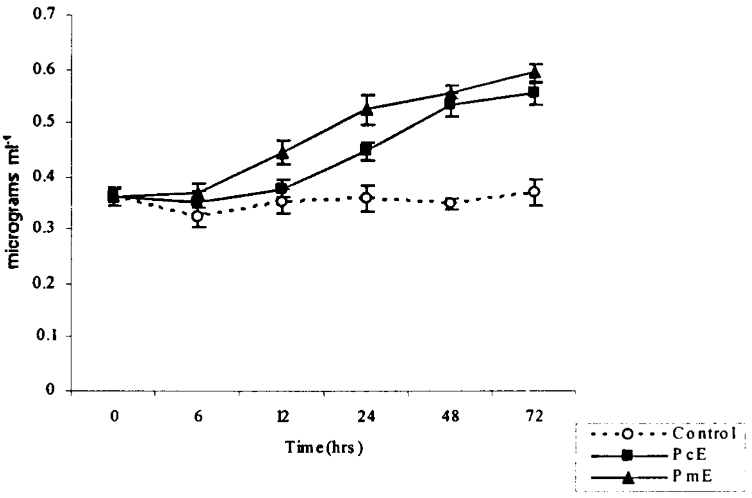


Fig.2.9: Extra-cellular phenolics in culture filtrates of black pepper cultures on elicitation. The plotted values are the average of 4 replicates

2.3.2.2. Extracellular alkalinization :

A significant increase in pH of the culture medium was induced by elicitor treatment. The increase in extra-cellular pH was gradual in *P. capsici* elicitor-treated cultures, while that in *P. meadii* was faster and higher (Fig.2.10). The initial pH of the cell suspensions at 0 hr, soon after inoculation, was 6.3-6.4. The increase in pH in elicited cultures was observed from 30 min. after addition of the elicitor. The response of *P. meadii* elicitor-treated cultures was stronger, with a pH increase of about 1.8 units, compared to *P. capsici* elicitor-treated cultures, which recorded a pH increase of about 1.0 unit, after 24hours of inoculation. At 24hrs after inoculation, the pH reached 8.1-8.25 in *P. meadii* elicitor-treated cultures while a pH of 7.5-7.7 was noted in *P. capsici* elicitor-treated cultures. The control cultures, which received mock inoculation, did not record any significant change in pH over the period of observation.

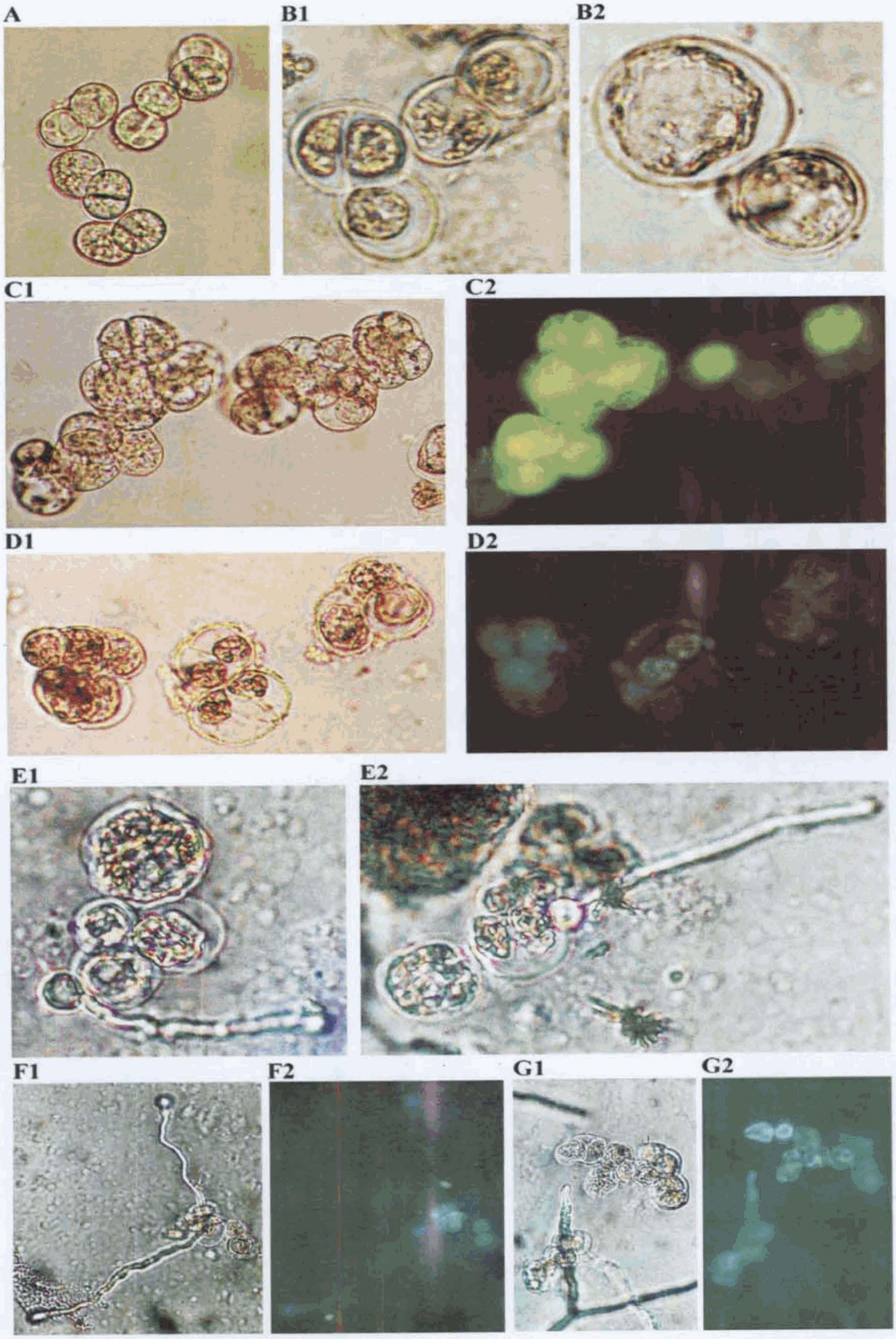


Fig. 2.13. Cytoplasmic aggregation (CA) in black pepper cells in response to elicitation or inoculation with the pathogen, P. capsici. A. Control non-elicited cells with intact cytoplasm; B1and B2. Elicitor (PcE-/ PmE-).treated cells showing shrunken cytoplasm; C1 and C2. FDA stained cell clumps of control cultures exhibiting green fluourescence; D1and D2. Elicited cell clumps showing cytoplasmic aggregation. The shrunken cytoplasm exhibits blue autofluorescence; E1 and E2. Black pepper cells exhibiting CA in response to invading hyphae of the pathogen, P. capsici. F1 & F2 and G1& G2. The shrunken cytoplasm in cells proximal to approaching hyphae of germinating zoospores of P. capsici showing blue autofluourescence. C1, D1, F1 & G1 are illuminated by fluourescent light of microscope, while C2, D2, F2 & G2 are the same images under UV light of the microscope.

2.3.2.4. Lipid peroxidation

Lipid peroxidation in elicitor-treated cultures was marginally higher than the control cutures at 24HAI. However, by 72 h after elicitation, the MDA content of the elicitor-treated cultures markedly increased over the control cultures. Among the elicited cultures, the hydroperoxide accumulation was higher in PmE-treated cultures than in PcE-treated cultures (Fig. 2.12).

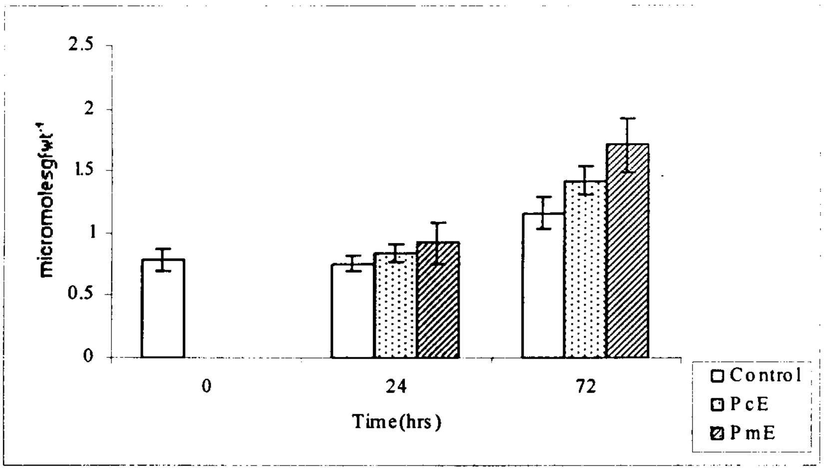


Fig.2.12: Lipid peroxidation in black pepper cells on elicitation. The values plotted are the average of 4 replicates. The Y-bars show the standard error.

2.3.2.5. Cytological changes in HWC-treated suspension cultures

The cytological studies in the present work aimed at tracing the events leading to and associated with hypersensitivity after elicitor treatment. The various observations made were:

2.3.2.5.1. Cytoplasmic aggregation

The cytoplasm of elicitor-treated cells appeared more granular than the control cells. The cytoplasmic streaming of elicited cells decreased and the cytoplasm shrank to a portion of the cell (Fig. 2.13: B1& B2). The proportion of cells showing cytoplasmic aggregation in relation to the total number of cells increased linearly and reached 60-68% by 24hours. The *P. meadii* elicitor-treated cultures showed a greater percentage of cells that exhibited cytoplasmic aggregation than *P. capsici* elicitor-treated cultures (Fig.2.14). Though the control cultures also had a few cells that showed cytoplasmic aggregation, there was little increase in their number with the incubation time. It was also noticed that shrunk cytoplasm of cells showing cytoplasmic aggregation auto-fluoresced blue under UV (330-380nm), and no longer stained with FDA (Fig. 2.13: D1&D2).

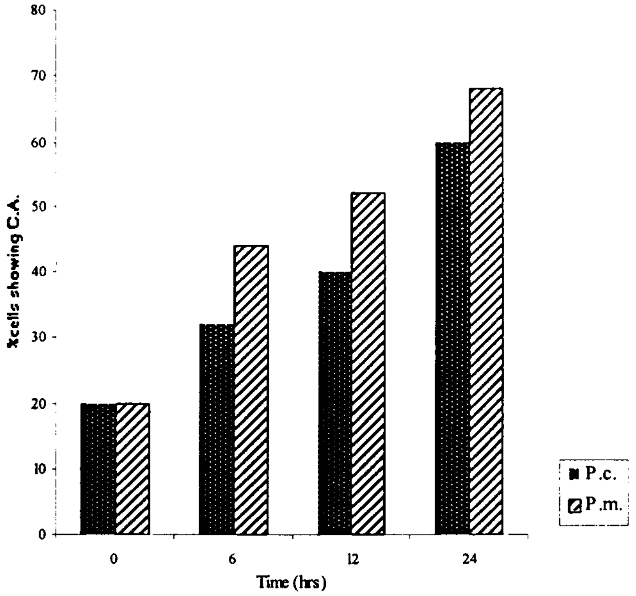


Fig.2.14: Cytoplasmic aggregation (C.A.) in black pepper suspension cultures in response to HWC from *P. capsici* and *P. meadii* (150μg/ml). The plotted values are the average of 4 replicates.

In another experiment, suspension cultures inoculated with zoospores of *P. capsici,* also showed cytoplasmic aggregation (Fig.2.13: E1& E2). The cells proximal to the fungal hyphae developing from the encysted cytospores exhibited shrinking of the cytoplasm to a portion of the cell. These cells also exhibited blue autoflourescence similar to the elicitor-treated cells showing CA. These observations confirm that the response of cells to elicitor treatment was similar to that of the presence of the pathogen.

2.3.2.5.2. Physical barrier formation

2.3.2.5.2.1 Callose:

Elicited cells exhibited formation of callose plugs on the cell wall, a feature seen during fungal invasion in plants. Callose fluoresced intense bluish white in elicited cells

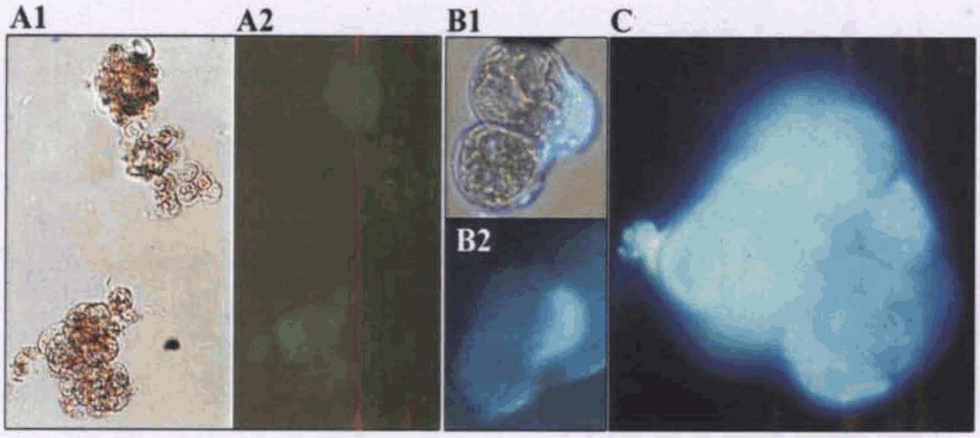


Fig. 2.15a

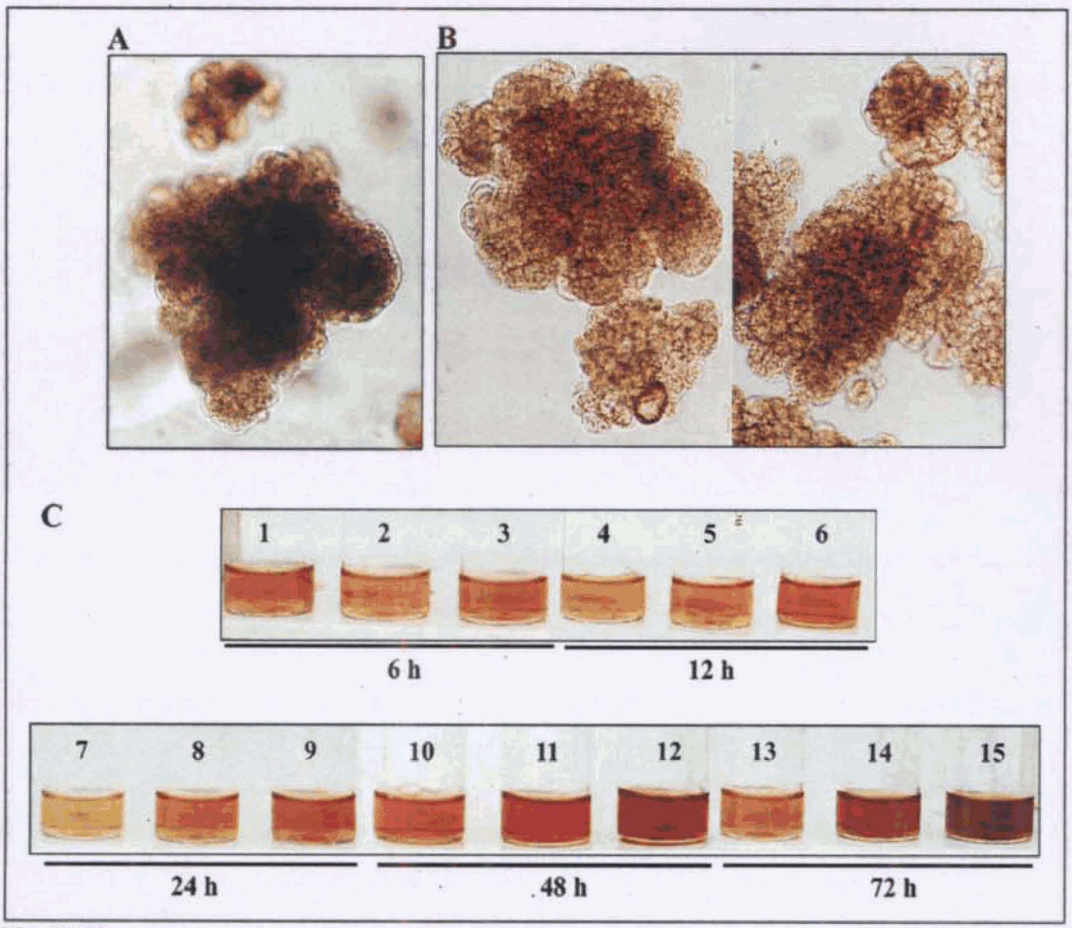


Fig. 2.15b

Fig. 2.15. Cell wall modifications in black pepper suspension cultures in response to elicitor treatment **Fig. 2.15a.** Callose formation - **A, B,** C show aniline blue staining of cells for callose. **A1** (under light and UV) & **A2** (UV alone) : Control cultures that do not show the characteristic bluish-white fluorescence of callose; **B1** (under light and UV) and B2 (UV alone) show the typical bluish-white fluorescence of callose. **C.** Another cell clump of elicited cultures showing the typical fluourescence of callose. **Fig. 2.15b.** Lignification: **A& B** show the neutral red staining for lignin. **A** shows a cell clump from non-elicited, control cultures showing very light red staining of the cells, while **B** shows cell clumps of elicited cultures showing the deep red staining of lignin. **C** shows the time-course accumulation of lignin complexes in control (1, 4, 7, 10, 13), PcE-treated- (2, 5, 8, 11, 14) and PmE-treated- (3, 5, 9, 12, 15) cultures. The vials show the lignin extracts of the cultures.

(Fig. 2.15a). Numerous individual free cells as well as those found in clumps showed callose plug formation in elicited cultures (Fig.2.15a: B1, B2, C). Though the cells/cell clumps of control cultures did exhibit light fluorescence with aniline blue, which may be due to the basal amount of callose already present, the cells did not show callose plug formation (Fig.2.15a: A1&A2).

2.3.2.5.2.2. Lignin:

Elicited cells stained deep red with phloroglucinol-HCl stain, indicative of the accumulation of cell wall-bound phenolics (Fig. 2.15b: A&B). The colour however faded rapidly with time. The control cells stained light, indicating a low level of synthesis of cell wall-bound phenolics.

Thioglycollic acid (TGA) extraction of cell walls was used to confirm if the increase in phloroglucinol-HCl staining of elicited cells is due to increase in synthesis of lignin or lignin-like polyphenolics. This also gave a quantitative measure of lignin accumulation for comparison between control and elicited cultures. It was observed that the elicited cultures recorded higher thioglycollic acid-extractable complexes than the control cultures at all intervals of observation (Fig.2.16). Further, increase in TGA-extractable complexes was more rapid and pronounced in cultures treated with *P. meadii* elicitor than in *P. capsici* elicitor-treated cells (Fig. 2.15b: C). The increase in the TGA-extractable complexes was significant over the control from the 6th hour after elicitation by both *P. capsici* and *P. meadii* elicitors, and increased consistently over the 72-hour period of observation. However, the increase in *P. capsici* elicitor-treated cells was gradual and to a lesser extent than in *P. meadii* elicitor-treated cells. It was observed that the content of TGA extractable complexes in *P. meadii* HWC treated cells was twice as high as that of *P. capsici* HWC treated cells and four times that of the control.

It was also observed that the UV- absorption spectrum of the TGA-extractable fraction of the cell wall showed a peak at 225nm rather than at 280nm, at which the TGA extractable complexes are quantified in the TGA assay of lignin described by Campbell and Ellis (1992). However, since the TGA- extractable complexes showed similar trends in their absorbance at both 225 and 280nm, the values at 280 nm have been represented in Fig.2.16.

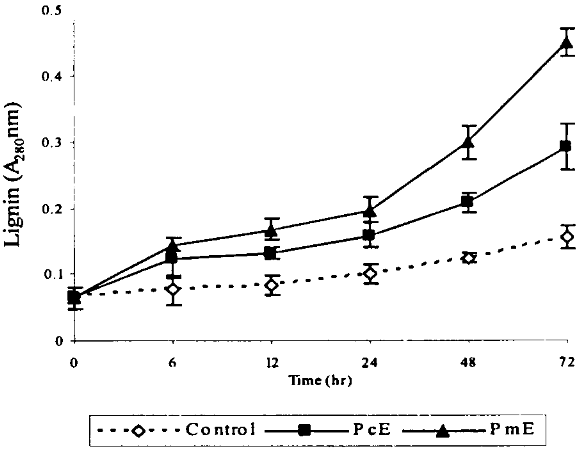


Fig. 2.16. Time course of lignification in black pepper suspension cultures in response to elicitation by hyphal wall elicitors from *P. capsici* and *P. meadii*. The values plotted are the average of 4 replicates.

2.3.2.6. Enzymatic defenses in response to elicitation in black pepper suspension cultures

The suspension cultures of black pepper were studied for the modulation in the levels of different defense enzymes, after elicitation with HWC of *P. capsici* and *P. meadii*.

2.3.2.6.1. Anti-oxidant enzymes

2.3.2.6.1.1. Superoxide dismutase (SOD)

The activity levels of total SOD remained low in elicitor-treated cultures until 24 hour hours after elicitation. The activity of both PcE- and PmE-treated cultures was either low or on par with the controls. Though PcE-treated cultures showed a marginal gain in increase in the activity at 24 hours after elicitation, the activity increased in PmE-treated cultures compared to PcE-treated cultures and control, thereafter. The activity in both PcE and PmE-treated cultures later declined after 48 hours after elicitation but remained higher than the control levels (Fig.2.17a).

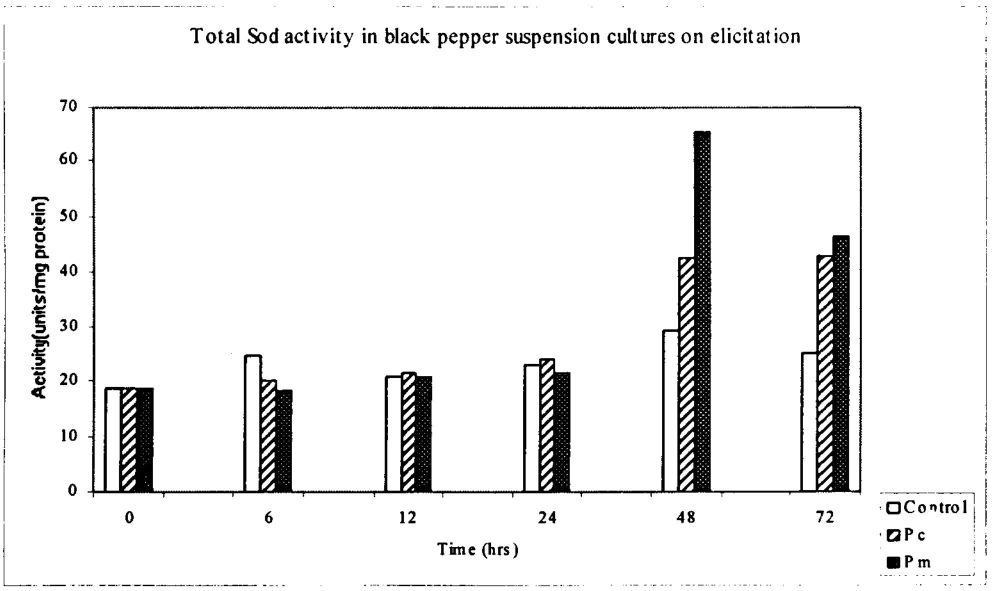


Fig.2.17a: Time-course change in total SOD activity in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*. The values plotted are the average of 4 replicates.

The activities of the different forms of SOD varied among the different treatments. In general, it was seen that the initial levels of different forms of SOD at 0hrs was in the order MnSOD>CuSOD>FeSOD. MnSOD activity of PcE-treated cultures recorded consistent and considerable increase over that of control and PmE-treated cultures from 6hrs of observation after elicitation in the present study. MnSOD activity of PmE-treated cultures was lower than the control levels till 24hours after eliciation, after which, the activity increased over the controls, but remained lower than PcE-treated cultures (Fig. 2.17b).

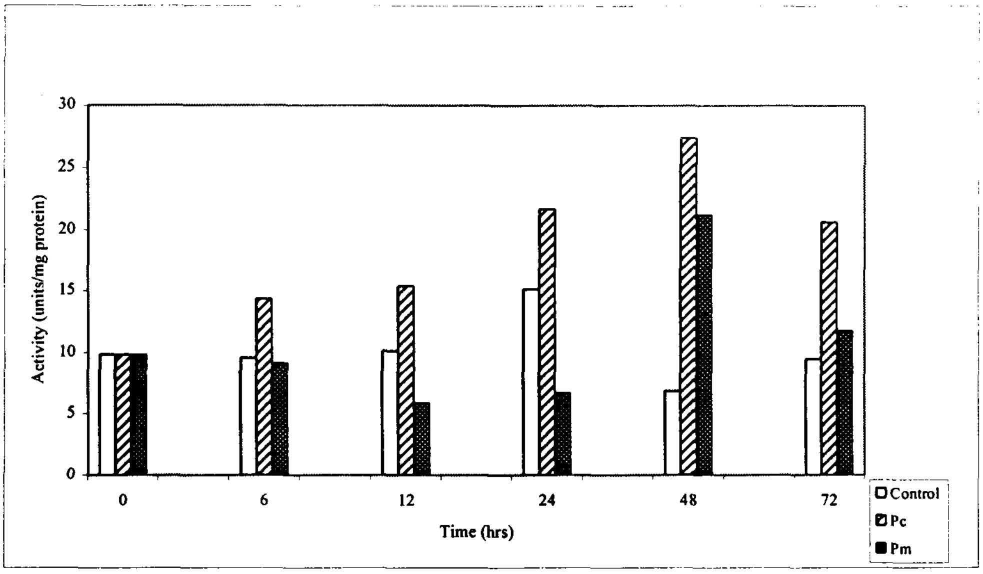


Fig. 2.17b: Time-course change in the activity of MnSOD in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*. The plotted values are the mean of 4 replicates.

While the major form of SOD in PcE-treated cultures was MnSOD, PmE-treated cultures had FeSOD as the major form of the enzyme. PmE-treated cultures showed a gain in FeSOD activity from the 6th hour of elicitation over control and PcE-treated cultures. The activity of FeSOD remained considerably low in control and PcE-treated cultures (Fig. 17c).

CuSOD was the dominant form of SOD in the control cultures throughout the course of the experiment. Though PcE- and PmE-treated cultures showed an increase in Mn and Fe SOD activity respectively, they gradually gained CuSOD activity in addition to their respective major form of SOD, 24 hours after elicitation (Fig.17d).

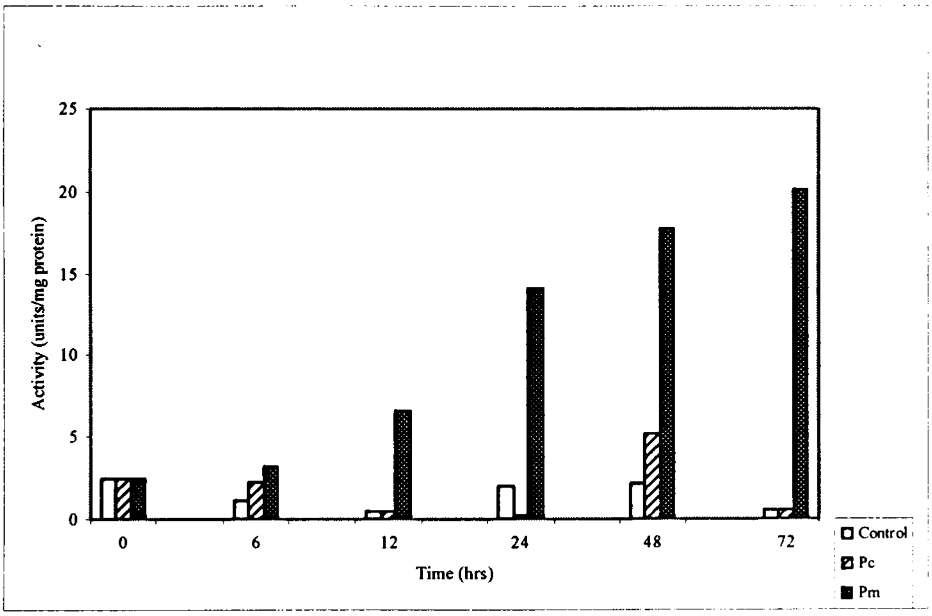


Fig. 2.17c: Time-course change in the activity of FeSOD in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*. The plotted values are the average of 4 replicates.

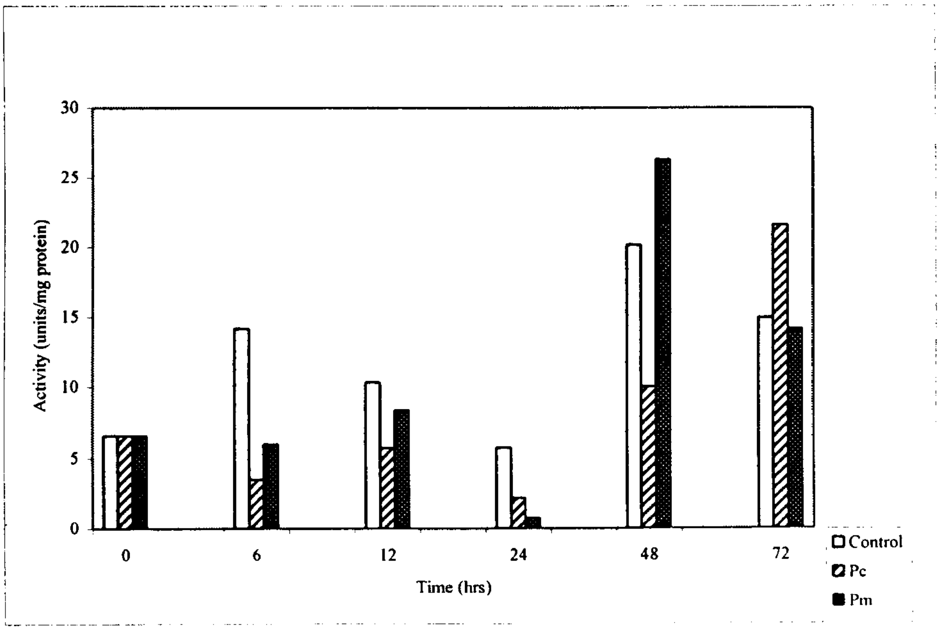


Fig. 2.17d: Time-course change in the activity of Cu/Zn SOD in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*. The plotted values are the average of 4 replicates.

2.3.2.6.1.1.1. Isozyme analysis of superoxide dismutase:

Activity staining of total SOD of suspension cultures under different treatments revealed, 4 bands corresponding to four alleles that represented the three different loci of the enzyme. The bands had an Rf value of 0.467, 0.558, 0.617 and 0.671 (Fig. 2.18). The PcE-treated cultures showed the presence of a prominent additional band of Rf value 0.72 On differential staining of SOD for studying the identity of the different bands obtained, it was observed that bands with Rf value 0.467 and 0.721 were MnSOD, while bands with Rf of 0.558 was FeSOD and bands of Rf 0.617 and 0.671 were CuSOD. The additional prominent band, that was anodic and present only in the PcE-treated cultures, was identified as another locus of MnSOD.

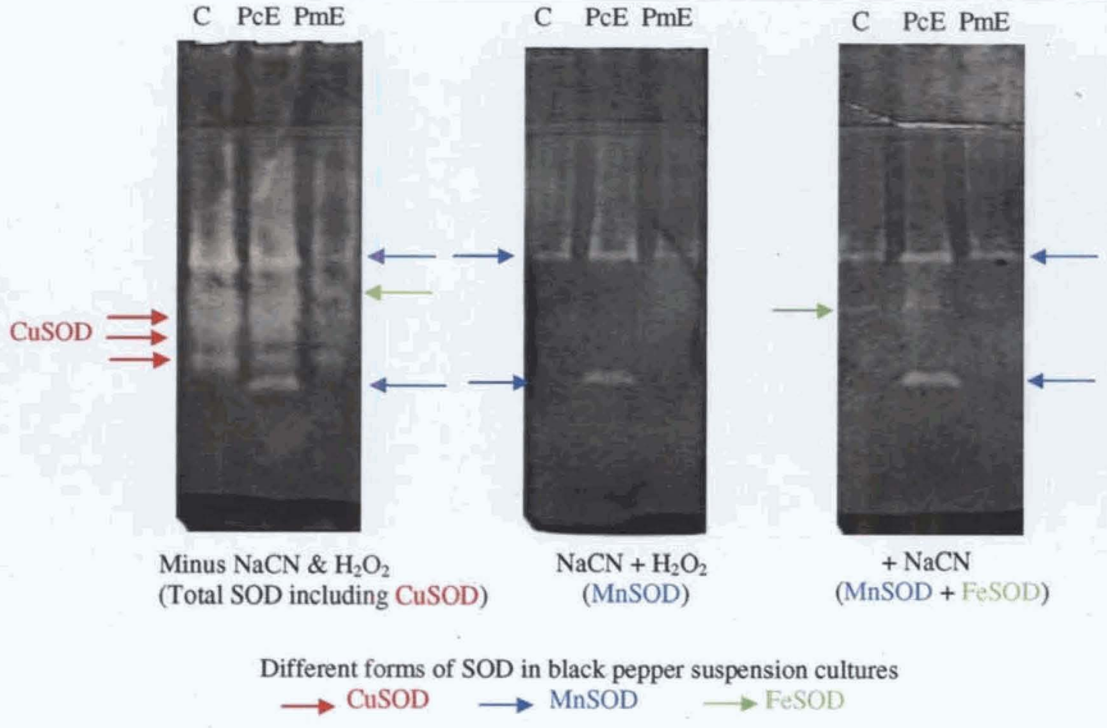


Fig. 2.18: Different forms of SOD in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii* as revealed by differential staining using NaCN and/or H_2O_2 as inhibitors of CuSOD and FeSOD.

2.3.2.6.1.2. Hydrogen peroxide-scavenging enzymes

2.3.2.6.1.2.1. Catalase

The activity of catalase was higher in PcE-treated cultures than in PmE-treated cultures and control (Fig. 2.19; Table 2.1.). The activity in PmE-treated cultures was well below the control levels until 12 hours after elicitation, after which it showed a marginal increase over the control cultures. On the other hand, the PcE-treated cultures showed an initial increase in catalase activity followed by a decline at 12 hours after eliciation, whereafter, the activity showed significant increase till 48 hours. The activity reached control levels at 72 hours after elicitation. Extracellular catalase activity however was negligible (data not shown) in all the treatments and hence was not recorded.

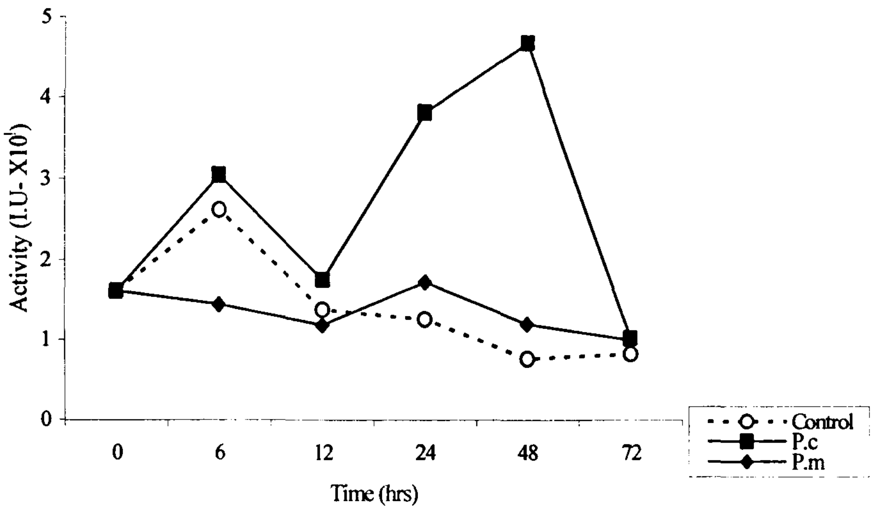


Fig. 2.19: Time-course change in catalase activity in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*.

Table 2.1: Catalase activity in black pepper suspension cultures on elicitation with hyphal wall components of *P. capsici* and *P. meadii.*

Tmt No	Tmt	Sp. Activity= I.U. (x 10^1µmoles H_2O_2 decomposed $min^{-1}mg\ protein^{-1}$)					
		0h	**6h**	**12h**	**24h**	**48h**	**72h**
1	**Control**	1.62 ± 0.2517	2.625 ± 0.1727	1.3815 ± 0.2027	1.258 ± 0.1184	0.765 ± 0.1853	0.828 ± 0.1253
2	**PcE**		3.053 ± 0.2834	1.758 ± 0.2016	3.825 ± 0.2629	4.68 ± 0.4921	1.025 ± 0.1680
3	**PmE**		1.45 ± 0.085	1.188 ± 0.2106	1.73 ± 0.1435	1.204 ± 0.118	1.0 ± 0.1322

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
3.00	24	1.3565	
1.00	24	1.3885	
2.00	24		2.6123
Sig.		.897	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

2.3.2.6.1.2.2. Peroxidase

The peroxidase activity in HWC–elicited suspension cultures showed increased levels than that in control cultures (Fig. 2.20; Table 2.2). However, the PmE-treated cultures showed higher activity than the PcE-treated cultures. The activity showed an initial decrease followed by consistent increase in the activity. The activity in PcE-treated cultures was on par with the control till 24 hrs after elicitation, thereafter showing an increase at 48 hours followed by a decline. The PmE-treated cultures also recorded a decline in activity 48 hours after elicitation.

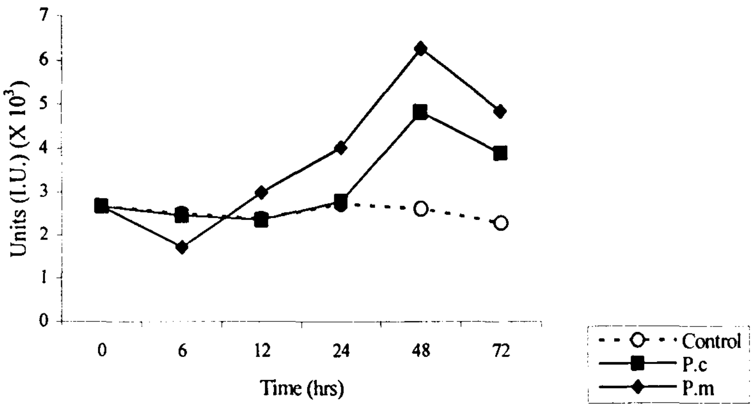


Fig. 2.20: Time-course change in the activity of peroxidase in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii.*

Table 2.2: Peroxidase activity in black pepper suspension cultures on elicitation with hyphal wall components of *P. capsici* and *P. meadii.*

Tmt No	**Tmt**	**Sp. Activity= I.U. (x 10^3 μmoles purpurogallin formed min^{-1}mg protein^{-1})**					
		0h	**6h**	**12h**	**24h**	**48h**	**72h**
1	**Control**	2.672 ± 0.5367	2.507 ± 0.459	2.386 ± 0.4105	2.72 ± 0.3475	2.611 ± 0.3262	2.281 ± 0.3201
2	**PcE**		2.46 ± 0.5743	2.348 ± 0.2577	2.78 ± 0.0445	4.83 ± 0.2566	3.901 ± 0.3931
3	**PmE**		1.719 ± 0.2015	2.98 ± 0.2836	4.033 ± 0.1167	6.279 ± 0.3164	4.853 ± 0.311

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan^a

Tmt	N	Subset for alpha = .05	
		1	2
1.00	24	2.4768	
2.00	24		3.1396
3.00	24		3.6998
Sig.		1.000	.081

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

2.3.2.6.1.2.2.1. Extra-cellular peroxidase

The general trend of extra-cellular peroxidases was an increase in levels of activity in elicitor-treated cells compared to control (Fig. 2.21; Table 2.3). However, the activity of peroxidase was significantly higher in culture filtrates of PmE-treated suspension cultures than that of PcE-treated cultures. The activity showed an initial decline over the controls, followed by a consistent increase in PmE-treated cultures reaching a maximum at 48 hrs after elicitation. The activity declined after 48 hours, consistent with the activity levels of intra-cellular peroxidases. The activity of extra-cellular peroxidases remained low in PcE-treated cultures than the PmE-treated suspensions. The control cultures showed low levels of activity.

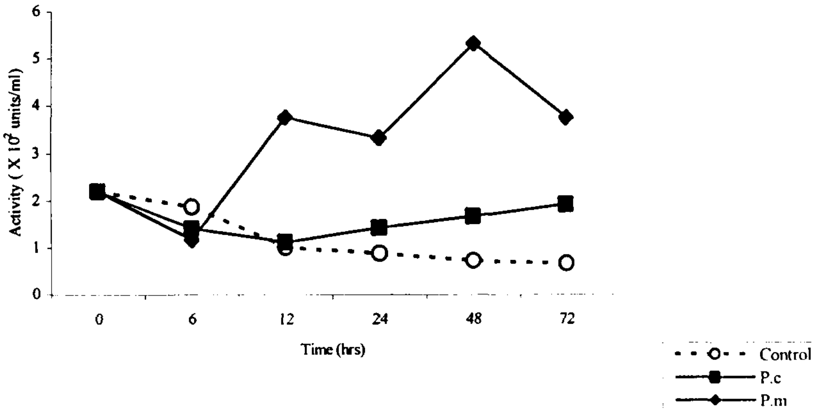


Fig. 2.21: Time-course change in extracelllar peroxidase activity in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*

Table 2.3: Extracellular-peroxidase activity in black pepper suspension cultures on elicitation with hyphal wall components of *P. capsici* and *P. meadii*.

Tmt No	Tmt	Sp. Activity= I.U. (x 10^2 μmoles purpurogallin formed min^{-1}mg protein^{-1})					
		0h	**6h**	**12h**	**24h**	**48h**	**72h**
1	Control	2.22 ± 0.0911	1.89 ± 0.1378	1.02 ± 0.5335	0.889 ± 0.063	0.731 ± 0.0645	0.67 ± 0.0788
2	PcE		1.42 ± 0.0781	1.13 ± 0.1954	1.44 ± 0.0447	1.69 ± 0.2544	1.95 ± 0.3436
3	PmE		1.18 ± 0.1799	3.77 ± 0.3785	3.34 ± 0.3067	5.34 ± 0.5173	3.77 ± 0.1616

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
1.00	24	1.2101	
2.00	24	1.6529	
3.00	24		3.2504
Sig.		.095	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

2.3.2.6.1.2.3. Enzymes of AGC-cycle

The cytoplasmic and chloroplastic fractions of suspension-cultured cells of the three different treatments were assayed for enzymes of the AGC-cycle, including ascorbate peroxidase and dehydroascorbate reductase.

2.3.2.6.1.2.3.1. Ascorbate Peroxidase

Ascorbate peroxidase activity remained low during the initial phases of elicitation in PmE-treated cultures in comparison with the PcE-treated and control cultures. The activity dipped significantly below the control levels at 12h after elicitation whereafter, it increased and remained marginally high over the control till the completion of the experiment. The AP activity in PcE-treated cultures was maintained higher over the other two treatments during most part of the time-course of the experiment. The enzyme activity in control cultures was maintained at similar levels throughout the experiment and did not change significantly over the time period (Fig. 2.22; Table 2.4).

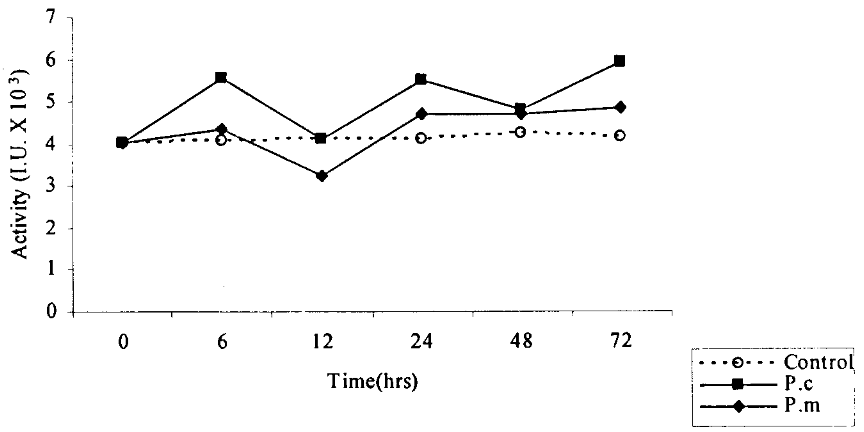


Fig. 2.22: Time-course change in activity of cytosolic AP in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*

Table 2.4: Cytosolic AP activity in black pepper suspension cultures on elicitation with hyphal wall components of *P. capsici* and *P. meadii.*

Tmt No	Tmt	Sp. Activity= I.U. (x 10^3μmoles AsA oxidized min^{-1}mg protein^{-1})					
		0h	**6h**	**12h**	**24h**	**48h**	**72h**
1	**Control**	4.023 ± 0.2188	4.085 ± 0.1825	4.126 ± 0.1651	4.13 ± 0.3298	4.28 ± 0.601	4.162 ± 0.2558
2	**PcE**		5.564 ± 0.4732	4.127 ± 0.569	5.531 ± 1.321	4.805 ± 0.9472	5.954 ± 0.3253
3	**PmE**		4.375 ± 0.541	3.25 ± 0.5836	4.745 ± 0.7401	4.726 ± 0.631	4.881 ± 1.4

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
1	24	4.10939	
3	24	4.33061	
2	24		4.94342
Sig.		.329	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

The AP activity of the chloroplastic fraction also showed similar trends among the different treatments. The PmE-treated cultures showed decreased activity of AP compared to that in PcE-treated cultures (Fig. 2.23; Table 2.5). The cultures showed an initial decrease in the activity of the enzyme till 12h after elicitation, followed by an increase till 48 HAE, after which the activity declined. The PcE-treated cultures however showed higher activity of chloroplastic AP throughout the time-course which was maintained significantly high over the other two treatments. The control cultures maintained low activity of the enzyme.

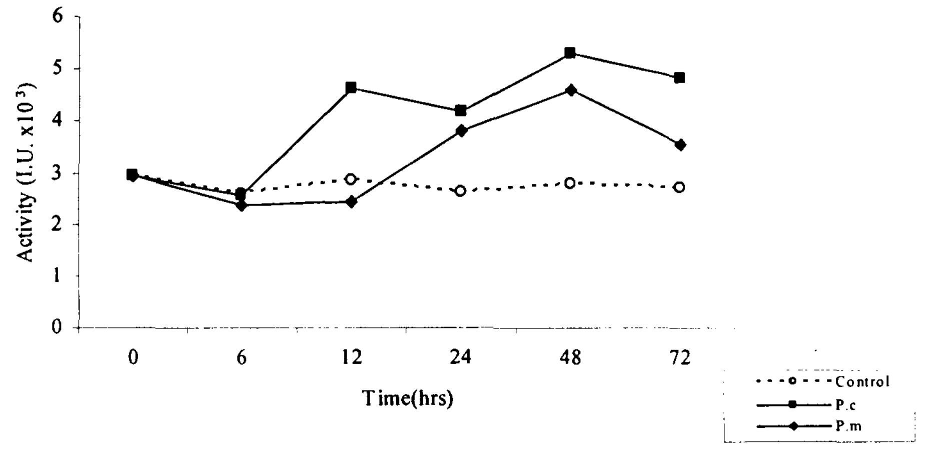


Fig. 2.23: Time-course change in activity of chloroplast fraction of AP in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*

Table 2.5: Chloroplastic AP activity in black pepper suspension cultures on elicitation with hyphal wall components of *P. capsici* and *P. meadii*.

Tmt	Tmt	Sp. Activity= I.U. (x 10^{3}µmoles AsA oxidized min^{-1}mg protein^{-1})					
		0h	**6h**	**12h**	**24h**	**48h**	**72h**
1	**Control**	2.955 ± 0.2876	2.592 ± 0.5571	2.887 ± 0.2304	2.659 ± 0.7101	2.8 ± 0.3937	2.73 ± 0.0785
2	**PcE**		2.585 ± 0.1086	4.629 ± 0.3562	4.186 ± 0.1885	5.294 ± 0.8386	4.807 ± 1.15
3	**PmE**		2.375 ± 0.4637	2.445 ± 0.3976	3.797 ± 0.3868	4.592 ± 1.503	3.524 ± 0.4775

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
1.00	24	2.8779	
2.00	24	3.3249	
3.00	24		5.1729
Sig.		.266	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

2.3.2.6.1.2.3.2. Dehydroascorbate reductase

PmE-treated cultures showed higher activities of cytosolic dehydroascorbate reductase compared to control and PcE-treated cultures. There was a sharp increase in levels at 6 hours after elicitation after which the levels dropped, though higher activity was maintained over the control and PcE-treated cultures till 72hrs (Fig.2.24; Table 2.6). The cytosolic DR activity in PcE-treated cultures was maintained on par with the control till 12 hours after elicitation. The activity subsequently dropped below the control at 24hrs after elicitation after which it increased and was maintained at marginally higher levels over the control.

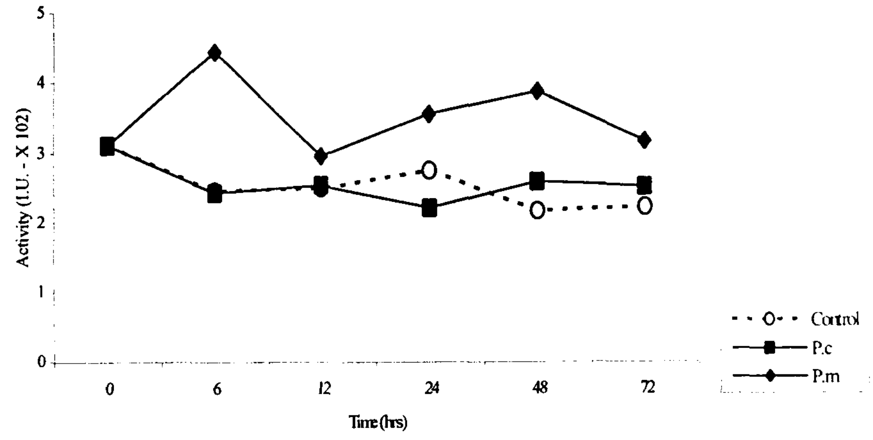


Fig. 2.24: Time-course change in activity of cytosolic DR in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*

Table 2.6: Cytosolic DR activity in black pepper suspension cultures on elicitation with hyphal wall components of *P. capsici* and *P. meadii.*

Tmt	Tmt	Sp. Activity= I.U. (x 10^2 μmoles AsA formed $min^{-1}mg\ protein^{-1}$)					
		0	6	12	24	48	72
1	**Control**	3.12 ± 0.2888	2.47 ± 0.5752	2.51 ± 0.2586	2.77 ± 0.3397	2.19 ± 1.0404	2.25 ± 0.2862
2	**PcE**		2.44 ± 0.3932	2.55 ± 0.2511	2.23 ± 0.3721	2.61 ± 0.4502	2.54 ± 0.8787
3	**PmE**		4.45 ± 0.4018	2.97 ± 0.3467	3.57 ± 0.2956	3.89 ± 0.42	3.19 ± 0.3457

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests
Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
2.00	24	2.6763	
1.00	24	2.7706	
3.00	24		3.4918
Sig.		.548	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

The chloroplastic dehydroascorbate reductase activity in the elicited cultures showed an initial decline in levels compared to control, where after, the PmE-treated cultures showed increased activity that reached a peak at 24 HAE, followed by a gradual decline to control levels. The PcE-treated cultures showed either lower levels of activity or were on par with the control cultures except at 48 HAE, when the cultures showed an increase in activity (Fig. 2.25; Table 2.7).

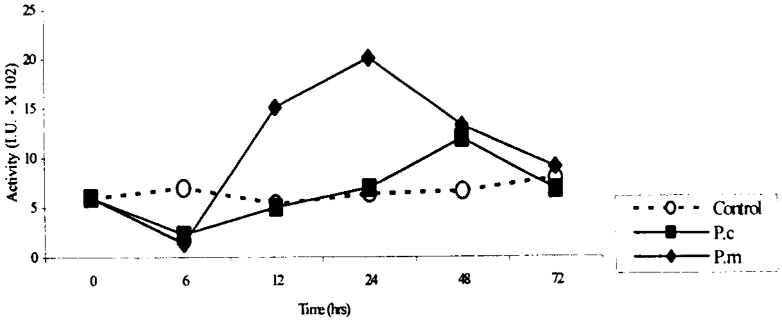


Fig. 2.25: Time-course change in activity of chloroplastic DR in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*

Table 2.7: Chloroplastic DR activity in black pepper suspension cultures on elicitation with hyphal wall components of *P. capsici* and *P. meadii.*

Tmt	**Tmt**	**Sp. Activity= I.U. (x 10^2 µmoles AsA min^{-1}mg protein^{-1})**					
		0	6	12	24	48	72
1	**Control**	5.94 ± 0.3299	6.95 ± 0.4553	5.37 ± 0.4025	6.35 ± 0.2897	6.63 ± 0.2644	7.99 ± 0.324
2	**PcE**		2.25 ± 0.5739	5.04 ± 0.2824	6.96 ± 0.3674	12.01 ± 0.4872	6.82 ± 0.3071
3	**PmE**		1.37 ± 0.4416	15.11 ± 2.9782	20.14 ± 1.0252	13.29 ± 0.7385	9.1 ± 0.7505

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan^a

Tmt	N	Subset for alpha = .05	
		1	2
1.00	24	6.5432	
2.00	24	6.5812	
3.00	24		10.9919
Sig.		.974	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

2.3.2.6.2. Other defense enzymes

2.3.2.6.2.1. β-1,3 glucanase

Elicited cultures showed higher levels of β-1, 3 glucanase compared to controls as early as 6h of elicitation. The increase was however marked after 24HAE and the levels remained high until 72 hrs. The PmE-treated cultures showed significantly higher activity than the PcE-treated cultures (Fig. 2.26; Table 2.8).

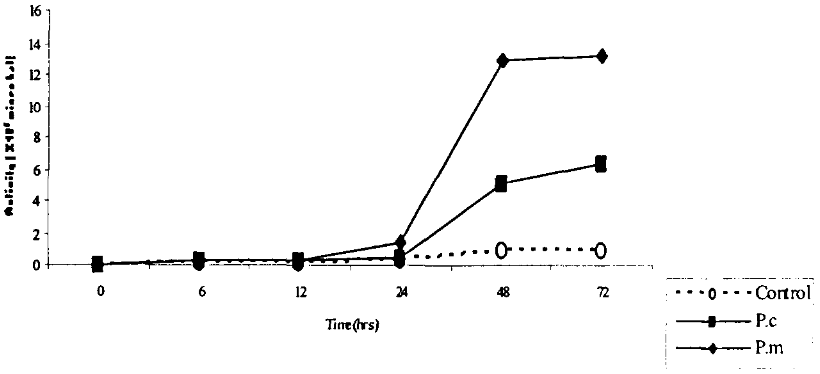


Fig. 2.26: Time-course change in β-1,3 glucanase activity in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*

Table 2.8: β-1,3 glucanase activity in black pepper suspension cultures on elicitation with hyphal wall components of *P. capsici* and *P. meadii*.

Tmt	**Tmt**	**Sp. Activity = x 10^1μ kat (μmoles glu. eq. formed sec^{-1} mg $protein^{-1}$)**					
		0	6	12	24	48	72
1	**Control**	0.06984 ± 0.0339	0.1221 ± 0.236	0.234 ± 0.3336	0.30 ± 0.4584	1.023 ± 0.6	0.891 ± 0.1872
2	**PcE**		0.302 ± 0.4714	0.347 ± 0.2623	0.426 ± 0.335	5.186 ± 0.6668	6.365 ± 1.505
3	**PmE**		0.364 ± 0.3975	0.376 ± 0.4823	1.396 ± 0.361	12.97 ± 0.0179	13.34 ± 2.1769

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
1.00	24	4.4602	
2.00	24	21.2098	
3.00	24		47.2122
Sig.		.133	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

2.3.2.6.2.2. Phenylalanine ammonia lyase

The PAL activity of elicited cultures was higher compared to that of control cultures, which maintained basal minimum activity (Fig. 2.27; Table 2.9). The activity in PmE-elicited cultures showed an increase after 6 hrs of elicitation and maintained higher levels till the end of the experiment. On the other hand, the PcE-treated cultures showed a very gradual increase in PAL activity after 12 hrs of elicitation, which peaked at 48 hrs after elicitation and declined gradually at 72 hours, when the experiment was completed.

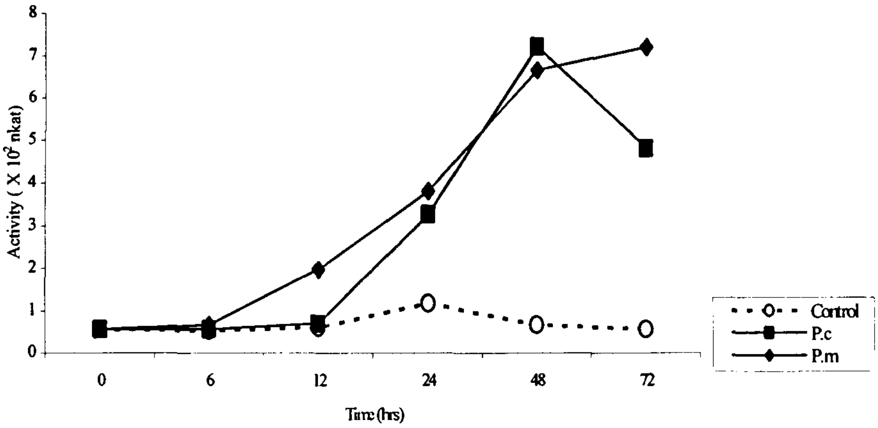


Fig. 2.27: Time-course change in PAL activity in black pepper suspension cultures on elicitation with HWC of *P. capsici* and *P. meadii*

Table 2.9: PAL activity in black pepper suspension cultures on elicitation with hyphal wall components of *P. capsici* and *P. meadii*.

Tmt	Tmt	Sp. Activity = μ kat (μmoles trans cinnamic acid formed sec^{-1} mg $protein^{-1}$)					
		0	6	12	24	48	72
1	Control	0.056 ± 0.0062	0.052 ± 0.0205	0.06 ± 0.0274	0.118 ± 0.065	0.0665 ± 0.032	0.056 ± 0.0466
2	PcE		0.056 ± 0.0235	0.069 ± 0.0107	0.327 ± 0.028	0.7231 ± 0.027	0.482 ± 0.0315
3	PmE		0.067 ± 0.0356	0.197 ± 0.038	0.381 ± 0.049	0.668 ± 0.0358	0.7219 ± 0.033

Each value represents the mean of 4 replicates ± S.D. The data were analyzed statistically by one-way ANOVA, with Duncan's multiple range post-hoc test.

One-way ANOVA Post-Hoc tests

Homogenous subsets

Duncan[a]

Tmt	N	Subset for alpha = .05	
		1	2
1.00	24	7.908E-02	
2.00	24		.2839
3.00	24		.3518
Sig.		1.000	.279

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 24.000.

2.3.3. Effect of water-soluble glucan pre-treatment on the response of cell suspension cultures to hyphal wall components

Cells pre-treated with WSG followed by treatment with HWC did not exhibit blue autofluorescence, characteristic of HWC-treated cells. The WSG-pre-treated cells appeared normal and did not exhibit hypersensitive browning on elicitation with HWC that usually results in blue auto-fluorescent cells. This suggests that the water-soluble glucans present in the germination fluid of *P. capsici* had a suppressive effect on the hypersensitive reaction elicited by HWC treatment. The WSG-treated cells/clumps consistently exhibited characteristic light yellowish green fluorescence, when stained with FDA. Addition of elicitor following WSG treatment also resulted in yellow-green fluorescent cells/clumps, while cells treated with elicitor alone, exhibited the characteristic blue auto-fluorescence of hypersensitive reaction (Fig. 2.28). The percentage cell clumps showing the fluourescent cells is shown in Fig. 2.29.

22510

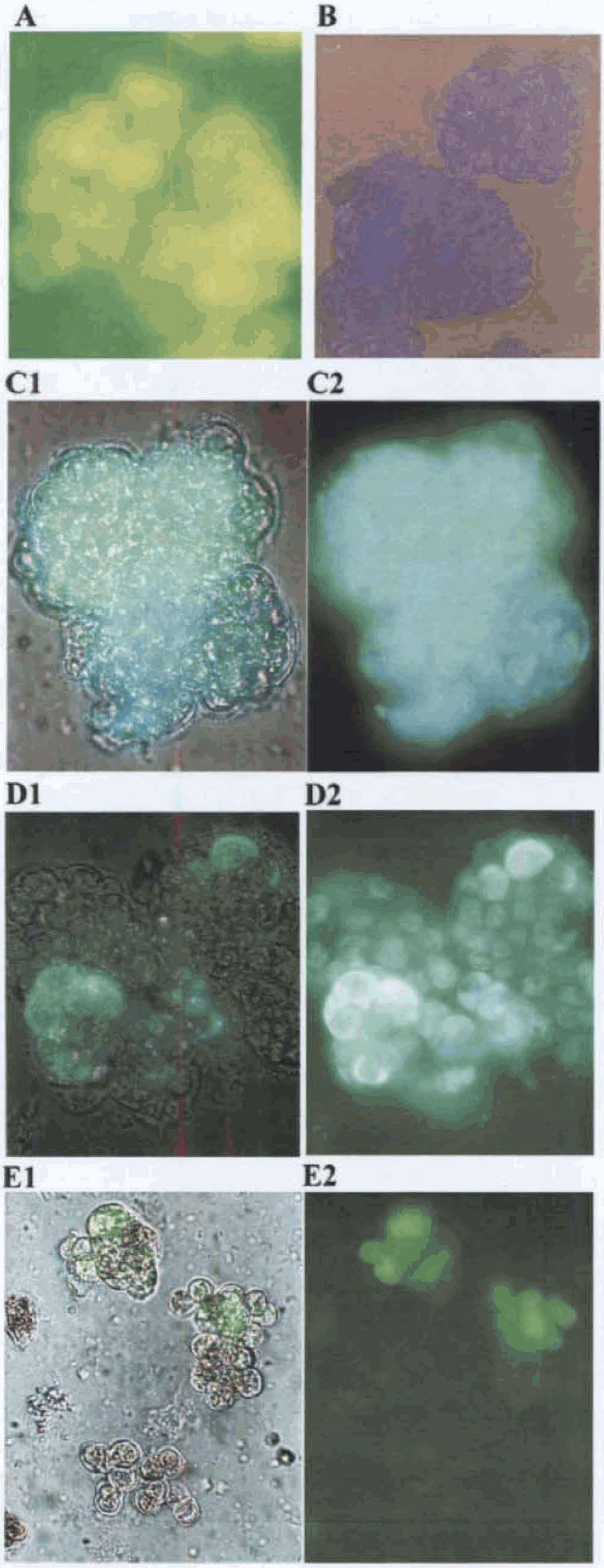


Fig. 2.28. Inhibition of HR by the suppressor factor from zoospore germination fluid of P.capsici. **A.** Typical green fluorescence of non-elicited cell clump; **B.** Typical blue autofluorescence of elicited cell clumps; **C1**(light+UV) &**C2** (UV alone). The dull greenish yellow fluorescence of WSG-treated cell clump. **D1**(light+UV) &**D2** (UV alone) show cell clumps of WSG pre-treated cultures treated with HWC from *P. capsici.* **E1** (light+UV) &**E2** (UV alone) show cell clumps of WSG pre-treated cultures treated with HWC from *P. meadii*.

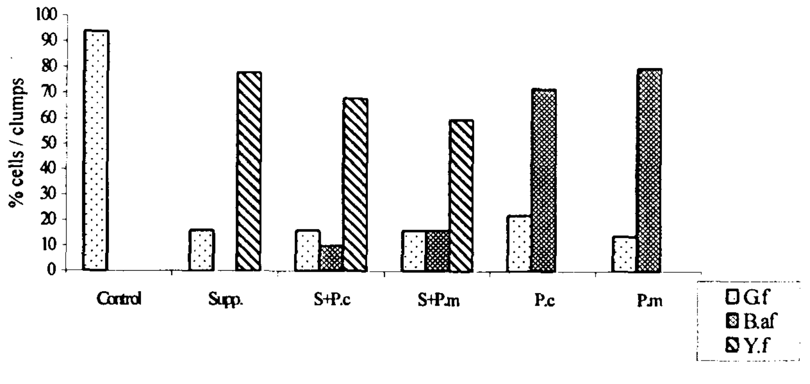


Fig. 2.29: Suppression of HR by water-soluble glucans isolated from germination fluid of *P. capsici*. The plotted values are the average of 4 replicates. G.f, B.af, Y.f denote, green, blue auto- and yellow fluorescence.

2.3.4. Effect of elicitor treatment of black pepper tissues on *P. capsici* growth

In an experiment set up to verify if the defense responses elicited in black pepper cultures treated with elicitors of *P. meadii* and *P. capsici* indeed had any inhibitory effect on the pathogen of black pepper, *P. capsici,* the elicited cultures were inoculated with zoospore suspension of the fungus. Microscopic observations made after 36 hours inoculation of the zoospores showed, that *P. capsici* growing in PmE-treated cultures exhibited cytoplasmic coagulation with disruption of continuity in the coenocytic cytoplasm (Fig. 2.30: E, F, G & H). However, the hyphae of *P. capsici* growing in control as well as PcE-treated cultures did not show any visible distortion in the cytoplasm (Fig. 2.30: B1, B2, C & D). This indicated that the defenses induced in PmE-treated cultures, indeed had a toxic effect on the pathogen. The results were further confirmed by leaf bioassay, in which elicitor treated cut leaves of black pepper were treated with zoospore suspension. The germlings of *P. capsici* colonized the petiole of the control leaves and formed a heavy growth around the petiole by 24hrs after incubation, while the growth was lesser on the petioles of PcE-treated leaves. The colonization was visibly absent on the petioles of the PmE-treated leaves (Fig. 2.31: A, B & C).

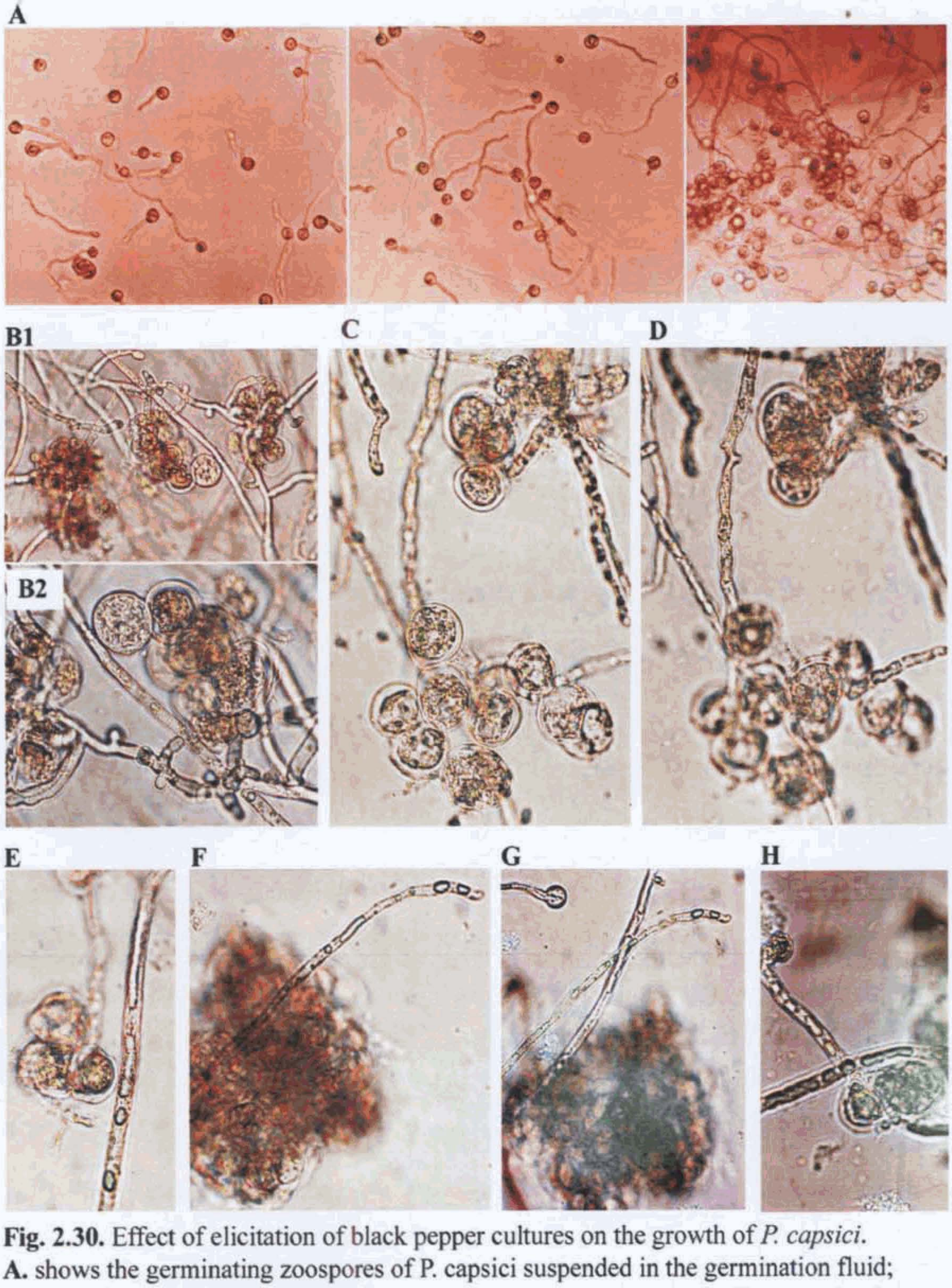


Fig. 2.30. Effect of elicitation of black pepper cultures on the growth of *P. capsici*. **A.** shows the germinating zoospores of P. capsici suspended in the germination fluid; **B1 & B2.** *P. capsici* hyphae in control, non-elicited cultures; **C & D.** *P. capsici* hyphae growing in PcE-treated cultures; **E, F, G & H.** *P. capsici* hyphae growing in PmE-treated cultures. The hyphae in PmE-treated cultures shows distortion and coagulation of the cytoplasm, while this was absent in control or PmE-treated cultures.

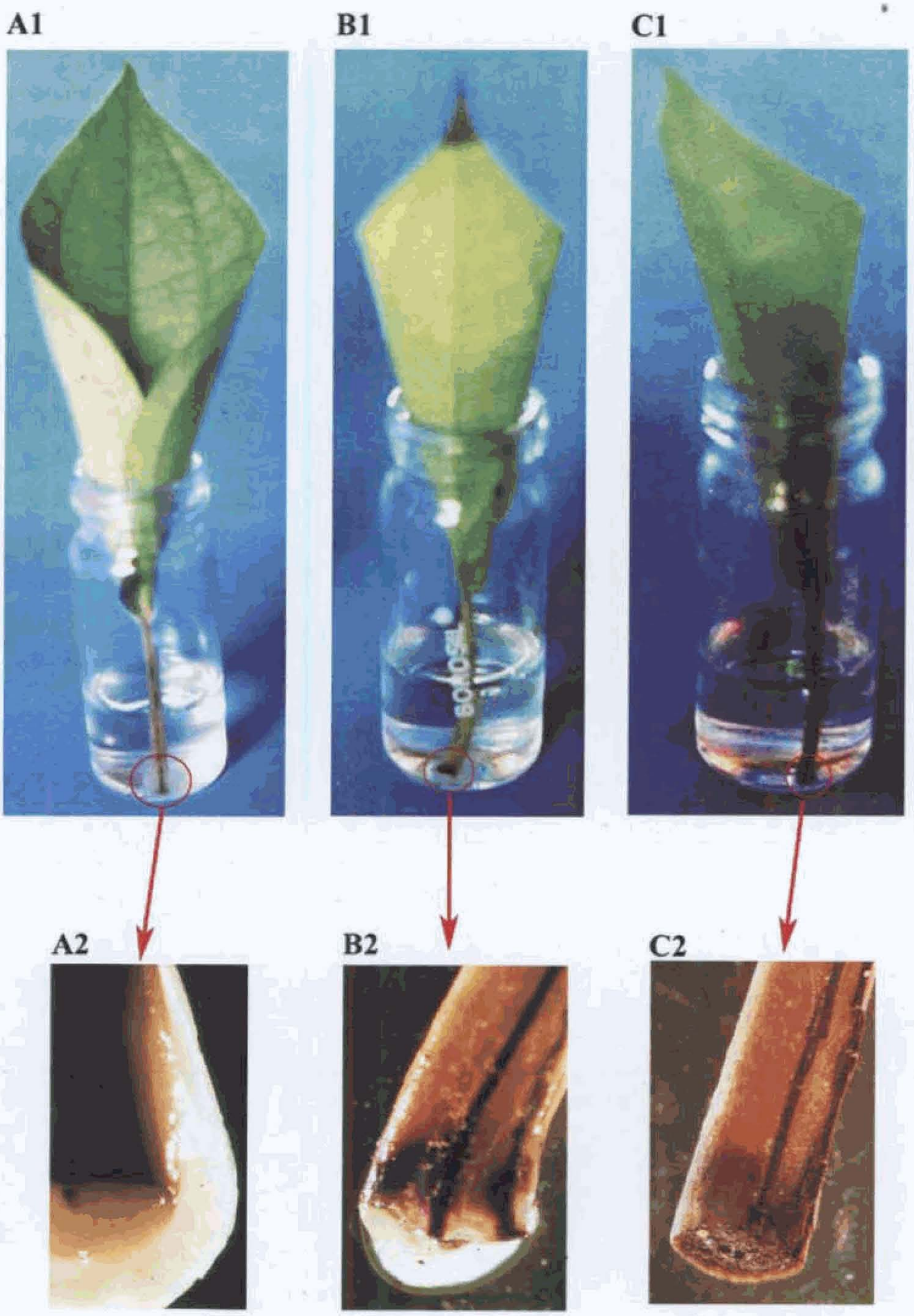


Fig. 2.31. Leaf bioassay showing suppression of growth of P. capsici on elicitor-treated leaves of black pepper. Petioles of control/elicitor-treated leaves were incubated in zoospore suspension of *P. capsici* for 24hrs. **A1 & A2** show heavy growth of *P. capsici* around the petiole of control leaves; **B1 & B2** show significant suppression of colonization of the fungus on petiole of PcE-treated leaf; **C1& C2** show the completesuppression of *P. capsici* growth on the petiole of PmE-treated leaf.

2.4 Discussion

Stress in any form alters the growth and physiology of all living beings. Environmental and biotic stresses commonly affect plant growth and development. The need to develop plants that can tolerate stress conditions gains more importance in crop plants, as the reduction in their productivity due to these stresses has adverse economic effects. The study of defense responses is vital in understanding the mechanisms by which a plant protects itself from the threat of any stress.

Black pepper is highly susceptible to *P. capsici* and improvement of the crop for disease resistance is important to minimize the huge crop losses caused by the foot rot disease caused by the fungus. Basic information on the responses of the plant against the pathogen is thus essential for future use in the improvement of the crop. Our ability to capitalize on inducible defenses and utilize them optimally in agriculture depends, in part, upon a fundamental knowledge of their biochemical nature (Bostock, 1999).

2.4.1. Study of defense responses

Defense responses of a plant during attempted infection, can be studied by two means:

1. By using a tolerant / resistant variety of the crop (here, *P. nigrum*), with the pathogen (here, *P. capsici*), to study its responses induced during infection and comparing them with those induced in a susceptible variety. Those responses that are induced in the tolerant/resistant host alone or expressed in greater magnitude in the tolerant than the susceptible host, would be the defenses that would protect the plant against the pathogen and could be exploited for the improvement of the crop. The defenses induced here are those of host-resistance.

2. In case there is no known tolerant/resistant variety existing in the crop, the putative defense responses can be induced in the available susceptible plant by interacting it with a non-pathogenic species of the fungus (i.e., in the present

study, any species of *Phytophthora* other than *P. capsici*) that induces a resistant reaction in the plant. In this case the responses of the plant, which make it resistant to the fungus (non-pathogenic species), are induced, or in other words, non-host resistance responses are induced. These responses can be compared with the responses in a susceptible reaction between the host and the pathogen to observe the differences between a non-compatible and a compatible reaction.

These defenses induced in the incompatible reaction that play a role in containing the fungal attack and to overcome the pathogen, are the candidate traits that are to be exploited in engineering a plant for resistance against a pathogen.

Though black pepper originated in the Western Ghats, a high degree of resistance to *P. capsici* is lacking in black pepper germplasm available in the country. However, a few tolerant lines have been identified (Sarma *et al.,* 1991). The absence of a solid resistant line to *P. capsici* impedes the comparative study of the responses between a susceptible and a resistant host, which is essential for identifying the factors responsible for susceptibility or resistance in a plant.

The unavailability of a highly tolerant/resistant variety for studying the defense responses can be circumvented, by studying and comparing the responses of the available susceptible plant induced during its interaction with a non-pathogen (incompatible interaction / resistant reaction) and a pathogen (compatible interaction / susceptible reaction). In both (a) tolerant/resistant plant-pathogen interactions and (b) plant-non-pathogen interactions, the incompatible reactions are induced. Or in other words, those responses, which prevent the ingress and establishment of the pathogen, are induced.

2.4.2. Leaf bioassay for testing virulence of different *Phytophthora* spp. on black pepper

A preliminary study to test the pathogenicity of different species of *Phytophthora* on black pepper showed that *P. parasitica* isolates from vinca and tomato and *P. meadii* isolate from cardamom failed to invade and establish inside the leaves. The HR they produced at the site of inoculation signifies that the host's defenses were activated on invasion by these species which halted their establishment inside the host tissue. This was seen lacking in black pepper- *P. capsici* interaction, resulting in infection or a compatible reaction.

Some of the cultivars of a host species are resistant while others are susceptible to a pathogen that causes a disease. This resistance of host is termed as host resistance, also commonly known as cultivar- or race-specific resistance. Resistance conferred by a non-host towards a non-pathogen is known as non-host resistance. The gene for gene hypothesis put forward by Flor states that "for each gene for resistance in the host, there is a corresponding gene for avirulence in the parasite".

A plant's capacity for the recognition of an incompatible pathogen (mediated by non-host or race-specific resistance) leads initially to a nearly universal defense reaction, the programmed hypersensitive death of cells in immediate vicinity of contact with the pathogen. This hypersensitive response (HR), in turn, is complemented by the expression of several other forms of induced resistance, including some local and systemic acquired resistance (LAR, SAR) (Graham and Graham, 1999). When soybean tissues are infected with an incompatible race of *Phytophthora sojae*, a series of phenylpropanoid defense responses are induced in a race-specific manner in healthy cells immediately adjacent (proximal) to the HR lesion (Graham, 1991). Parsley (*Petroselinum crispum*) plants are non-host disease resistant to the soybean-pathogenic fungus, *Phytophthora megasperma* f. sp. *glycinea* (Jahnen and Hahlbrock, 1988). Interaction of the two organisms causes a hypersensitive reaction of the plant, resulting in the formation of small local lesions around the infection site. When plants are infected with plant pathogens, various physiological changes occur.

Potato plants exhibit resistance reactions, and several phenomena related to defense in response to an incompatible race of *Phytophthora infestans*, the potato late blight fungus, or to treatment with hyphal wall components (HWC) prepared from *P. infestans*. Vleeshouwers *et al* (2000) examined the interaction between *Phytophthora* *infestans* and *Solanum* cytologically using a diverse set of wild *Solanum* species and potato (*S. tuberosum*) cultivars with various levels of resistance to late blight and observed that in wild *Solanum* species, in potato cultivars carrying known resistance (R) genes and in non-hosts, the major defense reaction appeared to be the hypersensitive response (HR) resulting in cell death. The hypersensitive response (HR) is associated with defense responses that are triggered in the hypersensitively reacting cells to repel the entry and establishment of the pathogen. Failure of plants to induce such defense reactions resulted in susceptibility to disease.

Similarly, it was observed in the present study that when black pepper leaves were inoculated with different species of *Phytophthora,* the host's defense mechanism was triggered by non-pathogenic *Phytophthora* species, namely, *P. parasitica* and *P. meadii* and remained active for a longer duration to control the spread of the non-pathogenic species inside the leaf tissue. In contrast, the defenses of the host were overcome by the pathogenic species of *Phytophthora* namely, *P. capsici*, making the host susceptible to the invasion by the fungus.

Of the three non-pathogenic species of *Phytophthora,* which produced HR on black pepper leaves in the present study, *P. meadii* was chosen as the non-pathogen for further studies, as it is an isolate, which infects another spice crop, cardamom. Thus, by studying the incompatible reactions between *Piper nigrum* and *P. meadii*, one is trying to understand the mechanism by which *P. nigrum* is able to resist invasion by *P. meadii* and how at the same time, is unable to overcome another species, *P. capsici*, of the same genus, *Phytophthora.*

The hypersensitive reaction observed in the incompatible reactions in the present study is a common occurrence in many resistant reactions among plants in response to an invading pathogen. The interaction between the phytopathogenic fungus *Phytophthora cryptogea* and the non-host tobacco (*Nicotiana tabacum*) leads to a hypersensitive reaction characterized by plant tissue necrosis and restricted growth of the fungus (Bonnet, 1985; Bonnet *et al.*, 1995). Parsley leaves respond hypersensitively to the soybean pathogen, *P. megasperma* f.sp. *glycinea*, and form microscopically small necrotic lesions upon infection with zoospores of the fungus (Knogge *et al.*,1987).

The defense responses in compatible and incompatible interactions can be studied using two methods: (1) by using the host plant and the pathogen/non-pathogen, under *in vivo* conditions and (2) by using host tissue (cell suspension cultures) and dead fungal components (hyphal wall elicitors) of pathogen/non-pathogen, *in vitro*. It has been suggested that elicitors mimic the pathogen by inducing defense responses, similar to those occurring in the *in vivo* plant, infected by a pathogen (Schmidt *et al.*, 1998; Szabo *et al.*, 1999).

The present study involves the study of the defense responses induced in black pepper cell-suspension cultures in response to hyphal wall elicitors from *P. capsici* and *P. meadii* to study the host - pathogen (compatible) and host - non-pathogen (incompatible) interactions, respectively.

2.4.3. Use of cell suspension cultures for studying black pepper-*Phytophthora* interactions

Response of a plant to any stress is the result of the cumulative effect of the responses of the individual cells. Studies on certain responses of the plant to stress under *in vivo* conditions are limited by the unresponsiveness of a majority of the cells in a mature plant to the imposed stress. Since the young responsive cells form only small sectors amidst the majority of the unresponsive, mature differentiated cells of the plant tissues, the magnitude with which the defenses are expressed is quite low. This limits the proper understanding of the mechanisms that are involved in response to the stress. Cell suspensions of plants have been widely used with fungal elicitors to study in detail, some of the host-pathogen interactions that are difficult to observe under *in vivo* conditions. Cell suspensions offer a homogenous system of responsive cells that can be uniformly exposed to the fungal elicitor to study the defense responses elicited in the cells. It has been observed by many researchers that many of the defenses induced in model systems like suspension cultures by elicitors of the pathogen *in vitro* are similar to those induced in the host by pathogen invasion *in vivo.*

Scheel *et al* (1986) suggested that cultured parsley cells treated with elicitor from *P. megasperma* f.sp. *glycinea* reflect atleast to some extent, the non-host interaction of parsley with invading hyphae of the pathogen. Takemoto *et al* (1997) suggested that potato cells in suspension culture are useful materials for studies of defense responses in response to HWC from *P. infestans*. Furuse *et al* (1999) successfully exploited potato suspension cultures to gain insight into the defense responses to inoculation with *P. infestans* or HWC of the fungus.

2.4.4. Defenses induced in black pepper suspension cultures on elicitation

On attack by a pathogen, plants elicit a complex set of defense responses to overcome the pathogen. This is possible by the initial recognition of the threat of pathogen invasion by the plant, which is mediated by the signal molecules of the pathogen, namely the elicitors. These elicitors, released by the plant enzymes bind to the receptors of the plant cells, triggering the defenses of the host. Thus, the host deceives the pathogen by making use of the pathogen's cell wall components to trigger the host's own defenses to kill or weaken the pathogen.

The present study involves the use of hyphal wall elicitors of two *Phytophthora* species, namely, *P. capsici* and *P. meadii*, isolated from the mycelial cell walls to study the defense responses elicited in black pepper cells. The logic of using two species of *Phytophthora* to study the defense responses in black pepper was based on the hypothesis that if *Phytophthora capsici* can infect and establish well in black pepper and another species of the same genus, namely, *P. meadii* produced a hypersensitive reaction and cannot cause infection, as mentioned in the above section, it can be assumed that the defense mechanisms of black pepper were overcome by the pathogen in the former interaction, while in the latter, the host defenses remained active which prevented the ingress of *P. meadii*. Hence, it could be suggested that in black pepper-*P. capsici* interaction, the host was susceptible, while in the black pepper-*P. meadii* interaction, the host was resistant to the invading fungus. Since the two fungi belonged to the same genus, it was hypothesized that black pepper responds to the different species of *Phytophthora* with similar set of defense responses and that while the host's defenses were breached by *P. capsici*, facilitating the establishment of the fungus, *P. meadii* did not succeed to overcome the defense machinery of the host plant. Thus, while black pepper-*P. capsici* interaction is a compatible interaction, the black pepper- *P. meadii* interaction is an incompatible interaction, and therefore, the defenses suppressed in the former interaction could be active in the latter. This work was therefore undertaken to study these defense responses occurring in black pepper cells in response to heat and alkali-soluble elicitors of *P. capsici* and *P. meadii.*

Some of the induced defenses observed in cell suspension cultures of black pepper by these hyphal wall elicitors in the present study include hypersensitive cell death and related changes in the cells, mechanical barrier formation and modulation of different enzymes associated with defense.

2.4.4.1. Hypersensitive cell death and associated cytological changes

The marked feature of black pepper suspension cultures on elicitation with hyphal wall components of *Phytophthora species*, observed in the present study was the hypersensitive browning and cell death. The anti-microbial response of both plants and animals is often accompanied by a coordinated activation of programmed cell death (PCD) and defense mechanism(s) (Greenberg, 1996). Characteristic features of the HR include the formation of a zone of dead cells around the infection site, the synthesis of salicylic acid and the accumulation of anti-microbial agents, such as pathogenesis-related proteins (PR) and phytoalexins (Bolwell *et al.*, 1995; Hammond-Kosack and Jones, 1996; Mehdy *et al.*, 1996).

It was observed in the present study that the hypersensitively reacting cells consistently fluoresced blue under UV light. This blue fluorescence exhibited by elicitor-treated cells on staining with fluorescein diacetate (FDA) was presumed to be due to lignification or phenolics accumulation occurring in cells on elicitation. This assumption was based on the fact that lignified sclerenchymatous cells of plant tissue also fluoresced blue under UV, with FDA. It was noted that these blue fluorescing cells also auto-fluoresced blue in the absence of FDA. The FDA staining in the present study therefore differentiated hypersensitively reacting cells from the control cells, with the former fluorescing blue unlike the latter, that fluoresced green with FDA. Thus, the blue auto-fluorescence served as a marker of elicitation in the present study.

MTT colorimetric assay developed by Mosmann (1980) has been used by many researchers to assess cell viability. Sanchez *et al* (1992) used the MTT- assay to determine cell death of tomato, sweet pepper and tobacco caused by the hyphal wall elicitors of *Phytophthora capsici, P. infestans and P. nicotianae.* The assay was also reliably used for assessing viability of cells in sweet pepper suspension culture cells, on treatment with hyphal wall components of *P. capsici* (Sanchez *et al.*, 1994). Since HWC at 200μg ml resulted in significant loss of viability of cells in *P. capsici* elicitor-treated cells, as revealed by the assay, a concentration of 150μg/ml was used in further studies for elicitation. At this concentration, considerable viability of the cultures was maintained that offered responsive cells, which reacted well to elicitor treatment by triggering their defense responses. Cell viability of tobacco suspension culture cells was not affected during incubation with the elicitor for at least 8hrs, judged from the ability of the cells to plasmolyse in hypertonic solution. These data indicated that tobacco suspension culture cells perceived and responded to the *Phytophthora* elicitor (Kamada and Muto, 1994).

Graham and Graham (1999), reviewed data that suggests that hypersensitive cell death is necessary to trigger the competency of surrounding living cells to respond to elicitors of defense responses. They also present the interesting hypothesis that these competent surrounding cells enter a hypersensitive cell death programme that is "rescued" by scavengers of active oxygen species and this is necessary for the cell to perform the metabolic functions associated with localized tissue defense.

A number of morphological, physiological and molecular changes have been identified that appear coincident with the rapid cell death component of HR. Altered ion fluxes across the plant cell membrane, generation of active oxygen species, changes in the phosphorylation state of regulatory proteins and transcriptional activation of plant defense systems are known to culminate in cell death at the site of infection, synthesis of lytic enzymes (Boller, 1987; Linthorst *et al.*, 1990), the production of pathogenesis-related proteins (Takahashi, 1975), protease inhibitors (Ryan, 1990), local accumulation of phytoalexins (Dixon, R.A., 1986; Paxton and Groth, 1994) and cell wall rigidification as a result of callose, lignin and suberin deposition (Hammond-Kossack & Jones, 1996; Yang *et al.*, 1997). These changes have been proposed to contribute alone or together to disease resistance. The multitude of biochemical processes associated with HR make the dead cells, and adjacent living cells, inhospitable for microbes (Kombrink and Somssich, 1995) and as a result, the pathogen is contained within the immediate vicinity of the infection site, thus preventing the spread to the non-infected parts of the plant. Thus, HR-related cell death has long been speculated as being directly responsible for limiting pathogen growth and development. The hypersensitive cell death observed in the black pepper cultures on elicitation in the present study could be culmination of a series of defense responses that were heightened in the cells after elicitation.

Several factors have been reported to be responsible for the execution of HR during a resistant response in plant-pathogen interactions. HR has been correlated with oxidative burst, membrane damage, ion fluxes, endonuclease activation, DNA cleavage and gene expression. However, it is not clear as to which of these events are primarily involved in the hypersensitive cell death.

Glazener *et al* (1996) using bacterial mutants defective in eliciting HR to infect tobacco plants found that cell death did not occur in tobacco cells infected with hrp$^-$ strain though the cells exhibited oxidative burst. Thus the study proved that oxidative burst need not always lead to cell death. However, Levine *et al* (1994) reported that H_2O_2 was indeed responsible for hypersensitive cell death in soybean suspension cultured cells on treatment with Pmg, a glucan wall elicitor from *Phytophthora megasperma* f. sp. *glycineae*. It was also observed by Levine *et al* (1994) that when H_2O_2 production was enhanced by inhibiting catalase during an HR elicited by avirulent bacteria, the amount of cell death was greatly increased. However, the fact that the kinase inhibitor K252A completely blocked oxidative burst by Pmg elicitor but did not completely inhibit the cell death caused by avirulent bacteria suggests that H_2O_2 may not act alone to trigger cell death. Thus the results of Glazener *et al* (1996) and Levine *et al* (1994) indicate that H_2O_2 alone is not sufficient to account for all the cell death occurring during resistant response. Results of Binet *et al* (2001) also indicate that AOS production is not involved in MT depolymerization and cell death and occurs independently to MT depolymerization in tobacco cells treated with cryptogein or plant oligogalacturonides. Sasabe *et al* (2000) characterized hypersensitive cell death of tobacco BY-2 cells that followed treatments with *E. coli* of INF1, the major elicitin secreted by the late blight pathogen *Phytophthora infestans* to investigate the involvement of the oxidative burst and/or the expression of defense genes in the signal transduction pathways leading to hypersensitive cell death. The results of the study suggested that the signal transduction pathways leading to cell death, oxidative burst and expression of defense genes branch differently in the very early stages that follow elicitin recognition by tobacco cells. In the present work, a detailed study about the role of active oxygen species in elicitor-induced cell death has not been done. It would be interesting to use the suspension cultures of black pepper to gain insight into the mechanism of cell death and the role of the oxidative burst in executing the HR.

Superoxide is also implicated in triggering HR-induced cell death (Doke and Ohashi, 1988) in tobacco leaves infected with TMV. The production of superoxide radicals during the initial phase of elicitation in black pepper cultures in the present study could have a role in partly bringing about cell death owing to the toxicity of these radicals. However, this is only a speculation and further studies are required to implicate these radicals in HR-induced cell death.

Ca^{2+} fluxes are also known to play an important role in the execution of HR. Levine *et al* (1996) observed that blocking of Ca^{2+} channels reduces cell death of soybean suspension cells in response to avirulent bacteria or H_2O_2. Treatment of these cells with

Ca^{2+} ionophores induced programmed cell death, proving that Ca^{2+} is necessary for cell death. Binet *et al* (2001) using lanthanum, a calcium channel blocker demonstrated that cryptogein-induced microtubule destabilization and cell death depends on calcium influx as reported for other cryptogein-induced events, namely AOS production, MAPK activation, plasma membrane depolarization, cytosol acidification, and chloride efflux. Using different concentrations of cryptogein, which triggered different rates of calcium influx and a saturating concentration of OGs inducing a low rate of calcium influx, the results of Binet *et al* (2001) suggest that a critical threshold level of intracellular Ca^{2+} concentration is essential to trigger MT depolymerization and cell death in cryptogein pathway. The differences of the calcium influx rate in cryptogein- or oligogalacturonides (derived from the plant cell wall)-treated cells could explain their efficiency or inefficiency respectively, to trigger MT destabilization and cell death. It has been observed by Mittler and Lam (1995) that Ca^{2+} is required for the activation of DNA endonucleases that are associated with the resistance response and hence it is possible that DNA cleavage is a necessary step in the suicide process. Though Ca^{2+} accumulation has not been studied in the present work, it would be an interesting area of research to analyze the role of these ions in triggering various defense responses and their possible role in cell death. The possible role of DNA endonucleases in the HR would also be worth exploring, to get an in depth understanding of the various regulatory mechanisms taking place during the hypersensitive response in black pepper cells.

In his review on programmed cell death in plants, Greenberg (1997) poses many questions that remain open regarding the regulation and execution of HR. Are O^{2-} and H_2O_2 obligatory signals in the induction of the HR?? If other signals exist, what are they? Could they be lipid peroxides?? What is the role of DNA cleavage? Are there HR-specific proteases as there are apoptosis-specific proteases in animals? What are the targets that become phosphorylated, dephosphorylated, or proteolyzed? Answering these questions should contribute to a full understanding of the regulation and function of pcd resulting from plant-pathogen interactions. Since suspension cultures of black pepper, as revealed in the present study offer an excellent system to study the cellular changes taking place during elicitation, it would be a great idea to exploit this system to gain more in depth understanding of the mechanisms of HR in black pepper.

2.4.4.2. Hypersensitive browning

Cell browning and medium darkening were the most marked symptoms of elicitation, associated with hypersensitive cell death in black pepper suspension cultures. Our observation is similar with that made in elicitor-treated cultures of sweet pepper (Garcia-Pérez *et al.*, 1998). Cell browning and death is a characteristic symptom accompanying hypersensitive reaction. Naton *et al* (1996) also reported rapid cell death as the major defense response in parsley cell cultures infected with germlings of *P. infestans.*

In the present study we also observed darkening of the medium accompanied with cell browning of cultures treated with the elicitor. Cell browning in pepper suspension cultures in response to elicitation by both lyophilized mycelium and fungus filtrate, was explained by Garcia-Pérez *et al* (1998) as being due to the oxidation of phenolics to quinones, which give rise to tannins and melanins that are responsible for darkening of the medium. Polyphenol oxidase (PPO) is known to catalyze the oxidation of phenolics to quinones, which can react with biological molecules, thus creating unfavorable environment for pathogen development (Leatham, *et al.*, 1980). Higher activities of PPO were reported in soybean in response to *Phytophthora megasperma* (Lazarovits and Ward, 1982). Vleeshouwers (2000) observed deposition of callose and extracellular globules containing phenolic compounds during the hypersensitive response of *Solanum* species to *P. infestans.* Since the extra-cellular globules were deposited near cells showing the HR, it was suggested that they might function in cell wall strengthening.

Groten and Barz (2000) observed accumulation of phenolic compounds in soybean suspension cultures treated with fungal wall elicitors from *Phytophthora sojae* and *Rhizoctonia solani.* Anderson *et al* (1991) also observed accumulation of phenolics in the culture medium of elicitor-treated bean suspension cultures. Increase in cellular and extra-cellular phenolics in the elicited black pepper cultures in the present study thus suggest a possible increase in the activity of polyphenol oxidase on elicitation. The inactivation of pathogen pectolytic enzymes by oxidized substrates of PPO is reported as part of host resistance mechanism (Spence, 1961). The intensity of browning of culture medium in the present study was observed to be significantly higher in PmE-treated cells than in PcE-treated cultures, suggesting that the defenses are induced to a greater extent in incompatible reactions. The participation of phenylalanine ammonia lyase in host plant resistance has been associated with the accumulation of aromatic fungitoxic compounds (Ampomah and Friend, 1988) and lignification (Cahill and McComb, 1992). PPO activity has also been reported to increase in black pepper leaves on infection with pathogen (Jebakumar *et al.*, 2000). Thus, in the light of the results obtained in the present study and the information already available in black pepper during pathogen attack (Jebakumar *et al.*, 2000), it could therefore be argued that high activities of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase may be responsible for the increased lignin and phenol contents observed in the elicitor-treated black pepper cultures, especially, in PmE-treated cultures.

2.4.4.3. Extracellular alkalinization

Other factors presumed to be involved in hypersensitive cell death are reactive oxygen species and possibly other toxic substances, including oxidized phenolics that are produced during the hypersensitive reaction. Consistent with these observations, we noticed in the present study that in addition to cell browning, there was an increase in the oxidative burst and extra-cellular pH in black pepper suspension cultures after elicitation.

The increased alkalinization of the medium observed in the present study could be due to the increased permeability of the cell membranes following elicitation that results in the release of intracellular electrolytes. Increase in pH of the extra-cellular medium, observed in suspension-cultures of sweet pepper on elicitation with elicitors from *P. capsici* (Garcia-Pérez *et al.*, 1998) was suggested to be a symptom of defense since it reflects the changes in the cell biochemistry as represented by phytoalexin production and increased cell browning seen in the elicited pepper cells. Cryptogein, a protein secreted by *Phytophthora cryptogea*, when added to tobacco cell suspensions, induces a rapid increase in extra-cellular pH and conductivity (Blein *et al.*, 1991). Anderson *et al* (1991) attributed the increase in extra-cellular pH of bean suspension-cultures treated with elicitor of *Colletotrichum lindemuthianum*, to the necrosis associated with hypersensitivity. It has been suggested by Rogers *et al* (1988) that lipid peroxidation occurring during hypersensitive reaction may damage the plasma membrane, resulting in the alteration of the ion fluxes that in turn alters the pH. In plant-pathogen interactions under *in vivo* conditions, alkalinization of apoplast has been shown to accompany pathogen recognition. It has been suggested that alkalinization of the apoplast also induces fast production of H_2O_2, an important active oxygen species involved in the oxidative burst during pathogen attack, from peroxidase enzyme (Bowell, *et al.* 1995, Salzer *et al.*, 1996). Stratmann *et al* (2000) reported alkalinization of the medium of suspension cultured cells of tomato on treatment with a peptide systemin and beta-glucan elicitor from *Phytophthora megasperma* and noted that this defense was inhibited by suramin, an inhibitor of growth factor receptor interactions in animal cells. Fellbrich (2000) observed that the Pp-elicitor from *Phytophthora nicotianae* var. *parasitica* induced influxes of protons signifying extracellular alkalinization, in cultured *Petroselinum crispum* cells. Groten and Barz (2000) also reported alkalinization of cultured medium in soybean suspension-cultured cells treated with cell wall extracts of fungal pathogens, *Phytophthora sojae* (Pmg-elicitor) and *Rhizoctonia solani* (Riso-elicitor).

Allan and Fluhr (1997) indicated that during pathogen invasion, the apoplast pH rises, favoring an H_2O_2 burst, and electrolyte leakage (that accompanies elicitor treatment) that may include many likely substrates for peroxidases and /or amine oxidases to produce sustained H_2O_2 production. The increase in alkalinity of the culture medium after elicitation of black pepper cultures observed in the present study may indicate a similar role in favouring higher H_2O_2 production by release of substrates for peroxidase enzyme that which was observed to increase significantly on elicitor treatment, especially in the PmE-treated cultures.

2.4.4.4. Superoxide anion generation

The hypersensitive reaction, a common response of a plant to an incompatible pathogen, initiates a number of metabolic changes in the host cells. Among these responses, oxidative processes can play a crucial role during the early steps of elicitation. One of the early events in plant-pathogen interactions is the rapid and transient production of active oxygen species ($O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$), called the oxidative burst (Baker and Orlandi, 1995). Although molecular oxygen, which is an essential element in aerobic metabolism, is relatively un-reactive, it is a potential source of reactive forms, such as free radicals or H_2O_2. Active oxygen species (AOS) are present at low levels in plant cells; they can be generated during electron transport or enzymatic processes involved in redox systems (Crane *et al.*, 1985).

In the present study, a general increase in the production of super-oxide ($O_2^{\cdot -}$) radicals was observed in black pepper suspension-cultures on elicitation with HWC of ***Phytophthora***. This is similar to the observations made by many researchers that active oxygen species are produced in plants during the hypersensitive reaction induced by elicitor treatment (Apostol *et al.*, 1989a) or pathogen-infection (Sutherland, 1991; Tzeng and DcVay, 1993; Mehdy, 1994). Involvement of $O_2^{\cdot -}$ generation reaction in the initiation of a resistance response triggered by infection with incompatible, but not compatible pathogens, or by treatment with an elicitor, was first reported in potato tuber tissues that were inoculated with incompatible races of *Phytophthora infestans* or treated with hyphal wall elicitors (Doke, 1983). Leaf tissues of tomato, tobacco and sweet pepper have been shown to generate $O_2^{\cdot -}$ upon infection by *P. infestans* (Chai and Doke, 1987). Activation of $O_2^{\cdot -}$ generating system has also been observed in potato leaf tissue and protoplasts, immediately after infection with incompatible race of *P. infestans* (Doke, 1985) or treatment with hyphal wall components (Doke, 1983). Rapid triggering of active oxygen species (AOS) that include $O_2^{\cdot -}$ and H_2O_2 was also observed in suspension-cultures of soybean (Linder *et al.*, 1988; Glazener *et al.*, 1991) and tobacco (Keppler andBaker, 1989; Keppler *et al.*, 1989) in response to elicitors from phyto-pathogenic bacteria. Nürenberger *et al* (1994) reported generation of reactive oxygen species in elicitor-treated cultured cells of parsley.

Many roles have been attributed to oxidative burst occurring during plant-pathogen interactions. Currently, it is considered that the reactive oxygen species of the oxidative burst are key mediators of programmed cell death (PCD) in plants (Bolwell & Wojtaszek, 1997). Naton *et al* (1996) demonstrated the accumulation of intra-cellular peroxides to be correlated with induced rapid cell death, and that this rapid cell death terminates growth of the fungal pathogen. Sanchez *et al* (1994) demonstrated the role of $O_2^{\cdot-}$ radicals in hypersensitive cell death of potato cells on treatment with HWC from *Phytophthora* spp. Pre-treatment of cells with Tiron, a $O_2^{\cdot-}$ radical scavenger followed by addition of HWC, resulted in the inhibition of hypersensitive cell death, indicating that activation of $O_2^{\cdot-}$ radical generating system is closely associated with the triggering of hypersensitive cell death. AOS has been reported to be related to lipid peroxidation (Keppler and Baker, 1989; El-Moshaty *et al.*, 1993) and both AOS production and lipid peroxidation are involved in mechanisms leading to cell death (Rustérucci *et al.*, 1996). The increased cell death observed in elicited cultures of black pepper in the present study can also be attributed to the AOS-induced lipid peroxidation.

AOS also function as a part of the signal transduction pathway leading to the induction of PR-proteins and systemic resistance in infected and non-infected parts of the plant. They play a prominent role in early and later stages of the plant pathogenesis response, and may serve as both cellular signaling molecules and direct anti-pathogen agents (Chen *et al.*, 1993; Green and Fluhr, 1995; Allan and Fluhr, 1997). Pathogen-induced oxidative bursts are harnessed by the plant cell both as a means of poisoning an invading pathogen and as an intra- and inter-cellular messenger of this invasion (Apostol *et al.*, 1988). Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) are the agents of this burst (Mehdy, 1994; Inzé and Van Montagu, 1995).

The source of ROS has been studied by many researchers. There are several hypotheses to explain the appearance of H_2O_2 in the medium of cultured cells and the apoplastic fluid of whole-plant tissues (Bolwell *et al.*, 1998). The earliest, proposed to explain the origin of H_2O_2 needed for formation of lignin in developing xylem of horseradish (*Armoracia lapathifolia*), involves the reduction of O_2 to superoxide by phenolic and $NAD^{\cdot}$ radicals produced by peroxidase (Yamazaki and Yokota, 1973; Elstner and Heupel, 1976; Gross et al., 1977; Halliwell, 1978). In this model the source of electrons in the apoplast is said to be malate, which is exported across the plasma membrane by a malate/oxalacetate carrier and used to reduce NAD^+ by apoplastic malate dehydrogenase. A second hypothesis, proposed for the oxidative burst induced in French bean cultured cells by *Colletotrichum lindemuthianum* cell wall elicitor, involves an apoplastic peroxidase in a more direct way (Bolwell et al., 1995). The O_2-heme complex of peroxidase is reduced to compound III by reductants exported from the cell. Under the proper conditions, i.e. elevated pH, the complex is effectively hydrolyzed to release H_2O_2. In this model the source of electrons has not been identified, but the release of a reductant from elicited cells has been observed. A third hypothesis, proposed for the oxidative burst induced in rose cultured cells by *Phytophthora* spp. elicitor (Auh and Murphy, 1995) and for other systems, does not involve peroxidase. Rather, a trans-plasma membrane superoxide synthase (NAD(P)H oxidase) transfers electrons from NADH or NADPH in the cytoplasm to O_2 to form superoxide. H_2O_2 is formed by the dismutation of superoxide. H_2O_2 in all the cases is formed by the dismutation of superoxide.

Thus, several enzymatic sources of ROS appear to exist in the plant cell. The plasma membrane-associated NAD(P)H oxidase is activated during the response of plants to pathogens (Jabs *et al.*, 1996) and is responsible for the production of super-oxide radicals ($O_2^{\cdot -}$) as explained above, which are then converted spontaneously or via superoxide dismutase (SOD) into H_2O_2 (Doke and Chai, 1985; Doke and Miura, 1995; Murphy and Auh, 1996). Another source of ROS, elicited during pathogen attack, is the cell wall-located peroxidase. Although peroxidases usually function to scavenge and remove H_2O_2 from the cells, it has been suggested that fast production of H_2O_2 also occurs from this enzyme during alkalinization of apoplast (Bowell, *et al.* 1995), which has been shown to accompany pathogen recognition (Salzer *et al.*, 1996). It is postulated that cell wall peroxidases function to cross-link walls and toxify the apoplast (the region between the plasma membrane and the cell wall). Otte and Barz (1996) suggested that

ROS produced by NAD(P)H oxidase drives peroxidase-catalyzed oxidative processes, showing that the ROS producing systems in plants may operate in tandem. Flavin containing oxidase such as xanthine / aldehyde oxidase has also been implicated as a source of ROS in plants (Allan and Fluhr, 1997), similar to that seen in mammalian systems. Yet another source of ROS in plants is the amines, which induce ROS production by acting as substrates for amine oxidases. Amine oxidases are a ubiquitous group of plant enzymes that catalyze the oxidation of a variety of monoamines, diamines and polyamines to the corresponding aldehyde and release NH_3 and H_2O_2 (Tipping and McPherson, 1995). Similarly, peroxidases are also known to produce H_2O_2, when supplied with an appropriate reductant (Bowell *et al.*, 1995).

Allan and Fluhr (1997) suggested that there are two phases of ROS induction in plant suspension cultures - Phase I, that includes very rapid responses within minutes, is involved in the initial elicitor- receptor interaction, and (2) Phase II, which includes later ROS production, lasting for many hours and correlates with the resistance or susceptibility of the plant to the pathogen. Phases I and II are presumed to differ in the source of ROS as well as in their kinetics. NADPH-dependent $O_2^{\cdot -}$ generation is one of the earliest defenses that takes place soon after elicitation and before the induction of defense genes. Induction of oxidative burst in elicited cells is rapid and does not require transcription, unlike most other plant defense responses, which involve induction of batteries of defense-related genes. In contrast to the elicitor-induced oxidative burst, these defense responses require at least several hours for cumulative transcription and translation before maximal response is observed, although initial transcriptional activation may be apparent within one hour (Dixon and paiva, 1995). In the present study, it was observed that the superoxide radical generation was more evident at 3h after elicitation and dropped thereafter until 12hrs. Though we did not follow the superoxide generation after 12hrs after elicitation, we assume based on higher activities of superoxide dismutase and other enzymes responsible for scavenging H_2O_2 that the superoxide radical generation would have continued, resulting in the triggering of various defense responses observed in the elicited cultures, discussed in this chapter.

Allan and Fluhr (1997) indicated that the increased pH that occurs during pathogen invasion, favors electrolyte leakage (that also accompanies elicitor treatment) that may include many likely substrates for peroxidases and /or amine oxidases to produce sustained H_2O_2 production. Thus, many plants are induced to accumulate higher levels of polyamines and amine-oxidizing systems during pathogen infection, and this positively correlates with resistance (Angelini *et al.*, 1993). Although moderately reactive themselves, much of the cellular damage caused by H_2O_2 and $O_2^{\cdot -}$ is the result of their conversion to even more reactive species; for example, $O_2^{\cdot -}$ is converted to H_2O_2 by the action of superoxide dismutases and H_2O_2 may in turn be converted in the presence of Fe^{2+} to the extremely toxic hydroxyl free radical ($OH^{\cdot}$) via the Fenton reaction. The oxidants may function directly, in cell wall cross-linking of structural proteins or as part of signaling mechanisms (Kuc, 1997).

Due to the speed of the response, oxidative burst is known to be one of the earliest defenses in plants, as it results in the production of H_2O_2 that might indirectly facilitate the process of serving as a second messenger of signal transduction (Apostol, 1988). H_2O_2 is considered as the most attractive candidate for signaling via ROS because of its relatively long life and high permeability across membranes (Allan and Fluhr, 1997). In general, it has been suggested that H_2O_2 could be involved in: (1) direct killing of the pathogen; (2) act as a second messenger in defense reactions (HR or synthesis of phytoalexins); (3) as a factor involved in plant cell reinforcement, a mechanism to render the cell wall less digestible by microbial enzymes, thus restricting pathogen development (Mehdy *et al.*, 1996). H_2O_2 has many of the properties expected of a defense-related signal transducer, since (a) it is formed rapidly upon elicitor treatment, (b) it can be rapidly destroyed, (c) it can independently stimulate some of the defense responses, like phytoalexin synthesis (d) its removal by catalase or other inhibitors blocks the defense response (Apostol, 1988). H_2O_2 in combination with cell wall peroxidase plays an important role in lignification (van Huystee, 1987). Though the production of H_2O_2 was not analyzed in the present study, it is presumed that the observed production of superoxide radicals would have resulted in increase in hydrogen peroxide by the action of superoxide dismutases that were seen to be active in elicited black pepper cultures. Furthermore, since peroxidases were seen to increase in the elicited cells it can suggested that their increase would have been due to the rise in hydrogen peroxide levels. This hypothesis gains support from the observation that there was increased lignification in elicited black pepper cells which could have been due to the oxidative processes involving peroxidases and H_2O_2. Apostol *et al* (1988) used cultured cells of soybean to study the role of oxidative burst, especially H_2O_2 in disease resistance. They studied that H_2O_2 production was rapidly induced in elicited cells, which along with peroxidases was responsible for the destructive oxidation of many compounds like IAA and pyranine, introduced in the medium.

Elicitation of tobacco leaves with cryptogein, resulted in cell death that was promoted by the generation of $O_2^{\cdot -}$ by xanthine oxidase. As cryptogein-induced cell death was not inhibited by catalase, it was suggested that $O_2^{\cdot -}$ and not H_2O_2, was responsible for the cell damage (Allan and Fluhr, 1997). Hence, it can be suggested from the results obtained in the present study with black pepper suspension cultures that the superoxide radicals generated during the initial phases of elicitation may in part be responsible for some of the cell death that occurred during the interaction of the elicitor and the suspension-cultured cells and that the action of SODs on these radicals would have resulted in the formation of H_2O_2, which in turn initiated the defense mechanisms in the elicited cells, possibly by a signal transduction mechanism. Further, it can be presumed that alkalinization of the extra-cellular medium observed in black pepper cells could be associated with the release of certain compounds which may be acted upon by enzymes like cell wall-bound peroxidases and amine oxidases, also resulting in the production of H_2O_2. These active oxygen species, i.e., H_2O_2, in addition to providing a hostile environment could also have participated in signalling the message of binding of elicitor from the surface of the cells after elicitor treatment, to the nucleus, triggering various defense responses including enzymatic changes, cell wall rigidification, and formation of secondary metabolites like phenolic compounds.

It was observed in the present study that the intensity of defense responses like cell browning, pH increase and phenolic exudation and cytoplasmic aggregation were more in the incompatible interaction of black pepper suspension cultures with the elicitor from *P. meadii* than in compatible interactions involving black pepper cultures and elicitor of *P. capsici.* This was similar to observations made by Garcia-Pérez *et al* (1998) in sweet pepper, in which the suspension cultures of *P.capsici* resistant cultivar, Smith-5, showed a more rapid and intense response to the elicitor preparations than the sensitive cultivars, Americano and Yolo Wonder. The higher activity of peroxidase observed in PmE-treated cultures could be responsible for these defenses through the increased production of hydrogen peroxide (eg. Lignification). On the other hand, the higher activity of catalase in PcE-treated cultures may have resulted in the scavenging of H_2O_2 formed by SOD, which may thus have become insufficient to trigger the defense responses efficiently.

2.4.4.5. Lipid peroxidation

The higher lipid peroxidation found in elicitor-treated cultures over the control signifies membrane damage, which may be due to increased active oxygen species during elicitor treatment. Active oxygen species have been reported to be involved in lipid peroxidation (Keppler and Baker, 1989; El-Moshaty *et al.*, 1993). Rustérucci *et al* (1996) observed that lipid peroxidation in cryptogein-treated leaves of *Nicotiana* was closely related with extensive necrosis, suggesting that necrosis appearance, lipid peroxidation and AOS formation, are related. In a latter study, Rustérucci *et al* (1999) observed that The LOX-dependent peroxidative pathway, responsible for tissue necrosis appeared to be one of the features of hypersensitive programmed cell death. Inhibition and activation of the LOX pathway was shown to inhibit or to activate cell death, and evidence was provided that fatty acid hydroperoxides are able to mimic leaf necrotic symptoms. A massive production of free polyunsaturated fatty acid (PUFA) hydroperoxides dependent on a 9-lipoxygenase (LOX) activity was characterized during the development of leaf necrosis

The increased lipid peroxidation in PmE-treated cultures could be related to the increased oxidative burst observed; leading to loss of membrane integrity and finally cell death, compared to that observed in PcE-treated cultures. The marginally higher activities of antioxidant enzymes observed in PmE-treated cultures during the latter part of elicitation therefore could be an effort by the cells to control the cell damage caused by active oxygen species. Maciejewska *et al* (2002) reported elevated ROS production, lipid peroxidation and lipoxygenase activity in *Solanum nigrum* treated with the pathogen *Phytophthora infestans*-derived elicitor in comparison with control plants indicating that oxidative stress took place. The authors postulated that PQ may be associated with mechanisms maintaining a tightly controlled balance between the accumulation of ROS and antioxidant activity that determines the full expression of effective defense.

2.4.4.6. Cytoplasmic aggregation

Another early response observed in black pepper cells to elicitor treatment in the present study is the aggregation of the cytoplasm. This observation is similar to the results obtained in several plant pathogen interactions. Kitazawa *et al* (1973) and Doke and Tomiyama (1980) observed similar response in elicitor-treated cultures of potato tissues and protoplasts and potato tissues to penetration by *P. infestans*. Furuse *et al* (1999) also observed cytoplasmic aggregation as one of the early resistance responses of potato suspension-cultures to infection by *P. infestans* or HWC treatment and that the aggregation of cytoplasm is connected with the association of actin filaments especially with actin polymerization.

Motility associated with cytoplasmic aggregation has been known to be dependent on the rearrangement of actin filament (Hazen and Bushnell, 1983; Gross *et al.*, 1993; Kobayashi *et al.*, 1994). By the use of inhibitors of the various components of the signal transduction like the protein kinase, Ca^{2+}, calmodulin, phospholipase A_2, it was found that the these components are associated with the decrease in monomer – total actin ratio that accompanies cytoplasmic aggregation during HWC treatment. Thus, the authors suggest that these results indicate that the factors involved in signal transduction might be connected with the cytoskeleton, especially actin filament association during the initiation of defense responses in potato cells. Thus, cytoplasmic aggregation in potato cells plays an important role in signal transduction in potato.

Tomiyama *et al* (1982) reported that treatment with cytochalasin B, inhibitor of the polymerization of actin, delayed hypersensitive cell death, also indicating that cytoplasmic aggregation is indispensable to defense responses. Cahill *et al* (2002) also observed complete disorganization of cell cytoplasm and re-orientation and loss of microtubules in the early stages of the incompatible interaction in association with cellular hypersensitivity in the hypocotyls cells of soybean cultivars resistant to *P. sojae*. The susceptible cultivar however did not show these responses. Thus, it was suggested that changes in microtubule orientation and state are among the first structural changes that are visible within cells during incompatibility in this system. Takemoto *et al* (1997) also suggested that the cytoplasmic aggregation observed in potato cell suspension cultures in response to HWC from *P. infestans* is caused by the rearrangement of microfilaments, since treatment of cells with cytochalasin D, an actin polymerization inhibitor, inhibited to a great extent, the aggregation of cytoplasm in cells. They further studied the molecular basis and function of cytoplasmic aggregation and found that the micro-filaments consisted of actin, a 43kDa protein that was bound to two other major proteins of 32kDa and 22kDa which were identified as basic chitinase and osmotin respectively. Actin filaments have been known to converge around the site of fungal invasion in cells of several plants (Gross *et al.*, 1993; Kobayashi *et al.*, 1994). Thus, Takemoto *et al* (1997) concluded that the association of osmotin and chitinase proteins with the actin filaments makes it possible for the translocation of these pathogenesis-related proteins with the actin filaments during cytoplasmic aggregation to assist the plant in its defense against the possible pathogen invasion.

Based on these studies, it can be supposed that cytoplasmic aggregation occurring in black pepper cells as an early response to elicitor treatment signifies a first line of defense along with other processes like oxidative burst and alkalinization of the extra-cellular medium. It has been observed in the present study that in addition to cells treated with elicitor, cytoplasmic aggregation was also found in black pepper cells infected with zoospores of *P. capsici.* Similar observations were made by Naton *et al* (1996) in parsley cells infected with germlings of *P. infestans*. It has been found in several plants that the functional proteins and components of signal transduction pathways are associated with the cytoskeleton (Calvert *et al.*, 1996; Dove *et al.*, 1994; Tan and Boss, 1992). Based on this and the information discussed above, it can be suggested that cytoplasmic aggregation could have a possible role in signal transduction and initiation of defense responses in black pepper cultures. However, the importance of cytoplasmic aggregation in defense responses in black pepper needs to be explored and proved by further studies.

Naton *et al* (1996) reported that the shrunken cellular content of the parsley cells exhibiting cytoplasmic streaming, on infection with *Phyotphthora infestans*, auto-fluoresced bluish under UV epi-fluorescent light and that the cells were no longer stained by the vital dye, FDA. Even single cells showed induced rapid cell death with the same appearance and at a rate similar to cells within micro-calli. Similar observations were made in the present study, in which the single cells and cell clumps and micro-calli of black pepper cultures treated with elicitor, auto-fluoresced blue. Very recently, Cahill *et al* (2002) reported blue auto-fluorescence in hypocotyls cells of resistant cultivar (Haro) of soybean, reacting incompatibly to *Phytophthora sojae*. These authors also noted that the auto-fluorescence was associated with granulation, aggregation and fragmentation of cytoplasm within single cells that were associated with hyphae. Though the authors observed auto-fluorescence of cell walls and cellular components in both compatible and incompatible interactions, the time of induction and extent of fluorescence differed. Significantly higher numbers of cells exhibited auto-fluorescence in incompatible interactions compared with compatible interactions. These results are similar to the observations made in the present study. The number of cells showing blue auto-fluorescence was more in black pepper suspension-cultures treated with elicitor of the non-pathogen, *P. meadii* than in the cultures treated with elicitor from the compatible pathogen, *P. capsici.* Knogge *et al* (1987) reported induction of furano-coumarins, the putative phytoalexins in parsley cotyledons, on infection with *Phytophthora megasperma* f.sp. *glycinea,* a fungal pathogen, to which parsley shows a non-host hypersensitive resistance response. It was observed that these furano-coumarins had inhibitory effects on the germination of the zoospores of *P. megasperma* f.sp. *glycinea.* Later, Kombrink and Hahlbrock (1986) also reported biosynthesis of furano-coumarins in cultured parsley cells on treatment with *P. megasperma* f.sp. *glycinea* elicitor. Further, it has been reported by Scheel *et al* (1986) that the lesions formed upon infection of parsley leaves with *P. megasperma* f.sp. *glycinea* were characterized by halos that showed the typical blue fluorescence of furano-coumarins, which accumulate in the infection droplets. Thus, it can be presumed that the blue auto-fluorescence exhibited by elicitor-treated cells in the present study, could also be due to the induction of similar secondary metabolites in black pepper. However, studies on the secondary metabolite induction in black pepper during elicitation or infection by pathogen/non-pathogen are needed to confirm this.

There have been many reports recently, about the occurrence of blue auto-fluorescence of cells in many plant-pathogen interactions (Hardham and Mitchell, 1998; Grant and Mansfield, 1999). More recently, Mohr and Cahill (2001) reported that auto-fluorescence of soybean walls is due to rapid lignification that occurred within 3hrs after inoculation and that it occurs to a greater degree in the incompatible response. Our results gain support from this report. It was noted in the present study that cells treated with elicitor from both compatible and incompatible pathogens, auto-fluoresce blue almost immediately, within 24 hours of elicitation, the number of cells auto-fluorescing being more in the cultures treated with elicitor from *P. meadii*, than in the cultures treated with the *P. capsici* elicitor. It was also observed that elicitor-treated cultures exhibited higher lignification than the controls, as early as 6 hours after elicitation, which may have been the reason for the presence of blue auto-fluorescing cells in the elicitor-treated cultures. The intensity of lignin accumulation was significantly higher in the cultures treated with elicitor from the incompatible pathogen, *P. meadii* than in cultures treated with elicitor from the compatible pathogen, *P. capsici,* which is in accordance with the higher number of cells showing auto-fluorescence in PmE-treated cultures than in PcE-treated cultures. Thus, the blue auto-fluorescence observed in elicitor-treated black pepper cells could be argued to be due to the lignin formation, similar to the observations made in soybean cells by Mohr and Cahill (2001).

It has been observed in the present study that responses like cytoplasmic aggregation and cell death occurred in single individual cells as well, similar to the defenses exhibited by cells in the micro-callus. Similar observations were made by

Naton *et al* (1996) in parsley cells. It was noted that the defense reactions, except the expression of defense-related genes were largely confined to individual infected cells within a micro-callus and occurred in the same manner in single cells, suggesting that the hypersensitive reaction including cell death was largely a cell-autonomous defense process that does not depend on the presence of adjacent cells. The cytoplasmic aggregation occurring in individual cells in elicited black pepper cultures and in cells of a micro-callus, that are close to the approaching hyphae of *P. capsici* in cultures inoculated with the fungus in the present study indicate that cytoplasmic aggregation in black pepper cells occurs in a largely cell-autonomous manner. Movement of nucleus towards or away from site of hyphal penetration has been reported by Freytag (1994) and Skalamera and Heath (1998). McLusky *et al* (1999) suggested that pathogen-derived signals are necessary for cytoplasmic and microtubule aggregation at the site of penetration and are possibly stimuli of nuclear movement. According to Cahill *et al* (2002), such signals may also be responsible for the auto-fluorescence of cell walls in both compatible and incompatible interactions and may be indicative of major changes or additions to the structure of wall components. Thus, the cytoplasmic aggregation and blue auto-flourescence observed in the black pepper cells in the present study on elicitation are consistent with the observations made by Freytag (1994) and Cahill *et al* (2002).

Defense reactions that precede rapid cell death, such as the synthesis of cell-wall material, translocation of cytoplasm and nucleus, and expression of defense-related genes (Freytag *et al.*, 1994) are all energy-demanding processes. Therefore the infected cells need to be supplied with sufficient energy to fulfill all the requirements to complete the hypersensitive reaction, including rapid cell death. Thus, HR-linked death in plants requires active plant metabolism and depends on the activity of host transcriptional machinery (He *et al.*, 1994) in contrast to cell death by necrosis, which does not require the active participation of the cell. It has been observed that the pathogen-infected tissue generally exhibits higher rate of respiration (Uritani and Asahi, 1980; Hutcheson and Buchanan, 1983). Naton *et al* (1996) demonstrated that the activation of energy metabolism and the accumulation of intracellular peroxides are correlated with induced rapid cell death, and that this rapid cell death terminates growth of the fungal pathogen.

Thus it can be said from the results obtained in the present study that the various defense responses triggered in the black pepper cultures, namely, cytoplasmic aggregation, deposition of secondary cell wall material and oxidative burst could be the result of increased metabolic activity after elicitation. These defenses ultimately could have led to the hypersensitive cell death, as observed by Naton *et al* (1996).

2.4.4.7. Cell wall barrier formation

Formation of mechanical barriers has also been known to be an early response to pathogen infection and elicitation. Black pepper cells exhibited increased lignification as early as 6 hours after elicitor treatment. Increased lignin synthesis has been reported by many researchers in plant-pathogen interactions. Nicole *et al* (1991) noticed tissue lignification of rubber tree on treatment with elicitors of *Rigidoporus lignosus*, a root rot fungus. This was correlated with increased cinnamyl-alcohol dehydrogenase (CAD) enzyme activity, a key enzyme of the phenylpropanoid pathway, implicated in lignin biosynthesis. Similar results were obtained by Hotter (1997) who reported increased lignification, concomitant with the increase in cinnamyl alcohol dehydrogenase in *Pinus radiata* suspension cultures treated with a cell wall elicitor of *Dothistroma pini*. Lignin, a polymeric cell wall phenolic, is synthesized by the phenylproapanoid pathway. The phenylpropanoids are derived from cinnamic acid, which is synthesized from phenylalanine by the action of phenylalanine ammonia-lyase (PAL), the rate-limiting entry point into phenylpropanoid metabolism. While PAL is the entry point into phenylpropanoid metabolism, CAD is specifically involved in the biosynthesis of lignin and related cell wall-bound polyphenolics (Hotter, 1997). Covalent cross-linking of lignin with carbohydrate and protein during lignin polymerization strengthens cell wall to a great extent that it interferes with mechanical penetration of plant tissue by fungal pathogens and enzymatic hydrolysis, thus making it resistant to cleavage of its compounds by pathogens. It also reduces the nutrient flow from plant to the pathogen and also the transport of myco-toxins from fungus to the plant (Nicholson and Hammerschmidt, 1992; Jaeck *et al.*, 1992). Moreover the accumulation of phenolic precursors and the subsequent free radicals formed during polymerization of lignin might affect the fungal growth. It is also believed that the hyphae in close proximity to lignifying host cells become lignified themselves and hence lose their plasticity (ability to extend) and cannot continue growth (Smart, 1991).

Peroxidases are also known to play a very important role in lignin synthesis. Peroxidases catalyze the oxidation of substrates like phenol and its derivates, by hydrogen peroxide. They are responsible for the radical dehydrogenation of sinapyl alcohol and coniferyl alcohol during the lignin synthesis (Lebeda *et al.*, 1999). Velazhahan and Vidyasekaran (1999) reported oxidation of coniferyl alcohol by peroxidases in *Rhizoctonia solani* elicitor-treated rice suspension cultures. The oxidation of coniferyl alcohol by peroxidase leads to synthesis of lignin. A higher peroxidase activity in elicitor-treated black pepper cells observed in the present study, especially in the incompatible interaction with *P. meadii* elicitor, may be the reason for the significantly higher lignin content in elicitor treated cultures compared to the control cultures. Our results are also in accordance with those obtained by Egea *et al* (2001) in sweet pepper. The authors reported accumulation of PR-proteins with peroxidase activity and the accumulation of lignin-like polymer associated with a hypersensitive reaction in cell suspension cultures of three varieties of *Capsicum annuum* in response to treatment with lyophilized mycelium and culture filtrate of *P.capsici*. The resistant pepper variety, Smith-5, showed a more intense lignification in response to the elicitor preparations than the sensitive varieties, Americano and Yolo Wonder. While elicitation reduced the level of total peroxidase activity in the susceptible varieties, such activity increased in resistant varieties, and was accompanied by de novo expression of acidic peroxidase isoenzymes in the extra-cellular and cell wall fractions. The increases in the activity of these peroxidases in the resistant variety were in concordance with the accumulation of lignin observed 24 h after inoculation by both elicitors from the fungus, suggesting a possible role of these isoenzymes in lignin biosynthesis, used to reinforce the cell walls against fungal penetration of the cells. The increased lignification observed in black pepper cells could also be due to the increased PAL activity observed after elicitation.

Oelofse and Dubery (1996) reported increase in lignin synthesis in *Phytophthora nicotianae* cell wall elicitor-treated tobacco cells as early as 4hr after elicitation. In the present study, lignification in black pepper cells was evident from 6hr, the very first interval of observation after elicitation. This signifies that the cell wall barrier formation also is a very early response to elicitor treatment. It is also to be noted here, that the thioglycolic acid-extractable complexes of the cell walls, generally regarded as a quantitative measure of lignin, consistently showed an absorbance peak at 225nm in the present study, the reason for which we are unable to answer. However, it may suggest that the lignin of black pepper has an absorbance maximum at 225nm rather than 280nm as reported by Campbell and Ellis (1992). It could also be due to the change in the lignin histochemistry after elicitation. Alteration in the histochemistry and structure of lignin was reported by Elfstrand *et al* (2002) intransgenic tobacco transformed with a defense-related cationic peroxidase gene from *Picea abies*.

Deposition of callose in cell walls is another defense response induced in plants against attempted invasion by a pathogen (Smart 1991). Black pepper suspension cultures responded to treatment with elicitor by the formation of callose plugs on the cell walls. This could suggest that callose formation may be another physical barrier induced in black pepper cells in response to invasion by *Phytophthora capsici* under *in vivo* conditions. Deposition of callose has been reported in several plant-pathogen ineractions. Naton *et al* (1996) observed that cultured parsley cells co-cultivated with germlings of *P. infestans* rapidly formed a local callose-containing barrier underneath the fungal appressorium. This local barrier prevented invasion by the fungus and halted the pathogen ingress. Gross *et al* (1993) also reported callose formation in parsley cultured cells. Epidermal cells of potato leaves also showed callose formation in response to *P. infestans*. Stromberg and Brishammar (1993) observed papilla formation, which was an assembly of β-glucans, in front of the appressorium formed by *P. infestans*, prior to perforation of the cell wall in the epidermal cells of potato leaves. These papillae could be stained with aniline blue confirming that these were callose formations and inhibited fungal invasion significantly. Vleeshouwers *et al* (2000) observed deposition of callose and extra-cellular globules containing phenolic compounds associated with the defense reaction during incompatible reactions of *Solanum* species with *P.infestans*. Mithofer *et al* (2002) also reported callose induction in the cell walls of soybean roots as a defense reaction, on infection with *Phytophthora sojae.* Fellbrich *et al* (2002) observed cell wall reinforcement due to the apposition of callose at the interface between necrotic and healthy leaf tissue of *Arabidoposis* 24h after application of a 24kD necrosis-inducing *Phytophthora* protein 1 (NPP1) purified from *P. parasitica*

Though control cultures of black pepper also stained with aniline blue, showing presence of residual callose, the fluorescence exhibited was to a lesser extent compared to that in elicited cells. Moreover, typical callose plug formation seen in elicited cells was absent in control cultures. Callose formation was observed to be one of the early defense responses occurring in black pepper - *P. capsici* interactions and helps in cell wall strengthening.

2.4.4.8. Enzymatic defenses

The health of plants is preserved not only by virtue of mechanical barriers or escape from infection but also through an active metabolic initiative, which activates plant defense responses (Dixon *et al.,* 1994). Metabolic changes in plants resulting from pathogen invasion have been reported in numerous host-pathogen systems. Many enzymatic defenses are induced during pathogen attack in plants and on elicitation with elicitors of pathogens. In the present study, we studied the modulation of activities of different antioxidant enzymes, hydrolases and phenylalanine ammonia lyase in suspension cultures of black pepper on elicitor treatment.

2.4.4.8.1. Anti-oxidant enzymes

One of the important events occurring in the early phase of plant-pathogen interactions is the rapid production of active oxygen species, which include $O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$. A plasma membrane-associated NAD(P)H oxidase is activated during the response of plants to pathogens (Jabs *et al* , 1996) resulting in the oxidative burst. The active oxygen species (AOS) thus produced play an important role in activating the defenses of the plant. In addition to their role in programmed cell death, they are also involved in the signal transduction leading to pathogenesis-related proteins (Chen *et al.,* 1993). However, the higher concentrations of oxygen free radicals produced during elicitation or attempted infection are toxic to the plant cells and hence plants possess a complex anti-oxidant system that detoxifies $O_2^{\cdot -}$ and H_2O_2 and may inhibit continued programmed cell death (PCD). The primary components of this system include anti-oxidant compounds namely, carotenoids, ascorbate, glutathione and tocopherols and the anti-oxidant enzymes, such as superoxide dismutase, catalase, peroxidase and the enzymes involved in the ascorbate-glutathione cycle (ASC-GSH cycle, Asada, 1992), namely, ascorbate peroxidase (AP), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR).

Information about the effect of *Phytophthora* infection on the activated oxygen-related enzymes in black pepper is meagre. In order to determine the relationship between the activity of the different anti-oxidant enzymes and the kind of response to *Phytopthora* infection in black pepper, the present study examined the changes in SOD isozymes, catalase, peroxidase AGC-cycle enzymes during elicitor treatment of black pepper suspension cultures. It was also an attempt to evaluate the usefulness of these antioxidant enzymes as biochemical markers of resistance of black pepper plants to *Phytophthora*.

2.4.4.8.1.1. Superoxide dismutase

Superoxide dismutases (SODs) are a group of metallo-enzymes that catalyze the disproportionation of superoxide free radicals ($O_2^{\cdot -}$) generated in different cellular compartments, to molecular oxygen and H_2O_2 (Rabinowitch and Fridovich, 1983). There are essentially 3 types of SOD, depending on the prosthetic metals they possess. Thus, there are MnSOD, FeSOD and Cu/Zn SOD, which have manganese, iron or copper/zinc respectively as their prosthetic metals. In higher plants, Cu/Zn and Mn-containing superoxide dismutases are widely distributed (Rabinowitch and Fridovich, 1983). Iron-containing SODs were earlier thought to be restricted to prokaryotic organisms and some eukaryotic algae, but, in recent years, Fe-SODs have been reported in several plant species (Droillard and Paulin, 1990, Daza x*et al.*, 1993; Becana *et al.*, 1989). Mn-containing SOD is found in mitochondria and peroxisomes, Cu/Zn-SOD is localized in chloroplasts, mitochondria, cytosol and glyoxysomes, and Fe-SOD is mainly present in chloroplasts, but recently was also found in mitochondria and peroxisomes (Droillard and Paulin, 1990).

Superoxide dismutases play an important role in protecting cells against the deleterious effect of superoxide free radicals (Rabinowitch and Fridovich, 1983). The activity increases when the cellular production of superoxide radicals is increased during stress situations. The increase in SOD activity following pathogen attack may be required to catalyze the formation of H_2O_2 and prevent accumulation of $O_2^{\cdot -}$ radicals. SOD may also serve to reduce the risk of the formation of the highly toxic $^{\cdot}OH$ radicals by a Fenton-type reaction, which would be accompanied by enhanced superoxide production (Halliwell and Gutteridge, 1989). The protective role of SOD during plant infection by pathogenic organisms such as fungi has been reported (Buonaurio *et al.*, 1987). In barley plants, whole-leaf SOD activity did not change after inoculation with *Blumeria graminis*, but a significant increase in apoplastic SOD was found both in resistant and susceptible lines compared with the non-inoculated controls (Vanacker *et al.*, 1998).

In the present study, higher activity of MnSOD was observed in PcE-treated cultures. Electrophoretic analysis of the different forms of SOD revealed an additional fast moving band of MnSOD unique to the PcE-treated cultures, which could thus have contributed to the higher activity of the enzyme in these cultures. In PmE-treated cultures however, Fe-SOD activity was observed to be higher, though electrophoretically, there was no difference in the pattern of isoforms between the different treatments. CuSOD was the most predominant form of SOD in control cultures.

The presence of Fe-SOD in *P. nigrum* adds to the list of the small number of higher plants in which the presence of this metalloenzyme has been demonstrated. FeSOD has been found in certain species of the plant families, Ginkgoaceae, Nympahaceae and Cruciferae (Bridges and Salin, 1981), in *Nicotiana tabacum* and *Nicotiana glutinosa* (Montalbini and Buonaurio, 1987), in tomato and bean leaves (Kwiatowski *et al.*, 1985) and in different cultivars of citrus plants and coffee, belonging to the family Rubiaceae (Almansa *et al.*, 1989; Daza *et al.*, 1993). Further, the very high activity of FeSOD in black pepper suspension cultures treated with *P. meadii* elicitor, is noteworthy, since Fe-SOD normally represents only between 10-20% of the total SOD activity in most of the higher plants (Daza *et al.*, 1993). FeSOD has been thought to be present mostly in prokaryotes. It has been hypothesized that the presence of prokaryotic FeSOD in higher plants may be due to the possibility of gene transfer from bacteria or algae to plants (Bridges and Salin, 1981). However, another evolutionary hypothesis points to the possibility that the occurrence of Fe-SODs in higher plants could be due to the expression of silent genes coding for Fe-SODs as a result of environmental pressures (Bridges and Salin, 1981). Moreover, results of Almansa *et al* (1991) and del Río *et al* (1991) suggest that the expression of Fe-SODs in plants could be regulated by control factors related to the plant's ability to develop adaptive protection responses against different types of stress conditions.

It was observed in the present study that there was a differential response in the SOD isozyme pattern in control, PcE- and PmE-treated cultures with respect to MnSOD isoform. The presence of an additional form of MnSOD unique to PcE-treated cultures, indicates that black pepper cells react differently, at least in terms of SOD activity, to *P. capsici* and *P. meadii.* Isozyme profiles might be used as a reference when the normal development of plants and pathological stages are compared, yielding valuable information about pathogenesis (Visedo *et al.*, 1990). In fact, it has been suggested that changes in the levels of particular isoforms of antioxidant enzymes, rather than changes in their total activity, may be more important in the stress response (Gómez *et al.*, 1999; Hernández *et al.*, 2000) as they reflect differential gene behaviour in response to the stress. The importance of the induction of an additional isoform of MnSOD in *P. capsici* HWC-elicited cultures can be verified by further studies to check if this can be used as a marker of resistance / susceptibility to *P. capsici.*

The higher activity of MnSOD, obtained by the enzyme assay and the consistent presence of an additional isozyme band of MnSOD in black pepper suspension cultures treated with *P. capsici* HWC, and further, the absence of the same in the control and

PmE-treated cultures, suggests some role for MnSOD in the response of black pepper cells to *P. capsici* HWC. However, in PmE-treated cultures, FeSOD activity was found to be predominant over the other forms of SOD, which was not observed in control cultures. The reason for the induction of different forms of SOD in response to the two different elicitors from *P. capsici* and *P. meadii* is not known. However, it can be hypothesized that since elicitors trigger the defense responses in plant cells, the induction of the different forms of SOD, which are not predominant in the control cultures, may have a defensive role in black pepper against the respective pathogens from which the elicitors were isolated. Thus, in response to *P. meadii* (incompatible spp.) elicitor, FeSOD seems to be the major form of superoxide dismutase, while MnSOD seems to be the dominant form of SOD in response to *P. capsici* (compatible spp.) elicitor. The results in this study, however, are in contrast to the results obtained in studies on coffee by Daza *et al* (1993). It was found by these workers that FeSOD was the most abundant enzyme in the leaves of coffee cultivar susceptible to the pathogen *Hemelia vastatrix* (Daza *et al.*, 1993) and Cu/Zn SOD, which showed additional isozyme bands in the resistant cultivar and showed greater activity compared to the susceptible cultivar, was implicated as being related to resistance against the fungal infection in coffee. An increase in SOD activity was observed in inoculated plants of apricot, susceptible and resistant to plum pox virus (Hernandez *et al.*, 2001). The SOD found in apricot was only CuSOD and it was observed that while four forms of CuSOD increased in the resistant cultivar, the susceptible cultivar showed the increase of only one form of CuSOD which did not increase in the resistant cultivar

In higher plants, the most abundant form of SOD in general, is the chloroplastic CuSOD (Rabinowitch and Fridovich, 1983). Thus, the abundance of CuSOD in control cultures in the present study is in agreement with this finding. The presence of three bands of CuSOD in the control and elicited suspension cultures of black pepper suggests, that in addition to the chloroplastic CuSOD, there are other isoforms of the enzyme, which may be localized in mitochondria and cytosol, where the generation of superoxide radicals has been demonstrated (Rabinowitch and Fridovich, 1983; del Río *et al.*, 1992). It can be speculated from the results of the present study that CuSOD is the general form of SOD in non-stressed black pepper cultures. However, elicitation with HWC from different species of *Phytophthora* results in the induction of other forms of SOD, namely, MnSOD and FeSOD. However, the exact roles of FeSOD and MnSOD in defense response of black pepper against *Phytophthora* needs to be further confirmed.

2.4.4.8.1.2. H_2O_2-scavenging enzymes

The action of superoxide dismutases on superoxide radicals results in yet another reactive oxygen species, the hydrogen peroxide. It has been known that hydrogen peroxide acts as a secondary messenger in the signal transduction. H_2O_2 has many of the properties expected of a defense-related signal transducer, since (a) it is formed rapidly upon elicitor treatment, (b) it can be rapidly destroyed, (c) it can independently stimulate some of the defense responses, like phytoalexin synthesis (d) its removal by catalase or other inhibitors blocks the defense response (Apostol, 1988). H_2O_2 in combination with cell wall peroxidase plays an important role in lignification (van Huystee, 1987). Though we did not study the generation of hydrogen peroxide in black pepper suspension cultures on elicitation, we presume based on the production of superoxide radical production and high activities of superoxide dismutases that the levels of hydrogen peroxide would have increased in elicitor-treated cells to trigger the defense responses. The different H_2O_2-scavenging enzymes analyzed in the present study are:

2.4.4.8.1.2.1. Catalase

Catalase is involved in plant defense reactions. This enzyme occurs in the peroxisomes and decomposes the hydrogen peroxide to water and oxygen. There are only few reports related to the role of catalase in plant defense processes. This enzyme competes with peroxidase for the same substrate. Buonario & Montalbini (1993) noted considerable decrease in catalase activity in tobacco plants in response to *Erysiphe cichoracearum* and this decrease correlated with very high peroxidase activity.

Similar results were obtained in our study, in which higher peroxidase activities in PmE-treated cultures were associated with low levels of catalase. Strong decrease of catalase during the early stages of pathogenesis of necrotrophs and bacteria is well correlated with increased H_2O_2 production (Fauth *et al.*,1996; Kuzniak *et al.*, 1999). Catalase activity decreased in relation to the controls in inoculated plants of both plum pox virus- susceptible and -resistant cultivars of apricot, though the breakdown of catalase was higher in the resistant cultivar than the susceptible plants ((Hernandez *et al.*, 2001). Thus, it can be presumed that the sustained low levels of catalase activity in incompatible black pepper-PmE interaction would have favoured accumulation of hydrogen peroxide, which through its well-established role in the signal transduction was responsible in triggering the various defense responses in Pm-E-treated cultures. The high levels of catalase in PcE-treated cultures on the other hand would have resulted in significant scavenging of this secondary messenger of signal transduction. The greater magnitude and speed of expression of defenses in PmE-treated cultures compared to PcE-treated cultures is a proof of this hypothesis.

Catalase is known to be an efficient scavenger of hydrogen peroxide. Apostol *et al* (1988) observed that addition of catalase before elicitation in soybean cultures resulted in a total inhibition of the oxidative burst that predominantly consisted of H_2O_2, which in turn resulted in significant reduction of glyceollin synthesis, a major defense response in soybean. However, when catalase was added 1hr after elicitation, no significant inhibition of the phytoalexin biosynthesis was observed, suggesting that catalase interferes with one of the initial events of elicitation, a step or process that is completed within 1hr of the initial extra-cellular stimulus, but well before any phytoalexins are formed. This also suggests the inhibition of signal transduction responsible for triggering the defense responses, including glyceollin synthesis, in which H_2O_2 plays a major role. Hence it can be hypothesized that higher levels of H_2O_2 were maintained in PmE-treated cultures compared to PcE-treated cultures, thus resulting in a more efficient transduction of the signal of the stimulus of elicitation in PmE-treated cultures.

Since catalase is a very efficient scavenger of H_2O_2, it is capable of totally blocking the oxidative burst. Hençe, from the results obtained in the present study, it can be supposed that lower levels of catalase in PmE-treated cultures may play a role in maintaining sufficient levels of H_2O_2 in the initial stages after elicitation of the black pepper cultures. This may help in transducing the signal of the stimulus of elicitation, thus triggering the defense responses in PmE-treated cell cultures at a faster rate. On the other hand, higher levels of catalase in PcE-treated cultures may be responsible for the slower rate of induction of these defense responses. However, the lower levels of catalase between 6 and 24hrs after elicitation would have facilitated the rise in H_2O_2 to levels that are necessary to trigger the defense responses in PcE-treated cultures, though with a delay than that observed in PmE-treated cultures. The delay in signal transduction, which may have been due to lower levels of H_2O_2 may be the possible reason for the delayed induction of defense responses seen in PcE-treated cultures.

2.4.4.8.1.2.2. Peroxidase

Peroxidases are frequently associated with plant defense against pathogens. Peroxidase is known to be involved in scavenging the toxic hydrogen peroxide produced in the tissues during active plant metabolism and pathogen attack. The increase in their activity generally coincides with increased H_2O_2 levels (Kuzniak *et al.*, 1999). The increased peroxidase activity observed in PmE-treated cultures in the present study could thus signify increased levels of H_2O_2, possibly by the action of superoxide dismutase on the superoxide radicals that were observed to be produced during the initial phases after elicitation.

Some authors suggest that the role of peroxidase in disease resistance may result from oxidation of phenols to fungi-toxic compounds (Cadena-Gomez and Nicholson, 1987; Urbanek *et al.*, 1987). Peroxidases remove the toxic hydrogen peroxide by utilizing it in oxidation of substrates like phenol and its derivatives, thus participating in synthesis of phenolic compounds of defense. The production of phenolic compounds include synthesis of flavones, stilbenes and other phenolic secondary metabolites such as chinons, tannins, melanins and also the polymerization of lignin and suberin composing monomers (Lebeda *et al.*, 1999). Tannins and melanins are dihydroxyphenol and chinon oligomers, which are toxic for pathogens. Thus the increased intra-and extracellular phenolics observed in elicited black pepper cultures may be due to these defensive actions of peroxidases. The higher production of phenolics in PmE-treated cultures coincides with the higher activity of this enzyme after elicitation. The production of phenolics in PcE-treated cultures was however lower than that found in PmE-treated cultures, consistent with the slow and low induction of the peroxidases in these cultures. This signifies that the wide range of defenses involving peroxidase activity mentioned above, in response to elicitor treatment was more efficient in the incompatible black pepper-PmE interactions, and that these defenses in the compatible interaction were induced to a lesser magnitude than in the incompatible interactions.

Peroxidases also participate in the building of intermolecular bonds during the organization of the cell wall at the sites of infection by pathogens (Repka &Slovakova, 1994). Generally, peroxidases enhance their activity after a pathogen attack, because they paricipate in defensive lignification and synthesis of phenolic compounds effective against pathogens (Nicholson & Hammerschmidt, 1992). Lignin and suberin are amongst these phenolic compounds produced by peroxidases that are involved in structural defenses. Lignin formation, a common response to pathogen attack (Kohle *et al*, 1984) is believed to require both H_2O_2 and peroxidases (van Huystee, 1987). Peroxidase is known to be involved in the oxidative polymerization (dehydrogenation) of hydroxycinnamyl alcohols namely the sinapyl and coniferyl alcohols, by using H_2O_2, to yield lignin (Vance *et al.*, 1980) and in the cross-linking of isodityrosine bridges in cell walls (Fry, 1982). These modifications of the cell wall act as barriers against pathogen invasion and hence constitute part of host resistance mechanism. The increased lignification in elicitor-treated cultures of black pepper in the present study supports these findings. The magnitude of lignification was higher in PmE-treated cultures, than the PcE-treated cultures, which correlated with higher activities of intra- and extra-cellular peroxidases in the former compared to that observed in the latter. The induction of peroxidases was more pronounced and at a greater speed in PmE-treated cultures whereas in PcE-treated cultures, the induction was slow and lesser in magnitude. These observations again are pointers to the fact that defensive responses are more active in incompatible interactions than in the compatible ones. Peroxidases also catalyze the production of hydroxyproline, and this is essential for biosynthesis of hydroxyproline-rich proteins, which may help stabilize cell walls against pathogen invasion (van Huystee, 1987). Thus, it was observed in the present study that the increase in peroxidases in the incompatible black pepper-PmE interactions, played a role in building up strong barriers in response to elicitation compared to the PcE-treated cultures, where lignification and possibly other cell wall barriers were comparatively less, which in turn could be due to a rather late increase in the activity of peroxidase.

Peroxidase also is known to produce free radicals (Mader *et al.*, 1980) and hydrogen peroxide (Peng and Kuć, 1992), which are toxic to several pathogens. Peroxidase also participates in the synthesis of ethylene, the concentration of which increases frequently in pathogenesis process (Tudzynski, 1997). The higher levels of peroxidases in PmE-treated cultures thus may be one of the reasons for the distortion of the mycelium of *P. capsici* found during co-cultivation of *P. capsici* with PmE-treated cultures. The inhibitory effect of the compounds produced by peroxidases may have been one of the factors responsible for the inhibition of mycelial growth on elicitor-treated cut leaves of black pepper. The higher magnitude of expression of peroxidase activity in PmE-treated leaves could have resulted in the complete inhibition of growth of *P. capsici* mycelium.

Peroxidases are known to increase in plants, eg. corn (Lagrimini and Rothstein, 1987), barley (Kerby and Somerville, 1989), Sorghum (Bhavanishonkara *et al.,* 1989), on infection with pathogen or elicitor (Hammerschmidt *et al.,* 1982; Kuc, 1987). Many resistant plants of wheat (Arora and Wagle, 1985), tomato (Brenneman and Black, 1979), lupin (Malolepsza *et al.*, 1989) have shown higher peroxidase levels. Malolepsza and Urbanek (1994) reported increased peroxidase activity in bean cell suspension cultures as a reaction to treatment with conidia and mycelium extract of *Bortrytis cinerea.* There are also reports suggesting that increases in peroxidase activity are not specifically associated with resistance (Nadolny and Sequeira, 1980; Czech-Kozlowska and Krzywanski, 1984, Elfstrand *et al.*, 2002).

Elicitor-treated cultures recorded an increase in extra-cellular peroxidase activity compared to the controls, with the PmE-treated cultures showing a greater activity than the PcE-treated cultures. Maolepsza and Urbanek (1994) attributed the increase in extra-cellular peroxidase activity in bean suspension cultures inoculated with conidia of *Botrytis cinerea* for the release of intracellular peroxidase into the medium due to cell damage caused by the growth of the pathogen. Elicitor-treated cultures in black pepper also exhibited cell death, which may have resulted in the release of intra-cellular peroxidases.

Cell wall-bound peroxidase participates in processes which occur in the extra-cellular matrix (Buonario & Montalbini, 1993). Extra-cellular peroxidases have been reported to utilize hydrogen peroxide in the oxidation of certain compounds by Apostol *et al* (1988). Extra-cellular peroxidases may also debilitate the pathogen by production of fungi-toxic compounds from the oxidation of phenols, which peroxidases are known to catalyze (Urbanek *et al.*, 1987). The significant increase in medium darkening is a proof to this hypothesis and could be another possible reason for the observed distortion of the hyphae of *P. capsici* growing in PmE-treated cultures. On the other hand, the extent of oxidation of phenols by peroxidases into quinines may not be high enough in PcE-treated cultures so as to affect the pathogen inoculated in these cultures.

2.4.4.8.1.2.3. Enzymes of the AGC-cycle

AGC-cycle consists of a set of enzymes, which act in tandem to scavenge hydrogen peroxide to maintain it within permitted levels to prevent cyto-toxicity. There are only few reports on the role of these enzymes in relation to biotic stress. Information on the role of these enzymes in the black pepper-*Phytophthora* interactions is nil. Hence, the present study was an attempt to observe the responses of these enzymes to elicitor treatment and to analyze the significance of the activities of these in the compatible and incompatible interactions, as reflected in the black pepper suspension cultures-*P. capsici* elicitor and black pepper cultures-*P. meadii* elicitor combinations, respectively.

Catalase is localized mainly in the peroxisomes (del Río, 1998). When the catalase activity drops, the peroxisomal H_2O_2 levels rise. H_2O_2 can diffuse through the peroxisomal membrane into the cytosol in a manner in which it diffuses through the mitochondrial and peroxisomal membranes by leakage, under stressful conditions (Jiménez *et al.*, 1998; del Río, 1998). The increase in H_2O_2 levels helps in the signal transduction of the plant-hypersensitve disease resistance response (Finket, 1998). Ascorbate peroxidase (AP) of the Ascorbate glutatione cycle (AGC-cycle) has a higher affinity for H_2O_2 than does CAT (Asada, 1992) and it has been shown that AP is present mainly in the cytosol (Gillham and Dodge, 1986).

Reactive oxygen species play an important role in the induction of defenses against pathogens. Suppression of active oxygen species (AOS)-detoxifying enzymes favour increased resistance in plant-pathogen interactions. Mittler *et al* (1999) reported that transgenic anti-sense tobacco plants with reduced capability to detoxify ROS (i.e., anti-sense APX or CAT) were found to be hyper-responsive to attack by *Pseudomonas syringae*. They activated programmed cell death (PCD) in response to low amounts of pathogens in comparison to control plants, which did not show activation of PCD in response to the pathogen. It was suggested that this suppression, occurring upon pathogen recognition and coinciding with an enhanced rate of ROS production, plays a key role in elevating cellular ROS levels, thereby potentiating the induction of PCD and other defenses. These findings supported the hypothesis that suppression of ROS-scavenging enzymes during the hypersensitive response plays an important role in enhancing pathogen-induced PCD.

The results obtained in the present study are also in accordance with the above suggestion. Ascorbate peroxidase activity was maintained at low levels in PmE-treated cutltures during the initial phase after elicitation. This, along with the significant reduction in catalase activity observed in these cultures could have resulted in the maintenance of high levels of H_2O_2, which in turn would have been the reason for the rapid induction of defense responses in PmE-treated cultures. However, a rise in H_2O_2 accumulation due to the dip in catalase and AP activities and also due to the oxidative burst on elicitation, would have resulted in an increasing risk of oxidative injury. The increased cell death observed in PmE-treated cultures is possibly a result of such an oxidative injury. The marginal increase of AP in PmE-treated cultures over the control cultures after 24 HAE could however be an attempt by the cells to mitigate to a limited extent, the damage caused by such enormous increases in the H_2O_2 levels. It was observed in the present study however, that the peroxidase levels were maintained high in PmE-treated cultures in comparison with the controls from the initial stages after elicitation. This probably could be a means to exploit the steep rise in H_2O_2 in these cultures to initiate various defense responses including oxidative processes that utilize H_2O_2 by the action of peroxidases. The decomposition of H_2O_2 during these processes would have thus checked the wasteful damage caused by these reactive oxygen species by utilizing them in triggering various defense responses. On the other hand, the increased activity of ascorbate peroxidase and catalase observed in PcE-treated cultures could have resulted in significant reduction in H_2O_2 levels in these cultures. The peroxidase activity in PcE-treated cultures was similar to the levels in control cultures till 24 HAE, after which, the levels rose, probably to participate in the defense responses. The low levels of H_2O_2 and the slow induction of peroxidase may have been responsible for the slow induction of the defense responses in PcE-treated cultures, which probably in turn could be due to the slow transmittance of the signal of elicitor treatment, in which H_2O_2 has an important role to play. Extrapolating these results observed in PcE-treated cultures to *in vivo* conditions, it can be suggested that such a situation *in planta* can facilitate proliferation and spread of the pathogen in the host in a compatible black pepper- *P. capsici* interaction.

H_2O_2 is known to induce PR defense genes. Chamnongpol *et al* (1996) reported that H_2O_2-induced oxidation was found to cause expression of PR genes in transformed tobacco plants deficient in the major CAT isoform, Cat I. The increase in H_2O_2 levels in PmE-treated cultures in the present study, with a concomitant decrease in catalase could have resulted in a similar induction of various defense genes including PR-genes. On the other hand, the low H_2O_2 levels in PcE-treated cultures during the early stages of elicitation could have resulted in the slow induction of defense responses in these cultures. Thus, the timing of the increase in levels of H_2O_2 is important in triggering the defense responses. The increase in H_2O_2 at the early stages could facilitate an early signal transduction.

Hernandez *et al* (2001) reported decrease in catalase activity in relation to the controls in inoculated plants of both plum pox virus- susceptible and -resistant cultivars of apricot, but the breakdown of catalase was higher in the resistant cultivar than in the susceptible plants. AP activity increased by 50% in the susceptible cultivar while the resistant cultivar did not show any significant changes in the AP activity compared to un-inoculated controls. Thus, an inverse correlation between AP induction and resistance to PPV seems to occur in apricot. On the other hand, in barley, an inverse correlation between CAT induction and resistance to *Blumeria graminis* was observed by Vanacker *et al* (1998). In the resistant barley plants, the fungus inoculation caused a significant decrease in AP activity, whilst CAT did not show significant changes. In the susceptible cultivar, on the other hand, inoculation with fungus induced a massive increase in CAT activity and caused no changes in AP activity. The results of the present study agree with the results of both these reports. While the PmE-treated cultures (in which the defenses were triggered rapidly) showed low levels of AP and CAT, the PcE-treated cultures (in which the defenses were induced less rapidly) showed an increase in activity of both these enzymes.

Mittler *et al* (1998) reported that viral-induced PCD in tobacco is accompanied by the suppression of cytosolic AP expression. This suppression leads to the reduction in the ability of the cells to scavenge H_2O_2, which in turn enables accumulation of this molecule. This finding supported the hypothesis that high H_2O_2 levels, together with suppression of anti-peroxidative activity of the cell causes the activation of PCD and the defense mechanisms (Mittler *et al.*, 1998). It has been suggested that the inhibition of AP and CAT activities by salicylic acid during the response of plants to invading pathogens results in the accumulation of H_2O_2 and the acceleration of PCD (Draper, 1997). Similar observations were made by El-Zhahaby *et al* (1995) who studied the reaction of barley cultivar with compatible and incompatible races of *Erysiphe graminis* f. sp. *hordei* and noted activation of several anti-oxidative processes in compatible host-parasite interactions of barley, which may diminish the damaging effects of oxidative stress. A substantial increase of AP and a decline of DHAR activities were also observed in mildew susceptible plants. Less-pronounced changes in the parameters were found in the resistant cultivar. As these anti-oxidative processes were less efficiently activated in the incompatible relationship, it was suggested that they may lead to an early necrotization in the resistant host. Our results are in agreement with these reports. The slow induction of SOD in the initial stages after elicitation in both PmE- and PcE-treated cultures would have promoted the formation of superoxide radicals, thus creating a toxic environment in these cultures. The induction of SOD 24 HAE, in both interactions would have played a role in the production of H_2O_2 that is essential for the signal transduction of the elicitor treatment. The lower levels of catalase in PmE-treated cultures would have resulted in maintenance of H_2O_2 levels that was required for the transmission of the signal of elicitation. The triggering of most of the defenses rapidly and to a greater extent in the PmE-treated cultures is in agreement with this hypothesis. On the other hand, the high activity of catalase in PcE-treated cultures would have resulted in the reduction of H_2O_2 levels, in turn limiting the signal transduction. Similarly, the activity of AP, which has greater affinity for H_2O_2 than catalase was also maintained at low levels in PmE-treated cultures compared to the control during the initial phases after elicitation. Though the activity increased from 24 HAE, the levels were maintained only at marginally higher levels than the control. The higher levels of SOD after 24 HAE also could have resulted in the rise in H_2O_2 levels in the elicited cultures. While the moderate levels of AP and heavy decline in catalase in PmE-treated cultures would have facilitated rapid signal transduction and ultimately cell death, increase in the activities of these enzymes in the PcE-treated cultures would have resulted in a significant reduction in the H_2O_2 levels, resulting in poor transduction of the signal of elicitation and hence the slow induction of defense responses in these cultures.

Velazhahan and Vidyasekaran (1999) however reported increase in ascorbate peroxidase and other antioxidant enzymes like peroxidase, catalase and superoxide dismutase as a defense response in rice suspension cultures treated with elicitor of *Rhizoctonia solani*, the rice sheath blight pathogen. Venisse *et al* (2001) observed that the bacterium *E. amylovora,* the causal agent of fire blight in *Pyrus communis* induced a sustained production of superoxide anion, lipid peroxidation, electrolyte leakage, and concomitant increases of several antioxidative enzymes (ascorbate peroxidases, glutathione reductases, glutathione-S-transferases and peroxidases), in tobacco, a non-pathogen. However, the compatible pathogen, *Pseudomonas syringae* pv. *tabaci*, did not cause such reactions in tobacco.

Dehydroascorbate reductase (DHAR) catalyzes the reduction of dehydroascorbate, the oxidized form of ascorbic acid. In the present study, PmE-treated cultures showed significantly higher levels of DHAR compared to controls and PcE-treated cultures, suggesting that the regeneration of ascorbate from dehydroascorbate is maintained by DHAR, thus serving to maintain the levels of ascorbate for use by ascorbate peroxidase, as suggested by Hernandez *et al* (2001). Foliar DHAR activity increased in inoculated plants of PPV-resistant and susceptible apricot cultivars, but the rise was higher in resistant cultivar (Hernandez *et al.*, 2001). In barley too, plants resistant to *Blumeria graminis* showed higher apoplastic DHAR activity resulting from fungal inoculation (Vanacker *et al.*, 1998).

DHAR catalyzes the regeneration of ascorbate from dehydroascorbate by using the reducing ability of reduced glutathione (GSH). The recovery of GSH from oxidized glutathione (GSSG) thus formed, is made possible by glutathione reductase (GR). Higher DHAR levels in PmE-treated black pepper cultures in the present study indicates that the AP activity was maintained to support the antioxidant system that sustained H_2O_2 at levels that were required to maintain the defense responses in PmE-treated cultures. It was observed in our studies, that the GR activities were quite low (data not shown) in all the treatments. A similar trend was reported by Hernandez *et al* (2001) in apricot, in which GR did not show statistically significant changes in both the inoculated resistant and susceptible plants. Thus, it can be speculated that a higher activity of DHAR may be due to the availability of sufficient GSH levels, which in turn is recovered from its oxidized form, due to the maintenance of GR activity, at least at control levels. There is also a possibility that the reduced glutathione necessary for DHAR activity may be supplied by another pathway, as suggested by Hernandez *et al* (2001) and its efficient transport from its site of synthesis. Thus, the maintenance of higher activity of DHAR in PmE-treated cultures compared with the PcE-treated cultures, seems to indicate that the PmE-treated cultures have a higher capacity to regenerate ascorbate and glutathione from their oxidized forms than the PcE-treated cultures and controls, thus acting against ascorbate oxidation. GSH, H_2O_2 and $O_2^{\cdot -}$ have been identified as messenger molecules in cellular signal transduction and also as factors in plant defense responses (Gómez *et al.*, 1999; Jiménez *et al.*, 1998). A relation between glutathione accumulation and pathogen resistance has been suggested by many researchers (Gullner *et al.*, 1999; Vanacker *et al.*, 1998 a, b). GSH has been identified as a putative long-distance signalling molecule (Foyer *et al.*, 1997) as this molecule is transported throughout the plant efficiently (Noctor and Foyer, 1988). Vanacker (1998) reported an increase in the glutathione content of leaves of barley resistant (Alg-R) to Blumeria graminis on pathogen attack, while the susceptible line (Alg-S) did not show such an increase. Though the levels of glutathione in elicited black pepper cultures were not analyzed in the present study, it can be presumed that these levels would have been maintained based on the fact that DHAR activity remained high in the cytosolic and chloroplastic fractions. The possible maintenance of optimum levels of GSH and H_2O_2 in PmE-treated cultures compared to the PcE-treated cultures due to the higher DHAR activity in the former may also contribute to the higher extent of defenses seen in PmE-treated cultures, than in the PcE-treated cultures or the un-elicited controls. Vanacker *et al* (1998a, b) reported that resistant barley and oat lines showed an increase in apoplastic GSH levels on inoculation with *B. graminis,* while there was no increase in the susceptible lines. Fodor *et al* (1997) suggested an important role for GR, SOD and the GSH levels in the systemic acquired resistance in tobacco. Vanacker *et al* (1999) studied the differential responses of three oat lines differing in their resistance to attack by biotrophic fungal pathogen *Blumeria graminis* and noted that resistance correlated with increased total foliar glutathione, an increase in the ratio of reduced to oxidized glutathione and decreased total activities of foliar ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase and monodehydroascorbate reductase. These results are similar to those observed in PmE-treated cultures and hence this report supports the suggestion in the present study that PmE-treated cultures exhibit a higher resistance response than the PcE-treated cultures.

Hernandez *et al* (2001) explained that the increase in DHAR activity in PPV resistant apricot plants, in which AP did not change, may reflect the requirement for an efficient functioning of ascorbate reduction, which could be used as a substrate, not only for AP, but also for different enzymes including cell wall peroxidases (Takahama and Okini, 1992) and ascorbate oxidase (Esaka *et al.*, 1989). He suggested that cationic peroxidases, using ascorbate as an electron donor, might attack the H_2O_2 or free radicals. In this way, extra-cellular peroxidases could play a detoxifying role as oxidant scavengers in resistant apricot plants. Similar observations were also made by Penel and Castillo (1989) in *Sedum album* under oxidative stress. It has also been suggested that the presence of ascorbate, together with apoplastic peroxidases, may regulate lignification processes in the cell wall (Takahama and Okini, 1992). Thus, it can be suggested from the results obtained in the present study that the increased activity of DHAR in PmE-treated cultures would have ensured high ascorbate content in the cells. However, since the AP activity was not very high in these cultures the utilization of ascorbate in relation to its production by DHAR activity could thus have been low. Since both extra- and intra-cellular peroxidases in PmE-treated cultures were high, it could be suggested that the net ascorbate content would thus have been utilized by these peroxidases as an electron donor in various defensive oxidative processes including lignification in turn, decomposing H_2O_2.

In summary, we observed that elicitation of black pepper suspension cultures with HWC of *P. capsici* and *P. meadii* induced differential responses of activities of the various antioxidant enzymes studied. The maintenance of SOD at minimal levels during the initial stages after elictiation, contributes to the initial accumulation of $O_2^{\cdot -}$ radicals, which may act as a repellant for the pathogen under *in vivo* conditions and may also help in the signal transduction of the stimulus of the elicitor. The increase in the SOD activity after 24 hrs in both PmE- and PcE-treated cultures however ensures the scavenging of $O_2^{\cdot}$ radicals and maintenance of these radicals at optimal levels. In addition SOD contributes to the formation of H_2O_2 during the process of disproportionation of $O_2^{\cdot -}$ radicals. H_2O_2 thus formed acts as a signal molecule and also plays a role in defense responses, by triggering physical barrier formation like lignin by the action of cell wall peroxidases, that utilize reductants like ascorbate, to oxidize them at the expense of H_2O_2, in turn reducing the harmful effects of these toxic reactive oxygen species. The increase in SOD activity may also prevent the accumulation of the toxic $O_2^{\cdot -}$ radicals, therefore reducing the risk of $^{\cdot}OH$ radical formation, that is highly toxic to the cells. Since H_2O_2 plays a significant role in the transduction of signal of elicitation to trigger the various defenses, the decrease in AP activity in PmE-treated cultures during the initial stages would have resulted in the rise in these molecules. The higher levels of DHAR in PmE-treated cultures compared to controls and PcE-treated cultures indicate that cells treated with *P. meadii* elicitor (incompatible interaction) are able to maintain higher levels of ascorbate that was necessary to maintain the AP activity in the latter stages of elicitation. In addition, higher activity of DHAR even during the initial phases of elicitation when the AP activity was not maintained high, also indirectly indicates maintenance of required levels of glutathione since DHAR uses GSH to reduce dehydroascorbate, the oxidized form of ascorbate, formed during the reactions catalyzed by AP activity. Maintenance of optimal levels of GSH for DHAR activity also indirectly indicates GR activity, at least at control levels, which catalyzes the recovery of glutathione from its oxidized form, GSSG. The GR activity in the present study was observed to be too low (data not presented) in all the treatments and hence, it is also possible that the GSH required for DHAR activity is replenished by its synthesis elsewhere in the cell, and its transportation across membranes, to the site of DHAR activity. The marginally high levels of chloroplastic AP and DHAR in PmE-treated cultures could function to maintain the electron transport chain in a reduced state, by scavenging excess H_2O_2 present in this organelle. Since GSH, H_2O_2 and $O_2^{\cdot -}$ radicals also function as signal molecules, it can be assumed that the possible maintenance of higher levels of these compounds, especially that of GSH and H_2O_2 in PmE-treated cultures, is responsible for the speed and magnitude at which defenses are triggered in these cultures compared to the PcE-treated cells.

The differential behaviour of catalase and peroxidase in PmE- and PcE-treated cultures could also be responsible for the differential activity of defenses in these two interactions. The very high levels of catalase in PcE-treated cultures (compatible interaction) may have resulted in significant reduction in H_2O_2 levels. Since H_2O_2 is known to play an important role in signal transduction and also participates in certain defense responses, the reduction of H_2O_2 concentration would have rendered the PcE-treated cells at a disadvantage since the low levels of H_2O_2 would have caused a delay in signal transduction, in turn reducing the speed and magnitude of the expression of defense responses to a considerable extent. On the other hand, the low activity of catalase in PmE-treated cultures (incompatible interaction) ensures maintenance of high levels of H_2O_2 required for the signal transduction as well as for triggering various defense responses. The high peroxidase activity in these cultures utilizes H_2O_2 in useful oxidative processes of defense, by oxidizing electron donors like ascorbate

2.4.4.8.2. Other defense enzymes

2.4.4.8.2.1. β-1, 3-glucanase

Another characteristic plant response to microbial attack is the production of endo-beta-1,3-glucanases, which are thought to play an important role in plant defense, either directly, through the degradation of beta-1,3/1,6-glucans in the pathogen cell wall, or indirectly, by releasing oligosaccharide elicitors that induce additional plant defenses.

In the present study, β-1, 3-glucanase showed higher activity in elicitor-treated black pepper cultures compared to the controls. The increase in activity was evident as early as 6 hours though it was markedly significant over the controls after 24hrs of elicitation. The PmE-treated cultures (incompatible interaction) exhibited rapid induction and more pronounced expression of the enzyme than the PcE- treated cultures (compatible interaction). Our results are similar to those obtained by Kombrink *et al* (1988) and Kim and Hwang (1994). Kombrink *et al* (1988) found that the major PR-proteins accumulating in the intercellular space of potato leaves following inoculation with *Phytophthora infestans* or treatment with *P. infestans*-derived elicitor were the 1,3-β-glucanases and chitinases and that the activities of these enzymes were stimulated strongly by infection or elicitor treatment, compared to controls. The rapid increase in these enzyme activities due to elicitor treatment than in *P. infestans*-inoculated leaves was explained to be due to the rapid uptake and distribution of elicitor throughout the leaf, as opposed to the time lag caused by germination and tissue penetration of the fungus. The similarity between the accumulation patterns of PR proteins in infected and elicitor-treated potato leaves reported by Kombrink *et al* (1988) supported their previous assumption that elicitor treatment closely mimics a true infection (Fritsemeier *et al.*, 1987).

Kim and Hwang (1994) implicated the two hydrolases, 1, 3-β-glucanases and chitinases, in disease resistance and symptom development, respectively, in pepper against *Phytophthora capsici*. *Phytophthora capsici* infection induced the synthesis and accumulation of β-1, 3-glucanases and chitinases in the stem tissues of sweet pepper plants earlier and to a higher extent in incompatible than in compatible interactions. They also observed relation of certain isoforms of glucanase to resistance in pepper. Tonon *et al* (2002) reported marked increase in GLU-39, a β-1, 3-glucanase, in potato cultivar with a high level of field resistance and was induced at 14h after inoculation and remained above basal levels at 38h after inoculation. The enzyme inhibited the germination of sporangia of *P. infestans*. However, in the susceptible cultivar, the enzyme was induced at lower levels and no differences were detected between wounding and infection. The localization, expression pattern and in vitro activity suggested that GLU-39 may have a major role in field resistance.

The massive gain in activity of 1, 3-β-glucanases in PmE-treated cultures observed in the present study may be one of the major defense responses in the resistance reaction of black pepper in the incompatible black pepper- *P. meadii* interaction. Though the PcE-treated cultures also showed increase in the activity, the levels were far less compared to those in p.m. treated cultures, which would have been responsible for the weak defense response elicited in these cells.

β- 1, 3- glucanases in higher plants have been implicated in disease resistance (Van Loon and Van Strein, 1999) owing to their ability to inhibit fungal growth. This glucanhydrolase acts in concert with other hydrolases such as chitinases and proteinases to disrupt the structural integrity of hyphal tips, thereby causing lysis of the fungal cells (Sathiyabama and Balasubramanian, 2000; Mauch *et al.*, 1989). β- 1, 3- glucan, the substrate of β- 1, 3- glucanase, is a major cell wall constituent of oomycetous fungi. β- 1, 3- glucanases are able to partially degrade fungal cell walls by catalyzing the hydrolysis of β- 1, 3- D-glucosidic linkages in β-D-glucans. They are considered to play an important role in plant defenses against a range of fungal pathogens. These are also known to be involved in plant resistance against oomycetous fungi (Graham and Graham, 1991) including different *Phytophthora* species (Kim and Hwang, 1997; Yi and Hwang, 1997). In hyphal tips of many fungi, including oomycetous fungi, 1, 3- β-glucans are exposed at the surface, and could be attacked easily by the β- 1, 3- glucanases (Boller, 1987). The oligosaccharides that are released from the fungal cell wall as a result of this hydrolytic activity could function as elicitors of various plant defense responses (Darvill and Albersheim, 1984). Thus, the increase in the activity of this enzyme in elicitor-treated black pepper suspension cultures observed in the present study could have an implication in the resistance of black pepper against its pathogen.

The restriction of colonization of *P. capsici* on black pepper leaves treated with *P. meadii*- derived elicitor in the present study may also suggest a role for the pronounced activity of 1, 3-β-glucanase in resistance of black pepper to *P. capsici* by its action on the pathogen. Though *P. capsici*-derived elicitor on cut leaves of black pepper also resulted in inhibition of colonization of the fungus compared to the control, the inhibition was far less than that found in the PmE-treated cut leaves. This may be attributed to the weak defenses elicited in PcE-treated leaves, which may possibly be due to the gradual induction and low activity of the enzyme, as seen in PcE-treated cultures.

Extrapolating these results to the *in vivo* situation, the rapid induction of β- 1, 3-glucanase in the incompatible black pepper –*P. meadii* interaction could result in earlier recognition of the elicitor by the host. The rapid accumulation of β- 1,3- glucanase at the site of penetration could play an important role in lysing the 1,3- β-glucans exposed at the surface of the hyphal tips of *Phytophthora capsici* thus helping in the defense mechanism of the plant during the black pepper – *Phytophthora* interactions.

It is to be noted here, that the colorimetric assay of β- 1,3- glucanase, as done in the present work may not be sufficient for determining a possible role for the enzyme as it does not distinguish the isoforms of β- 1,3- glucanase that may be associated with different metabolic functions during the elicitor treatment or infection *in vivo* (Yi and Hwang, 1996). Since different isoforms can have different sub-cellular localization and functions and can be differentially regulated, the study of the role of specific isoforms of is crucial to understanding the features in pathogen/host plant interactions (Pozo *et al.*, 1999). β- 1, 3- glucanases show a complex isozyme pattern with isoforms differing in their biochemical characteristics, primary structure, antigenicity, enzyme activity, sub-cellular localization and anti-fungal properties (Simmons, 1994). Hence, it is important to study the different isoforms of the β- 1,3- glucanase induced during black pepper-*Phytophthora capsici* interactions to study which of the isoforms play a role in plant resistance. It also needs to be determined whether the β- 1, 3- glucanase induced by *P. meadii* elicitor treatment and treatment by *P. capsici* elicitor are the same, to understand the induction of weak defenses in *P. capsici* elicitor-treated cells. The isozyme profile of these glucanases could give some information.

A role for 1, 3-β-glucanase in disease resistance of soybean to *Phytophthora megasperma* f.sp. *glycinea* was reported by Yi and Hwang (1996). The accumulation of 1, 3-β-glucanase in soybean hypocotyls in response to infection by compatible race of *Phytophthora megasperma* f.sp. *glycinea* was more pronounced and was suggested to be associated with symptom development. But, the induction and accumulation of the enzyme in response to the incompatible race was suggested to be associated with the restriction of symptom development as the enzyme activity was enhanced more rapidly up to 66hrs after inoculation compared to 30hrs after infection in the compatible interaction. A previous study by Ham *et al* (1991) indicated that the 1, 3-β-glucanase activity induced in soybean leaves on infection with *Phytophthora megasperma* f.sp. *glycinea* functions in defense by releasing a phytoalexin elicitor from the mycelial walls of the fungus (Yi and Hwang, 1996).

β- 1, 3- glucanase solubilizes elicitor-active glucan molecules from the cell walls of invading fungal pathogens (Mauch and Staehelin, 1989), in turn inducing its own production and that of other defense enzymes involved in the synthesis of antimicrobial phytoalexins and cell wall barriers. The cell wall bound β- 1, 3- glucanase plays an important role in recognition and the release of elicitors. In pepper- *P. capsici* interactions, it has been observed that *P. capsici* grows initially in the intercellular space of pepper plants (Hwang *et al.*, 1989), making contact with the β- 1, 3- glucanase located on the middle lamella along the air spaces. Upon contact with the hyphae, the β- 1, 3-glucanases are postulated to release oligosaccharide fragments from the β-1, 3- glucan-containing fungal cell walls. These act as elicitors, which may bind to some receptors of the cells, thus triggering the production of more amounts of β- 1, 3- glucanase and other defenses. Kim and Hwang (1997) suggest that the accumulation of the phytoalexin, capsidiol in the resistant reactions of pepper stems to *P. capsici* may be related to the induction of certain β- 1, 3- glucanase isoforms in defense response against the fungal infection. Egea *et al* (1999) reported more pronounced accumulation of β- 1, 3-glucanase in stem tissues of resistant cultivar of sweet pepper, Smith-5 (S-5) than in the susceptible cultivar, Yolo Wonder (YW) on infection with *Phytophthora capsici.* One transcript, size 2.0 kb, was detected in both cultivars, and accumulated very close to the inoculation site between 1 and 3 days after inoculation, but to a greater extent in the resistant cultivar than in the susceptible cultivar. These data suggest that an early and rapid accumulation of beta-1, 3-glucanase transcript is associated with the defense reaction that develops in pepper stems infected with *P. capsici.*

The results obtained in the present study indicate that the elicitors of *P. capsici* and *P. meadii* trigger the induction of β- 1, 3- glucanase in black pepper suspension cultures. The rapid and pronounced expression of glucanase activity in the PmE-treated cultures (incompatible interaction), compared to the low activity in PcE-treated cultures (compatible interaction) is consistent with the results obtained by other researchers mentioned above. Jebakumar *et al* (2001) reported increase in β- 1,3- glucanase activity in black pepper under *in vivo* conditions, on infection with *P. capsici.* The authors reported that though all the cultivars exhibited an increase in β- 1,3- glucanase activity on infection, higher levels of the enzyme were observed in the tolerant line-black pepper interaction (which can be considered as a mild incompatible interaction) compared to that found in the susceptible reactions, suggesting a possible role for the enzyme in tolerance to *P. capsici.* The results of the present study in which the incompatible reaction between black pepper cells and *P. meadii* elicitor resulted in higher activities of the enzyme, are consistent with the results obtained by Jebakumar *et al* (2001). This supports the hypothesis that the response of black pepper suspension cultures to elicitor *in vitro* are similar to the *in vivo* responses of black pepper. The asumption of the possible role of β-1, 3- glucanase in defense is supported by the observation in the present study that the elicitor-treated black pepper leaves inhibited colonization of *P. capsici*. While the colonization of *P. capsici* on the petioles of PcE-treated leaves was greatly inhibited, the PmE-treated cut leaves of black pepper did not support the colonization of *P. capsici* altogether.

2.4.4.8.2.2. Phenylalanine ammonia lyase

Fungal elicitors are known to induce the *de novo* synthesis of anti-microbial compounds, the phytoalexins, which are involved in the defense against phytopathogenic microorganisms. The enhancement of PAL and accumulation of phytoalexins has been observed in many plant-pathogen interactions. In the present study also, PAL activity was found to increase significantly in elicited cultures over the controls, and the expression of the activity was more pronounced in PmE-treated cells. The fact that there was an increase in both extra- and intra-cellular peroxidases in PmE-treated cultures and intracellular peroxidases in PcE-treated cultures, and the hypothesis that the peroxidases may play a role in polymerization of cell wall phenolics, it seems necessary that their activity can be sustained only by the increased biosynthesis and export of phenylpropanoid substances. Such an increase in secondary metabolites is possible only by the increased activity of the enzymes of the phenylpropanoid pathway, in which PAL has an important role to play. Hence the increase in PAL activity in PmE- and PcE-treated cultures in the present study indicates a role for the enzyme in the production of defense-related secondary metabolites that may help in strengthening the cell wall.

Similar increase in PAL activity, closely linked to the flavonoid synthesis, was found to be induced by elicitor treatment in cell suspension cultures of *Pisum sativum* (Wlodzimiez *et al*, 1997). Szabo *et al* (1999) also observed a very high increase in the specific activities of PAL and in the accumulation of rosmarinic acid in suspension cultures of *Coleus blumei*(1999) treated with an elicitor preparation from the culture medium of the phytopathogenic oomycete, *Pythium aphanidermatum*. Knogge *et al* (1987) observed high activities of PAL and 4 CL in parsley, concomitant with the increase in furanocoumarin synthesis. Treatment of suspension-cultured potato cells with an elicitor from *Phytophthora infestans* induced an increase in the accumulation of soluble and cell wall-bound phenolics (Keller *et al.*, 1996). Schmidt *et al* (1998) observed that elicitor treatment of the suspension-cultured potato cells dramatically stimulated activity of PAL and other enzymes involved in the phenylpropanoid metaboism. The enhancement of the enzymes correlated well with the increases in amounts of wall-bound and excreted phenolics. Kamada and Muto (1994) observed increasing PAL activity in tobacco suspension-cultured cells from 2hr after treatment with elicitor of *Phytophthora nicotianae.* The two enzymes of the phenylpropanoid metabolism, L-phenylalanine ammonia lyase (PAL) and 4-coumarate: CoA ligase (4CL) were shown to be induced by gene activation in elicitor-treated parsley cells (Knogge *et al.*, 1997). Orsomando *et al* (2003) showed that pure phytotoxic protein PcF from *Phytophthoras cactorum -fragaria* stimulates the activity of phenylalanine ammonia lyase in tomato seedlings, similar to other known fungal protein elicitors involved in plant-pathogen interaction.

The fact that there was an increase in both extra- and intra-cellular peroxidases in PmE-treated cultures and intracellular peroxidases in PcE-treated cultures, and the hypothesis that the peroxidases may play a role in polymerization of cell wall phenolics, it seems necessary that their activity can be sustained only by the increased biosynthesis and export of phenylpropanoid substances. Campbell and Ellis (1992) observed that the level of PAL activity rapidly increased in elicited *Pinus banksiana* cell cultures during the course of accumulation of TGA-extractable complexes suggesting that *de novo* synthesis of the phenylpropanoid product, lignin might be taking place. Similar observations were made in our study, in which increase in PAL was concomitant with the increase in lignin content of the elicited cells. Similar time course of lignin accumulation and induction of PAL supports this hypothesis. Lignin accumulation in elicited cells was noted from the 6th hour after elicitation and increased consistently till 72 hrs. The extent of lignification in PmE-treated cultures was far more higher compared to that in PcE-treated cultures, which correlated with similar trends in the PAL activity.

2.4.5. Significance of the induced defenses

It was observed that many of the defense responses of black pepper suspension cultures analyzed in the present study were modulated differently by elicitors from *P. capsici* and *P. meadii.* Though some of the major defense responses were induced in both PcE- and PmE-treated cultures, the speed and magnitude of expression of these responses was more pronounced in the *P. meadii* elicitor-treated cultures. This supports the findings of many researchers that the major determinant of the success of a resistance strategy lies in its speed of expression (Keen and Bruegger, 1977; Kuc and Rush, 1985). Thus if significant levels of defense products appear before pathogen colonization is achieved, the attack can be successfully repelled. A delayed resistance response however would result in the activation of counteracting mechanisms by the pathogen, which ultimately takes control of the host machinery before the host can overcome the pathogen. Under such a situation, the pathogen establishes inside the host making it susceptible.

It has often been observed that the expression of defense-related components is higher in incompatible than in compatible interactions between plants and their pathogens as has been seen in the case of β- 1,3- glucanase (Manandhar *et al.*, 1999) peroxidase (Chung and Chung, 1982; Matsuyama, 1983) and phenylalanine ammonia-lyase (Zhang *et al.*, 1987). This is consistent with our observations in the present study, in which the defenses were more pronounced in the incompatible black pepper-*P. meadii* elicitor interactions than in the compatible interaction involving *P. capsici* elicitor.

It can be hypothesized here that the difference in the response of black pepper suspension cultures to the two different elicitors lies in the recognition mechanism that may play an important role in the induction of the defenses. The higher intensity of defenses induced by *P. meadii* elicitor points to the possibility that receptors of the cells may be more configured for the elicitor of *P. meadii* than for the *P. capsici* elicitor, which results in a more efficient transduction of the signal of elicitor perception in PmE-treated cultures. The high glucanase activity in PmE- treated cells may directly inhibit the growth of the invading fungi, or may also act indirectly by releasing fungal wall elicitors that trigger more defense responses of the host. Glucanases of sweet pepper have been known to act indirectly by releasing from fungal cell walls, elicitors that can stimulate phytoalexin accumulation in the host plant (Keen and Yoshikawa, 1983). It needs to be determined whether the lack of high resistance of PcE- treated cells or leaves to *P. capsici* ingress observed in the present study is due to the induction of glucanase to a lesser extent than that found in the PmE-treated tissues. It can be postulated that the lower activity of glucanase in PcE-treated cultures may result in the release of fewer elicitors, which in turn would result in triggering other host defense responses to a lesser extent. The binding efficiency of the *P. capsici* elicitor to possible receptors of black pepper cells also needs to be looked into, since the results obtained in the present study, in terms of the triggering of various responses, point to the possibility that the receptors of black pepper cells are not configured for *P. capsici* elicitor, resulting in poor binding of the elicitor to the receptor, which in turn would have resulted in an inefficient signal transduction, thus resulting in the weaker defense responses in PcE-treated tissues. The weaker defenses in turn could be the reason for the lesser ability of the cells to inhibit the invading fungus. The reason for postulating that the weak defense responses in PcE-treated cells were due to the less efficient binding of the *P. capsici* elicitor to the receptor is due to the fact that the defense responses of a plant are known to be triggered after the cells receive the stimulus of the attack of the pathogen. This stimulus is perceived by the binding of the elicitors of the pathogen by the receptors of the host cells (Suzuki *et al.*, 1995), which in turn sets off a chain of events that transmit the signal from the surface of the cell to the nucleus, where the gene expression is altered to face the threat of the imminent stress.

Our assumption that the difference in the intensity of defense responses elicited in black pepper suspension cultures by the two different elicitors may be due to the difference in recognition mechanism is supported by the findings of Groten and Barz (2000) who compared the responses of suspension-cultured soybean cells of four cultivars to different fungal and bacterial elicitors that included crude cell wall elicitors of *Phytophthora sojae* and *Rhizoctonia solani* and two isolates of bacterial pathogen, *Pseudomonas syringae* pv *glycinea.* Cells of all four cultivars of soybean showed the same elicitor-induced defense responses. However, the reactivity of the cultivars was not identical in terms of time courses and intensities of the defenses. Furthermore, the ability of the various elicitors to induce defense responses varied markedly. The authors suggest that these differences indicate that (1) cells of the same species but of different cultivars are equipped with the same array of perception systems to recognize various stimuli but (2) the sensitivity of these perception systems or later steps in the signal transduction seem to be stimulated to a different extent in the analyzed cultivars. Doke and Tomiyama (1980) also suggested that the reactivity of tissue cells to hyphal wall components might reveal the intensity of resistance of each type of host.

To evaluate whether defense responses elicited in black pepper cultures treated with elicitors of *P. meadii* and *P. capsici* indeed had any inhibitory effect on the pathogen, *P. capsici,* the elicited cultures were inoculated with zoospore suspension of the fungus. Microscopic observations made at 24hours after inoculation of the zoospores showed, that *P. capsici* growing in PmE-treated cultures exhibited discontinuity in the coenocytic cytoplasm and cytoplasmic coagulation. However, the hyphae of *P. capsici* growing in control as well as PcE-treated cultures did not show any visible distortion in the cytoplasm. This indicated that the defenses induced in PmE-treated cultures, indeed had a toxic effect on the pathogen. The results were further confirmed by leaf bioassay, in which elicitor treated cut leaves of black pepper were treated with zoospore suspension. The germlings of *P. capsici* colonized the petiole of the control leaves and formed a heavy growth around the petiole, while the growth was lesser on the petioles of PcE-treated leaves. The colonization was visually absent on the petioles of the PmE-treated leaves.

The distortion of cytoplasm of the hyphae of *P. capsici* growing in PmE-treated cultures, *in vitro*, shows that the *P. meadii* elicitor does induce defense responses in black pepper cells, and the rapidity and magnitude of the induced defenses in *P. meadii* elicitor-treated cultures provide a more hostile environment for the pathogen than the PcE-treated cultures. The inhibitory effect of the defenses induced by *P. meadii* elicitor was further confirmed by the total inhibiton of *P. capsici* colonization at the cut surface of the petioles of leaves treated with *P. meadii* elicitor. The inhibition of colonization of the germlings of *P. capsici* on petioles of elicitor-treated leaves suggests that defense responses are triggered in the leaves on treatment with the elicitors and that the *P. meadii* elicitor induced greater resistance to the ingress by the pathogen.

Naton *et al* (1996) observed a correlation between cell death of parsley cells and formation of secondary fungal hyphae of *P. infestans.* The actual mechanism by which rapid cell death halts the pathogen is unclear, though a few studies suggest that the major reason for the termination of pathogen growth may be the collapse of the intracellular fungal structure immediately following the collapse of the host cell, as was observed by Freytag *et al* (1994) in the potato- *P. infestans* interaction. Other factors presumed to be involved are reactive oxygen species and possibly other toxic substances that are produced during the hypersensitive reaction and liberated following the collapse of the host cell.

Similarly, the distortion of cytoplasm of *P. capsici* hyphae occurring during co-cultivation with black pepper suspension cultures treated with *P. meadii* hyphal wall elicitor can be attributed to many of the defenses occurring in the cells, leading to host cell death. The oxidative burst, one of the early responses to elicitation could be one of the reasons for the collapse of the fungal structure, as observed by Freytag (1994) in the potato-*P. infestans* interaction. The oxidative burst may serve both as intra-and inter-cellular messenger as well as a means of poisoning the invading pathogen. H_2O_2, one of the main components of the oxidative burst has been implicated in direct killing of pathogen cells and host cells in addition to its role in the oxidative cross-linking of plant cell wall proteins to render the wall less digestible by microbial enzymes (Mehdy *et al.*, 1996). Thus, a possible oxidative burst accompanied with the strengthening of the cell wall by peroxidase-mediated oxidative reactions would have inhibited the invasion of the pathogen in elicitor-treated cut leaves of black pepper. Further detailed studies are however needed to support this hypothesis.

Extra-cellular peroxidases may also debilitate the pathogen by production of fungi-toxic compounds from the oxidation of phenols, which peroxidases are known to catalyze (Cadena-Gomez and Nicholson, 1987; Urbanek *et al.*, 1987). The significant increase in medium darkening is a proof to this hypothesis and could be another possible reason for the observed distortion of the hyphae of *P. capsici* growing in PmE-treated cultures. The extent of oxidation of phenols by peroxidases into quinines and other phenolic compounds may not be high enough in PcE-treated cultures so as to affect the pathogen completely. The high increase in intra-and extra-cellular phenolics observed in the PmE-treated cultures would have possibly affected the approaching pathogen.

Peroxidase is also known to produce free radicals (Mader *et al.*, 1980) and hydrogen peroxide (Peng and Kuc, 1992), which are toxic to several pathogens. Peroxidase also participates in the synthesis of ethylene, the concentration of which increases frequently in pathogenesis process (Tudzynski, 1997). The higher levels of peroxidases in PmE-treated cultures thus may be one of the reasons for the distortion of the mycelium of *P. capsici* found during co-cultivation of *P. capsici* with elicitor-treated cultures. The inhibitory effect of the compounds produced by peroxidases may have been responsible for the inhibition of mycelial growth on elicitor-treated cut leaves of black pepper. The complete inhibition of growth of mycelium on *P. meadii* elicitor-treated leaves may be due to the higher magnitude of expression of peroxidase activity induced by *P. meadii* elicitor.

Another possible reason for the inhibition of growth of *P. capsici* on elicitor-treated leaves could be the action of glucanase. Kim and Huang (1997) reported inhibition of the hyphal growth of *Phytophthora capsici* by a basic 34kDa β-1,3-glucanase. They observed that untreated hyphal cells of *P. capsici* appeared well preserved and showed no growth in alteration, whereas β- 1, 3- glucanase from pepper stems lysed the hyphal walls of the fungus. Cell walls of zoospores and hyphae were distinctly degraded. Thus, the cytoplasmic distortion observed in *P. capsici* hyphae growing in PmE-treated black pepper cultures may be due to the lysis of cell wall components of *P. capsici* by β- 1,3- glucanase that is released by the hypersensitively dying cells. It could also be due to the action of wall- bound extra-cellular glucanase and other defenses induced rapidly and to a higher extent in PmE-treated tissues than in PcE-treated ones.

The inhibition of colonization of the germlings of *P. capsici* on petioles of elicitor-treated leaves suggests that defense responses are triggered in the leaves also, on treatment with the elicitors. The near total inhibition of colonization of the fungal mycelium around the cut end of the petioles of PmE-treated black pepper leaves suggests that the *P. meadii* elicitor induced greater resistance to the ingress of the pathogen.

2.4.6. Suppression of HR by water-soluble glucans

The hypersensitive reaction caused by hyphal wall elicitors of *P. capsici* and *P. meadii* in black pepper suspension cultures was found to be suppressed by a factor present in the zoospore germination fluids of *Phytophthora capsici*. This factor generally considered as water-soluble glucan, has been known to suppress the hypersensitive reaction occurring in incompatible interactions. Such observations have been made in potato - *P. infestans* interactions (Sanchez *et al.*, 1993; Sanchez *et al.*, 1992). Hyphal wall components of *Phytophthora* spp. cause the hypersensitive reaction of the solanaceous plant cells regardless of host and non-host or susceptible or resistant type (Sanchez *et al.*, 1992). However, water-soluble glucans (WSG) that are released during spore germination (Doke *et al.*, 1980) inhibit the hypersensitive cell death as well as phytoalexin production in a race-cultivar specific manner (Doke *et al.*, 1979).

Despite the fact that *Phytophthora* species possess numerous cell wall elicitors that can potentially be recognized by plants, they are aggressive plant pathogens. One of the possible reasons for this is that *Phytophthora* produces suppressors, i.e. compounds that prevent recognition of elicitors by the host. These are mainly water-soluble glucans. In potato, compatible races of the late blight fungus prevent the hypersensitive death of infected cells, which is usually seen during the interaction with incompatible races. This is possibly through water-soluble inhibitor glucans (Currier, 1981; Doke & Tomiyama, 1980; Doke *et al.*, 1979; Garas *et al.*, 1979). Hence, compatible races are able to go on to colonize and destroy their host.

Sanchez *et al* (1994) detected neutral and anionic suppressor glucans in the germination fluids of the cytospores of *P. capsici,* suggesting that *P. capsici* also releases water-soluble glucans to establish susceptibility in the host plants similar to *P. infestans.* These water-soluble glucans were found to suppress the elicitor-induced death of suspension-cultured cells of susceptible cultivars of host plants namely pepper and tomato but not resistant cultivar of pepper and non-host plant, tobacco. Sanchez (1994) also reported that zoospores of *P. capsici* encyst, germinate and form appressoria on the surface of host leaves after inoculation, and then the fungus penetrates in the epidermal cells. It later grows through the intercellular spaces of the host tissue forming few haustoria. Thus, *P. capsici* grows inter-cellularly, making contact only with the epidermal cells. The authors suggested that host-selectivity of *P. capsici* may be determined in the leaf epidermal cells where the suppressor glucans released during infection effectively suppressed the occurrence of hypersensitive reaction. The suppressor glucans may not work in the invaded epidermal cells of resistant host and non-host leaves resulting in localization of invading fungus with concurrence of hypersensitive reaction.

The suppressive action of the water soluble glucan isolated from the germination fluids of *P. capsici* of the hypersensitive cell death in black pepper suspension cultures in the present study supports the finding of Sanchez *et al* (1994) that *P. capsici* releases suppressor glucans for the establishment of susceptibility in host plants. The characteristic browning of cells usually seen in elicited cultures of black pepper was seen lacking when the cultures were pre-treated with suppressor. The blue auto-fluorescence, characteristic of elicited cells was also seen lacking when the cells were pre-treated with the water-soluble glucan. Thus, the characteristic hypersensitive cell death triggered by hyphal wall components of *P. capsici* and *P. meadii* was inhibited by the pre-treatment of cultures with water-soluble glucans, suggesting that the suppressor indeed plays a role in inhibiting the defense responses triggered by the hyphal wall elicitors.

Suppression of hyphal wall components-induced hypersensitive response of protoplasts from susceptible potato tissues by water-soluble glucans isolated from the germination fluids of *Phytophthora infestans* was also reported by Doke and Tomiyama (1980). They suggested that the selective activity of glucans in suppressing the hypersensitive response of protoplasts from susceptible potato tissues is related to the establishment of a compatible interaction between the fungus and potato tissues by binding of glucans to some receptor on the protoplasmic membrane. They further explained that glucans possibly occupy the binding site for hyphal wall components on the protoplasmic membrane or cause a configurational change of the binding site, resulting in no effective binding of hyphal wall components to the receptor. The results obtained in the present study also support these findings that the factor isolated from the germination fluids of *P. capsici* has some role in preventing the induction of defenses in black pepper cultures on elicitation with HWC. It may also be hypothesized from the findings of researchers described above that the suppressor may bind to a putative receptor on the plasma membranes of the black pepper cells, in turn preventing the induction of defenses by hyphal wall components, characterized by the hypersensitive reaction.

Sanchez *et al* (1993) observed that different *Phytophthora* species infecting solanaceous plants produce water soluble glucans that suppress the activation of $O_2^{\cdot-}$ generation responsible for triggering hypersensitive cell death in their respective hosts, and thus enable the establishment of a compatible interaction. Thus it can be presumed that suppressor glucans isolated in the present study may inhibit some such processes which may be the superoxide-generating system in the plasma membrane, responsible for triggering hypersensitivity or defense reactions. However, further studies are needed to confirm this hypothesis.

The recognition mechanism of the host plays an important role in deciding whether it is susceptible or resistant to a fungal attack. Hence, in a compatible interaction, the pathogen evades the recognition mechanism of the host, gaining control of the host metabolism. On the other hand, in an incompatible interaction, the host identifies the non-pathogen, and through a series of signal transduction steps, overcomes the invading fungus. The faster and greater the induction of defense response, the more tolerant/resistant a plant is, to a pathogen. It is assumed from the results obtained in the present study that the defense responses elicited are the same in susceptible and resistant reactions but the time and extent of triggering of these responses is what determines resistance or susceptibility to challenge by a particular *Phytophthora* species. These responses occur at a greater speed and magnitude in a resistant host, where the recognition mechanism is more efficient than in the susceptible host.

Research into the possible presence of f a receptor in black pepper cells in the recognition of the surface structures of *Phytophthora* that would transduce the signal of elicitor perception into cellular response would be interesting. It would be an interesting area of research to study the possible presence of such receptors in black pepper cells that perceive and transduce the signal of attack by *Phytophthora* species. Very recently, Fliegmann *et al* (2004) observed that soybean exploits a specific molecular pattern, a 1,6-beta-linked and 1,3-beta-branched heptaglucoside (HG), present in cell walls of the oomycetal pathogen *Phytophthora sojae*, as a signal compound eliciting the onset of defense reactions. The authors noted that the specific and high affinity HG-binding site is contained in the beta-glucan-binding protein (GBP), which in turn is part of a proposed receptor complex. The ability to perceive and respond to *Phytophthora* cell wall-derived beta-glucan elicitors was formerly considered to be exclusive to plants that belong to the Fabaceae. But more recently, genes encoding GBP-related proteins have been found to exist in presumably all plant species. The high affinity HG-binding activity in soybean was reported to be exclusively membrane-localized (Mithofer and Ebel, 1999). Furthermore, Fliegmannn *et al* (2004) showed that the GBP is composed of two different carbohydrate active protein domains, one containing the beta-glucan-binding site, and the other related to glucan endoglucosidases of fungal origin. The function of GBP would comprise a prime attack on the pathogen by degrading the cell walls resulting in the release of soluble cell wall components. The glucan hydrolase displays most likely an endo-specific mode of action, cleaving only 1,3-beta-d-glucosidic linkages of oligoglucosides consisting of at least four moieties. Thus, the intrinsic endo-1,3-beta-glucanase activity of the GBP is perfectly suited during initial contact with *Phytophthora* to release oligoglucoside fragments enriched in motifs that constitute ligands for the high affinity binding site present in the same protein. More recently, a number of pathogenesis-related proteins have been shown to be glucanases or chitinases, which may play an active role during plant defense by attacking fungal cell walls. However, according to Fliegmann *et al* (2004), in contrast to "classical" 1,3-β-glucanases, which partly have been classified as pathogenesis-related proteins, the GBP displays a unique capability to use the products of the intrinsic hydrolytical activity as ligands of a disparate binding site localized in the same protein as part of the pathogen receptor. The results of the present study indicate the possible presence of similar perception mechanism, since the black pepper cells treated with cell wall elicitors of *P. meadii* react rapidly and more intensely to the elicitor than the cells treated with elicitor from *P. capsici*. In the present study, hyphal wall elicitors of *P. capsici* and *P. meadii* simulated compatible and incompatible interactions respectively of black pepper suspension cultures to *Phytophthora.*

Significance of the present work

The present study on the responses of black pepper suspension cultures to elicitor treatment is an attempt to shed light and provide cues on some of the events taking place at the cellular level, which are difficult to observe under *in vivo* conditions and to reflect on the possible implications of these observations. These cues would help adopt and pursue future lines of work to gain more insight into these events. These studies would further help to understand the possible role of the various events in defense against *Phytophthora capsici* to improve the crop for resistance against this fungus. This study is also to assert that suspension cultures can be successfully used as tools to study in detail, some of the defense responses that occur in black pepper against *Phytophthora capsici* which are otherwise difficult to study in the whole-plant pathogen interactions under the *in vivo* conditions.

Suspension cultures of black pepper can also be exploited for studying molecular changes occurring in the cells on elicitation. Suty *et al* (1995) employed cell suspension cultures of tobacco as a good tool to investigate the earliest modulations in gene expression following the addition of cryptogein elicitor. Suspension cultures are being popularly used to study the signal transduction mechanisms occurring in plant cells in response to hyphal wall elicitors (Mithofer *et al.*, 2001; Fellbrich *et al.*, 2000; Kirsch *et al.*, 2001), as they offer homogenous, responsive cells that can be handled easily to dissect the various components of the signal transduction pathway which would be otherwise difficult to study under *in vivo* conditions since the tissues *in planta* consist of cells at different stages of growth and differentiation.

Based on the vast information generated from the various biochemical responses of plants followed by pathogen attack, several strategies have been proposed to engineer disease resistance against fungal pathogens. The studies on biochemical responses to elicitor and disease has helped in cloning the genes expressed during these treatments such as those coding for avirulence, disease-resistance, PR proteins, antifungal proteins, toxic secondary metabolites, rate limiting enzymes controlling oxidative burst, cysteine-rich thionins. Such studies have paved way for designing strategies to confer resistance to a wide range of fungal pathogens infesting crops. It has also been possible to over-express these defense genes constitutively in several transgenic crop systems. Inducible/temporal expression of these genes opens up another area and adds to the basic repertoire of defense strategies. For eg., Li and Fan (1999) transformed potato plants through *Agrobacterium*-mediated transformation using harpin protein gene from apple fire blight pathogen *Erwinia amylovora* and potato prp1-1 promoter as main DNA elements, to induce hypersensitive response to ward off the pathogens. The constitutive and pathogen infection-induced expression of harpin protein gene in the transgenic potato reduced the lesion growth rate of *Phytophthora infestans.* The results highlighted that engineered hypersensitive response in plants was a very promising approach to produce fungal disease resistant genotypes.

Thus basic studies on the defense responses induced during pathogen attack or elicitor treatment hold great promise in adopting new strategies to improve crop plants for disease resistance. Such basic studies on the defense responses in black pepper would result in the identification of important factors that are induced in response to the pathogen. Some of these factors could well be associated with defense reaction of black pepper against *P. capsici.* The present study was mainly focused to highlight and provide cues on the different events taking place in black pepper cells on elicitation and further work on these lines would provide a greater insight into the mechanism of cell responses to elicitor treatment. These results can then be possibly extrapolated to *in vivo* conditions to formulate strategies to engineer and improve black pepper for resistance against *P. capsici.* The embryogenic suspension cultures used in this study would offer an excellent system to carry out investigations on finer details of cellular events triggered on elicitation with *Phytophthora* cell wall elicitors like the signal transduction pathway, the role of microtubules, active oxygen species and cell wall modifications etc. in the defense against *P. capsici.*

Since such studies have not been taken up in black pepper and since there are miles to go before any meaningful strategy for resistance against *P. capsici* in black pepper can be formulated, it would be worthwhile to undertake more fundamental studies into each aspect of the different events taking place in black pepper cells during the black pepper- *P. capsici* interaction. It can be once more emphasized here that black pepper suspension cultures hold a great promise and exploiting this model system in such studies would provide more insights into the molecular events taking place in black pepper cells in response to elicitors or pathogen, so as to improve the crop for disease resistance. The fact that some of the defenses studied in the present study using suspension cultures of black pepper mimic those occurring in plants *in vivo*, supports our strong view that these model systems can be conveniently used to study some of the responses of black pepper cells to the fungus, in much more detail.

Summary

Y. Anuradha “Improvement of Black pepper (Piper nigrum L.) through induction of stress tolerance in vitro” Thesis. Indian Institute of Spices Research, University of Calicut, 2004

Summary

Piper nigrum L., popularly known as black pepper is an important spice crop that earns huge foreign exchange for the country. However, the yield of black pepper is affected by environmental and biotic factors that result in considerable losses for the country's exchequer. Abiotic stress in the form of drought is one of the major constraints affecting black pepper production as it affects both growth and development of the plants. The most important developmental phase of black pepper that is affected by moisture stress is the flowering phase which commences during May-June. Delayed monsoon delays flowering. Moreover, once the flowering commences, the crop requires continuous availability of moisture, the lack of which results in spike shedding resulting in yield loss. In addition to drought, biotic stress in the form of insect, nematode and fungal attacks severely affects the crop. Among these biotic factors, the fungal disease namely, foot rot disease of black pepper caused by *Phytophthora capsici* is a major disease that results in severe damage of the crop. The constraints of black pepper yield due to these abiotic and biotic factors have drawn the attention of many researchers to initiate studies on the various aspects of these stresses and their effect on the crop.

Breeding for tolerance/resistance for these stresses has been a major strategy for protecting the crop against the damaging effects of these stresses. The present work is a step in this direction and was mainly initiated to understand the biochemical responses of black pepper to biotic (*Phytopthora capsici*) and abiotic (drought) stress, in an effort to understand the mechanism of tolerance to the imposed stress. Embryogenic black pepper suspension cultures were exploited in the present study to provide a model system to dissect the responses of black pepper cells to the imposition of drought and disease stress. The postulation in these studies was that the response of black pepper plant as a whole to biotic or abiotic stress is the sum total of the responses of individual black pepper cells to these stresses. Since suspension cultures provide a homogenous system of uniformly growing cells, they form ideal systems for studying the cell responses to a particular imposed stress. In addition to studying the responses of black pepper cells to biotic and abiotic stresses, the present work was also aimed at undertaking *in vitro* studies to select water stress-tolerant cell lines in black pepper.

This dissertation is divided into two main chapters: Chapter 1 describes the studies carried out on abiotic stress and Chapter 2 is dedicated to studies on biotic stress. The following is the summary of the results obtained:

ABIOTIC STRESS

Drought stress was simulated *in vitro* by amending the liquid nutrient medium of the suspension cultures with polyethylene glycol 6000 (PEG 6000). Before the studies on water stress on black pepper cells could be initiated, the dose response curve of growth of black pepper cells to PEG 6000 was worked out to find the intensity of water stress caused by different concentrations of PEG. It was observed that PEG at concentrations 15, 25 and 30% resulted in 50, 82.4 and > 100% decrease in growth respectively, compared to control. Hence, 15, 25 and 30% PEG were used to induce mild, severe and lethal water stress for further studies. It was interesting to note that PEG at 5% did not affect the growth of black pepper cells significantly and was on par with the control treatment.

The studies on biochemical responses of black pepper cells to PEG-induced water stress included osmolyte accumulation, modulation of antioxidant enzymes and induction of water stress- and ABA-induced proteins.

During moisture stress, plant cells tend to lose water to the outside environment due to the osmotic gradient created due to depleting moisture outside. Hence, plant cells accumulate solutes to maintain the osmotic potential and to conserve water. The accumulation of some of these solutes (osmolytes) was studied in the present work. Among the different osmolytes, accumulation of both organic and inorganic osmolytes was analyzed. Among the organic osmolytes, total free amino acids, reducing sugars and proline were studied. It was observed that free amino acids increased in all the water-stressed black pepper suspension cultures compared to the control. Among the water stress treatments, 15% PEG-exposed cultures showed significant increase over the control and other water stress treatments during the initial phase of the water stress period but later declined after the 15th day. However, 25% PEG-treated cultures showed moderate increases which was sustained till the end of the stress period. The 30% PEG-treated cultures however showed very marginal increase in total free amino acids during the first 10 days of the stress period, after which the levels fell below that of the control cultures. Since water stress significantly increased the total free amino acids in 15 and 25% PEG-treated cultures, the amino acid profile of these cultures was examined to determine which of the amino acids contributed to the increase in the amino acid pool. It was observed that water stress induced a general increase in most of the amino acids compared to the control cultures. The PEG-treated cultures (15 and 25%) showed an increase in alanine, glutamine, phenylalanine, serine and threonine. Of these, glutamine levels were found to be maintained at higher levels in cultures exposed to severe and mild water stress till the end of the stress period. Phenylalanine and alanine were abundant in cultures exposed to severe water stress (25%PEG) till the 20th day of the stress period. Threonine was found to increase gradually in 15% PEG-treated cultures and remained the dominant amino acid by the end of the stress period. However, 25% PEG-treated cultures did not show an increase in threonine. The increase in amino acids in the PEG-treated cultures however showed a decline by the end of the stress period. The control cultures showed a dominance of alanine and phenylalanine during the initial stages of growth while glutamine was found to be the predominant amino acid towards the end of the experiment. However the levels of these amino acids remained far below that of the PEG-treated cultures. Quantitative analysis of proline revealed that the imino acid increased under mild water stress conditions mediated by 15% PEG. However, the levels were on par with the control during the initial stages of the imposition of stress with the increase being significant only from the 15th day of the stress period. However, in cultures exposed to 25 and 30%, there was a significant decrease in proline initially, which later increased but remained low compared to the control cultures.

Reducing sugars were found to increase significantly in all the water stress-treated cultures, the increase being proportional to the severity of the stress. However, the significant increases seen in cultures exposed to severe stress, namely, 25 and 30% was short lived as they declined progressively with time. On the other hand, the content of reducing sugars in cultures exposed to mild water stress was lower than that of the cultures exposed to severe and lethal water stress, but the levels were maintained steady till the end of the stress period.

Among the inorganic osmolytes, an increase in K^+ ions was noted in the water-stressed cultures, especially in cultures exposed to 15 and 25% PEG, the increase being proportional to the severity of the stress. The 15% PEG-treated cultures however showed an initial massive increase in comparison with all other treatments which later decreased and remained above the control cultures. The 25% PEG-treated cultures showed greater increase in the K^+ levels than the cultures exposed to 15% PEG. The K^+ levels in 30% PEG-treated cultures however remained low. The Na^+ content was found to increase in water stress-treated cultures, the increase being significant in cultures exposed to 25 and 30% PEG. There was no significant variation in Mg^{2+} content in the cultures on imposition of water stress. The values remained on par with the control treatment or remained low, especially in 30% PEG-treated cultures. On the other hand, the Ca^{2+} content decreased in all the water stress treatments compared to the control. Thus, black pepper cells in general accumulated significant concentrations of reducing sugars, free amino acids and K^+ ions during PEG-induced water stress. In addition, the black pepper cells also recorded moderate increase in proline levels in response to water stress.

One of the main consequences of water stress is oxidative damage caused by free radicals that disrupts membrane integrity and consequently all the physiological processes. Hence, the modulation of the different antioxidant enzymes that act to contain oxidative damage was analyzed in the present study. The enzymes studied were the superoxide radical scavenging enzymes and the hydrogen peroxide scavenging enzymes. In the former, the total activity of superoxide dismutase (SOD) was studied. It was observed that the SOD levels increased in all the PEG-treatments which later declined and reached below the control levels after the 15th day of the stress period, especially in 30% PEG-treated cultures. It was observed that the increase in total SOD was gradual in 15% PEG-treated cultures and did not fluctuate significantly throughout the stress period unlike the cultures under severe and lethal water stress. In addition to the total SOD, the expression of the constituent isoforms of SOD was also studied. It was observed that Cu/Zn SOD was the major isoform in the control cultures. Mn SOD was the predominant isoform in black pepper cultures exposed to mild water stress. However, with increase in the duration of the stress, Cu/Zn SOD also gained activity. In the cultures under severe and lethal water stress, the activities of all the isoforms of SOD were active. Cu/Zn and Mn SOD were higher from the initial stages of the stress period, which was later followed by an increase in FeSOD with progression of time. FeSOD activity was found to increase with prolonged exposure to intense water stress but was negligible under mild water stress conditions. While the modulation in the activities of the different isoforms of SOD was observed there were no differences in the electrophoretic pattern of these isoforms between the cultures of different treatments.

Enzymes involved in the scavenging of H_2O_2 formed during the action of SOD were also studied. Among the H_2O_2 –scavenging enzymes, the activities of catalase, peroxidase and the enzymes of the ascorbate-glutathione (AGC) cycle were analyzed. Catalase activity was observed to be severely inhibited under severe and lethal water stress conditions. However, the cultures exposed to mild water stress (15% PEG) showed an initial increase in catalase activity which later declined and was maintained at only marginally higher levels compared to the control. Peroxidase activity increased in all the water-stressed cultures during the initial phase of the drought period. The activity was higher in cultures exposed to 25 and 30% PEG compared to those under mild water stress (15% PEG). However, the activity in these cultures dropped below the control by the 15th day of stress. The 15% PEG-exposed cultures on the other hand showed a gradual increase in activity and sustained higher levels than the controls till the end of the stress period.

It was noticed in the present study that the enzymes of the AGC were more active than the catalases and peroxidases in all the water stress-treated cultures of black pepper. It was observed that cytoplasmic and chloroplastic fractions of ascorbate peroxidase (AP) increased significantly under water stress. While the cultures exposed to severe and lethal water stress showed significant increase over the control during the initial period of the stress, the cultures exposed to mild water stress (15% PEG) recorded a gradual increase which peaked on prolongation of the stress. The levels in these cultures remained higher than that in all other treatments till the end of the stress period. Dehydroascorbate (DHAR) activity showed different trends in the cytosolic and chloroplastic fractions of the cultures. Cytosolic DHAR activity increased in all water-stressed cultures compared to the control, the increase being higher in cultures exposed to severe and lethal water stress. The cultures exposed to mild stress recorded only a gradual increase in activity. While the levels in cultures exposed to 25% PEG maintained high levels throughout the stress period, the levels in cultures under lethal stress conditions declined immediately after the initial increase. By the end of the experiment, all the water-stressed cultures maintained higher activity over the control, the activity in the cultures in the decreasing order being 25, 15 and 30% PEG respectively. In the case of the chloroplastic fraction of DR, 30% PEG-exposed cultures showed higher activity than all the other treatments from the very beginning of the stress period and maintained higher till the 20th day of the stress period. However, 15 and 25% PEG-exposed cultures showed a gradual increase in activity. By the end of the stress period, the activity was maintained high above the control only in the 15% PEG-exposed cultures, while the activity declined below the control levels in cultures exposed to severe and lethal stress. Glutathione reductase, the last enzyme of the AGC pathway showed higher activity in all the water stress treatments compared to the control. However, only the cultures exposed to mild water tress maintained higher enzyme activity over the control throughout the stress period. The activity in the cells exposed to severe and lethal water stress declined by the 10th day of the stress period and remained below the control levels till the end of the experiment. Thus, it can be said, in general that the antioxidant enzyme activity in black pepper cultures under mild stress was maintained steady and higher till the end of the stress period compared to the cultures exposed to severe and lethal water stress in which the enzyme activities recorded an initial increase after which they declined soon and failed to maintain the levels till the end of the stress period.

Consistent with the maintenance of steady and higher activities of the different antioxidant enzymes of black pepper cultures exposed to mild water stress, it was noted in the present study that lipid peroxidation was lower in these cultures compared to that found in cultures exposed to severe and lethal water stress, in which the activities of antioxidant enzymes were highly inhibited.

The phenolic content of black pepper cultures under water stress also recorded a similar trend. Cultures exposed to severe and lethal water stress recorded a significant increase over the control but the levels declined on progression of the stress. However, the cultures under mild water stress conditions showed gradual increase in the levels which were maintained high till the end of the stress period.

Black pepper cultures were also analyzed for the alterations in protein profiles during water stress. The possible role of the plant hormone, Abscisic acid (known to have a role in mediating drought responses) in mediating these changes was also analyzed. SDS-PAGE analysis of protein extracts of the cultures exposed to different water stress regimes revealed the induction of three new polypeptides with molecular weights, 39.94, 26.67 and 17.71. These polypeptides were induced under all the water stress treatments. It was also interesting to note that the plant hormone, abscisic acid (ABA) also induced the same set of proteins induced by water stress, possibly denoting that ABA may have a role in mediating water stress response in black pepper cultures. It was also observed in the present study that small polypeptides with molecular weights, 9.8 and 6.45 were induced in cultures exposed to PEG as well as ABA-pre-treated cultures exposed to PEG but were missing in cultures treated with ABA alone. Further, it was observed that two major polypeptides of molecular weight 40.94 and 32.8, present in the control cultures disappeared on treatment with PEG or ABA.

In vitro selection

Before any cell selection for water stress tolerance could be initiated, a few preliminary studies on the effect of the water stress agent, polyethylene glycol 6000, on the growth and differentiation of black pepper cells were taken up.

It was observed that growth and differentiation of black pepper suspension cultures decreased with increasing concentrations of polyethylene glycol (PEG). Cultures exposed to 5% PEG exhibited growth on par with the control treatment. PEG at concentrations above 10% affected these parameters significantly. As mentioned earlier, PEG at concentrations 15, 25 and 30% caused 50, 82.4 and >100% reduction in growth respectively. However, it was noted that though the fresh weight gain in black pepper cultures was inhibited on exposure to higher concentrations of PEG, the PEG-exposed cultures maintained higher dry matter compared to the control.

Similar to the trends shown in terms of growth, the black pepper cultures also showed reduction in the ability to differentiate into embryos with increasing concentrations of PEG. The inhibition of differentiation was highly significant at concentrations 20% and beyond. At lower concentrations however, though the differentiation ability of the cultures was affected, there were reasonable number of embryo forming units. It was interesting to note that similar to the growth characteristics, 5% PEG exposed cultures also differentiated on par with the control cultures after their return to the control medium. Further, it was observed that on maintenance in PEG-amended medium, 5% PEG promoted differentiation of the cultures which formed more embryo forming units compared to the control cultures. Thus, growth and differentiation of black pepper cultures was not significantly affected by PEG in the range 5-15%, while these parameters were highly inhibited by concentrations 20% and beyond. 5% PEG promoted growth and differentiation in black pepper cultures. It was also observed that black pepper cells exhibited decreased cell size on prolonged exposure PEG-mediated water stress, compared with the cells in the control medium.

In vitro selection for water stress tolerance was carried out by both step-wise and direct selection methods. In the step-wise selection, black pepper cultures were exposed to a mild water stress mediated by 15% PEG for a period of 6 months followed by a further exposure of the cultures to 25% PEG for another 6 months. The surviving cells did not show further growth in the PEG-amended medium and hence were multiplied on the control medium for enough biomass. The cells and cell clumps experienced an initial osmotic shock on their transfer to control medium. However, they multiplied and differentiated after a period of 40 days. Some clumps gave rise to hard callus which did not show any signs of regeneration When these differentiating clumps were returned back to the 25% PEG-amended medium to test their tolerance, it was found that the growth of these clumps was highly inhibited similar to the behaviour of non-selected cells in 25% PEG-amended medium. The differentiating clumps were maintained in the 25% PEG medium for 2 months. However, since the clumps did not show further signs of growth, they were returned to the control medium. After an initial osmotic shock of 30-40 days, the blackened embryogenic clumps put forth fresh embryos. These were multiplied on the control medium. To test the tolerance of these embryos to the PEG regime in which they were selected, they were inoculated in 0, 10, 15 and 25% PEG-amended media. The growth of the embryos was inhibited in 10 and 15% PEG-amended medium compared to that in the control medium. The inhibition however was very high and significant in 25% PEG-amended medium. Hence it was concluded that the cells that underwent step-wise selection were merely adapted to high intensity water stress mediated by 25% PEG on prolonged exposure and that they were not truly tolerant genotypes.

In the direct selection method, black pepper cells were directly exposed to severe water stress mediated by 25% PEG. After an exposure period of 6 months, the cultures consisted of callus and embryogenic clumps, the development of which was suppressed. The surviving calli and embryogenic clumps were transferred back to the control medium for multiplication. The callus which had a characteristic brown colour multiplied but the embryos failed to develop further. The callus was tested for its tolerance by transferring it to 15, 25 and 30% PEG-amended medium for a period of 45 days. It was observed that the growth of the callus was inhibited in the PEG-amended medium compared to that in the control medium. When these cultures were plated on to solid control medium, it was observed that the growth of the callus that was exposed to 15 and 25% PEG was highly inhibited and that exposed to 30% PEG showed minimal growth on the control medium. Thus, step-wise selection resulted in embryos which were not tolerant to the imposed water stress and the callus obtained by direct selection method also did not exhibit tolerance. However, from the studies undertaken, it was observed that on prolonged exposure to stress, the black pepper cells survived the stress through physiological adaptation.

The callus obtained by both direct and indirect selection methods was characterized biochemically for osmolytes, lipid peroxidation and phenol accumulation. The trends observed were similar to that observed during the general responses of black pepper cells to PEG-mediated water stress. Among inorganic ions, it was observed that in general, , K^+ and Na^+ ions increased while Mg^{2+} and Ca^{2+} decreased significantly in black pepper cells exposed to water stress compared to the cells in the control medium. The trend was similar in all the cultures irrespective of whether they were subjected to selection regimes of PEG or were non-selected control cells. Increase in K^+ accumulation was however significantly higher in the selected cell lines compared to the control, non-selected cell lines under the imposed water stress. Among the organic osmolytes, total free amino acids, proline and reducing sugars increased upon imposition of water stress in both the selected cell lines as well as the control cells. While the increase in reducing sugars and proline was only marginally high than the control non-selected cell lines, the increase in total free amino acids was significantly high in the selected cell lines. Among the selected clones, the cell line selected by the step-wise method recorded higher concentrations of total free amino acids than the cell line selected by the direct method, in the PEG-amended medium. In the case of proline however, the cell line obtained by the direct selection method seemed to accumulate higher levels of the imino acid compared to the cell line obtained by step-wise selection. In the case of lipid peroxidation, the selected cell lines and non-selected cultures showed similar increase in MDA content under water stress. In terms of phenolics accumulation, the adapted cell lines accumulated greater levels of phenolics than the non-adapted cell lines, with the cell line selected by the step-wise selection method showing higher levels than the cell line selected by the direct method.

Thus, the attempt to select truly tolerant cell lines in black pepper for PEG-mediated water stress was unsuccessful. However, the cells of black pepper had the ability to adapt to increasing water stress conditions by physiological adaptation.

BIOTIC STRESS

Under biotic stress, host pathogen interactions were studied with the help of a model system in which the fungus, *Phytophthora* was reduced to hyphal wall components of the fungus and the plant was represented by the suspension cultures of black pepper. The suspension cultures were inoculated with the hyphal wall components of *Phytophthora capsici* for studying the cellular changes taking place during host-pathogen interactions.

Studies on the defense responses of any plant species to a pathogen should ideally involve a tolerant and a susceptible plant variety so that one can study and compare the responses in a compatible and incompatible interaction and identify the true defense responses of the plant against the pathogen. Since, there are no truly tolerant varieties of black pepper that show a high degree of resistance to the fungus, use of a non-pathogenic species of *Phytophthora* was considered a good idea to simulate an incompatible interaction. Hence, a leaf bioassay was done as a preliminary study to identify a *Phytophthora* spp. which did not cause infection on black pepper leaves. It was observed that the isolates of *P. parasitica* and *P. meadii* used for the study did not cause the diseased symptoms on black pepper leaves. Instead, they caused a hypersensitive reaction, typical of an incompatible interaction. Hence, it was decided to use *P. meadii* in further studies to simulate an incompatible interaction.

Hyphal wall components (HWC) from the pathogen, *P. capsici* and non-pathogen, *P. meadii* were used with the black pepper suspension cultures to study the compatible and incompatible interactions respectively. Before using the hyphal wall components in studies on the host-pathogen interactions, the ideal concentration of each of the HWC of *P. capsici* and *P. meadii* to be used with black pepper suspension cultures was determined by assessing the cell viability by the MTT colorimetric assay, FDA cell viability assay and in terms of decrease in fresh weight of cells. The FDA cell viability assay was especially useful as it served as a marker for visualizing the elicitation of cells. The elicited cells fluoresced blue under UV while the control cells fluoresced green, typical of viable cells. The control cultures did not exhibit blue fluouresence. The elicited cells also autofluoresced blue under UV even without FDA staining.

The different defense responses elicited by black pepper cells on treatment with the two elicitors were studied. The most characteristic feature of elicited cells was hypersensitive cell browning, the response being more marked and faster in *P. meadii* elicitor-treated cells than in PcE-treated cells. All the associated defenses were faster and higher in magnitude in PmE-treated cultures compared to the PcE-treated cultures. Phenolics exudation was also visible, with the response being quicker in PmE-treated cells which was as early as 12hrs. The intensity of phenolics exudation increased with incubation time and was higher in PmE-treated cultures than in PcE-treated cultures. Another immediate response to elicitor treatment was extra-cellular alkalinization. While the increase in the pH of the culture medium was gradual in PcE-treated cultures, it was faster and higher in PmE-treated cultures. The cultures also showed a spurt of superoxide anion production as early as 10 min. after elicitation, compared to control cultures, with the response being marginally higher in PmE-treated cultures than that observed in PcE-treated cultures. Lipid peroxidation was higher in elicited cultures compared to the control cultures. The PmE-treated cultures showed higher MDA content than the PcE-treated cultures. The cytological changes observed in black pepper cultures in response to elicitation were cytolasmic aggregation and physical barrier formation, namely lignification and callose formation. It was also observed that lignification was rapid and quantitatively more pronounced in PmE-treated cultures than that observed in PcE-treated cultures.

The enzymes associated with defense were also studied. Among the antioxidant enzymes, modulations in the activities of superoxide dismutase, catalase, peroxidase and the enzymes of the ascorbate-glutathione cycle were analyzed. Total SOD activity remained low in elicitor-treated cultures until 24 hours after elicitation after which it increased and remained higher than control levels in both PcE- and PmE-treated cultures. However, the activity was higher in PmE-treated cultures. Among the different isoforms of SOD, MnSOD was found to be higher in PcE-treated cultures compared to control and PmE-treated cultures, with the increase being evident as early as 6hrs post elicitation. It was observed that while the major form of SOD in PcE-treated cultures was MnSOD, PmE-treated cultures had FeSOD as the major isoform. CuSOD was the dominant form of SOD in the control cultures throughout the course of the experiment. Though PcE- and PmE-treated cultures showed an increase in Mn and FeSOD activity respectively, they gradually gained CuSOD activity in addition to their respective major SOD isoform, 24hrs post elicitation. Differential staining of the different isoforms of SOD activity using PAGE revealed the induction of a distinct locus of MnSOD in PcE-treated cultures that was seen lacking in both control and PmE-treated cultures.

Catalase activity was found to be significantly high in PcE-treated cultures as early as 6hrs post elicitation. However, the activity in PmE-treated cultures was lower than the control cultures during the initial phase after elicitation, after which it rose and was maintained only marginally higher than the control cultures. The peroxidase activity was higher in elicited cultures compared to that found in the control cultures. However, in terms of speed and magnitude of induction, the activity was higher in PmE-treated cultures compared to the PcE-treated cultures. The extra-cellular peroxidase activity also increased significantly on elicitation, with the activity being more pronounced in the PmE-treated cultures.

Among the enzymes of the ascorbate-glutatione cycle, cytosolic ascorbate peroxidase remained low in PmE-treated cultures during the initial stages of elicitation, after which it increased but remained only marginally high than the control cultures. The PcE-treated cultures however showed higher activity of the enzyme than the control and PmE-treated cultures during most part of the entire time-course of the experiment. Similar trend was observed in the activity of the chloroplastic fraction of ascorbate peroxidase. The PmE-treated cultures showed decreased activity during the initial phases of elicitation, after which the activity increased but remained low compared to that found in PcE-treated cultures. The activity remained high in PcE-treated cultures throughout the experiment. Dehydroascorbate reductase activity in the cytosolic fraction of the cultures remained high in PmE-treated cultures. However, PcE-treated cultures showed low or marginally higher levels compared to the control cultures. Similar trends were observed in the DR activity in the chloroplastic fractions of the cultures. The PmE-treated cultures showed higher activity while the activity in PcE-treated cultures was low or on par with the control cultures.

Among the other defense enzymes studied, β-1, 3 glucanase and phenylalanine ammonia lyase were found to be increase significantly in the elicited cultures over the controls. The induction of β-1, 3 glucanase was induced as early as 6hrs after elicitation. The activity was induced faster and in significantly higher magnitude in PmE-treated cultures compared to the PcE-treated cultures. The increase in phenylalanine ammonia lyase was observed in both the elicited cultures, with the PmE-treated cultures showing a faster and steady increase in enzyme activity. The PcE-treated cultures showed only a gradual increase in PAL activity which dropped by 72 hrs post elicitation.

The effect of water soluble glucans (WSG) of the pathogen, *P. capsici* on the elicitation of the characteristic hypersensitive response by the hyphal wall components of *Phytophthora* in black pepper suspension cultures was studied. It was observed that the water soluble glucans present in the germination fluids of *P. capsici* suppressed the hypersensitive reaction elicited by hyphal wall components. Cells pre-treated with WSG followed by HWC treatment appeared normal and did not exhibit hypersensitive browning or the characteristic blue auto-fluorescence that is usually observed in black pepper cells on elicitor treatment.

To verify if the defenses induced in black pepper cultures by the hyphal wall components of *P. capsici* and *P. meadii* indeed could have an inhibitory effect on the pathogen, *P. capsici*, the PmE- and PcE-treated cultures were inoculated with the zoospores of the pathogen, *P. capsici*. Microscopic observations made 36hrs after inoculation of the zoospores revealed that the hyphae of *P. capsici* growing in PmE-treated cultures exhibited cytoplasmic coagulation with disruption of continuity in the coenocytic cytoplasm. The hyphae growing in PcE-treated and control cultures however did not show any visible distortion in the cytoplasm showing that the defenses induced in PmE-treated cultures indeed had a toxic effect on the pathogen. These results were further confirmed by a leaf bioassay in which the elicitor-treated cut leaves of black pepper were inoculated with the zoospore suspension of *P. capsici*. It was observed that the germlings of *P. capsici* colonized the petiole of the control leaves heavily while the growth of the mycelium was inhibited significantly in PcE-treated cultures. The PmE-treated leaves however showed no visible colonization of the fungus at the petiole. This again showed that the defenses induced in PmE-treated leaves had a highly inhibitory effect on the pathogen, while those induced in PcE-treated leaves were not high enough to completely inhibit the pathogen.

APPENDIX

1. SH nutrient medium (Schenk & Hildebrandt, 1972)

1. $(NH_4)H_2PO_4$ - 300 mg
2. KNO_3 - 2500 mg
3. $CaCl_2.2H_2O$ - 100 mg
4. $MgSO_4.\ 7H_2O$ - 280 mg
5. $FeSO_4.7H_2O$ - 15 mg
6. Na_2EDTA - 20.0 mg
7. Microelements stock(1000X) - 1 ml
8. Nicotinic acid - 5.0 mg
9. Pyridoxine-HCI - 0.5 mg
10. Thiamine-HCI - 5.0 mg
11. Inositol - 1000 mg
12. Sucrose - 30 mg

The above ingredients are dissolved in distilled water and the volume made up to 1 litre.

<u>Microelements stock</u> (1000X) – 100ml contains:

1. $MnsSo_4.H_2O$ - 1000 mg
2. $ZnSo_4.H_2O$ - 100 mg
3. H_3BO_3 - 500 mg
4. KI - 100 mg
5. $Na_2MoO_4.H_2O$ - 10 mg
6. $CuSO_4.5H_2O$ - 20 mg
7. $CoCI_2.6H_2O$ - 10 mg

2. MS nutrient medium (Murashige & Skoog, 1962)

1. NH_4NO_3 - 825.00 mg
2. KNO_3 - 950.00 mg
3. $CaCl_2.2H_2O$ - 220.00 mg
4. $MgSO_4.\ 2H_2O$ - 185.00 mg
5. KH_2PO_4 - 85.00 mg
6. $FeSO_4.\ 7H_2O$ - 13.90 mg
7. Na_2EDTA - 18.65 mg
8. Microelements stock(1000X) - 1 ml
9. Nicotinic acid - 0.5 mg
10. Pyridoxine-HCl - 0.5 mg
11. Thiamine-HCl - 1 mg
12. Glycine - 2.0 mg
13. Inositol - 100.0 mg
14. Sucrose - 30 mg

The above ingredients are dissolved in distilled water and the volume made up to 1 litre.

Half-strength Microelements stock (1000X) – 100ml

1. $Mnso_4.H_2O$ - 1115.0 mg
2. $ZnSo_4.7H_2O$ - 530.0 mg
3. H_3BO_3 - 310.0 mg
4. KI - 41.5 mg
5. $Na_2MoO_4,2H_2O$ - 12.5 mg
6. $CuSO_4.5H_2O$ - 1.25 mg
7. $CoCl_2.6H_2O$ - 1.25 mg

3. Native polyacrylamid gel (PAGE) electrophoresis

A discontinuous gel was used to separate the isozymes. The composition of the separating and stacking gels are given below:

Separating gel - 10%, 15ml

1. Acrylamide : Bisacrylamide (30 : 0.8)	-	5.0 ml
2. Resolving gel buffer Stock (1.5 M Tris-Cl,pH 8.8)	-	3.8 ml
3. Distilled water	-	6.05 ml
4. 10% Ammonium persulfate (APS)	-	0.15 ml
5. TEMED	-	0.02 ml

Stacking gel – 5%, 6 ml

1. Acrylamide : Bisacrylamide (30 : 0.8)	-	1.0 ml
2. Stacking gel buffer stock (1 M Tris-Cl,pH 6.8)	-	0.75 ml
3. Distilled water	-	4.19 ml
4. 10% APS	-	0.06 ml
5. TEMED	-	0.02 ml

Electrode buffer : 0.025 M Tris, 0.192 M glycine, pH8.3

The gels were casted in Hoefer Mightly Small (Amersham Co.Pvt Ltd.) vertical electrophoresis unit and the gel was run at a constant current of 20mA/gel with the tank buffer in a cold room.

Native Sample Buffer – 4X

1. Stacking gel buffer stock (1.0M Tris-Cl, pH6.8)	-	5.0 ml
2. Glycerol	-	4.0 ml
3. Bromophenol blue	-	20 mg
Vol. made up with distilled water to		10 ml

4. Activity staining for different sod isofrms (Hemandez *et al.*, 1999)

Stain solution - 150 ml

1. 100mM Na_2 EDTA, pH 8.0	-	0.335 ml (=11.25 mg EDTA)
2. Riboflavin	-	6.0 mg
3. Nitroblue tetrazolium (NBT)	-	15 mg
4. 1 M Tris-Cl, pH 8.0	-	7.5 ml

Volume made up to 150 ml and stored in dark. Stain solution is prepared fresh

Staining procedure

1. Gels were covered with stain solution @ 75 ml/gel in plastic trays.
2. The gels were incubated in dark at 37^{o}c for 20 min.
3. The gels in the sain solution were then exposed to light until clear bands appeared against a blue back gournd.
4. The stain solution was poured off and the gels were stored in 7% acetic acid or distilled water.

5. SDS-PAGE

The composition of the separating and stacking gels are given below:

<u>Separating gel</u> - 12%, 15ml

1. Acrylamide : Bisacrylamide (30 : 0.8)	-	6.0 ml
2. Resolving gel buffer Stock (1.5 M Tris-Cl,pH 8.8)	-	3.8 ml
3. Distilled water	-	4.88 ml
4. 10% SDS	-	0.15 ml
5. 10% Ammonium persulfate (APS)	-	0.15 ml
6. TEMED	-	0.02 ml

<u>Stacking gel</u> – 5%, 6 ml

1. Acrylamide : Bisacrylamide (30 : 0.8)	-	1.0 ml
2. Stacking gel buffer stock (1 M Tris-Cl,pH 6.8)	-	0.75 ml
3. Distilled water	-	4.19 ml
4. 10% APS	-	0.06 ml
5. TEMED	-	0.02 ml

Tank buffer : 0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH8.3

The gels were casted in Hoefer Mightly Small (Amersham Co.Pvt Ltd.) vertical electrophoresis unit and were run at a constant current of 20mA/gel with the tank buffer.

SDS Sample Buffer – 2X

			Fin. Conc.
1. Stacking gel buffer stock (1,0M Tris-Cl, pH6.8)	-	5.0 ml	0.125M Tris-Cl
2. 10% SDS	-	4.0 ml	4% SDS
3. Glycerol	-	2.0 ml	20% v/v
4. Bromophenol blue	-	20 mg	0.2%
5. 2-,ercaptoethanol (added just before use)	-	0.2 ml	2 %
Volume made up with distilled water to		9.8 ml (excluding mercaptoethanol)	

5.1. Coomassie Brilliant Blue (CBB R250) stain for SDS -gel

- Methanol : glacial acetic acid solution :

Methanol	-	500 ml
Distilled water	-	400 ml
Glacial acetic acid	-	100 ml

The staining soltion is prepared by dissolving 0.25 g CBB R250 per 100 ml of methanol :. acetic acid solution.

The gels are stained by immersing them in the solution for a minimum of 4 hrs or incubated overnight on a rocking platform. For destaining, the gels the stain solution is rmoved and soaked in the methanol : acetic acid solution without the dye and incuaed for 4-8 hrs on a rocking platform, by changing the destain solution 3-4 times.

6. PVPH – medium (Tsao, and Guy, 1977)

Cornmeal	-	17 g/l
Pimaricin	-	10 ppm 10 micrograms/ml
Vancomycin Hcl	-	200 ppm (245.5 mg/100 ml)
PCNB 75% (Pentachloronitrobenzene)	-	100 ppm (146.3 mg/100 ml)
Hymexazol (Purity 94.4%)	-	50 ppm

7. Carrot Agar medium (Ribeiro, 1978)

Carrot agar medium is prepared by blending 200g corrots in distilled water nd straining the juice through muslin, and making up the volume of the juice to 1 litre. The medium is solidified with 2% agar and autoclaved after dispensing in conical flasks.

8. Modified GYP liquid for Mass multiplication of Phytophthora (Hall *et al.*, 1969)

One litre medium contains the following ingredients

L-Asparagine	-	2.0g
$FeSO_4.7H_2O$	-	0.001g
$CaCl_2.2H_2O$	-	0.010 g
$MgSO_4. 7H_2O$	-	0.10 g
KH_2PO_4	.	0.470 g
K_2HPO_4	.	0.260
Thiamine-HCl	-	0.001 g
$ZnSO_4. 7H_2O$	-	0.001 g
$CuSO_4. 5H_2O$	-	0.00002 g
$NaMoO_4.2H_2O$	-	0.00002 g
$MnCl_2.4h_2O$	-	0.00002 g
Glucose	-	30.0 g

Preparation :

A basal medium ws prepared by dissolving all the above ingredients excluding glucose in distilled water and the volume made up to 900 ml. A glucose stock was prepared by dissolving 30g glucose in distiiled water and the volume made up to 100ml. The Glucose stock and the basal medium were autoclaved separately since in the presence of asparagines, glucose formed a toxic yellow substance on autoclaving, which inhibits the fungal growth. The glucose stock was then added to the basal medium @ 10ml/90ml basal medium under sterile conditions in the laminar flow chamber.

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