BIOCHEMICAL STUDIES IN RELATION TO GLUCOSE–6–PHOSPHATE DEHYDROGENASE IN *PIPER NIGRUM* L.

Thesis submitted to the University of Calicut
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

By

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CERTIFICATE

This is to certify that the thesis entitled **"Biochemical studies in relation to Glucose-6-phosphate Dehydrogenase in *Piper nigrum* L."** submitted to the University of Calicut by Sri. Mani C.J. in partial fulfillment for the award of the degree of Doctor of Philosophy in Botany is a bonafide record of the research work carried out by him under my co-guidance. No part of the work has formed the basis for the award of any other degree or diploma previously.

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Dr. S. Nandakumar

DECLARATION

I hereby declare that the thesis entitled **"Biochemical studies in relation to Glucose-6-phosphate Dehydrogenase in *Piper nigrum* L."** submitted by me for the award of the degree of **Doctor of Philosophy** of the University of Calicut is an original research work carried out by me in the Department of Botany, University of Calicut. No part of the work has formed the basis for the award of any other degree or diploma.

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C.J. Mani

DEDICATED TO MY TEACHERS
AND
STUDENTS

INTRODUCTION

Mani C.J. “Biochemical studies in relation to glucose-6-phosphate dehydrogenase in piper nigrum l.” Thesis. Department of Botany , University of Calicut, 2004

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INTRODUCTION

CONTENTS

1.1. General account

1.1.1. *Piper nigrum* L.

Piper nigrum L. belongs to the *Piperaceae* family and it is of pantropical distribution, concentrated mainly in South Asia and South and Central America. Western Ghats of Southern India is considered as the centre of origin of *P.nigrum*, since most of the species closely related to *P.nigrum* are present there and most of them are endemic to these regions. Some of the economically important species of *Piperaceae* along with *P. nigrum* are *P. betle* L. (betel vine), cultivated extensively in India where the leaves are used for chewing; *P. longum* L. (Indian long pepper), *P. retrofactum* Vahl., *P. mullesua* Don. the fruits and roots of which are used in indigenous medicines, and *P. cubeba* L.f. (tailed pepper) used in indigenous medicines.

Black pepper (known as the king of spices, the most important and widely used spice in the world) is the dried, mature berries of *P. nigrum* L. This spice with its characteristic pungency and flavour is an ingredient in many food preparations. It was used for different purposes by different people in the past, and continues to be so currently and will remain so in future as well. For the western people it is a spice, an essential additive to their food; for the ancient Egyptians it was an ingredient in the embalming mixture; for the ancient Aryans it was a valuable drug and for the Indians, pepper is a spice as well as an important component of many Ayurvedic preparations. Black pepper was originally a wild produce in the forest. Cultivation of pepper was started much later.

1.1.2. Centers of pepper cultivation

Due to the increasing demand of black pepper, efforts to grow it have been made in many tropical countries. Initially it was cultivated in the Malabar Coast and from there it was taken to the Indonesian islands, probably by the first century A.D. or even earlier. From there, black pepper cultivation might have spread to various Pacific island nations, South East Asian countries, and later to tropical Africa and America. Now pepper is grown in about 26 countries. In India, Kerala is the original home of pepper. The state of Kerala accounts for nearly 95 percent of the total production of the country. The states of Karnataka and Tamilnadu contribute the remaining part. Small quantities of pepper are also produced in the states of Goa, Andhra Pradesh, and Orissa. Recently Andaman and Nicobar islands are recognized as a potential centre for pepper cultivation. (*c.f.*. Ravindran, 2000).

Pepper is grown as a mixed crop as well as a pure crop. The largest producer of pepper was India (*ca*. 60,000 metric ton in 1996 - 97). Recently Vietnam overtook India in the production of black pepper. In India, pepper is cultivated in about 191,426 ha. Even though India stands first in the area of cultivation of pepper, in productivity, it is the lowest in the world with just 303 kg /ha. India, Indonesia and Vietnam are the major exporters of pepper. (*c.f.* Ravindran, 2000).

The projected consumption of the world of black pepper is estimated to be around 230,000 metric tons and by the year 2020, it will be around 280,000 metric tons while the current production is approximately 180,000 metric tons. An increase in the production of around 100,000 metric tons of black pepper is required to meet the demand for the next two decades. The increasing demand can only be met by large-scale cultivation of varieties of pepper, which can withstand both biotic and abiotic stresses, including tolerance / resistance to the pests and diseases (*c.*f. Ravindran, 2000).

Over one hundred cultivars of *P. nigrum* L. have been identified so far. Many of them are on the verge of extinction due to various reasons like devastation of pepper cultivation by diseases like foot rot and slow decline and replacement of the traditional cultivars by a few high-yielding varieties. The cultivars of black pepper might have originated from the wild ones through domestication and selection.

Kerala is gifted with the richest cultivar diversity for black pepper, followed by the state of Karnataka. During the turn of the 20th century extensive plantations of tea, coffee and cardamom were established in the hilly tracts of Western Ghats. Large numbers of people migrated from the plains to these hilly areas mainly in search of agricultural land during the post-second world war period; carrying along with them, pepper cultivars. The efforts of these hard-working people were responsible for the selective spread of certain high yielding cultivars, and they became very popular in all pepper-cultivated regions (*c.f.* Ravindran, 2000).

In Kerala, the most popular cultivar is 'Karimunda'. The Indian Institute of Spices Research (IISR), located at Kozhikode, Kerala, carried out a clonal selection in this cultivar. Based on yield and quality, two lines were selected for release to farmers. These lines were named 'Sreekara' and 'Subhakara'. The yields realized from these two lines were much higher than what was obtained from the other established cultivars (Ratnambal *et al.*, 1990).

1.1.3. Diseases of black pepper

Black pepper, *P. nigrum* L. is affected by several diseases, which are caused by fungi, bacteria, virus, and mycoplasma, and by nutritional disorders. Crop losses due to diseases and pests are identified as the major causes of low productivity of black pepper in India (Sarma and Anandaraj, 1997). The earliest record of diseases of black pepper in India was that of Barber (1903). Butler (1906) also recorded the death of pepper, and Rao (1929) isolated *Phytophthora* from the diseased pepper plants. In India the major disease of black pepper causing death of the plants is the wilt disease or foot rot disease, and is caused by *Phytophthora capsici* (Sarma and Anandaraj, 1997). *Phytophthora capsici* infects all parts of the plant. The expression of symptoms depends upon the site of infection (Anandaraj and Sarma, 1995). On leaves, one or more dark spots having characteristic fimbriate advancing margins occur, which later coalesce leading to defoliation. Infection on runner shoots (stolons), occur both on the tender leaves and shoots. Infection on tender shoots upon reaching the stem, causes collar infection and results in the sudden wilting of the plant. Drying and defoliation are the results of infection on branches. Infection on spikes causes blackening of developing fruits and peduncle and in due course the affected spikes are shed. Infection on the underground parts such as collar and roots is fatal. Foot rot results in sudden wilting and death of the plants. Although extensive studies have been carried out on the epidemiology of the foot rot disease, very few studies have been made on the biochemical pathways and the enzymological aspects of both the host and the pathogen.

When plant tissues are infected by pathogens, there is an alteration of the metabolism and in particular an increase in respiration. The increased respiration is termed infection-induced respiration. Studying the alterations in the metabolism and changes in the levels of activities of the concerned enzymes of the host provides us vital information in understanding the pathogenesis-related metabolic changes (Uritani and Asahi, 1980). In general, parasitic infection induces a marked respiratory increase in the host tissues.

1.2. Pentose phosphate pathway and pathogenic infection

When plant tissues are mechanically wounded, the rate of respiration increases considerably. The phenomenon is called "wound respiration". When plant tissues are infected by pathogens a similar increase in the rate of respiration is noted. This increase is termed "infection-induced respiration". The increase in respiratory activity due to infection is much higher than in the case of mechanical wounding (Muto and Uritani, 1970).

The remarkable respiratory increase in infected tissue is the result of many biochemical events triggered by the plant-cell recognition of mechanical wounding or pathogenic infection (Uritani, 1976). The plant cells actively decompose carbohydrate to supply chemical energy in the form of ATP and to produce the precursors for the formation of secondary metabolites such as phenyl propanoids and phytoalexins (Uritani, 1976). The initial increase in respiration due to infection by the fungus usually occurs with the appearance of visible symptoms, rises to a maximal rate coincident with sporulation of the fungus and then declines. The respiratory quotient of the diseased tissue is usually about 1.0. It has frequently been suggested that respiratory increase is due to the respiration of the host and that the contribution of the parasite in pathological respiration is negligible. However, the extent of the fungal contribution is not yet clear (*c.f.* Goodman *et al.*, 1986).

The activity of the pentose phosphate pathway is more enhanced than that of glycolysis in response to mechanical wounding or parasitic infection. (*c.f.* Uritani and Asahi, 1980). This is reasonable, since pentose phosphate pathway produces the precursors of nucleic acids and phenyl propanoids, whose synthesis is greatly increased in response to mechanical wounding or pathogenic infection. Ribose-5-phosphate is required for the biosynthesis of nucleic acids and erythrose-4-phosphate is converted to numerous phenyl propanoids *via* shikimate pathway. The pentose phosphate pathway also produces NADPH, which is often required for the hydroxylation reactions involved in the formation of some phenylpropanoids (e.g., chlorogenic acid and lignin) and terpenes (e.g., some phytoalexins). The enhancement of the rate of pentose phosphate pathway is greater in infected tissues than in wounded tissues (Uritani and Akazawa, 1959; Shaw, 1963; Daly, 1976).

Activation of the pentose phosphate pathway by wounding or infection may contribute significantly to the respiratory increase, since glyceraldehyde-3- phosphate which is an intermediate of the pathway, can then be metabolised to pyruvate through glycolysis.

Plants infected by fungi, bacteria or viruses may display a common response, namely, an increase in the rate of respiration, which seems to be one of the most general phenomena in the physiology of diseased plants (Verleur, 1968). However, this is a non-specific one, as not only plant pathogens but also numerous chemicals and mechanical injury are able to induce it (*c.f.* Goodman *et al.*, 1986).

Activation of the pentose phosphate pathway is one of the most characteristic features of the metabolism of the diseased plants. It has been determined, however, that the activation of pentose phosphate pathway represents a non specific plant response that is also evoked by wounding, nitrogen starvation, excision of plant organs, viral infections and toxic chemicals (Kiraly, 1964). As might be expected, the activity of the two key enzymes of the pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, is heightened in rust, mildew diseased plants and also in potato tubers infected with *Phytophthora infestans*. Thus, the activation of the pentose phosphate pathway and also its key enzymes in the infected plants may be considered as a non-specific and generally occurring alteration of the respiratory pattern; nonetheless, it is a response by the plant to infection (*c.f.* Goodman *et al.*, 1986).

1.3. Enzymes of the pentose phosphate pathway

The pentose phosphate pathway is a very complex and multifunctional pathway. It is considered that the primary function of pentose phosphate pathway is to supply NADPH for the reductive biosynthetic reactions in the cytoplasm. Other important functions are the provision of pentose phosphates, particularly ribose-5-phosphate for nucleotide and nucleic acid biosynthesis and the production of erythrose-4- phosphate, which can lead to shikimic acid and aromatic amino acids. The requirements of the cell for NADPH and the other products will vary during situations like mechanical injury and pathogenic infection. The first two enzymes, namely, glucose-6-phosphate dehydrogenase and 6- posphogluconate dehydrogenase, called, as the shunt dehydrogenases are very important. Activation of the pentose phosphate pathway in both wounded and infected tissues is due to an increase in the amounts of the enzymes involved (Scott *et al.,* 1964).

1.3.1. Glucose -6- phosphate dehydrogenase, (EC.1.1.1.49)

The reaction catalysed by this enzyme leads to the formation of 6-phospho gluconolactone. 6-phosphogluconolactonase hydrolyzes this lactone, although the non-enzymatic hydrolysis of 6-phosphogluconolactone is relatively rapid.

D-Glucose-6-phosphate + $NADP^+$ $\leftrightarrows$ 6-Phospho-D-gluconolactone + NADPH + H^+ (1)

6-Phospho-D-gluconolactone+ H_2O $\rightarrow$ 6-Phospho-D-gluconate (2)

The overall reaction is not reversible.

There is evidence that glucose-6-phosphate dehydrogenase in higher plants is under coarse control. Muto *et al.,* (1969), have found a marked increase in the activity of the enzyme in sliced sweet potato root during aerobic incubation. The activity of 6-phosphogluconate dehydrogenase also rose. In pea root, glucose-6-phosphate dehydrogenase increased relative to phosphofructokinase, with distance from the apex (Fowler and ap Rees, 1970). There were indications that pentose phosphate pathway activity increased simultaneously (*c.f.* Uritani and Asahi, 1980).

It is likely that the enzyme is also under fine control. Using the 290-fold purified sweet potato glucose-6-phosphate dehydrogenase, Muto and Uritani (1970) observed negative cooperativity with the substrate glucose-6-phosphate. NADPH was a competitive inhibitor (with $NADP^+$), when glucose-6-phosphate was saturating (Muto and Uritani, 1972). Ashihara and Komamine (1976), purified glucose-6-phosphate dehydrogenase 160-fold from the hypocotyles of *Phaseolus mungo* seedlings. NADPH inhibited the enzyme and the degree of inhibition was decreased by an increase in pH. The saturation curve with $NADP^+$ was of Michaelis-Menten type but became sigmoidal in the presence of NADPH. There seems little doubt that the pentose phosphate pathway will be regulated at the glucose-6-phosphate dehydrogenase step. The changes in the amount of glucose-6-phosphate dehydrogenase in higher plants represent a coarse control of the enzyme activity (i.e. regulation by induction and repression of enzyme synthesis). A fine control (i.e. regulation by activation and inhibition of enzyme activity) will bring about more rapid variation in enzyme activity. The key of this fine control appears to be NADPH and it is probable that glucose-6-phosphate dehydrogenase activity is determined by the NADPH / NADP$^+$ ratio (*c.f.* Turner and Turner 1980)

1.3.1.1. Substrate specificity of glucose-6-phosphate dehydrogenase

Olive *et al., (*1971) have stressed that glucose-6-phosphate dehydrogenase from various organisms can be distinguished on the basis of the nucleotide (co-enzyme) specificity. The first group, the enzyme from yeast and *Escherichia coli*, reacts exclusively with NADP$^+$; the second group, the enzyme from animal sources, reacts with NADP$^+$ mainly, but also have weak activity with NAD$^+$; the third group, including the enzymes from *Leuconostoc*, *Pseudomonas, Hydrogenomonas, and Thiobacillus* can react approximately equally well with NAD$^+$ and NADP$^+$. For the plant enzymes, Mayer *et al.*, (1966), reported that lettuce glucose-6-phosphate dehydrogenase could react with both NAD$^+$ and NADP$^+$. NADP$^+$ specific glucose-6-phosphate dehydrogenase has also been found in bamboo (Higuchi and Shimada, 1967), and sweet potato (Muto *et al.,* 1969). Speer (1974) reported that glucose-6-phosphate dehydrogenase from lettuce ('Grand Rapids'), the same variety as used by Mayer *et al.,* is NADP$^+$ specific and this cannot be replaced by NAD$^+$. Ashihara and Komomine

(1976) reported that glucose-6-phosphate dehydrogenase from some plant materials (including lettuce seedlings) react exclusively with NADP$^+$. These results led to the conclusion that almost all plant glucose-6-phosphate dehydrogenases are NADP$^+$ specific.

1.3.1.2. Sub unit nature of glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase is usually an active 200-230 kDa homotetramer of 50-60 kDa subunits, which disaggregates into lower activity-dimers or monomers under high reducing-power levels (Wright *et al.*, 1997). Esposito *et al.*, (2001) in their studies on the kinetic properties and localization of the isoforms of glucose-6-phosphate dehydrogenase in barley roots reported a subunit molecular weight of 57.5 ± 1.2 kDa for glucose-6-phosphate dehydrogenase II and 52.1 ± 1.3 kDa for glucose-6-phosphate dehydrogenase I.

1.3.1.3. Glucose- 6- phosphate dehydrogenase and stresses

Many reports suggest that the activity of glucose -6- phosphate dehydrogenase changes as a response to various stresses like water stress (Ford and Simon, 1972; Tsai, 1973; Sheoran *et al.,* 1988; Argandona and Pahlic, 1991), temperature stress (Onyia and Gahan, 1985; Kuroda and Sagisaka, 1998; Fukuoka and Enomoto, 2001), chemical stress (Peterkova, 1978; Decleire and Roey, 1979; Barchietto *et al.,* 1992; Kwok and Shetty, 1998; Shetty *et al.,* 2001)), salt stress (Ritambhara *et al.,* 1995; Misra and Dwivedi, 1995; Ashihara *et al.,* 1997), metal stress (Assche *et al.,* 1988;

Usha Keshan *and* Mukherji, 1995; Slaski *et al.*, 1996; Lagriffoul *et al.*, 1998; Mocquot *et al.*, 1996; Samantaray *et al.*, 1998) and oxygen stress (Ivanov *et al.*, 1989; Meillon *et al.*, 1990; Sehmer *et al.*, 1998).

1.3.1.4. Glucose-6-phosphate dehydrogenase and wounds

Wound respiration is induced when plant tissues, especially plant storage tissues such as tuberous roots are mechanically cut into slices and incubated under conditions of moderate temperature and high humidity (Kahl, 1974).

Gerbrandy and Verleur (1975) observed two forms of glucose-6-phosphate dehydrogenase and noted that wounding of potato tubers enhance the relative activity of the slower moving isoform.

Gahan *et al.*, (1997) in their studies on wounded roots of peas, and cotyledons of *Solanum aviculare* demonstrated a sharp increase in glucose-6-phosphate dehydrogenase activity.

1.3.1.5. Glucose-6-phosphate dehydrogenase and infection

Glucose-6-phosphate dehydrogenase, the first enzyme of the pentose phosphate pathway is a key enzyme in the regulation of this pathway. It is well established that the activities of glucose-6-phosphate dehydrogenase and also 6-phosphogluconate dehydrogenase (EC.1.1.1.43), are elevated considerably as a response to the pathogenic infection. Activation of pentose phosphate pathway in both wounded and infected tissue is due to an increase in the amount of the enzymes involved. Thus the increased activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are due to increases in the amounts of these enzymes (Scott *et al.,* 1964). It has been found that an injury (either mechanical or pathogenic) results in a marked localized increase in the activity of glucose-6-phosphate dehydrogenase, but not glucose phosphate isomerase, indicating diversion of glucose breakdown from glycolysis to the pentose phosphate pathway (*c.f.* Palmer, 2001).

Wu (1973) studied the cell free extracts from the seeds of *Phaseolus aureus* after two hours exposure to *Rhizoctonia solani* and reported an increase in the activity of glucose-6-phosphate dehydrogenase and suggested the involvement of the hexose monophosphate pahway during the early stages of pathogenesis. Increased activities of glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, hexokinase, phosphoglucomutase and phosphoglucoisomerase were reported in the tissue adjacent to the pustule, tissue between pustules and also in pustule tissue by Tschen and Chen (1974) in the primary leaves of *Phaseolus vulgaris* infected with *Uromyces phaseoli* typica. Enhancement of activity of glucose-6-phosphate dehydrogenase was observed by Schipper (1975) in *Populus tremuloides,* infected with *Hypoxylon mammatum.*

Makovcova and Sindelar (1977) studied the activity of TMV- infected tobacco plants and reported that glucose-6-phosphate dehydrogenase activity was directly related to the rate of viral multiplication. But studies in yellow dwarf virus infected rice plants showed a reverse trend. Tschen *et al.*, (1979), noted a lower activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the infected leaves than in the healthy leaves. In his studies on the changes of the metabolism of rape plants after infection with *Plasmodiophora brassicae* Wor., Linss (1977) observed a parallelism between the enhancement of the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and in the vegetative growth of the fungus.

Kabsch (1982), reported an increase in the catalytic activity of glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, during the first stages of pathogenicity in cucumber leaves infected with *Sphaerotheca fuliginea.* He also observed a decrease in glucose and fructose in the infected leaves and an increase in the phosphorylated derivatives, glucose-6-phosphate and fructose-6-phosphate. Sindelar (1986), also observed a markedly higher activity of the enzyme glucose-6-phosphate dehydrogenase in tobacco plants infected with potato virus Y, and the variation was correlated with virus multiplication.

In their studies on the responses of cultured parsley cells to elicitors from phyto-pathogenic fungi, Kombrink, and Hahlbrock (1986) observed variations in activities of three enzymes participating in primary metabolism. Glucose- 6- phosphate dehydrogenase activity was increased by the treatment with fungal elicitors. Studies on the effect of epiphytic microorgaisms on the activity of enzymes of glycolysis and hexose monophosphate pathway in the process of maize seed germination by

Churikova and Nesterenko (1986) have shown a marked increase in the activities of aldolase and glucose-6-phosphate dehydrogenase during the first 24 hours. They also observed that the activity of the enzymes in seeds germinating in the presence of microorgamisms was higher than in sterile seeds.

In a study on the effect of *Azospirillum brasilense* inoculation on enzyme activity in maize root seedlings, Fallik *et al.*, (1988) observed increased activities of the enzymes alcohol dehydrogenase, acid phosphatase, glutamine synthetase, isocitrate dehydrogenase, malate dehydrogenase, pyruvate kinase and shikimate dehydrogenase. But, *A. brasilense* inoculation did not affect the activity of phenylalanine ammonia lyase and glucose-6-phosphate dehydrogenase that are generally elevated in plants as a result of bacterial, fungal or viral infection. They suggested that *A. brasilense* did not behave as a pathogenic rhizosphere bacterium.

Resistant varieties of plants show higher activity of glucose-6-phosphate dehydrogenase than the susceptible varieties as a response to infection. Kabsch (1988) studied the enzymic changes taking place in *Cucumis sativus* infected with *Sphaerotheca fuliginea.* Fungal infection resulted in a higher activity of phosphofructokinase and glucose-6-phosphate dehydrogenase in resistant varieties than in susceptible varieties.

Long-term activation of the oxidative pentose phosphate pathway following elicitation could serve to maintain the pools of substrates necessary during activation of the shikimic acid pathway, leading to the production of defensive compounds. Norman *et al.*, (1994) treated parsley cell cultures with an elicitor from *Phytophthora megasperma* f.sp. *Glycenea,* which resulted in an immediate increase in the rate of respiration in the dark. The enhanced rate of respiration corresponded with the activation of phosphofructokinase and glucose-6-phosphate dehydrogenase, the key enzymes in the regulation of carbohydrate flow to glycolysis and oxidative pentose phosphate pathway respectively. The increased rate of activation of the two enzymes was maintained for a long time.

Argandona (1994) reported increased activities of the enzymes glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glutathione reductase in the aphid infested leaves and roots of barley and wheat. Glucose-6-phosphate dehydrogenase activity was increased about 66 per cent in leaves and 16 per cent in roots of plants infested for three days, and the activity decreased about 33 and 50 per cent in roots and leaves respectively after 9 days of infestation.

Tecsi *et al.*, (1994) observed a large stimulatory effect on the capacity for oxidative pentose phosphate pathway in the cotyledons of *Cucurbita pepo* infected by cucumber mosaic virus. The infection not only stimulated the activities of glucose-6-phosphate dehydrogenate and 6-phosphogluconate dehydrogenase but also the enzymes of glycolysis and tricarboxylic acid cycle and anapleurotic reactions.

Metabolic changes and quantitative changes in enzyme activities and metabolite pools occur during the formation of ectomycorrhizal association.

Blaudez *et al.*, (1998) studied the metabolic changes during the formation of mycorrhiza. When seedlings of *Betula pendula* were grown in the presence of *Paxillus involutus*, glucose-6-phosphate dehydrogenase activity was higher in infected roots than in non-infected roots. Changes in enzyme capacities and metabolite pools indicated that mycorrhiza formation caused a re-arrangement of the main metabolic pathways during the very early stages following contact, which might be related to the structural changes.

The activation of pentose phosphate and glycolytic pathways as a result of infection with sheath blight fungus, *Rhizoctonia solani* in genetically related resistant and susceptible rice plants were studied by Danson *et al.*, (2000). The response of the key regulatory enzymes of pentose phosphate pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were very high. The activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were increased more than two-fold in both resistant and susceptible plants. The activity was higher in resistant than in susceptible plants. They suggested that the altered carbohydrate metabolism in sheath blight infection might play an important role in modulating the response of rice plants to infection.

Georgieva *et al.*, (2000) studied the metabolic changes in tomato fruits and seeds after viral, bacterial and fungal infection. Tomato fruits and seeds were separately infected with cucumber mosaic virus, *Pseudomonas syringae* pv.*tomato* and *Botrytis cinerea*. They observed that the specific responses depend on the type of the pathogen. Glucose 6- phosphate dehydrogenase activity was enhanced as a result of viral infection.

1.3.1.6. Glucose-6-phosphate dehydrogenase and phenolics

Many experimental data in patho-physiological research support the theory that the phenolics level and the level of other secondary metabolites is higher in diseased plants than in healthy ones. In association with phenol biosynthesis, the activities of phenylalanine ammonia lyase and tyrosine ammonia lyase are enhanced. Both enzymes are involved in the deamination of aromatic amino acids and, therefore, in the synthesis of phenolics. The enhanced aromatic biosynthesis probably resulted from the accompanying tissue necrosis. It must be stressed, however, that the enhanced aromatic biosynthesis and the stimulated enzyme activities are characteristic not only of infection but also of injury and accelerated ageing. These responses are, therefore, not specific for infection-based injury (*c.f.* Goodman *et al.,* 1986).

Yamauchi *et al.,* (1978) studied the effect of ascorbic acid on the metabolism of phenolic compounds in the seeds of capsicum associated with chilling injury. They observed an increase in the contents of phenols and ortho-diphenols, which was consistent with the enhanced activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and shikimate dehydrogenase.

Elicitors from pathogenic fungi induce the formation of coumarin derivatives (phytoalexins). In their study on the responses of cultured parsley cells to fungal elicitors, Kombrink and Hahlbrock (1986) observed the formation of phytoalexins of coumarin derivatives when treated with elicitors from several different phyto-pathogenic fungi. The activities of two hydrolytic enzymes, chitinase and 1,3-betaglucanase increased strongly in elicitor-treated cells. The activities of three enzymes participating in the primary metabolism, glucose-6-phosphate dehydrogenase, phosphofructokinase and fructose-6-phosphate phosphotransferase were affected differently. The activity of glucose-6-phosphate dehydrogenase was increased, that of phosphofructokinase remained almost constant, while the activity of fructose-6-phosphate phosphotransferase declined drastically.

A correlation between high polyphenol content and increased activity of glucose-6-phosphate dehydrogenase in the rejuvenated shoots of walnut was observed by Jay Allemand and Drouet (1989).

The relation between the enhanced activity of glucose-6-phosphate dehydrogenase and increased synthesis of phenolic compounds was demonstrated by Moskova *et al.,* (1993). When high yielding 'Vrana 123' and relatively low yielding 'Antei' tomato cultivars were treated with phenylalanine, tyrosine, sodium pyruvate and sucrose, all at 10^{-3} M for 48 hours, the varieties differed appreciably in their capacity to utilize these compounds. Vrana 123 used phenylalanine and tyrosine mainly for the synthesis of soluble proteins, while 'Antei' used them to increase the synthesis of total phenols and chlorogenic acid. The treatment inhibited glutamate dehydrogenase activity in Vrana 123, but stimulated it in 'Antei'. 'Antei' used sodium pyruvate *via* an increase in glutamate dehydrogenase and glucose-6-phosphate dehydrogenase activity and content of phenolic compounds and chlorogenic acid, while Vrana 123 showed lower or unchanged values in these cases after treatment.

The correlation between enhanced glucose-6-phosphate dehydrogenase activity and increased content of total phenolics was reported by Kwok and Shetty (1998) in shoot clones of *Thymus vulgaris* L. When proline analogues like hydroxyproline and azetidine-2-carboxylate were supplied exogenously to shoot cultures of *Thymus vulgaris* L., proline synthesis was promoted which activated the pentose phosphate pathway and the phenyl propanoid pathway.

Shetty *et al.,* (2001) reported an enhancement of total phenolic, L-DOPA, and proline content in germinting fava bean *Vicia faba* in response to bacterial elicitors. Food grade elicitor, gellan gum, a polysaccharide from *Pseudomonas elodea* stimulated the glucose-6-phosphate dehydrogenase activity and also the synthesis of total phenolics and proline content. The glucose-6-phosphate dehydrogenase activity was higher upto the 3rd day in seeds treated with gellan gum and decreased after the 3rd day of treatment. There was an increase in the phenolic and proline contents. This increase was negatively correlated with glucose-6-phosphate dehydrogenase activity. They suggested that the precursors produced by the glucose-6-phosphate dehydrogenase in the early periods of treatment might have acted as feed back inhibitors for glucose-6-phosphate dehydrogenase and this might have resulted in the decrease in its activity. At later stages the increase in phenolic and proline contents may be due to the utilization of the precursors.

1.3.1.7. Glucose-6-phosphate dehydrogenase and proline

The link between pentose phosphate pathway and proline synthesis / accumulation as well as the correlation between the enhancements of glucose-6-phosphate dehydrogenase and proline accumulation is well established. Free proline accumulates in plants in response to a wide range of biotic and abiotic stresses like water stress, salinity, temperature, pathogenic infection, pollution and nutrient deficiency / toxicity.

An increase in proline content as a response to infection by *Alternaria cucumerina* in the susceptible variety of watermelon was reported by Chopra and Jhooty (1974). Raggi *et al.,* (1974) studied the changes in the total free amino acids, and proline contents in stems and leaves of susceptible and resistant varieties of tomato infected with *Fusarium oxysporum.* There was an increase in the content of valine, isoleucine, leucine and phenylalnine and in the most advanced stages of infection, histidine, glycine, and proline. Rudgard and Wheeler (1985) reported an increase in proline concentration in field - grown Brussels sprouts due to infection by *Erysiphe cruciferarum.*

Based on their studies on the control of proline accumulation in senescing leaf discs in *Vigna unguiculata* L., Swamy *et al.,* (1989) suggested that the availability of NADPH produced during pentose phosphate pathway could be a limiting factor for proline biosynthesis. A direct relationship between proline content and glucose-6-phosphate dehydrogenase activity was established by Argandona and Pahlich (1991). Free proline accumulation in response to infection by *Colletotrichum falcatum* in sugarcane was reported by Sinha *et al.,* (1984) and Singh *et al.*, (1993). Free proline accumulation may also occur as a result of the effect of toxins produced by pathogens. Bandurska *et al.,* (1994) observed an increase in free proline in the seedling leaves of Polish winter wheat cultivars as a response to application of vomitoxin (deoxynivalenol) , a toxic metabolite produced by *Fusarium culmorum*.

Oncel *et al.,* (1996) reported an increase in proline content in the resistant cultivars of *Capsicum annuum* L. as a response to the infection caused by *Phytophthora capsici.* They have observed that the proline accumulation was lesser in the roots, stems and leaves of susceptible genotypes when compared to the resistant genotypes. El Rahim *et al.,* (1998) compared proline accumulation in *Zea mays* infected with *Cephalosporium maydia* under water stressed conditions. The leaves of diseased plants had a higher content of proline than those of non-stressed healthy plants.

Many workers also reported formation of proline rich proteins as a response to infection by pathogens. Millar *et al.,* (1992) isolated a major cell wall glycoprotein rich in proline from the cultured *Phaseolus vulgaris.* The protein itself was bound to the growing hyphal tips of the pathogen *Colletotrichum lindemuthianum.* While studying the effects of alfalfa mosaic virus infection on the expression of stress responsive genes in bean, Zhang and Chai (1998) discovered a proline rich protein along with other pathogenesis-related proteins like dehydrin and polyubiquitin. They suggested that these proteins play an important role in protecting the plant from the virus.

Chai and Zhang (1999) studied the effect of various biotic and abiotic stresses in *Phaseolus vulgaris.* They reported the formation of a stress related proline rich protein as a response to virus infection and various abiotic stresses. The gene coding this protein, PvSR1 (*Phaseolus vulgaris* stress related protein) was expressed in the primary leaf tissue of bean. They suggested that PvSR1 genes might play an important role during the stress, in maintaining cellular integrity by forming strong linkage with the cell wall.

1.3.1.8. Isoenzymes of Glucose-6-phosphate dehydrogenase and their significance

Schnarrenberger *et al.,* (1973) reported two isoenzymes of glucose-6-phosphate dehydrogenase in spinach leaves, one in chloroplasts and one in the cytosol. This suggests that leaf cells have a pentose phosphate pathway in the chloroplasts as well as in the cytosol. It is generally accepted that the two isoforms play different roles in plant metabolism. According to Dennis *et al.,* (1997), the cytosolic enzyme is involved in the production of precursors for nucleic acid biosynthesis and the plastid glucose-6-phosphate dehydrogenase plays a major part in the production of reducing power for nitrite reduction and fatty acid biosynthesis; hence, glucose-6-phosphate dehydrogenase might be the main site of reducing power for glutamine oxoglutarate aminotransferase (GOGAT) in nonphotosynthetic tissues (Bowsher *et al.,* 1992).

Changes in the protein profile and isozyme patterns due to the infection by *Monochaetia mali* in apple fruits were studied by Naik and Powell (1973). Even though there were no changes in the total protein bands from infected and non-infected fruits regardless of maturity, the isozyme patterns exhibited changes. The number of polyphenol oxidase and malate dehydrogenase bands increased in the infected mature fruits. They also reported an additional band for both glucose-6-phosphate dehydrogenase and acid phosphatase in infected fruits.

Hadacova (1974) reported five glucose-6-phosphate dehydrogenase isoenzymes with activity differences in the root growth zones of *Vicia faba*. The specific activity of glucose-6-phosphate dehydrogenase increased with cell maturation and differentiation. He also observed that the changes in the total activity of glucose-6-phosphate dehydrogenase were not associated with the change in the number of glucose-6-phosphate dehydrogenase isoenzymes.

While studying the effect of wounding on glucose-6-phosphate dehydrogenase in the tuber tissue of *Solanum tuberosum,* Gerbrandy and

Verleur (1975) observed two forms of glucose-6-phosphate dehydrogenase, and wounding of tuber tissue enhanced the relative activity of the slower moving enzyme.

Hoover *et al.*, (1977) identified four isoenzymes of glucose-6-phosphate dehydrogenase in the preparations from tobacco suspension cultures. These enzymes were named glucose-6-phosphate dehydrogenase I (G6PDH I), glucose-6-phosphate dehydrogenase II (G6PDH II), glucose-6-phosphate dehydrogenase III (G6PDH III), and glucose-6-phosphate dehydrogenase IV (G6PDH IV). They have studied the kinetic properties of the two distinct isoenzymes out of the four forms namely glucose-6-phosphate dehydrogenase I and glucose-6-phosphate dehydrogenase IV in detail. Based on their studies they have suggested that the glucose-6-phosphate dehydrogenase I with a molecular weight of 85,000 to 91,000 contained no subunit structure and glucose-6-phosphate dehydrogenase IV had a dimeric structure with a subunit molecular weight of 54,000 to 59,000 and a native molecular weight of 115,000.

Herbert *et al.,* (1979) separated the isoenzymes each of glucose phosphate isomerase, phosphoglucomutase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from 13 species, which included C_3, C_4, and CAM plants and green algae. They concluded that the isoforms of the four enzymes investigated were present in both chloroplast and cytosol in C_3, C_4, and CAM plants and green algae.

The relations between the multiple forms of some dehydrogenases like, lactate, malate, and alcohol, glutamate and glucose-6-phosphate dehydrogenase and polyploidy was studied by Talyshinskii (1980) in seeds of mulberry polyploids. He observed that changes in the number, pattern and electrophoretic mobility of enzymes occurred with increasing ploidy, but the increase in enzyme activity was not proportional to the increase in ploidy.

Valenti *et al.,* (1984) purified the two different forms of glucose -6-phosphate dehydrogenase from etiolated and green leaves of 6-day old maize seedlings. They have determined the subunit molecular weight as 54,000. The active protein had a molecular weight of 114,000, which doubled to MW 209,000 in the presence of $NADP^+$. They also observed that the association behaviour of enzyme from green leaves was similar, and the MW of the catalytically active protein was also similar to the form of etiolated leaves.

In a study on the isoenzymes of glucose-6-phosphate dehydrogenase from the plant fraction of soybean nodules, Hong and Copeland (1991) separated two isoenzymes of glucose-6-phosphate dehydrogenase from the plant fraction of soybean nodules by $(NH_4)_2SO_4$ gradient fractionation, chromatography, chromatofocussing and affinity chromatography. The two isoforms, glucose-6-phosphate dehydrogenase I and glucose-6-phosphate dehydrogenase II were specific for $NADP^+$ and glucose-6-phosphate, and had an optimum activity at pH 8.5 and 8.1 respectively. The apparent molecular weight was estimated to be approximately 110,000 in the absence of $NADP^+$ and double this size in the presence of $NADP^+$. They have also determined the Km values for both the substrates.

Huppe and Turpin (1996) reported novel isoforms in *Chlamydomonas reinhardtii* during growth on nitrate. They observed that the extractable glucose-6-phosphate dehydrogenase activity was higher from nitrogen limited *Chlamydomonas reinhardtii* cells than from nitrogen sufficient cells. Native gel electrophoresis revealed that the isoform complexity varied depending upon the form of nitrogen supplied. The isoforms associated with NO_3^- growth appeared within two hours of switching cells from NH_4^+ to NO_3^-. They have suggested that the novel isoforms may represent specific isoenzymes of glucose-6-phosphate dehydrogenase or the formation of a complex of the glucose-6-phosphate dehydrogenase species already present.

Sindelarova and Sindelar (1991) determined the subcellular localization of glucose-6-phosphate dehydrogenase isoenzymes in mesophyll protoplasts of tobacco. Chloroplastic and cytosolic proteins were determined by differential centrifugation. They obtained 16.8 ± 2.1 % of the total activity of glucose-6-phosphate dehydrogenase in the chloroplast fraction and rest in the cytosolic fraction.

Isoenzymes of glucose-6-phosphate dehydrogenase can be effectively used to distinguish certain species of pathogenic fungi, for example, *Aphamomyces chladogamus*, from other species. While studying the soil- borne root pathogens of spinach in southern Sweden, Larsson

(1994) observed that *Aphanomyces chladogamus* could be distinguished from other species by the analysis of isoenzymes, particularly glucose-6-phosphate dehydrogenase and malate dehydrogenase.

Baes and van Cutsem (1993) analysed eleven enzymatic systems of chicory by native polyacrylamide gel electrophoresis and suggested that these enzyme systems could be used as biochemical markers in breeding works. The eleven enzyme systems included glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. In a similar experiment Metz and Hymowitz (1993) identified the potential use of glucose-6-phosphate dehydrogenase isozymes as markers in hybridization experiments. Leaf materials of F1 hybrids of *Glycine max* x *Glycine tomentella*, and BC1 backcrosses with *G. max* were screened initially using 19 isozymes. Six enzyme systems out of this 19 were identified as potentially useful markers, particularly malate dehydrogenase, glucose-6-phosphate dehydrogenase and phosphoglucomutase whose banding patterns allowed the clear identification of hybrids.

The isoenzyme characters can be used to identify the isolates of certain pathogenic fungi like *Phytophthora citrophthora.* Mchau and Coffey (1994) conducted an integrated study of morphological and isoenzyme patterns found within a worldwide collection of *Phytophthora citrophthora.* A total of 14 enzyme systems were used to compare the isolates. Based on the isoenzyme patterns, the species *Phytophthora citrophthora* was separated into three distinct isoenzyme sub-groups such as CTR 1, CTR 2, and CTR 3. The migration patterns for both glucose-6-phosphate dehydrogenase and hexokinase were highly useful in differentiating the sub-group CTR 3.

The isoenzymes of glucose-6-phosphate dehydrogenase are valuable in chemotaxonomic investigations in *Vitis vinifera* L. Scienza *et al.,* (1994) screened extensively the seed proteins and isoenzymes of various enzymes in the cultivars of *Vitis vinifera* L. subspecies *sativa* and *sylvestris*, from several Italian localities. The results showed that only patterns of storage protein sub-units, isoenzymes of acid phosphatase, esterase and glucose-6-phosphate dehydrogenase were informative in the chemotaxonomic investigations.

Ammonium metabolism can stimulate the activity of glucose-6-phosphate dehydrogenase. Esposito *et al.*, (1998), reported two isoforms of glucose-6-phosphate dehydrogenase and the activity of one of which was increased by the supply of ammonium in barley.

In their study Esposito *et al.,* (2001), separated and purified two isoforms of glucose-6-phosphate dehydrogenase from barley roots by anion chromatography. The two glucose-6-phosphate dehydrogenase (glucose-6-phosphate dehydrogenase I and glucose-6-phosphate dehydrogenase II) enzymes purified from barley roots showed different molecular weights of the sub-units, 52.1 kDa for glucose-6-phosphate dehydrogenase I and 57.5 kDa for glucose-6-phosphate dehydrogenase II, and suggested that each enzyme is a tetramer with molecular weights of 208.4 kDa and 230 kDa respectively. They have also shown that 17 per cent of the total activity could be ascribed to glucose-6-phosphate dehydrogenase I, which is suspected to be plastidic. Based on the kinetic data they have suggested plastid localization for glucose-6-phosphate dehydrogenase I, and a cytosolic localization for glucose-6-phosphate dehydrogenase II.

Isoenzymic studies can also be used in the identification of cultivar of yams (*Dioscorea cayenensis* / *Dioscorea rotundata* complex). Mignouna et al., (2002) proposed a key for the cultivar groups by employing six enzymatic systems (glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase, aspartate aminotransferase, shikimate dehydrogenase, phosphoglucomutase and esterase). They have reported that the clustering of accessions of yams based on isoenzyme data was very close to the one obtained using morphological characters.

1.4. 6-Phosphogluconate dehydrogenase and infection

The activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase is heightened in plant diseases caused by pathogenic fungi like rust and mildew fungi. Activation of the pentose phosphate pathway in both wounded and infected tissues is due to an increase in the amounts of these enzymes. In most of the instances when glucose-6-phosphate dehydrogenase is activated as a result of pathogenic infection there is a concomitant activation of the 6-phosphogluconate dehydrogenase. This is reasonable because, 6-phosphogluconate dehydrogenase acts on the product of glucose-6-phosphate dehydrogenase reaction.

Tschen and Chen (1974) reported an increase in activity of 6-phosphogluconate dehydrogenase along with the glucose-6-phosphate dehydrogenase as a response to infection by *Uromyces phaseoli* typica, in the leaf tissues of bean.

Linss (1977), observed increased activity of 6-phosphogluconate dehydrogenase in rape roots infected with *Plasmodiophora brassicae* Wor. Noel and Mc Clure (1978) reported greater specific activities of the multiple forms of 6-phosphogluconate dehydrogenase and peroxidase in cotton roots infected with the nematode *Meloidogyne incognita* than in the uninfected roots. A reverse trend was noticed in the virus-infected leaves of rice. Tschen *et al.*, (1979) reported that, when symptoms of yellow dwarf virus appeared on the first leaf, the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucomutase and phosphogluco isomerase were lower than in healthy leaves. They suggested that the pentose phosphate pathway had not increased in infected plants.

Kabsch (1982) reported an increase in the catalytic activity of glucose-6-phosphate dehydrogenase accompanied with that of 6-phosphogluconate dehydrogenase in the first stages of pathogenicity caused by *Sphaerotheca fuliginea* in the cucumber leaves.

Danson *et al.*, (2000) studied the activation of pentose phosphate and glycolytic pathways as a result of infection with sheath blight fungus, *Rhizoctonia solani* in genetically related resistant and susceptible rice plants. The responses of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were very high. The activity was higher in resistant than in susceptible plants. The activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase increased more than two-fold in both resistant and susceptible plants.

1.5. Objectives of the present study

The Objectives of the present study are to investigate:

1. The properties of the key enzyme of pentose phosphate pathway, glucose-6-phosphate dehydrogenase in healthy plants,
2. The changes in the levels of activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase-the first two enzymes of pentose phosphate pathway-as a result of infection,
3. The changes in the levels of total phenolics, total free amino acids, and total free proline during infection, and
4. The multiple forms of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the control and infected plants.

The results obtained from the present study might serve as a valuable indictor to link primary metabolism with secondary metabolism during the process of infection. A better understanding of the various biochemical phenomena occurring during the course of infection will be valuable in devising possible methods of plant protection and, improvement of plants, tolerant or resistant to pathogens. The alterations of metabolism under pathological conditions provide opportunities for the evaluation of various fundamental biochemical principles.

MATERIALS AND METHODS

Mani C.J. “Biochemical studies in relation to glucose-6-phosphate dehydrogenase in piper nigrum l.” Thesis. Department of Botany , University of Calicut, 2004

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MATERIALS AND METHODS

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2.1. General

2.1.1. Propagation of experimental plants

For all the experiments outlined below, *Piper nigrum* L. 'Sreekara' was used. Authentic plants were obtained from the Indian Institute of Spices Research (IISR), Kozhikode, Kerala, India. An efficient technique of propagation called rapid multiplication technique (also called as bamboo-method) was adopted in the present study.

In this method, a long trench of 60 cm deep and 40 cm wide was made. The trench was filled with rooting medium containing soil, sand and dried powdered cow dung in the ratio 1:1:1. Split halves of bamboo with septa having 1.25 to 1.5 m length and 8-10 cm diameter were fixed at 45° angle on a strong support. The bamboo splits were arranged touching one another. Rooted cuttings of pepper plants were planted in the trench, one each per bamboo split. The lower portion of the bamboo splits were filled with the rooting medium composed of weathered coir dust and dried powdered cow dung in the ratio 1:1. The growing vines were tied to the bamboo splits with jute threads. The vines were irrigated well. As the vines grew up, the remaining portions of the bamboo splits were filled up with the rooting medium. Rooting occurred at every node. When the vine reached the top, the entire vine was removed from the rooting medium and the vine was cut into numerous pieces in between each node. The nodal cuttings with the bunch of roots were planted in polyethylene bags filled with potting mixture composed of soil, sand and dried powdered cow dung in the ratio 1:1:1. The polyethylene bags with cuttings were kept in a cool humid place. The buds started developing in about three weeks and the polyethylene bags were transferred to an area covered with 60 % shade net. Three-month-old plants of uniform growth with 5-6 leaves were used for all the experiments. The third leaf from the tip that was of medium maturity was regularly used for the analyses.

2.1.2. Selection of tissue.

Enzymic studies were carried out in the leaf tissue. The presence of glucose-6-phosphate dehydrogenase was also confirmed in stem and root tissue (data not presented) of *Piper nigrum*. The leaf samples were collected between 9 and 10 am every time. Care was taken to prevent the loss of moisture by keeping the samples immediately on collection in polyethylene bags and storing in thermocol box containing cracked ice. Enzyme extraction was conducted within 10 minutes after the collection of samples. The leaves were washed in tap water followed by distilled water and blotted with filter paper to remove the adhering water. The petiole was removed and the tissue was cut into small pieces and was randomized. The required quantity of tissue was accurately weighed in a chemical balance.

2.2. Studies on glucose-6-phosphate dehydrogenase in the leaves of *P. nigrum*

2.2.1. Standardization of the homogenizing medium

2.2.1.1. Tissue homogenisation

A 5.0 % (w/v) homogenate was usually prepared. 10 % (w/v) homogenates were prepared for studies on enzyme purification and electrophoresis. *P. nigrum* is rich in phenolics. Crude tissue homogenates without the supplements such as 2-mercaptoethanol and ethylenediamine tetraacetate showed 'browning' due to phenolic oxidation. Hence the following experiments were carried out.

2.2.1.2. Medium for homogenization

For the preliminary experiments, to standardize the homogenizing medium in order to minimize the effect of phenolics on enzyme activity measurements, an ice-cold basal medium consisting of 50 mM Tris-HCl buffer pH 7.5, 20 mM 2-mercaptoethanol, 50 mg polyvinyl polypyrrolidone (PVPP) / g tissue and 10 mM ethylenediamine tetraacetate (EDTA) was used. The tissue was homogenized in a pre-chilled glass mortar using a pestle. Acid-washed sand was used as an abrasive to increase the efficiency of grinding. The mortar was kept in a plastic bowl surrounded by cracked ice to prevent inactivation of the enzyme due to increase in temperature. The finely ground tissue was then squeezed through two layers of muslin and the volume of the filtrate was made up to 10 ml. All steps during extraction were conducted at 4 to 8^0 C. The filtrate was centrifuged at 5000 X *g* for 15 minutes at 0^0 C in a Kubota 20,000 T refrigerated centrifuge and the supernatant was collected.

2.2.1. 3. Sephadex G-25 Gel filtration

This step became necessary following the observation that spectrophotometric measurements of enzyme activity using the 5000 X *g* supernatants of extracts of *P. nigrum* gave very high background absorption. This was due to the presence of substances endogenously present in the supernatant, which absorbed strongly in the UV region. By the removal of the endogenous, UV -absorbing molecules by gel filtration, the assays could be rendered sensitive and hence this step was resorted to. The following is the protocol of gel filtration:

Sephadex G-25 (coarse) was allowed to swell overnight in excess double distilled water. The suspension was stirred and the fine particles were removed by successive washing and decantation. Water was drained away and the swollen gel was suspended in a medium consisting of 50 mM Tris-HCl, pH 7.5 and 10 mM 2-mercaptoethanol. After stirring and equilibration overnight in the cold, the gel was packed into a glass column, 20 cm in length and 1.8 cm in diameter. The gel was packed to a height of 10cm leaving about 10cm space above (total bed volume was about 25ml). The column was fixed vertically and the top layer of the gel was made perfectly horizontal. A filter paper disc was placed above the gel surface and the column was transferred to a refrigerator and allowed to cool. The equilibration medium above the filter paper disc was drained off by operating the stopcock. The void volume was determined using Blue Dextran. 2.0 ml of the 5000 X *g* supernatant of enzyme extract was carefully layered above the filter paper using a pipette and an equal volume of the equilibration medium was drained off. The top of the column was carefully rinsed with a small quantity of equilibration medium and the same was drained off as before. Proteins were eluted from the column using excess equilibration medium. A total effluent volume equal to the void volume was drained off at a flow rate of 0.5 ml / minute. Proteins were collected as a single fraction of 4.0 ml following the exclusion of the void volume, with one-fold dilution. The gel filtrate thus obtained which was almost colourless served as the source of enzyme for the preliminary studies.

2.2.1.4. Assay system

An assay system patterned by Hoover *et al.,* (1977), with minor modifications was initially used for preliminary standardization experiments of glucose-6-phosphate dehydrogenase. The assay system consisted of 0.5 ml 100 mM Tris-HCl buffer pH 7.9, 0.1 ml 5.0 mM $MgCl_2$, 0.2 ml enzyme, 0.1 ml 20mM glucose-6-phosphate, and 0.1 ml 2.0 mM $NADP^+$ in a total volume of 1.0 ml. The reaction was initiated with $NADP^+$. The components were mixed in a 1.0 ml quartz cuvette and the O.D at 340 nm was measured directly in a Shimadzu Model UV 1601 double beam spectrophotometer. The assay was carried out at room temperature (25 ± 1°C). The change in absorbance in 3.0 minutes was noted. Water was used as the reagent blank.

Linearity of enzyme reaction was verified by noting the O.D at 30-second interval.

2.2.1.5. Effect of additives during homogenization

The standardized medium of homogenization for *Piper nigrum* leaf tissue was formulated after evaluating the effects of different additives such as 2-mercaptoethanol, ethylenediamine tetraacetate (EDTA) and polyvinyl polypyrrolidone (PVPP) on the activity of glucose-6-phosphate dehydrogenase. For this purpose, different concentrations of the above-mentioned additives were incorporated systematically in the homogenizing medium and the enzyme activities were monitored. The optimum concentrations of 2-mercaptoethanol, EDTA, and PVPP during homogenization were found out and subsequent experiments were performed using the optimized medium.

2.2.1.6. Determination of optimum concentration of 2-mercaptoethanol in the homogenizing medium

2-mercaptoethanol has been widely used as an antioxidant / reducing agent and to prevent the oxidation of phenolics. The basal medium for homogenization in the present study for a 5.0% (w/v) homogenate consisted of 50 mM Tris HCl buffer pH 7.5, 50 mg PVPP per gram tissue, and 10 mM EDTA. This was supplemented with 2-mercaptoethanol at different final concentrations of 10 mM, 20 mM and 50 mM. High concentrations of 2-mercaptoethanol were necessary since the phenolic content of *Piper nigrum* was considerable and the tissue became brown in colour during homogenization (the concentration of 2-mercaptoethanol used in the medium of homogenization for various plant tissues cited in literature varied between 2 to 30 mM). Tissue homogenates were prepared as described earlier and activities of glucose-6-phosphate dehydrogenase were determined using the supernatant obtained after centrifugation and Sephadex G-25 gel filtration. Once the optimum concentration of 2-mercaptoethanol was determined, this was kept constant in the subsequent experiments. Concentration higher than 50 mM were not tested because the activities obtained at 20 mM and 50 mM did not show much difference.

The results are recorded in the Results (see section **3.1.1**).

2.2.1.7. Determination of optimum concentration of ethylenediamine tetraacetate in the homogenizing medium

The supplementation of EDTA in the homogenization medium has proved efficacious for complexing with divalent cations and with higher valence metal ions (the latter in general are inhibitors of enzymes). EDTA also blocks the enzymatic and / or non-enzymatic oxidation of phenolics. For determining the optimum concentration of EDTA, the basal medium supplemented with optimum concentration of 2-mercaptoethanol was further supplemented with different final concentrations of neutralized EDTA namely, 5.0 mM, 10 mM and 20 mM. The homogenate was prepared as explained earlier. Controls were also kept which did not receive any EDTA.

The results of these experiments are reported in Results (see section **3.1.2**).

2.2.1.8. Determination of optimum concentration of polyvinyl polypyrrolidone in the homogenizing medium

Polyvinyl polypyrrolidone (PVPP, or insoluble PVP) is an agent, which effectively fixes the phenols. The incorporation of PVPP in the homogenizing medium is often found to be beneficial. The basal medium supplemented with optimum concentrations of 2-mercaptoethanol and EDTA (determined from earlier experiments) was further supplemented with varying concentrations of PVPP like, 50 mg, 100 mg, 150 mg / g tissue for homogenization. Glucose-6-phosphate dehydrogenase activity was determined as described earlier and the optimum concentration of PVPP was arrived at.

The results of these experiments are reported in Results (see section **3.1.3**).

2.2.1.9. Standardized medium for homogenization

From the above experiments it was possible to formulate a medium for homogenization suitable for eliciting maximum activity of glucose-6-phosphate dehydrogenase. The optimum concentration of each additive like 2-mercaptoethanol, EDTA, and PVPP, which were determined separately were kept constant and incorporated into the basal Tris-HCl buffer medium.

2.2.2. Stadardization of the assay system for glucose-6-phosphate dehydrogenase in the leaves of *P. nigrum*

2.2.2.1. Enzyme activity substrate concentration relationship

The activity of glucose-6-phosphate dehydrogenase at different concentrations of both the substrates was tested.

2.2.2.1.1. NADP$^+$

Final NADP$^+$ concentrations in the assay system ranging from 0.1mM to 0.6 mM (0.1mM, 0.2, 0.3, 0.4, 0.5, and 0.6 mM) at a fixed concentration of 2.0 mM glucose-6-phosphate were employed for determining the relationship between the enzyme activity and substrate concentration. Activity measurements were carried out for six minutes for each assay and substrate saturation curve for NADP$^+$ was plotted and the concentration of NADP$^+$ at which maximum velocity was obtained was determined. This saturating concentration was employed in the subsequent experiments.

2.2.2.1.2. Glucose-6-phosphate

Final concentrations glucose-6-phosphate in the assay system ranging from1.0 mM to 6.0 mM (1.0 mM, 2.0, 3.0, 4.0, 5.0, and 6.0 mM) at a fixed concentration of 0.2 mM NADP$^+$ were employed for determining the relationship between the enzyme activity and substrate concentration. Activity measurements were carried out for six minutes for each assay and substrate saturation curve for glucose-6-phosphate was plotted and the concentration of glucose-6-phosphate at which maximum velocity was obtained was determined. This saturating concentration of glucose-6-phosphate was employed in the subsequent experiments.

2.2.2.2. Effect of Mg^{2+}

Many reports point to the fact that Mg^{2+} activate glucose-6-phosphate dehydrogenase. The inclusion of Mg^{2+} in the assay system is reported to be effective by many workers (Ashihara and Komamine, 1976, Hoover *et al.,* 1977, Esposito *et al.,* 2001). The optimum concentration of Mg^{2+} was determined by the systematic incorporation of Mg^{2+} in the form of $MgCl_2$ in different concentrations to the basal assay system which consisted of 0.3 ml 500 mM Tris-HCl, pH 8.1, 0.2 ml enzyme extract, 0.1 ml 20 mM glucose-6-phosphate, and 0.1 ml 2.0 mM $NADP^+$, in a total volume of 1.0 ml. Controls were also kept which did not receive any Mg^{2+}. The reaction was initiated by the addition of $NADP^+$. The enzyme activity was determined. The optimum concentration of Mg^{2+} in the assay system was fixed and was supplied routinely in the subsequent experiments described later.

2.2.2.3. Determination of optimum pH

The optimum pH for glucose-6-phosphate dehydrogenase was determined using the Sephadex G-25 gel filtrate in the initial experiments and later confirmed by using the pooled active fractions obtained from Sephadex G-200 gel filtration. Tris-acetate buffer in the pH range 6.0 to 7.2 and Tris-HCl in the pH range 7.5 to 9.0 were used. The assay system consisted of 0.3 ml 500 mM buffer of various pH values, 0.2 ml enzyme preparation, 0.1 ml 100 mM $MgCl_2$, 0.1 ml 20 mM glucose-6-phosphate, 0.1 ml 2.0 mM $NADP^+$, and 0.2 ml water in a total volume of 1.0 ml. The reaction was initiated by $NADP^+$. The measurements of enzyme activity were done by direct spectrophotometry as explained earlier. The change in optical density in three minutes was measured. The optimum pH was determined by plotting a graph with activities on the Y-axis and pH on the X-axis.

2.2.2.4. Determination of enzyme proportionality range

Enzyme proportionality was tested at the predetermined optimum pH and at saturating concentration of substrates. The Sephadex G-25 gel filtrate in the range of 0.05 ml to 0.3 ml (0.05, 0.1, 0.15, 0.20, 0.25 and 0.30ml) was incorporated in the assay system. Measurements of activity were carried out as described earlier and a graph was plotted with velocity on Y-axis and volume of enzyme on X-axis.

2.2.2.5. Relationship between incubation period and enzyme activity

The assay was carried out at room temperature (25 ± 1° C) and using the already optimized conditions with respect to the substrates, enzyme concentration, pH and Mg^{2+}. Reaction was allowed to proceed for 10 minutes. Activities in terms of Δ O.D were measured at the end of each minute and a graph was plotted with velocity on the Y-axis and reaction time on the X- axis. The linearity relationship was studied.

2.2.2.6. Incubation temperature

During the entire course of the present study, glucose-6-phosphate dehydrogenase activities were measured at room temperature (25 ± 1^{0}C). A separate determination of the optimum temperature for the purified enzyme was also carried out (se section **2.2.5.2**)

2.2.2.7. Standardized assay system

After the standardization of optimum conditions for glucose-6-phosphate dehydrogenase activity from the leaves of *P. nigrum*, an assay system fulfilling all these parameters was finalized and followed in the subsequent studies.

The standard assay system consisted of 0.3 ml 500 mM Tris-HCl buffer pH 8.1, 0.2 ml enzyme, 0.1 ml 100 mM $MgCl_2$, 0.1 ml 20 mM glucose-6-phosphate, 0.1 ml 2.0 mM $NADP^+$, and 0.2 ml water in a total volume of 1.0 ml. The reaction was started by the addition of $NADP^+$ and the activity for 3 minutes was measured by direct spectrophotometry at room temperature.

Unless otherwise specified, the above assay system was employed for the various experiments outlined below.

2.2.2.8. Verification of endogenous activity

Since glucose-6-phosphate dehydrogenase activity was measured as change in optical density at 340 nm it was felt necessary to verify whether the Δ O. D. observed was solely due to the enzyme reaction or whether non-enzymatic and / or other endogenous reactions also contributed to the overall increase in the optical density. This was verified by withholding one or the other substrate in the assay system. The change in the optical density was measured for 3.0 min. In one experiment, only glucose-6-phosphate was added to the assay system withholding $NADP^+$ and the change in O.D. was monitored. In a similar experiment, only $NADP^+$ was supplied withholding glucose-6-phosphate. No change in O.D was noted when one or the other substrate was withheld; the reaction commenced only in the presence of both the substrates.

2.2.2.9. Substrate specificity

The specificity of the enzyme glucose-6-phosphate dehydrogenase towards $NADP^+$ was verified. This was done by substituting NAD^+ for $NADP^+$. NAD^+ was supplied at a final concentration of 0.2 mM. The assay system was the same as explained earlier. The change in optical density was insignificantly low and confirmed that $NADP^+$ could not be substituted by NAD^+.

2.2.3. Estimation of proteins

During the studies on glucose-6-phosphate dehydrogenase in healthy and diseased plants in the present investigation, the determination of protein formed a routine procedure. This was necessary for calculating the specific activity of the enzyme at every stage of the study, for calculating the fold enrichment during enzyme purification and to assess the change in protein, if any, as a result of infection. In all the studies, protein was estimated in the tissue preparation following precipitation with trichloroacetic acid (TCA). Protein estimation was essentially according to Lowry *et al.,* (1951) as modified by Khanna *et al.,* (1969).

2.2.3.1. Detailed account of protein estimation

For the estimation of protein, aliquots of samples (usually 1.0 ml) were taken in centrifuge tubes and mixed with 1.0 ml of 10 % (w/v) TCA and the mixture was kept in the cold for flocculation. After 30 minutes the suspension was centrifuged for 5.0 minutes in a Remi T8 tabletop centrifuge and the supernatant was decanted off.

The subsequent treatment of the protein precipitate was according to Khanna *et al.,* (1969). The precipitate was washed twice with 2.0 % (w/v) TCA and centrifuged. The resulting precipitate was again washed with 1.0 % TCA and centrifuged. The residue was then washed with 100% acetone and centrifuged. This was followed by washing with 80 % (v/v) acetone and centrifugation. The residue obtained after washing with 80 % acetone was suspended in a fixed volume of (usually 4.0 ml) 0.1 N NaOH and heated in a bath of boiling water for 5 min and cooled. The supernatant was used for the estimation of protein after centrifugal clarification.

The total protein in the sample was estimated by the colorimetric method of Lowry *et al.,* (1951). Suitable aliquots were drawn and made up to 1.0 ml with distilled water and 5.0 ml alkaline copper sulphate reagent was added and the tubes were kept for 10 min. Following this, 0.5 ml of 1.0 N Folin-Ciocalteu phenol reagent was added and mixed well. Colorimetric measurements were made after 30 min. The colorimeter was set to 100% transmittance with the reagent blank containing 1.0 ml distilled water, 5.0 ml alkaline copper sulphate reagent and 0.5 ml of 1.0 N Folin-Ciocalteu reagent. Colour intensity was measured at 750 nm. Bovine serum albumin (BSA) was used as the standard. A working standard containing 100 μg / ml BSA was used for this purpose.

2.2.4. Purification of glucose-6-phosphate dehydrogenase

In order to study the properties of glucose-6-phosphate dehydrogenase, an enzyme fraction which was relatively pure was necessary. Attempts were made to purify the enzyme as detailed below.

During the course of standardization studies it was found that the 5000 × *g* supernatant was light green in colour. However, by increasing the centrifugal force to 20,000 × *g* the colouration could be almost completely removed and hence this step was resorted to in the purification experiments.

The muslin cloth filtered homogenate, following centrifugal clarification at 20,000 × *g* for 20 min in a Kubota 20,000 T refrigerated centrifuge, was subjected to purification procedures such as ammonium sulphate fractionation, Sephadex G-200 gel filtration and ion exchange chromatography. After preliminary evaluation of the enrichment achieved, together with the quantitative recovery of the enzyme activity in these steps the most suitable method was adopted for the purification of glucose-6-phosphate dehydrogenase in *P.nigrum* leaf tissue.

2.2.4.1. Ammonium sulphate fractionation

One of the most versatile steps in the purification of enzymes is the 'salting out' using ammonium sulphate. The protocol of a typical experiment is explained below:

25 ml of a 10 % homogenate was prepared as described earlier. The homogenate was centrifuged in the cold at 20,000 × *g* for 20 min and the supernatant was collected. A small portion (5.0 ml) was set aside for the estimation of protein and basal enzyme activity measurements, and the bulk (20 ml) of the supernatant was taken in a beaker and kept surrounded with cracked ice. Saturated solution of ammonium sulphate (enzyme grade) was prepared and the pH was adjusted to near neutrality by the addition of ammonia. The solution was cooled in ice and measured volumes were added to the supernatant drop wise with continuous mixing by means of a magnetic stirrer, to bring the ammonium sulphate concentration to 30 % saturation. The suspension was kept in the cold to allow flocculation of proteins. It was then centrifuged at 15,000 × *g* for 15 minutes in a refrigerated centrifuge. The supernatant was collected and the precipitate (F_1) was suspended in a medium, which contained 50 mM Tris-HCl buffer, pH 7.5, and 10 mM 2-mercaptoethanol.

The supernatant was brought to 60 % ammonium sulphate saturation by the further addition of saturated neutralized ammonium sulphate solution. After centrifugation, the precipitate (F_2) was collected and suspended in the suspending medium which contained 50 mM Tris-HCl buffer, pH 7.5, 10 mM 2-mercaptoethanol. The supernatant was collected and the volume was measured. Required amount of powdered ammonium sulphate was now added to bring the ammonium sulphate concentration to 70 %. The pH during the addition of powdered ammonium sulphate was maintained around 7.0 by the addition of cold dilute ammonia solution at regular intervals. The 70 % saturated supernatant was centrifuged and the precipitate (F_3) was suspended in appropriate volume of suspending medium. The supernatant was subjected to ammonium sulphate saturation with required amount of powdered ammonium sulphate to bring the concentration to 90 %. After centrifugation the precipitate (F_4) was collected and suspended in the suspending medium. The final supernatant was discarded. All the suspensions (F_1, F_2, F_3, and F_4) were again centrifuged and only the supernatants were collected and were used for enzyme studies. Enzyme activities in the four residual fractions and in the original supernatant were determined after appropriate dilutions. The results are recorded in the **Results** (see section **3.4.1**).

2.2.4.2. Routine procedure for ammonium sulphate fractionation

From the preliminary experiments on ammonium sulphate fractionation, four saturation levels were tested for enzyme activities, namely from 0-30 %, 30-60 %, 60-70 %, and 70-90 % ammonium sulphate saturation. Of these, the 30-60 % fraction contained the bulk of activity of glucose-6-phosphate dehydrogenase. In order to recover most of the enzyme in a single fraction, but at the same time to exclude unwanted proteins, the following procedure was adopted. The ammonium sulphate saturation was brought initially to 30 % and the precipitate was discarded. The saturation was then raised to 70 % and the proteins were collected. This 30 to 70% fraction was used in the subsequent experiments.

2.2.4.3. Stability of the enzyme during storage

For the detailed studies on the various properties of the enzyme, a relatively pure enzyme preparation was necessary in adequate amounts. The multiple steps of purification procedure and the time involved could result in loss of catalytic activity of the enzyme. Therefore as a first step, the stability of the enzyme had to be verified. Attempts at stabilization of the enzyme under storage condition also had to be made. The following experiments were performed in this context.

During the initial experiments it was found that the enzyme activity diminished considerably during storage. Experiments were carried out to determine the extent of loss of activity of glucose-6-phosphate dehydrogenase during storage. The tissue was homogenized and centrifuged as explained earlier, and a small portion of the supernatant was kept aside. The remaining supernatant was subjected to fractionation and the 30-70 % saturated ammonium sulphate fraction was recovered. 2.0 ml of this was desalted by gel filtration through a Sephadex G-25 column as explained earlier and 4.0 ml was collected (F_2 gel filtrate). The original supernatant, ammonium sulphate fraction and the gel filtrate were kept surrounded by cracked ice inside a refrigerator. The zero-day activities of all the preparations were measured using the standardized assay system. The activity measurements were carried out after 24 hours and 72 hours. The loss of activity was determined.

2.2.4.4. Stabilization of the enzyme by the addition of $NADP^+$

The importance of incorporation of $NADP^+$ in the enzyme preparation to stabilize the isozymes of glucose-6-phosphate dehydrogenase was reported by many workers (Hoover *et al.,* 1977; Hong and Copeland, 1991; Esposito *et al.,* 2001).

Enzyme preparations such as 20,000 X *g* supernatant and F_2 gel filtrate were prepared as explained earlier. All the preparations were kept in a beaker surrounded by cracked ice. Enough $NADP^+$ solution was added to each tube to make the final concentration 1.0×10^{-5} M $NADP^+$. The activity was measured. The activities were measured at zero-day and after storage in the cold for 24 hours and 72 hours.

The extent of the stabilization of the enzyme by the addition of $NADP^+$ was determined.

The results are reported in **Results** (see section **3.4.3**).

2.2.4.5. Sephadex G-200 column chromatography.

The active fraction (30-70 %) obtained from ammonium sulphate fractionation was then subjected to further purification. Ion exchange chromatography using phosphocellulose was attempted but was found to be unsatisfactory since the recovery of the enzyme following chromatography was very low. Hence this step was left out. It was decided to purify the enzyme by gel filtration by using Sephadex G-200 column chromatography.

2.2.4.6. Preparation of the gel.

The procedure for the column preparation and chromatographic separation was according to Fischer (1969).

The dry Sephadex G-200 gel was allowed to swell in water at room temperature for 72 hours. After the removal of fine particles the gel was equilibrated overnight in 50 mM Tris-HCl buffer pH 7.5 containing 10 mM 2-mercaptoethanol. The gel was then packed into a glass chromatographic column (2.5 x 45 cm). The gel was packed to a height of 30 cm.

2.2.4.7. Protocol for enzyme purification.

The protocol for the purification of the glucose-6-phosphate dehydrogenase by Sepahdex G-200 is as follows:

The 20,000 × *g* supernatant (as described earlier) from a 10 % (w/v) homogenate was subjected to ammonium sulphate fractionation. The ammonium sulphate concentration was made to 30 % and allowed to remain for flocculation in the cold. This was centrifuged at 15,000 × *g* for 15 min and the supernatant was collected. The ammonium sulphate concentration was further raised to 70 % and allowed to remain in the cold for flocculation. This was again centrifuged at 15,000 × *g* for 15 min. and the residue was collected. The residue was taken up in a small volume of suspending medium consisting of 50 mM Tris-HCl buffer, pH 7.5, and 10 mM 2-mercaptoethanol and centrifuged again. The supernatant was collected. A sample of this enzyme preparation (usually 3.0 ml) was then applied to a pre-equilibrated 2.5 X 30 cm Sephadex G-200 column and eluted with 50 mM Tris-HCl buffer, pH 7.5 and 10 mM 2- mercaptoethanol at a flow rate of 1.0 ml / 4 min. The first 50 ml of the eluant was discarded as the void volume. Following this, fifteen 5.0 ml. fractions were collected. These fractions were separately used for the determination of enzyme activity and estimation of proteins. The fractions having high activities were pooled and used in the subsequent studies.

2.2.5. Kinetic studies and properties of the purified glucose-6-phosphate dehydrogenase in the leaves of *P. nigrum*

For all the studies on the properties of the purified glucose-6-phosphate dehydrogenase, the Sephadex G-200 gel filtrate was used as the source of enzyme. The standardized assay system consisted of 0.3 ml 500 mM Tris-HCl buffer pH 8.1, 0.2 ml enzyme, 0.1ml 100 mM $MgCl_2$, 0.1 ml 20 mM glucose-6-phosphate, 0.2 ml distilled water, and 0.1ml 2.0 mM $NADP^+$ in a total volume of 1.0 ml. The reaction was started with $NADP^+$. For the studies on the effects of various additives on the purified glucose-6-phosphate dehydrogenase, the assay system used contained 0.3 ml 500 mM Tris-HCl buffer pH 8.1, 0.2 ml enzyme, 0.1ml 100 mM $MgCl_2$, 0.1 ml 20 mM glucose-6-phosphate, 0.1 ml distilled water, 0.1ml additive and 0.1ml 2.0 mM $NADP^+$ in a total volume of 1.0 ml.

2.2.5.1. Determination of Michaelis-Menten constant (Km value)

2.2.5.1.1. Determination of Km value for $NADP^+$

The activities of purified glucose-6-phosphate dehydrogenase at different concentrations of $NADP^+$ in the assay system ranging from 0.02 to 0.3 mM (0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.2, and 0.3 mM) with fixed concentration of glucose-6-phosphate were measured. The standardized assay system was used. The Km value for $NADP^+$ was determined using the double-reciprocal method of Lineweaver-Burk (*c. f.* Dixon and Webb, 1979).

2.2.5.1.2. Determination of Km value for glucose-6-phosphate

The activities of purified glucose-6-phosphate dehydrogenase were measured at varying concentrations of glucose-6-phosphate in the assay system ranging from 0.1 to 2.0 mM (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 2.0, mM) at a fixed concentration of $NADP^+$ The assay was carried out using the standardized system. The Km value for glucose-6-phosphate was determined using the double-reciprocal method of Lineweaver-Burk (*c.f.* Dixon and Webb, 1979)

2.2.5.2. Optimum temperature for the purified glucose-6-phosphate dehydrogenase activity

This was tested over a temperature range of 20^0 C to 55^0 C, while keeping the other assay conditions constant. The pooled active fractions of Sephadex G-200 gel filtrate were used as the source of enzyme. The assays were carried out by incubating the system for ten minutes at different temperatures such as 20^0 C, 25^0 C, 30^0 C, 35° C, 40^0 C, 45° C, 50^0 C and 55^0 C using constant temperature water bath. At the end of the 10th minute the tubes were chilled in ice for 2 minutes and then transferred to a bath of boiling water to arrest the reaction. After 2 minutes of heating, the tubes were again kept in ice bath for 10 minutes for the flocculation of precipitated proteins. The substrate was added to the controls after arresting the reaction. The tubes were centrifuged and the absorbance was measured at 340 nm using water as reagent blank.

2.2.5.3. Effect of some phenolic compounds on the activity of glucose-6-phosphate dehydrogenase

The effect of selected phenolic compounds on the activity of glucose-6-phosphate dehydrogenase was tested using the purified enzyme preparation following Sephadex G-200 gel filtration. The procedure adopted for ammonium sulphate precipitation and gel filtration was the same as that explained earlier. The fractions were collected and tested for the enzyme activity and the most active fractions were pooled. Phenolic compounds were dissolved in the 500 mM Tris-HCl buffer pH 8.1 to get stock solutions of

100 mM each. The effect was tested at two different concentrations, namely, 0.1 mM and 0.5 mM in the assay system. Compounds that were insoluble were not tested. Some of the phenolic compounds that gave very high absorption at 0.5 mM concentration were tested at 0.1mM and 0.05mM concentrations. Appropriate volumes of the solutions of phenolic compounds were added to the standardized assay system and pre-incubated for five minutes. At the end of the 5th minute 0.1ml 2.0 mM $NADP^+$ was added to start the reaction and the activity was measured. Controls without the additives were also kept in the same manner. The activities of controls and experimentals were compared and the percentages of activation / inhibition were determined.

2.2.5.4. Effect of metal ions

The effect of selected metal ions on the purified glucose-6-phosphate dehydrogenase was tested generally at final concentrations of 1.0 mM and10 mM. Lower concentrations of metal ions were also tested in the case of those that gave high inhibition of the enzyme and those that gave very high absorbance at 340 nm. Appropriate volumes of the salt solutions of metals were added to the standardized assay system and pre-incubated for five minutes. At the end of the 5th minute 0.1 ml 2.0 mM $NADP^+$ was added to start the reaction and the activity was measured. Controls were also kept in the same manner. The activation / inhibition based on optical density change was determined and the percentage of activation / inhibition was calculated.

2.2.5.5. Effect of sugar phosphates on the activity of glucose-6-phosphate dehydrogenase

The effect of selected sugar phosphates on the activity of glucose-6-phosphate dehydrogenase was tested using various sugar phosphates at a final concentration of 10 mM. Stock solutions of sugar phosphates were prepared in 500 mM Tris-HCl buffer pH 8.1. Appropriate volumes of the solutions of sugar phosphates were added to the standardized assay system and pre-incubated for five minutes. At the end of the 5th minute 0.1 ml 2.0 mM $NADP^+$ was added to start the reaction and the activity was measured. The activation / inhibition of enzyme activity was determined and the percentage of activation / inhibition was calculated by comparing with the controls as before.

2.2.5.6. Effect of some important metabolic intermediates such as pyruvate, citrate, 2-oxoglutarate and acetate on the activity of glucose-6-phosphate dehydrogenase

The effect of pyruvate, citrate, 2-oxoglutarate, and acetate on the activity of glucose-6-phosphate dehydrogenase was tested at a final concentration of 10 mM. All the compounds were dissolved in 500 mM Tris-HCl pH 8.1 and were incorporated into the standardized assay system and pre-incubated for 5 minutes. The reaction commenced with the addition of 0.1 ml 2.0 mM $NADP^+$. The activation / inhibition was determined by comparing with the controls as explained in the previous experiment.

2.2.5.7. Effect of some plant growth regulating substances on the activity of glucose-6-phosphate dehydrogenase

Effect of some plant growth regulating substances like indole-3-acetic acid (IAA), gibberellic acid (GA_3) and 2. 4-dichlorophenoxy acetic acid (2,4-D) on the activity of glucose-6-phosphate dehydrogenase was tested at 1.0 mM final concentration of each. Appropriate volumes of the solutions of IAA, GA3 and 2,4-D were added to the assay system and pre-incubated for five minutes. At the end of the 5th minute 0.1 ml 2.0 mM $NADP^+$ was added to start the reaction and the activity was measured. The activation / inhibition was determined and the percentage of activation / inhibition was calculated by comparing with the controls.

2.2.5.8. Effect of some amino acids on the activity of glucose-6-phosphate dehydrogenase

Effect of some standard amino acids on the activity of glucose-6-phosphate dehydrogenase was tested at a final concentration of 10 mM. The stock solutions were prepared by dissolving the various standard amino acids in 500 mM Tris-HCl pH 8.1. Precipitation was observed in the case of cysteine when it was incorporated into the assay system. Amino acids were dissolved in 500 mM Tris-HCl pH 8.1 and were incorporated into the standardized assay system and pre-incubated for 5 minutes. The reaction commenced with the addition of 0.1 ml 2.0 mM $NADP^+$. The activation / inhibition was determined by comparing with the controls as explained in the previous experiments.

2.2.5.9. The effect of selected anions on the activity of glucose-6-phosphate dehydrogenase

The effects of selected anions on the activity of glucose-6-phosphate were tested at a final concentration of 10 mM. Salts of the respective anions were dissolved in 500 mM Tris-HCl pH 8.1 and were incorporated into the standardized assay system and pre-incubated for 5 minutes. At the end of the 5th minute 0.1 ml 2.0 mM $NADP^+$ was added to start the reaction and the activity was measured. Controls were also kept in the same manner. Activation / inhibition was calculated by comparing with the controls as explained before.

2.2.5.10. The effect of dithiothreitol and glutathione (reduced) on the activity of glucose-6-phosphate dehydrogenase

The effect of dithiothreitol, and glutathione (reduced) on the activity of glucose-6-phosphate dehydrogenase was tested at a final concentration of 10 mM. Solutions of these compounds were prepared in water and incorporated into the standard assay system and pre-incubated for 5 minutes. The reaction was initiated by the addition of 0.1 ml 2.0 mM $NADP^+$. The activation / inhibition was calculated by comparing with the controls.

2.2.5.9. The effect of selected nucleotides and nucleotide derivatives on the activity of glucose-6-phosphate dehydrogenase

Selected nucleotides and nucleotide derivatives were used to study their effects on the activity of glucose-6-phosphate dehydrogenase. Stock solutions of selected nucleotides and nucleotide derivatives were dissolved in cold distilled water. The effect of NADPH, NADH and cyclic AMP (cAMP) were tested at a final concentration of 0.1 mM, and that of NAD^+ and ATP were tested at a final concentration of 0.2 mM and 2.0 mM respectively. The effects of additives were compared in the presence and absence of Mg^{2+} in the assay system. The additives were added to the standardized assay system and pre-incubated for 5 minutes. The reaction was initiated with the addition of 0.1 ml $NADP^+$. The percentage of activation / inhibition was determined by comparing with the controls.

2.2.6. Electrophoretic sepaaration of the isoenzymes of glucose-6-phosphate dehydrogenase

2.2.6.1. Electrophoresis

Electrophoresis was carried out to verify if isozymic forms of glucose-6-phosphate dehydrogenase are present in the leaf tissues of *Piper nigrum*. Electrophoretic studies were also performed to see whether there is any change in the isozymic profile of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as a result of disease infection. A 10 % (w/v) homogenate was prepared using the standardized homogenization medium as explained earlier. The volume was made up to 10 ml. The homogenate was centrifuged at 20,000 × *g* for 20 min in a refrigerated centrifuge and the supernatant was collected. Powdered ammonium sulphate was added to make the ammonium sulphate saturation to 90 % in the cold. After flocculation the protein precipitate was separated by centrifugation at 15,000 × *g* in the cold and suspended in the suspending medium composed of 50 mM Tris HCl, pH 7.5, 10 mM 2-mercaptoethanol 1.0 × 10^{-5} M $NADP^+$ [The importance of incorporation of $NADP^+$ in the enzyme preparation to stabilize glucose-6-phosphate dehydrogenase was mentioned in an earlier section (see section **2.2.4.4)**]. This was centrifuged again and 2.0 ml of the supernatant was gel filtered as explained before using a 10 cm x 1.8 cm Sephadex G-25 column. The flow rate was adjusted to elute 1.0 ml per 2 minutes. The proteins were eluted using the medium that consisted 50 mM Tris HCl, pH 7.5, and 10 mM 2-mercaptoethanol 1.0 ×10^{-5} M $NADP^+$. 4.0ml was collected with one-fold dilution. The gel filtrate was taken in a dialysis bag and the bag was placed in a beaker containing 25 % pre-cooled polyvinyl pyrrolidone (mol.wt. 40,000) for reverse dialysis. The beaker was kept surrounded by cracked ice in a refrigerator for 6-8 hours. After the reverse dialysis, the bag was profusely washed on the outside with chilled distilled water. The concentrated enzyme preparation was taken out and powdered analytical grade sucrose was added to make the final sucrose concentration to 20 %. The enzyme preparation so obtained was used for electrophoresis.

Electrophoretic studies were also conducted using the most active fractions obtained from the Sephadex G-200 gel filtration to identify the number of isozymes of glucose-6-phosphate dehydrogenase present in the purified enzyme preparation. The 20,000 × *g* supernatant (as described earlier) from a 10 % (w/v) homogenate was subjected to 30-70 %

ammonium sulphate saturation as explained earlier (see section **2.2.4.1**) The residue was taken up in a small measured volume of suspending medium which consisted of 50 mM Tris-HCl buffer pH 7.5, 10 mM 2-mercaptoethanol and 1.0 × 10^{-5} M $NADP^+$ and centrifuged again. A sample of this enzyme preparation (usually 3.0 ml) was then subjected to Sephadex G-200 gel fitration and eluted in the cold with 50 mM Tris-HCl buffer pH 7.5, 10 mM 2-mercaptoethanol and 1.0 × 10^{-5} mM $NADP^+$ at a flow rate of 1.0 ml / 4 min. The first 50 ml of the eluant was discarded as the void volume. Subsequently a total of 15 fractions were collected each of 5.0 ml. All the fractions were tested for enzyme activity and the most active fractions (usually 3 or 4 fractions) were pooled together. The pooled active fractions were concentrated by reverse dialysis as described earlier. The concentrated enzyme preparation was collected and powdered analar grade sucrose was added to make the sucrose concentration to 20 %. The enzyme preparation so obtained was used for electrophoresis.

2.2.6.2. Gel casting

Discontinuous polyacrylamide gel electrophoresis (PAGE) was carried out to separate the isoenzymes. Native gel electrophoresis was performed at 4 ° C in the polyacrylamide gels according to the method of Gall *et al.*, (1980) with minor modifications.

The stacking gel (2.8 % acrylamide) was prepared in 200 mM Tris-Phosphate (pH 6.8). A 4.0 ml stacking gel mixture contained the following components. 0.373 ml Acrylamide / bisacrylamide (30 % T, 2.67 % C), 1.0

ml 200 mM Tris-phosphate buffer pH 6.8, 10 µl 10 % ammonium persulphate, 2.0 µl tetramethyl ethylene diamine (TEMED) and water to 4.0 ml. The resolving gel / separating gel (5.0 % acrylamide) was prepared in 1.0 M Tris-phosphate pH 8.5. A 10 ml mixture for resolving gel contained the following components. 1.665 ml acrylamide / bisacrylamide (30 % T, 2.67 % C), 5.0 ml 1.0 M Tris-phosphate buffer pH 8.5, 50 µl 10 % ammonium persulphate, 5.0 µl TEMED and water to 10ml.

The gels were cast in a Biorad mini vertical electrophoresis gel-casting unit. The resolving gel mixture was poured first and the top layer was layered with a small volume of water to avoid contact with air. After the completion of polymerization, water was removed using filter paper strips. After placing the comb, the stacking gel mixture was added and the top region was layered with water. After the completion of polymerization the comb was removed carefully and the wells were washed twice with the electrophoretic buffer. 80 µl of enzyme concentrate containing 20 % sucrose was added in duplicate to each well. Bromophenol blue (0.025 %) was used as the tracking dye in one of the wells. Electrophoresis was carried out using the electrophoretic buffer Tris-glycine, pH 8.4. Gels were maintained at 4° C inside a cold room during the 3 hours of electrophoresis at a voltage of 80 V for 45 min followed by 120 V for 2.0 hours and 15 min.

At the end of the electrophoretic run the gel was carefully removed and washed profusely with chilled distilled water. The gel was cut into two equal halves vertically. One-half was used to detect the enzyme activity and the other half for protein staining.

2.2.6.3. Detection of enzyme activity in the gels

The gel, after washing, was immediately transferred to the reaction mixture which contained 2.5 ml 500 mM Tris-HCl pH 8.1, 2.5 ml 100 mM $MgCl_2$, 7.5 g nitro blue tetrazolium, 0.625mg phenazine methosulphate, 2.5 ml 20 mM glucose-6-phosphate, 2.5 ml 2.0 mM $NADP^+$, and water to 25 ml. The colour development was carried out at room temperature in the dark. The gels were rocked gently for 15 - 30 min, until the reaction was complete, and then rinsed with distilled water for a few hours and was then photographed.

2.2.6.4. Detection of proteins by silver staining

Silver staining method was adopted since staining using Coomassie brilliant blue did not show any detectable colour for protein bands corresponding to glucose-6-phosphate dehydrogenase. This may be due to the low content of enzymic proteins present in the sample. Silver staining, a more sensitive protein staining procedure was carried out according to the method of Hames and Rickwood (1994). The stain solution was prepared immediately before use. All the stock solutions were prepared in glassware since plastic containers may leach organic materials, which interfere with silver straining. Silver stain solution was prepared by mixing the two solutions-Solution A and Solution B. Solution A was prepared by dissolving

0.8 g silver nitrate in 4.0 ml distilled water. Solution B was prepared by mixing 21 ml of 0.36 % NaOH with 1.4 ml of freshly prepared 14.8 M ammonium hydroxide. The gel was taken and washed thoroughly in distilled water and was soaked in 50 % (v/v) methanol for at least 1.0 hour in a glass dish. The silver stain solution was prepared by adding solution A drop wise to solution B with constant stirring and made up to 100 ml with distilled water. The gel was stained in the stain solution for 15 min with gentle agitation. The gel was then washed with distilled water for five minutes with gentle agitation and was then soaked in the developing solution until the appearance of bands (bands usually appeared in less than 10 minutes). The developing solution was prepared just before use by mixing 2.5 ml of 1.0 % citric acid with 0.25 ml 37 % formaldehyde and made up to 500 ml with distilled water. After the development of bands the gel was washed with water and placed in 50 % methanol to stop darkening of the background gel. The development time was standardized and was followed in the subsequent experiments. The protein bands were visualized as black bands. The gel was then photographed.

2.3. Biochemical studies in disease-induced plants of *P. nigrum*

These studies were carried out to assess the biochemical changes if any that may take place as a result of infection by *Phytophthora capsici.* The studies included the changes in the total phenolics, free amino acids, proline and changes in the levels of activities of the first two enzymes of the pentose phosphate pathway namely glucose-6-phosphate dehydrogenase and 6-

phosphogluconate dehydrogenase. Studies were also conducted to identify whether there is any change in the isozymic profile of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as a result of infection. These studies were carried out on the leaves of three-month-old *P. nigrum* plants.

2.3.1. Inoculation with *Phytophthora capsici* on the leaves of *P. nigrum*

2.3.1.1. Fungal culture

The fungal isolate named as Isolate No. 02-1 (Chelavoor isolate) was obtained from the repository of *Phytophthora capsici* maintained at the Plant Pathology Division, Indian Institute of Spices Research, Kozhikode. The isolate of *P. capsici* was cultured as follows.

Stock cultures of *P. capsici* were maintained in 20 % carrot agar medium as explained by Ribeiro (1978) for 72 hours. The culture was maintained by sub culturing the inoculum. Discs of 5.0 mm diameter were cut from the margins of the culture. 20 % carrot agar medium was prepared and poured in glass petri plates. One disc of fungal inoculum was carefully kept at the centre of the culture medium. Discs of 5.0 mm diameter were cut after 72 hours growth and were used for the inoculation on the leaves. Fresh cultures were obtained from IISR, for sub culturing and to replace the older and contaminated cultures.

2.3.1.2. Preparation of carrot agar medium

Fresh carrot (200 g) was sliced and 500 ml of distilled water was added and ground for 40 seconds at high speed in a homogenizer. The extract was squeezed through four layers of cheesecloth. The filtrate was made up to 1000 ml and transferred to a conical flask and 16 grams of agar was dissolved in it. The medium was autoclaved for 10 min at 1.0 kg /cm^2. After cooling to room temperature the medium was stored under refrigeration.

2.3.1.3. Inoculation Procedure

Discs of 5.0 mm diameter of the fungal inoculum were cut and used for inoculating the lower side of the previously moistened leaves. The mycelial side of the disc was placed in contact with the lower side of the third leaf from the tip of the experimental plants. Three discs were used per leaf for inoculation and the discs were covered with cotton and moistened. The plants were then placed in a chamber specially fabricated for the purpose to maintain humidity. The chamber exclusively fabricated for this purpose was a metal framework. The bottom of the chamber was layered with sand and wetted thoroughly. The chamber was covered with transparent polyethylene sheet on all sides. The chamber was then sealed to maintain the humidity with wet sand on all sides. The un-inoculated control plants were also maintained in a similar chamber.

2.3.2. Sampling of infected leaves

Leaf samples of "Sreekara' were collected in duplicates 24, 48 and 72 hours after inoculation (HAI). The necrotic tissue was cut and removed. Margins of the necrotic areas were cut and pooled and used for the analyses. The sampling was regularly done between 9:00 am and 10:00 am. Leaf tissue from the leaves of control plants were also taken and pooled. From the pooled leaf samples, 500 mg each was used for enzyme extraction, for the extraction of total phenolics, amino acids and proline. About 1 to 2 g was used for the determination of dry weight. Weighed samples of leaf tissues for the analysis of total phenolics, amino acids and proline were stored in 80% (v/v) ethanol in stoppered tubes till used. Enzyme extraction was done immediately after the collection of samples but extraction of total phenolics, amino acids and proline was done at a later time.

2.3.3. Determination of dry weight

Weighed samples (of about 1.0 to 2.0 g) of the tissues from infected and control leaves were transferred to clean pre-weighed weighing tubes and heated in a hot air oven maintained at 100° C. At the end of 1.0 hour, the tubes were transferred to another oven maintained at 60° C. Heating was continued for a period of 3 to 4 days with regular checking of the weight every day till constant weight was attained. The percentage dry weight was calculated.

2.3.4. Preparation of ethanolic extract for analyses of total phenolics, total amino acids and proline from the leaves of control and infected plants of *P. nigrum*

Leaf tissues of control and 24 h, 48 h and 72 h infected plants were collected and the necrotic tissues were removed. The tissue near the necrotic areas was cut and pooled. 500 mg of this tissue was kept in 80 % (v/v) ethanol in stoppered tubes. The tissue with ethanol was quantitatively transferred to a glass mortar and ground with a pestle. Acid washed sand was added as abrasive to facilitate efficient grinding. More of ethanol was added in small lots as grinding progressed. The finely ground suspension was carefully transferred to a 250 ml round-bottom flask. The mortar and the pestle were washed with small volumes of 80 % (v/v) ethanol and the washings were added to the flask. A total of about 25ml ethanol was required for each sample. The flasks were fitted with water condensers and refluxed over a steam bath for 2.0 hours. The flasks were then cooled, contents transferred to centrifuge tubes and centrifuged in a tabletop centrifuge. The residue was quantitatively transferred using 80 % ethanol and reground in a mortar with a pestle. Following refluxing for 1.0 hour, the extract was clarified by centrifugation as before and the supernatant was combined with the original. The final volume of the alcoholic extract was noted (generally between 40-50 ml). 5.0 ml of this was set aside for the estimation of total phenolics and amino acids. The remaining extract was used for the estimation of proline.

2.3.5. Estimation of total phenolics in control and infected plants

Total phenolics in the leaf tissues of control and infected plants were colorimetrically determined in the ethanolic extracts according to the method of Folin and Denis (1915) as modified by Swain and Hillis (1959) and Goldstein and Swain (1963). Aliquots of alcoholic extracts (usually 0.2 ml) were used for the colorimetric determination of phenolics. Tannic acid was used as the standard.

2.3.6. Estimation of total free amino acids in control and infectedplants

The amino acids in the 80 % ethanolic extract were separated by ion exchange chromatography using Dowex-50W-X8, (20-50 US. Mesh (Na^+)) ion exchanger. The ion exchanger was first converted into the H^+ form by treatment with 0.1N HCl, and washed with distilled water till it became neutral. 2.0 ml of the 80 % ethanolic extract was evaporated in an evaporating dish on a steam bath. The residue left was dissolved in 2.0 ml of 0.1 N HCl, centrifuged and the supernatant was loaded into a 17cm x 1.25 cm ion exchanger column. The column was washed with 100ml of double distilled water. The washings were discarded. The column was eluted with 50ml of 5.0 % (v/v) ammonia solution. The ammonia fraction was collected and evaporated in an evaporating dish over a steam bath. The residue obtained on evaporation was dissolved in 3.0 ml of 10 % (v/v) isopropyl alcohol. Appropriate aliquots (usually 0.2 ml) in triplicates were drawn and were used for the determination of total free amino acids.

The total free amino acids were colorimetrically estimated according to the method of Lee and Takahashi (1965). The amino acid standard used was an equimolar mixture of four amino acids, namely, DL- alanine, DL-tryptophan, L- histidine and DL- aspartic acid.

2.3.7. Estimation of total proline in control and infected plants

The 80 % (v/v) ethanolic extract prepared as explained earlier was used for the determination of total proline. The ethanolic extract remained after the estimation of total phenolics and amino acids was measured and evaporated in an evaporating dish on a steam bath.

Total proline in the leaf tissues of control and infected plants was colorimetrically determined according to the method of Bates (1973). The dry residue was dissolved in 5.0 ml of 3.0 % (w/v) aqueous sulfosalicylic acid. 2.0 ml aliquot was used for colour development. The sample was heated with 2.0 ml of acid ninhydrin and 2.0 ml of glacial acetic acid in a bath of boiling water for 1.0 hour. The reaction was terminated in an ice bath. 4.0 ml toluene was added to the reaction mixture and mixed vigorously for 15 to 20 seconds. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance was read at 520 nm using toluene as reagent blank. Proline was used as the standard.

2.4. Studies on the levels of activities of glucose-6-phosphate dehydrogenase in disease-induced plants of *P. nigrum*

The changes in the levels of activities of glucose-6-phosphate dehyrdogenase were studied in disease-inducecd *P. nigrum* plants caused by *Phytophthora capsici*. Infection was induced as explained earlier (see section **2.3.1.3**). Leaves were collected from the diseased plants 24, 48 and 72 hours after inoculation. The necrotic lesions were cut and removed. The surrounding tissues were cut and pooled. 500 mg of tissue was used in each enzyme extraction experiment. The tissue was homogenized in a pre-chilled mortar with pestle using the standardized medium (see section **2.2.1.9**). Acid washed sand was added as an abrasive. The homogenate was squeezed through 2 layers of muslin and the volume was made up to 10 ml. The filtrate was centrifuged at 20,000 × *g* for 20 min. in a refrigerated centrifuge. The supernatant was collected and 2.0 ml was gel filtered as explained earlier (see section **2.2.1.3**) using a 10 cm x 1.8 cm Sephadex G-25 column at a flow rate of 0.5 ml per minute. The proteins were eluted as a single 4.0 ml fraction using a medium, which consisted of 50 mM Tris- HCl, pH 7.5 and 10 mM 2-mercaptoethanol. The gel filtrate so obtained was used as the source of the enzyme. The assay system contained 0.3 ml 500 mM Tris HCl, 0.1ml 100 mM $MgCl_2$, 0.2 ml enzyme, 0.1 ml 20 mM glucose-6-phosphate, 0.1 ml 2.0 mM $NADP^+$ and 0.2 ml water to make 1.0 ml. The reaction was started with the addition of $NADP^+$ .The amount of proteins present in the gel filtrate of each experiment was determined as explained earlier (see section 2.2.3.1). The specific activity of the enzyme was calculated.

2.5. Studies on the levels of activities of 6-phosphogluconate dehydrogenase in disease- induced plants of *P. nigrum*

It was mentioned in the **Introduction** that when glucose-6-phosphate dehydrogenase is activated, there is a concomitant activation of 6-phosphogluconate dehydrogenase as a result of pathogenic infection. This is reasonable because 6-phosphogluconate dehydrogenase acts on the product of glucose-6-phosphate dehydrogenase reaction.

Experiments were carried out to study the levels of activities of 6-phosphogluconate dehydrogenase at different stages of infection (24 HAI, 48 HAI and 72 HAI). Standardized conditions for homogenization and assay of glucose-6-phosphate dehydrogenase were adopted in the case of 6-phosphogluconate dehydrogenase also and no separate standardization experiments were conducted. The same gel filtrate which was used for determining the activity of glucose-6-phosphate dehydrogenase was used for the assay of 6-phosphogluconate dehydrogenase also.

The activity measurements were made as in the case of glucose-6-phosphate dehydrogenase. The assay system contained 0.3 ml 500 mM Tris HCl, 0.1 ml 100 mM $MgCl_2$, 0.2 ml enzyme, 0.1 ml 2.0 mM 6-phosphogluconate, 0.2 ml water and 0.1 ml 2.0 mM $NADP^+$. The reaction was started with the addition of $NADP^+$. Specific activities were calculated in each experiment as before after determining the protein content in the gel filtrate.

2.6. Identification of isoenzymes of glucose-6-phosphate dehydrogenase in the leaves of control and *P. capsici* infected *P. nigrum*

Electrophoresis of glucose-6-phosphate dehydrogenase isoenzymes in control and disease-induced plants was carried out using the Sephadex G-25 gel filtrate. A 10 % (w/v) homogenate of the leaves of both control and infected (48 hours after inoculation) plants was prepared as explained earlier and centrifuged in the cold at 20,000 × *g* for 20 minutes. Ammonium sulphate precipitation at 90 % saturation was carried out. The precipitate after flocculation and centrifugation at 15,000 × *g* for 15 minutes was suspended in a medium which consisted of 50 mM Tris HCl, pH 7.5, 10 mM 2-mercaptoethanol 1.0 × 10^{-5} M $NADP^+$. The suspension was again centrifuged in the cold at 15,000 × *g* for 15 minutes. 3.0 ml each of the enzyme preparation (control and infected leaves) was gel filtered separately through a 1.8 x 10 cm Sephadex G-25 column. The gel filtrate was concentrated separately using 25 % (w/v) polyvinyl pyrrolidone by reverse dialysis as explained earlier (see section **2.2.6.1**). The concentrate was mixed with analar grade sucrose powder to make the final concentration of sucrose to 20 %. 50 μl each of enzyme concentrate was loaded in alternate wells of the slab gel and electrophoresis carried out. The preparation of gel, electrophoretic buffer and reaction mixture were the same as described earlier (see section **2.2.6.1**). The electrophoresis was carried out at 100 V for 1.0 hour followed by 130 V for 2.0 hours. The enzyme activity in the gel slabs was detected as described earlier.

2.7. Identification of isoenzymes of 6-phosphogluconate dehydrogenase in the leaves of control and *P. capsici* infected *P. nigrum*

This was carried out using the same enzyme preparation used for the electrophoresis of the isozymes of glucose-6-phosphate dehydrogenase in control and disease induced plants as explained (see section **2.6**). All the procedures and conditions were the same as mentioned above except that the reaction mixture for gel slabs contained 0.1 ml, 2.0 mM 6-phosphogluconate, instead of glucose-6-phosphate.

2.8. Unit of enzyme activity

One unit of glucose-6-phosphate dehydrogenase / 6-phosphogluconate dehydrogenase is defined as the amount of enzyme, which reduces 1.0 μ mole of $NADP^+$ in one hour at 25 ± 1^0C under the specified conditions of assay.

2.9. Specific activity

The specific activity is defined as units of enzyme per mg protein in the enzyme preparation.

2.10. Presentation of experimental data

Enzyme activities are presented both as units per gram fresh tissue and units per gram dry tissue.

2.11. Statistical evaluation

All the analyses described in the dissertation were carried out in replicates (6 to 10) and the values are expressed as mean ± standard deviation.

2.12. Statistical significance

The statistical significance was tested using the t-test. Values of $p < 0.05$ were taken as statistically significant.

2.13. Chemicals

All the reagents and chemicals were of analytical grade wherever necessary. Special chemicals such as polyvinyl polypyrrolidone and 6-phosphogluconate were obtained from Sigma, USA.

RESULTS

3

RESULTS

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3.1. Standardization of homogenizing conditions

The effects of additives like 2-mercaptoethanol, EDTA, and PVPP in the homogenization medium were tested (see sections **2.2.1.6**, **2.2.1.7** and **2.2.1.8**) to optimize the homogenizing medium for glucose-6-phosphate dehydrogenase measurements.

The results of the above are presented below.

3.1.1. Determination of optimum concentration of 2-mercaptoethanol in the homogenization medium.

Tissue homogenates, which were prepared without 2-mercaptoethanol, showed 'browning' due to phenolic oxidation and consequent protein precipitation as a result of phenolic-protein interaction. The protective effect of 2-mercaptoethanol at different concentrations in the homogenization medium was tested and the results of a typical experiment are recorded in Table 1.

Table 1

There was no demonstrable activity of glucose-6-phosphate dehydrogenase in the absence of 2-mercaptoethanol. Incorporation of 2-mercaptoethanol in the homogenization medium resulted in an increase in enzyme activity. Compared to the activity at 10 mM concentration of 2-mercaptoethanol, there was a marked increase at 20 mM (45 %) and a further marginal increase of 13 % (of that at 20 mM) at 50 mM. At 70 mM concentration, there

Table 1

Effect of 2-mercaptoethanol during homogenization on glucose-6-phosphate dehydrogenase activity from the leaves of *P.nigrum.*

2-Mercaptoethanol, mM	Glucose-6-phosphate dehydrogenase activity, Δ OD / h	Percentage activity.
10	0.937	(100)
20	1.357	145
50	1.537	164
70	0.921	98

The data are from a typical experiment. 5.0 % (w/v) homogenate of *P. nigrum* was prepared. The basal medium which consisted of 50 mM Tris-HCl buffer pH 7.5,. The activity at 10 mM 2-mercaptoethanol is taken as reference and is represented in parenthesis. Details of assay are as reported in **Materials and Methods.**

was a decrease, to reach a value of about 98 % that of 10 mM. Based on the results, the optimum concentration of 2-mercaptoethanol was fixed at 50 mM for a 5.0 % (w/v) fresh tissue homogenate.

3.1.2. Determination of optimum concentration of ethylenediamine tetraacetate in the homogenization medium

Incorporation of neutralized EDTA in the homogenization medium as a chelating agent for divalent metal ions was tested at final concentrations of 5.0, 10 and 20 mM.

Increase in activity of about 47 % at 5.0 mM and 66 % at 10 mM were noted when compared to the control. The increase was only 64 % at 20 mM. The optimum concentration of EDTA was fixed at 10 mM and was used in all subsequent experiments.

The results of a typical experiment is represented in Table 2.

Table 2

3.1.3 Determination of optimum concentration of polyvinyl polypyrrolidone during homogenization

Different concentrations (50, 100, and 150 mg / g fresh tissue) of polyvinyl polypyrrolidone were incorporated during homogenization of tissue and the enzyme activities were compared with the control (without PVPP). Supplementation of PVPP during homogenization resulted in a higher enzyme activity when compared to the control experiments. At 50 mg / g and

Table 2

Effect of EDTA during homogenization on glucose-6-phosphate dehydrogenase activity from the leaves of *Piper nigrum.*

EDTA , mM	Glucose-6-phosphate dehydrogenase activity Δ OD / h	Percentage activity.
0	1.090	(100)
5	1.602	147
10	1.812	166
20	1.792	164

The data are from a typical experiment. 5 % (w/v) homogenate of *P. nigrum* tissue was prepared in the basal medium, which consisted of 50mM Tris-HCl buffer pH 7.5, and 50 mM 2-mercaptoethanol. Neutralized EDTA was incorporated at final concentrations as indicated. The value for control is represented in parenthesis. Details of assay are as reported in **Materials and Methods.**

100 mg / g fresh tissue there was 38 % and 66 % increase in activity respectively. At 150 mg / g tissue, the maximum concentration tested, there was no further increase in activity. In all future experiments, the concentration of PVPP was maintained at 100 mg / g fresh tissue. It may be mentioned, inter alia, that the incorporation of PVPP during homogenization has also resulted in a decrease in the background absorption of assay system at 340 nm

The results from a typical experiment are recorded in Table 3.

Table 3

3.2. Standardized homogenizing medium for the assay of glucose-6-phosphate dehydrogenase

From the results of the foregoing experiments, a standardized medium for homogenization was formulated for the detailed study of glucose-6-phosphate dehydrogenase from leaf tissues of *Piper nigrum.* The standardized medium consisted of 50 mM Tris-HCl pH 7.5, 50 mM 2-mercaptoethanol, 10 mM neutralized EDTA and 100 mg PVPP / g fresh tissue. Using these conditions, for a 5 % (w/v) homogenate of fresh tissue of *P. nigrum* leaves, optimum activity of glucose-6-phosphate dehydrogenase was elicited.

Table 3

Effect of polyvinyl polypyrrolidone during homogenization on glucose-6-phosphate dehydrogenase activity from the leaves of *Piper nigrum.*

PVPP, mg / g tissue	Glucose-6-phosphate dehydrogenase activity, Δ OD / h	Percentage activity.
0	1.426	(100)
50	1.975	138
100	2.369	166
150	2.341	164

The data are from a typical experiment. 5.0 % (w / v) homogenate of *P. nigrum* leaf tissue was prepared in the basal medium which consisted of 50 mM Tris-HCl buffer pH 7.5, 50 mM 2-mercaptoethanol and 10 mM EDTA. PVPP was incorporated at final concentrations as indicated. The value for control is represented in parenthesis. Details of assay are as reported in **Materials and Methods.**

3.3. Standardization of assay system

3.3.1. Determination of optimum pH

The optimum pH for the assay of glucose-6-phosphate dehydrogenase using the Sephadex G-25 gel filtrate as the source of enzyme was tested as described earlier (see section **2.2.2.3**). This was tested over a pH range of 6.0 to 9.0 using Tris-acetate buffer in the pH range 6.0 to 7.2 and Tris-HCl in the pH range 7.5 to 9.0. The assay system consisted of 0.3 ml 500 mM buffer of various pH values, 0.2 ml enzyme preparation, and 0.1 ml 100 mM $MgCl_2$, 0.1ml 20 mM glucose-6-phosphate, and 0.1ml 2.0 mM $NADP^+$, and 0.2 ml water. The reaction was initiated by the addition of $NADP^+$. Glucose-6-phosphate dehydrogenase from *Piper nigrum* showed a sharp pH optimum of 8.1. On either side of this pH there was marked decrease in enzyme activity. In the subsequent experiments the assays were conducted at the optimum pH of 8.1.

Results from a typical experiment are recorded in Figure 1.

Fig.1

3.3.2. Verification of endogenous activity

As was explained earlier (see section **2.2.2.8**) it was necessary to verify the contribution of endogenous reaction, which may affect the measurement of the activity of glucose-6-phosphate dehydrogenase. No change in absorption was observed when the substrates were withheld separately and the reaction commenced only in the presence of both the substrates (Data not represented). This experiment ensured that the enzyme activities reported in the present work are truely due to glucose-6-phosphate dehydrogenase.

3.3.3. Substrate specificity towards NADP$^+$

The need for performing this experiment was explained earlier (see section **2.2.2.9**). No enzyme activity was noted when NAD$^+$ was used as the substrate in place of NADP$^+$ showing that the enzyme is very specific for NADP$^+$ as the hydrogen acceptor (Data not represented).

3.3.4. Incubation period-enzyme activity relationship

Measured over a period of ten minutes at one-minute interval, the course of the reaction exhibited linearity. Therefore, assays were carried out routinely for 3 min in subsequent experiments.

Results from a typical experiment are recorded in Figure 2.

Fig. 2

3.3.5. Determination of enzyme proportionality range.

Enzyme proportionality range was tested at pH 8.1. Using the Sephadex G-25 gel filtrate (2.5 % w/v tissue equivalent) a linear relationship was obtained between the volume of the enzyme preparation and initial reaction velocity up to 0.3 ml, the maximum volume tested. In subsequent experiments the amount of enzyme was maintained within the proportionality range.

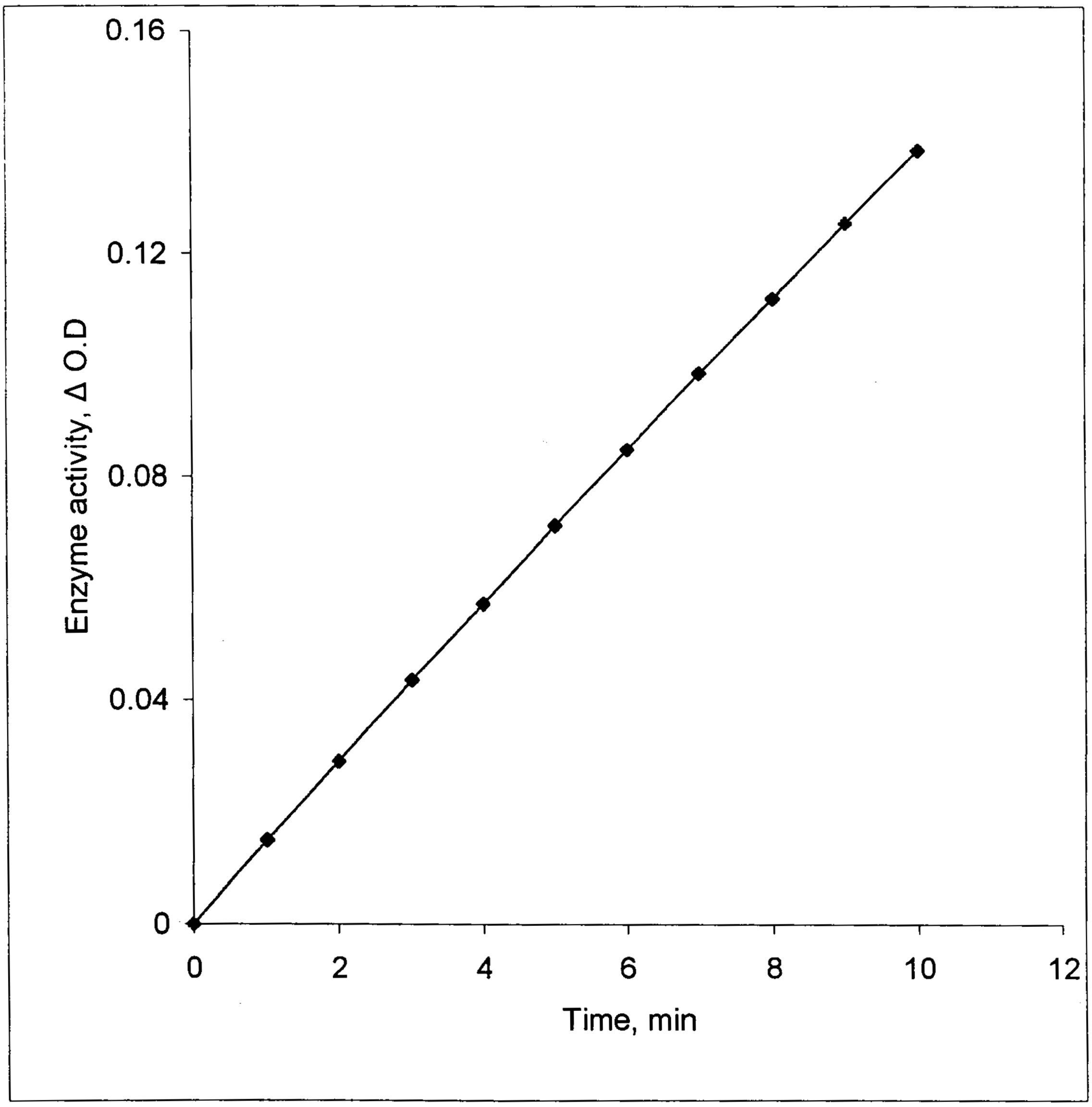

Fig. 2. Incubation period - enzyme activity relationship of glucose-6-Phosphate dehydrogenase from the leaves of *P.nigrum.* (Details as described in **Materials and Methods**)

Results from a typical experiment are recorded in Figure 3.

Fig 3

3.3.6. Enzyme activity- substrate concentration relationship

The reaction velocity with respect to the concentrations of substrates in the assay system was determined for both NADP$^+$ and glucose-6-phosphate.

3.3.6.1. NADP$^+$

Tested in the range 0.1 mM to 0.6 mM final concentration of NADP$^+$ in the assay system, it was found that the enzyme was nearly saturated at 0.2 mM. The Vmax remained more or less unchanged at higher concentrations. Based on the result, the concentration of NADP $^+$ in the assay system was maintained at 0.2 mM for all subsequent experiments.

The results from a typical experiment are recorded in Figure 4.

Fig. 4

3.3.6.2. Glucose-6-phosphate

Using glucose-6-phosphate at varying final concentrations in the range 1.0 mM to 6.0 mM in the assay system, the initial velocity showed an increase with an increase in the substrate concentration. The enzyme was saturated at 2.0 mM glucose-6-phosphate. Thereafter, the velocity remained nearly the same at higher concentrations. For all future experiments the

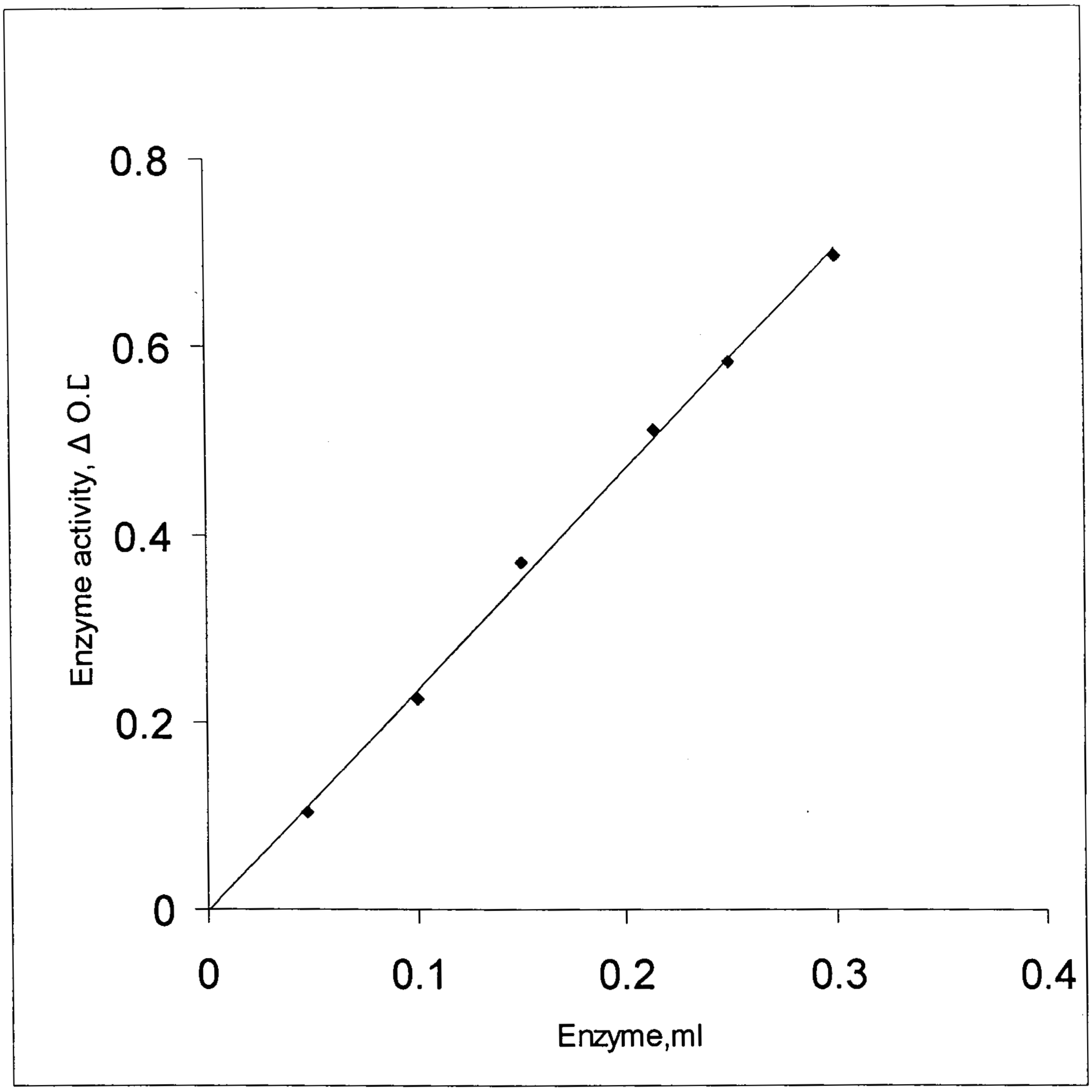

Fig.3. Enzyme concentration-reaction velocity relationship of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum* (Details as described in **Materials and Methods**).

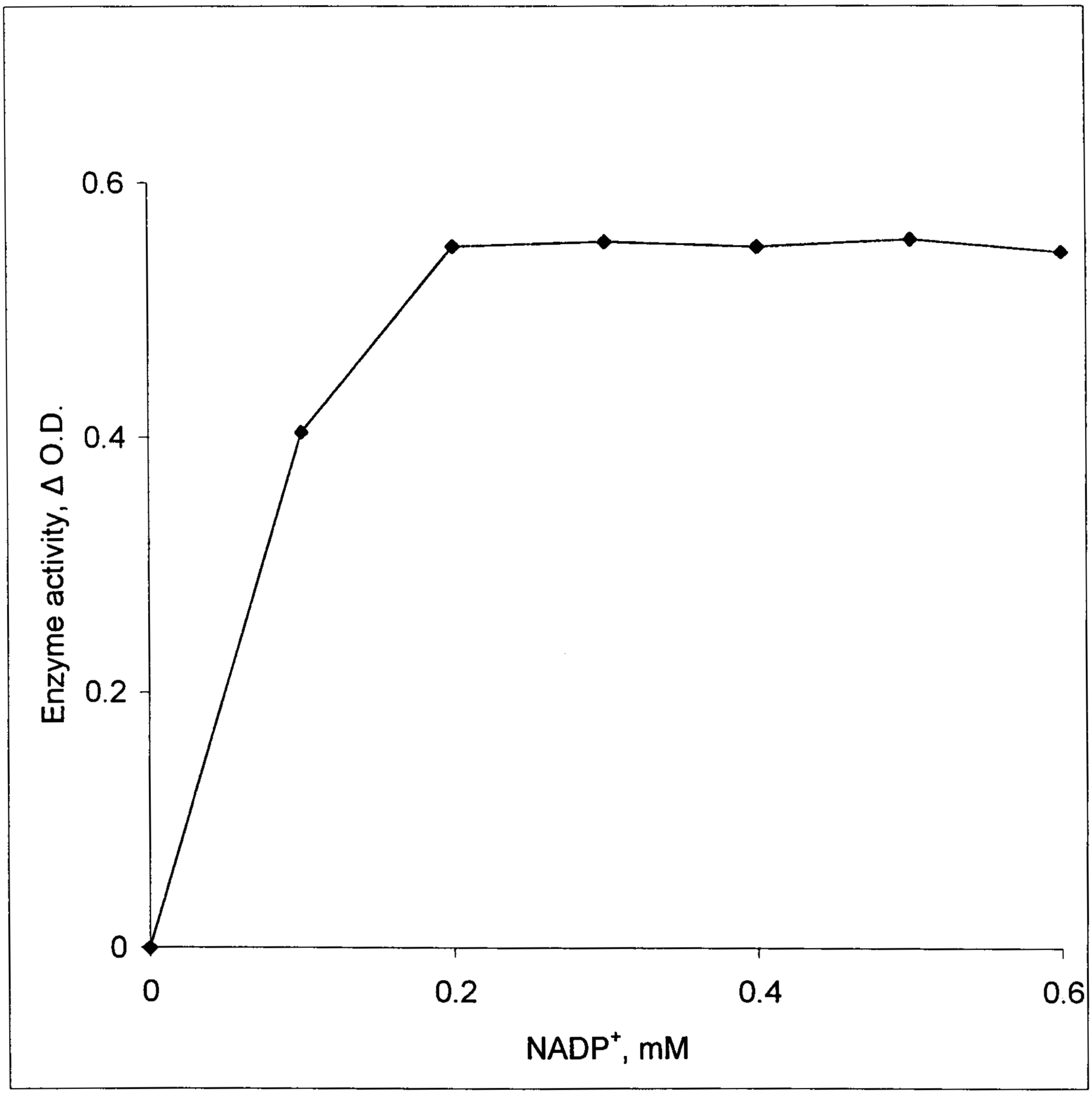

Fig. 4. Enzyme activity-NADP$^+$ concentration relationship of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum* (Details as described in **Materials and Methods**)

assays were carried out using saturating concentration of substrate (2.0 mM glucose-6-phosphate).

The results from a typical experiment are recorded in Figure 5.

Fig.5

3.3.7. Effect of Mg^{2+} on the activity of glucose-6-phosphate dehydrogenase

The effect was tested using $MgCl_2$ in the range 0.0 to 15 mM in the assay system at saturating concentrations of substrates. The enzyme activity steadily increased with an increase in Mg^{2+} concentration up to 10 mM. At 10 mM Mg^{2+}, there was an increase of 96 % over the control. Thereafter, the enzyme activity remained the same. The optimum concentration of Mg^{2+} in the assay system was fixed as 10 mM and was supplied routinely in the subsequent experiments.

The results from a typical experiment are recorded in Figure 6.

Fig. 6

3.4. Purification of glucose-6-phosphate dehydrogenase

3.4.1. Ammonium sulphate fractionation

In the preliminary experiments for enzyme purification using the 20,000 × *g* supernatant of homogenates, the fractionation was carried out at 4 saturation levels of ammonium sulphate namely 0 –30, 30-60, 60 –70 and

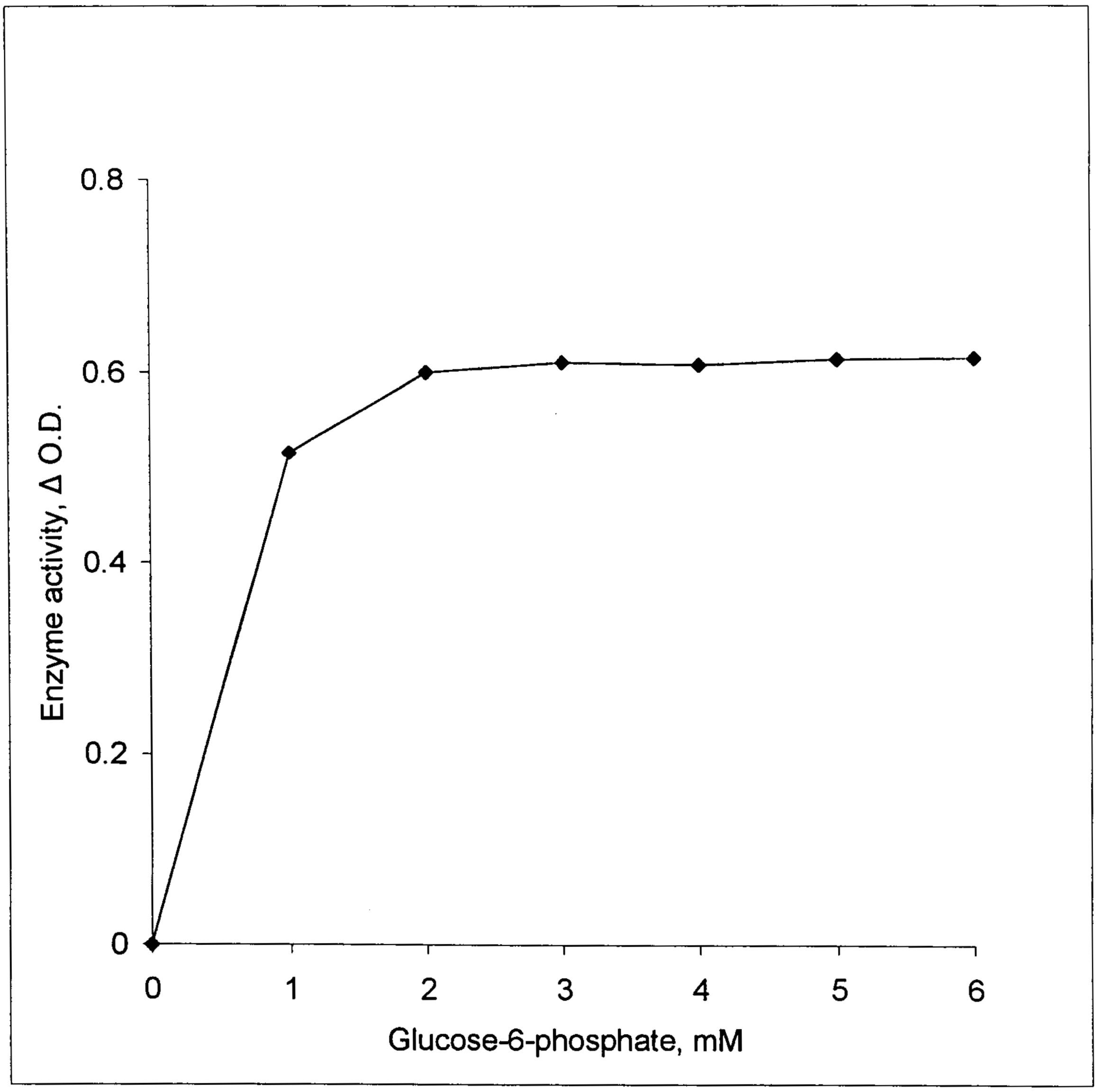

Fig.5. Enzyme activity-substrate concentration relationship of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum* (Details as described in **Materials and Methods**)

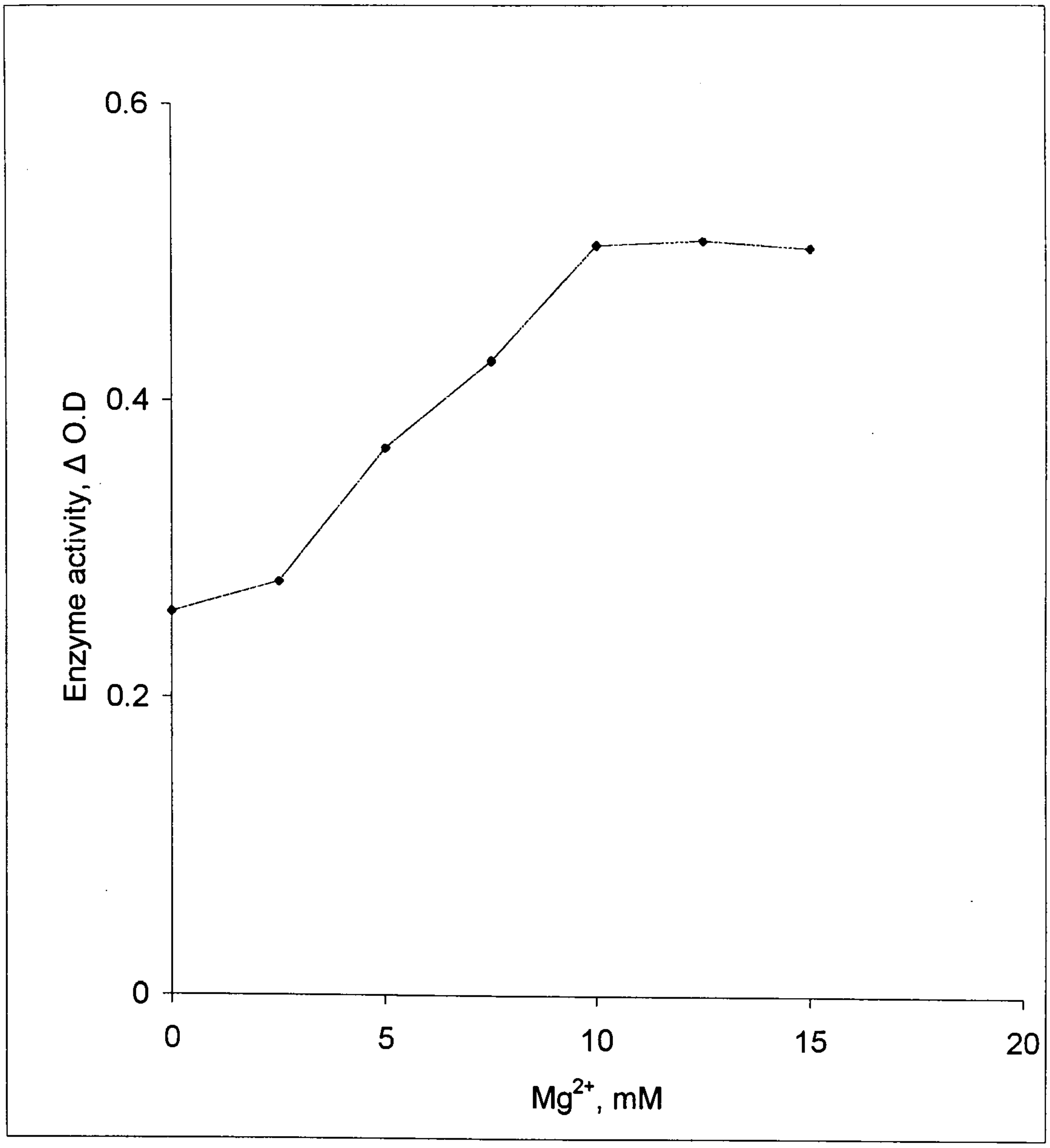

Fig. 6. Effect of Mg^{2+} on the activity of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum* (Details as described in **Materials and Methods**)

70-90 per cent (see section **2.2.4.1** for details). Enzyme activities, percentage recovery, fold purification and specific activity from each fraction was calculated.

Results from a typical experiment are recorded in Table 4.

Table 4

Of the four fractions, the 30-60 % fraction contained the maximum activity with a recovery of over 75 % of the 20,000 × *g* supernatant, and fold purification of 1.33. This fraction also showed the highest specific activity (1.054). The 0-30 %, 60-70 % and 70-90 % showed negligible recovery of activities (3.64, 5.31 and 0.92 per cent respectively). Based on the above data, the ammonium sulphate fractionation was carried out in the range 30-70% saturation for all subsequent purification procedures.

3.4.2. Stability of the enzyme during storage

The need for the verification of stability of the enzyme under storage was explained earlier (see section **2.2.4.3**). This was tested using the supernatant (20,000 × *g*) and Sephadex G-25 gel filtrate of the 30-70 % ammonium sulphate fraction (F_2 gel filtrate). The supernatant and F_2 gel filtrate (Sephadex G-25) retained 62 % and 50 % respectively of their original activities after 24 h. The value dropped to 47 % and 30 % respectively at the end of 72 h. The results indicated that the 20,000 × *g* supernatant was more stable than the gel filtered ammonium sulphate fraction. However, for the study of the properties of the enzyme this preparation was unsuitable

Table 4

Ammonium sulphate fractionation of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum*

Enzyme source	Enzyme activity, units / g fresh tissue	Total protein, mg	Specific activity, units / mg protein	Recovery of enzyme activity, %	Fold Purification
Supernatant 20,000 x *g*	15.39	19.36	0.795	(100)	(1)
0-30 % saturation (F_1)	0.560	1.03	0.544	3.64	0.68
30-60 % saturation (F_2)	11.65	11.05	1.054	75.70	1.33
60-70 % saturation (F_3)	0.817	0.860	0.95	5.31	1.19
70-90 % saturation (F_4)	0.141	0.367	0.384	0.92	0.48

The data presented are from a typical experiment. Supernatant from a 5 % (w / v) homogenate was subjected to ammonium sulphate fractionation. Residual suspension of each fraction was then tested for enzyme activity and protein. Other experimental details are given in **Materials and Methods.**

(presence of endogenous substances) and hence, ammonium sulphate fractionation was carried out regularly and the active F_2 fraction or 24 h-old fraction was routinely used in the subsequent experiments.

Results of a typical experiment are recorded in Table 5.

Table 5

3.4.3. Stabilizing effect of NADP$^+$

The protective effect of NADP$^+$ if any, on the enzyme was tested using the F_2 gel filtrate. The enzyme preparation was stored in the cold and the activity was measured at the end of 24 and 72 hours. At the end of 24 hours there was a decrease in enzyme activity. Only 50 % enzyme activity was retained in the absence of added NADP$^+$, whereas 67 % activity was retained in the enzyme preparation that contained 1.0×10^{-5} M NADP$^{+\cdot}$ There was a further decrease in enzyme activity after 72 hours. Only 30 % of the activity remained in untreated enzyme sample whereas 38 % remained in the presence of 1.0×10^{-5} M, NADP$^+$. Fresh enzyme samples or 24 hour-old samples with 1.0×10^{-5} M, NADP$^+$ were used routinely in all subsequent experiments.

The results of a typical experiment are recorded in Table 6.

Table 6

Table 5

Stability of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum* during storage.

Enzyme source	Total enzyme activity, units		
	0 h	24 h	72h
Supernatant (20,000 x *g*)	28.26 (100)	17.49 (62)	13.28 (47)
F2- (Sephadex G-25 gel filtrate)	23.74 (100)	11.75 (50)	7.06 (30)

The data presented are from a typical experiment. The enzyme preparations were kept under cold condition and the activities at 0 h, 24 h and 72 h were measured. The values in parenthesis represent the % activities remaining. Details are as explained in **Materials and Methods.**

Table 6

Stabilizing effect of $NADP^+$ on glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum.*

Enzyme source	Total enzyme activity, units		
	0 h	24 h	72 h
F2 gel Filtrate	24.6 (100)	12.18 (50)	7.32 (30)
F2 gel Filtrate + $NADP^+$ (1.0×10^{-5} M)	27 (100)	18.06 (67)	10.26 (38)

The data presented are from a typical experiment. The enzyme was stored in the cold in the cold in the presence of 1.0×10^{-5} $NADP^+$. The values in parenthesis represent the % activities remaining. Details are as explained in **Materials and Methods.**

3.4.4. Sephadex G-200, column chromatography

The partially purified enzyme preparation obtained after ammonium sulphate fractionation (30-70 % fraction) was subjected to further purification by molecular sieving in a Sephadex G-200 column as described earlier (see section **2.2.4.5**). Each fraction thus collected was assayed for glucose-6-phosphate dehydrogenase activity. Protein in each fraction was also determined. The results of a typical experiment carried out for enzyme purification using Sephadex G-200 column chromatography are recorded in Table7 and Figure7.

Table 7

Figure 7

Of the 15 fractions, the first 2 fractions were devoid of protein and enzyme activity. The fractions 8, 9, 10, 11, and 12 were very active. These fractions also showed high specific activity and fold purification. The other fractions were only feebly active.

Fractions 8-12 contained appreciable recovery of enzyme, fractions 10 and 11 showing the maximum activity. These two fractions together constituted 27.43 % of the loaded enzyme activity. These fractions also revealed high specific activities and fold enrichment. Fractions 3 to 7 showed only very low enzyme activity. 32 % of the total loaded proteins got eluted in fractions 1 to 7. Fractions 8 to 12 contained 22.06 % of the total

Table 7

Sephadex G-200 column chromatographic separation of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum*.

Fraction No.	Protein, mg	Enzyme activity, units	Specific activity.	Enzyme recovery, %
1	0.026	0.000	0.000	0.00
2	0.102	0.000	0.000	0.00
3	0.487	0.236	0.485	0.84
4	0.706	0.420	0.595	1.49
5	0.857	0.643	0.750	2.28
6	1.100	0.536	0.487	1.90
7	1.073	0.590	0.550	2.09
8	0.795	1.350	1.698	4.78
9	0.645	1.758	2.726	6.22
10	0.535	3.279	6.129	11.60
11	0.528	3.233	6.123	11.44
12	0.520	1.760	3.385	6.23
13	0.343	0.943	2.749	3.34
14	0.319	0.622	1.950	2.20
15	0.305	0.257	0.843	0.91

The 30-70 % ammonium sulphate fraction was used for chromatography. A total of 23.74 units of enzyme in 13.702 mg protein were loaded into a 2.5 X 30 cm Sephadex G-200 column which was equilibrated with 50 mM Tris-HCl buffer pH 7.5 containing 10 mM 2-mercaptoethanol and eluted with 50 mM Tris-HCl buffer pH 7.5 and 10 mM 2- mercaptoethanol. The percentage of recovery is in relation to the enzyme loaded. Details are given in **Materials and Methods.**

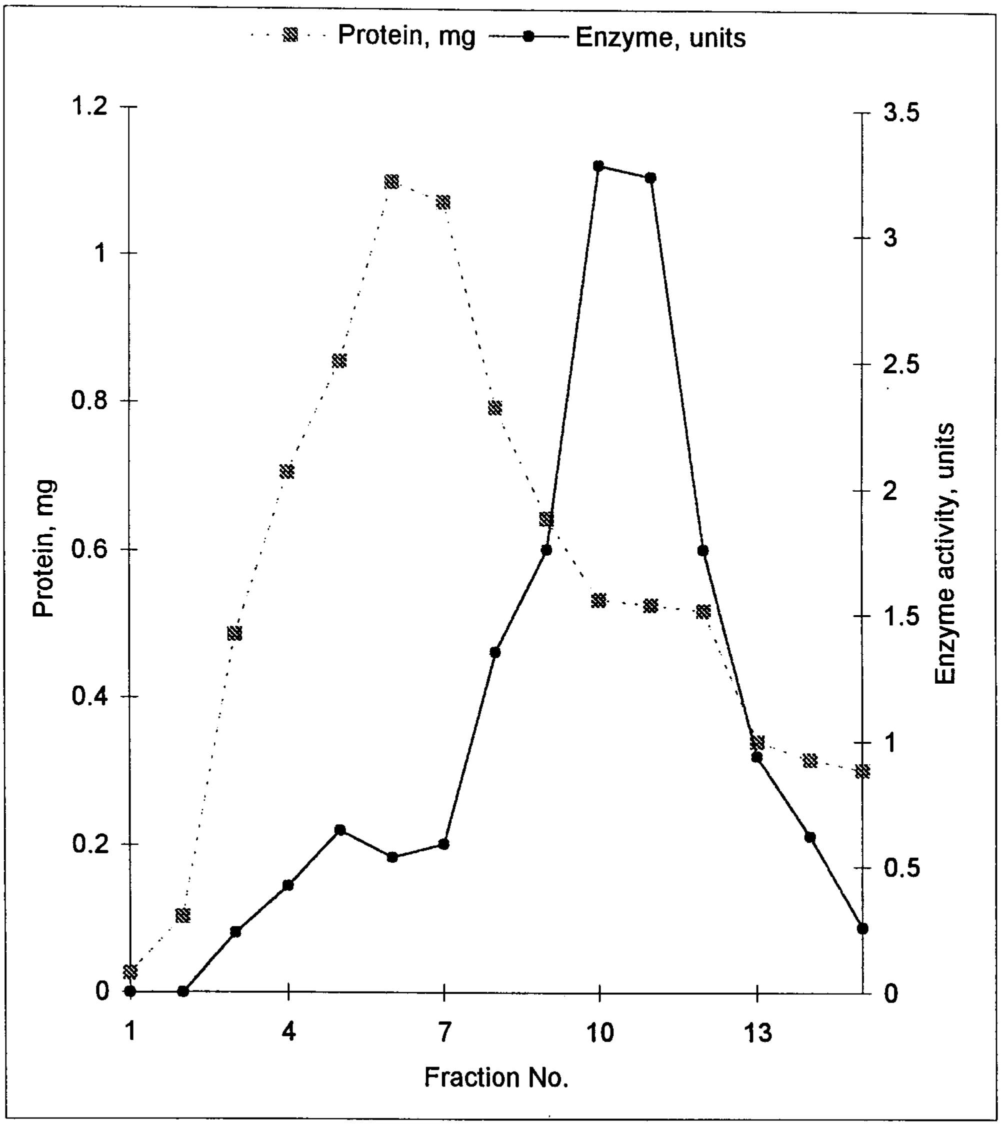

Fig.7. Sephadex G-200 column chromatographic separation of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum*. (Details as described in **Materials and Methods**)

loaded proteins and 48 % of the loaded enzyme activity. When compared to the 20,000 × *g* supernatant, these fractions recorded a fold purification of 4.

About one third of the loaded protein was eluted in fractions 1 to 7. Several 5.0 ml fractions were collected after the 15th fraction; however, they showed no glucose-6-phosphate dehydrogenase activitiy (data not shown in the table).

A summary for the purification of glucose-6-phosphate dehydrogenase from *P. nigrum* leaves is recorded in Table 8

Table 8

3.5. Kinetic studies and properties of purified glucose-6-phosphate dehydrogenase

3.5.1. Lineweaver-Burk plot and Km value determination

3.5.1.1. NADP$^+$ as substrate

The Km value of NADP$^+$ was calculated from the Lineweaver-Burk plot (1/*v* vs 1/*s*) using varying concentrations of NADP$^+$ and saturating concentration of glucose-6-phosphate (see section **2.2.5.1.1**). The purified glucose-6-phosphate dehydrogenase was found to have a Km value of 6.08 × 10^{-5} M for NADP$^{+.}$

The results of a typical experiment are recorded in Figure 8.

Figure 8

Table 8

Protocol of the purification of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum*

Step	Total enzyme activity, units	Total Protein, mg	Specific activity	Fold Purification	Enzyme recovery, %
Supernatant. 20,000 x g	28.26	18.510	1.527	(1)	(100)
30-70 % Ammonium sulphate fraction	23.74	13.702	1.733	1.135	84.00
Sephadex G-200 Fractions.					
Fraction No.8	1.350	0.795	1.698	1.112	4.78
Fraction No.9	1.758	0.645	2.726	1.785	6.22
Fraction No.10	3.279	0.535	6.129	4.013	11.60
Fraction No.11	3.233	0.528	6.123	4.009	11.44
Fraction No.12	1.760	0.520	3.385	2.217	6.23

The data are from a typical experiment. Supernatant of tissue homogenate was subjected to ammonium sulphate fractionation in the range 30 -70 % saturation and the precipitated protein was suspended in a minimum quantity of suspending medium and centrifuged. The supernatant was then subjected to Sephadex G-200 column chromatography as described in Table 7. The experimental details are given in **Materials and Method.**

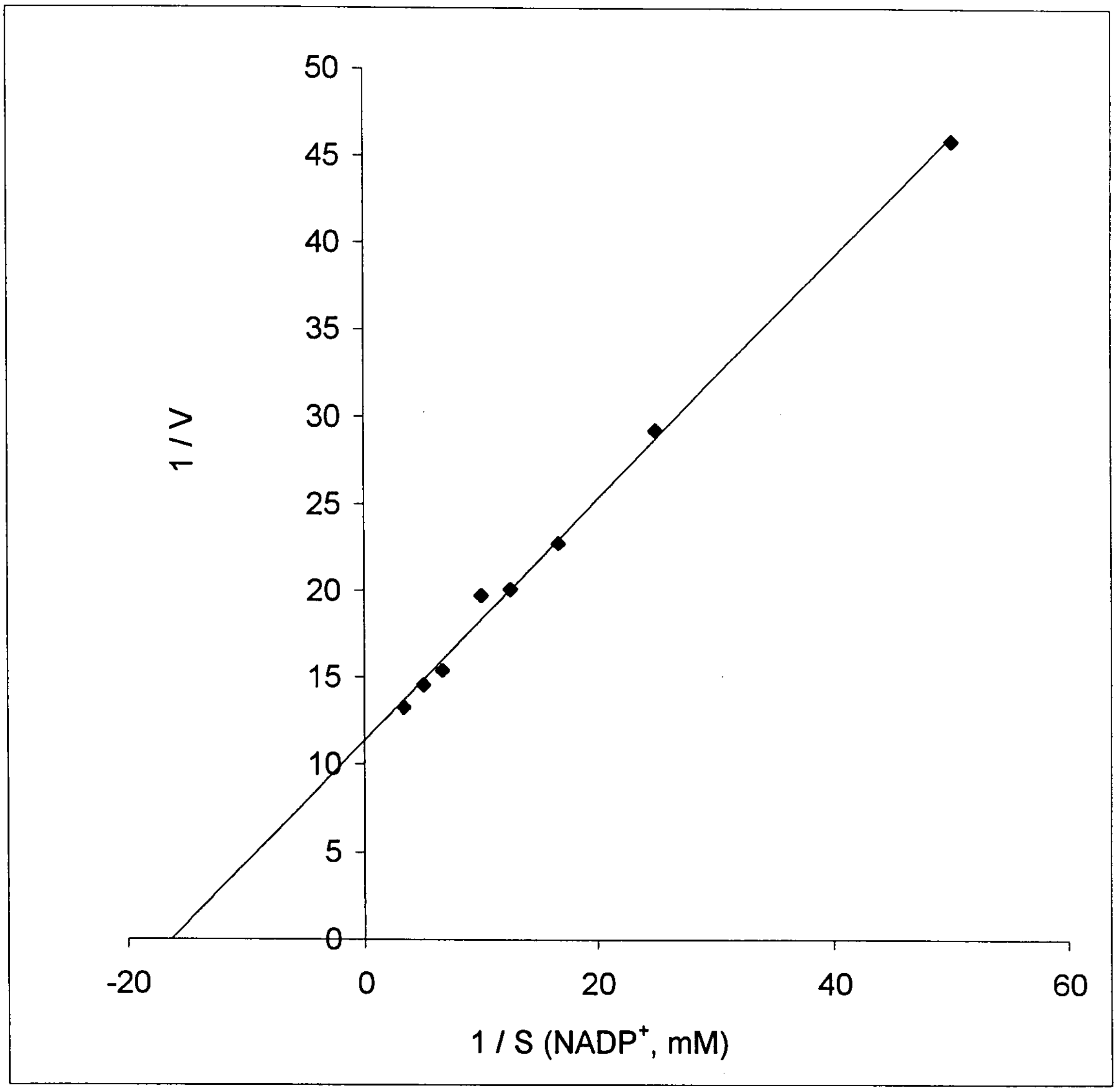

Fig.8. Lineweaver-Burk plot for NADP$^+$ of the purified glucose-6-phosphate dehydrogenase activity from the leaves of *P. nigrum* (Details as described in **Materials and Methods**).

3.5.1.2. Glucose-6-phosphate as substrate

The Km value of glucose-6-phosphate was calculated from the double reciprocal (1/*v* vs 1/*s*) plot using varying concentrations of glucose-6-phosphate but at saturating concentration of $NADP^+$ as explained earlier (see section **2.2.5.1.2)**. The purified glucose-6-phosphate dehydrogenase had a Km value of 2.857×10^{-4} M for glucose-6-phosphate.

The results of a typical experiment are recorded in Figure 9.

Figure 9.

3.5.2. Optimum temperature for the activity of purified glucose-6-phosphate dehydrogenase

The enzyme showed a progressive increase in activity up to 40° C. Thereafter the activity declined gradually and at 55° C only 40 % of the activity at 40° C remained. Thus, the optimum temperature of the enzyme under the conditions of the experiment was 40° C. The activity of glucose-6-phosphate dehydrogenase was doubled at 30° C when compared to that at 20° C. Further increase in temperature from 30 to 40° C resulted in 36 % increase in activity. The results indicated that the enzyme is sensitive to high temperature beyond 40° C.

The results of a typical experiment are recorded in Figure 10.

Figure 10

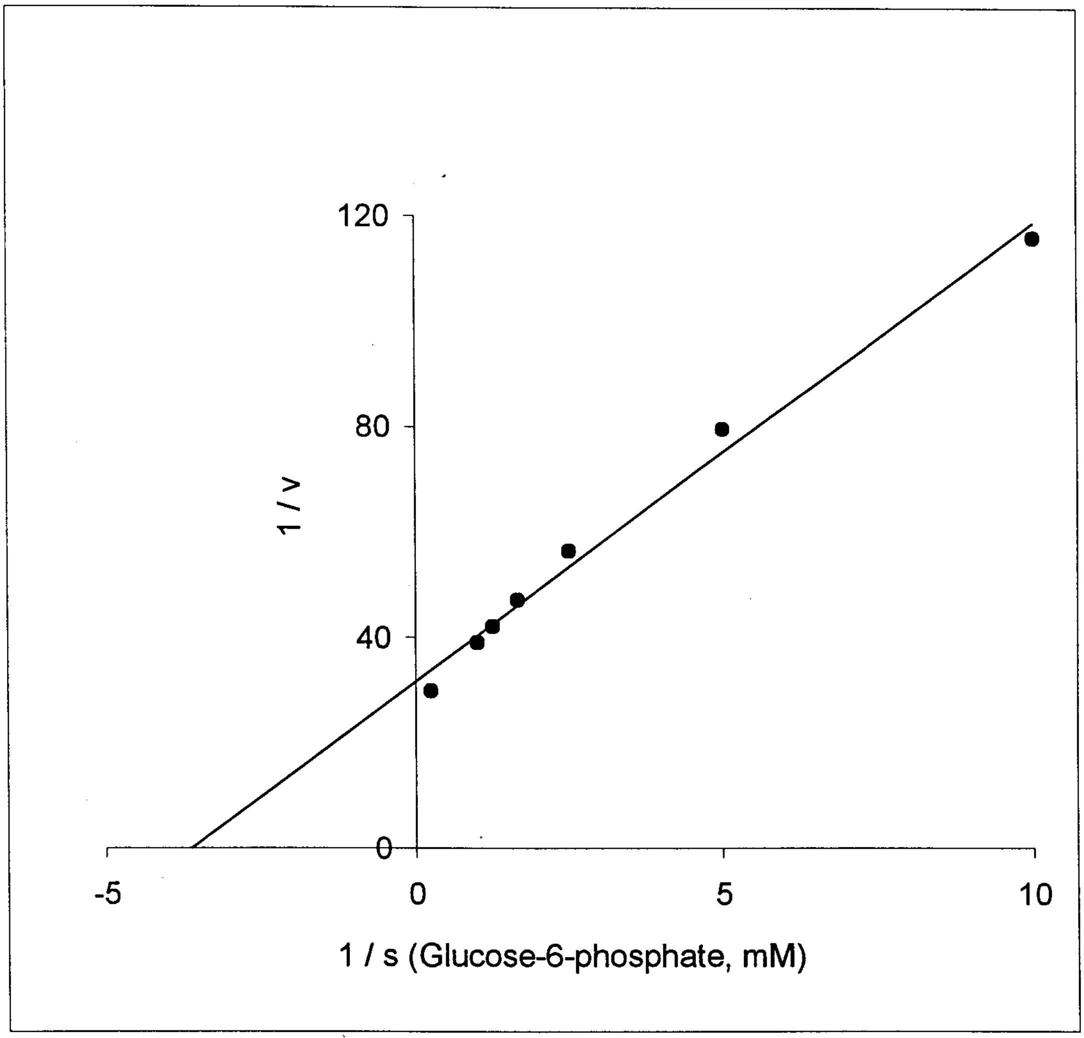

Fig.9. Lineweaver-Burk plot for glucose-6-phosphate on the purified glucose-6-phosphate dehydrogenase activity from *P. nigrum* (Details as described in **Materials and Methods**).

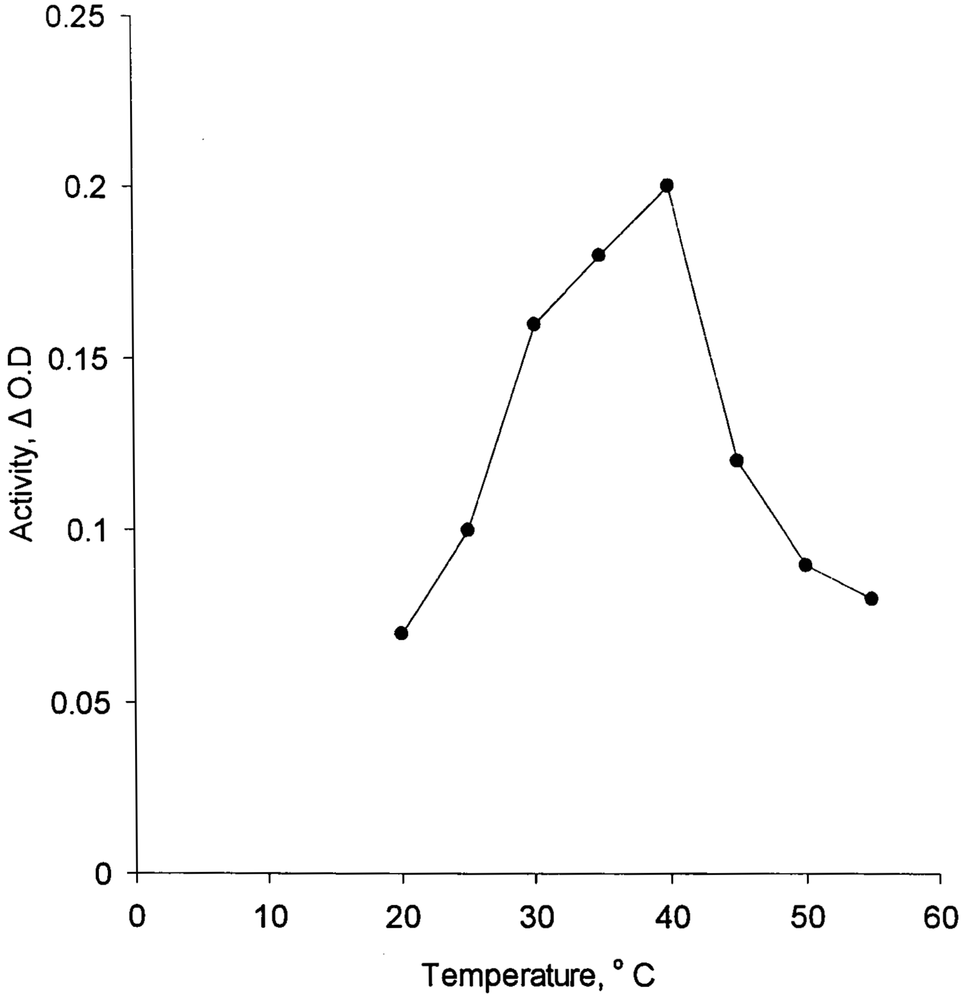

Fig.10. Determination of optimum temperature for glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum* (Details as described in Materials and Methods)

3.6. Effect of additives on the activity of glucose-6-phosphate dehydrogenase

3.6.1. Effect of phenolic compounds

Fourteen phenolic compounds, namely caffeic acid, catechol, pyrogallol, esculin, gallic acid, trans-cinnamic acid, resorcinol, p-coumaric acid, ferulic acid, chlorogenic acid, synapic acid, salicylic acid, shikimic acid, and p-aminobenzoic acid, were included in the assay system at 0.1 and 0.5 mM final concentrations, to test the effect of these compounds (see section **2.2.5.3**). Of the phenolic compounds tested, caffeic acid recorded maximum inhibition, 74 % at 0.1 mM final concentration followed by catechol (58 %). At 0.5 mM, inhibition by catechol was 72 %. Pyrogallol showed an inhibition of 35% at 0.1mM, which increased to 49 % at 0.5mM. Chlorogenic acid elicited 14 % inhibition at 0.05 mM and 17 % at 0.1 mM. All the other phenolic compounds tested showed some degree of inhibition on the activity of glucose-6-phosphate dehydrogenase even at 0.1mM.

The results are recorded in Table 9.

Table 9

3.6.2. Effect of cations

The effect of cations namely, Cd2^{+}, Ni^{2+}, Mo^{2+}, Ca^{2+} and Mn^{2+} were tested generally at 1.0 mM and 10 mM final concentrations. Lower concentrations of metal ions were tested in the case of Hg^{2+}, Zn^{2+}, and Cu^{2+}. Mercury and zinc were found to be the most potent inhibitors of glucose-6-

Table 9

Effect of phenolic compounds on the activity of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum.*

Compound	Concentration, mM	Inhibition, %
Caffeic acid	0.1	74
Catechol	0.1	58
	0.5	72
Pyrogallol	0.1	35
	0.5	49
Esculin	0.1	32
Gallic acid	0.1	26
	0.5	34
trans-Cinnamic acid	0.1	21
	0.5	28
Resorcinol	0.1	16
	0.5	27
p-Coumaric acid	0.1	15
Ferulic acid	0.1	15
Chlorogenic acid	0.05	14
	0.1	17
Synapic acid	0.1	13
Slicylic acid	0.1	13
	0.5	15
Shikimic acid	0.1	11
	0.5	17
p-Aminobenzoic acid	0.1	6
	0.5	13

Percentage of inhibition is calculated based on the activity of the control. Other details are as explained in **Materials and Methods** phosphate dehydrogenase. Mercury recorded an inhibition of 94 % at 0.1mM final concentration and zinc showed an inhibition of 94 % at 0.5 mM final concentration. Copper recorded an inhibition of 87 % at 0.5 mM final concentration. Cadmium and nickel ions elicited 79 % and 65 % inhibition respectively at 1.0 mM concentration. The other ions showed lower degrees of inhibition.

The results of a typical experiment are recorded in Table 10

Table 10

3.6.3. Effect of sugar phosphates

Fructose 1, 6-diphosphate and glucose-1-phosphate showed 20 and 17 % inhibitions respectively at 10 mM concentrations. Fructose-6-phosphate on the other hand showed no inhibitory effect.

The results of a typical experiment are recorded in Table 11.

Table 11.

3.6.4. Effect of some important metabolic intermediates

At a final concentration of 10 mM, pyruvate recorded 24 % inhibition and 2-oxoglutarate and acetate recorded 15 and 11 % inhibition respectively. Citrate showed only 8 % inhibition.

The results of a typical experiment are recorded in Table 12.

Table 12

Table 10

Effect of cations on the activity of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum.*

Compound	Concentration, mM	Inhibition, %
$HgCl_2$	0.01	94
	0.1	95
$ZnSO_4.7H_2O$	0.5	94
	1.0	96
$CuSO_4.5H_2O$	0.5	87
	1.0	87
$CdCl_2$	1.0	79
	10.0	86
$NiSO_4.7H_2O$	1.0	65
	10.0	89
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.0	29
	10.0	62
$CaCl_2.2H_2O$	1.0	17
	10.0	48
$MnSO_4.H_2O$	1.0	6

The data are from a typical experiment. Pooled active fractions of Sephadex G-200 gel filtration was used as the source of enzyme. The values are expressed as % of enzyme inhibition of the control (in the absence of metal ions). Details are as explained in **Materials and Methods.**

Table 11

Effect of sugar phosphates on the activity of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum*

Compound	Concentration, mM	Inhibition, %
Fructose-1, 6-diphosphate	10	20
Glucose-1-phosphate	10	17
Fructose-6-phosphate	10	01

The data are from a typical experiment. Sugar phosphates were incorporated in the assay system at a final concentration of 10 mM. Pooled active fractions of Sephadex G-200 gel filtrate was used as the source of enzyme. Details are as explained in **Materials and Methods**.

Table 12

Effect of some important metabolic intermediates on the activity of glucose-6-phosphate dehydrogenase

Compound (as sodium salts)	Concentration, mM	Inhibition, %
Pyruvate	10	24
2-oxoglutarate	10	15
Acetate	10	11
Citrate	10	8

The data are from a typical experiment. The compounds were incorporated in the assay system at a final concentration of 10 mM. Pooled active fractions of Sephadex G-200 gel filtration was used as the source of enzyme. Details are as explained in **Materials and Methods**.

3.6.5. Effect of some plant growth regulators

GA_3 recorded 20 % inhibition while IAA and 2, 4-D recorded 15 % and 12 % inhibition respectively when tested at a final concentration of 0.1 mM. Higher concentrations were not tested.

The results are recorded in Table 13.

Table 13

3.6.6. Effect of some amino acids

All the amino acids were tested at a final concentration of 10 mM in the assay system. The highest inhibition was observed for isoleucine, phenylalanine, and proline (32 %). Histidine, valine, tryptophan, glutamic acid, leucine and serine showed inhibition in the range 26-21 %. Asparagine, glutamine, and lysine had no effect on glucose-6-phosphate dehydrogenase. All the other amino acids showed intermediate inhibitory effects.

The results are recorded in Table 14.

Table 14.

3.6.7. Effect of some anions

Tested at a final concentration of 10 mM all the anions showed some degree of inhibition. Sulphite recorded the highest inhibition (41 %) followed by fluoride and metabisulphite 32 % and 31 % respectively. The other anions showed lower inhibitory effects on glucose-6-phosphate dehydrogenase.

Table 13

Effect of some plant growth regulators on the activity of glucose-6-phosphate dehydrogenase from the leaves of P. nigrum

Compound	Concentration, mM	Inhibition, %
GA3	0.1	20
IAA	0.1	15
2,4-D	0.1	12

The results are from a typical experiment. All the compounds were incorporated in the assay system at a final concentration of 0.1 mM. Pooled active fractions of Sephadex G-200 gel filtrate was used as the source of enzyme. Details are as explained in **Materials and Methods**.

Table 14

Effect of some amino acids on the activity of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum*.

Amino acid	Concentration. mM	Inhibition, %
Isoleucine (Ile)	10	32
Phenyl alanine (Phe)	10	32
Proline (Pro)	10	32
Histidine (His)	10	26
Valine (Val)	10	24
Tryptophan (Trp)	10	22
Glutamic acid (Glu)	10	21
Leucine (Leu)	10	21
Serine (Ser)	10	21
Arginine (Arg)	10	17
Alanine (Ala)	10	15
Methionine (Met)	10	15
Hydroxyproiline (OH)Pro	10	14
Glycine (Gly)	10	9
Threonine (Thr)	10	7
Aspartic acid (Asp)	10	4
Asparagine (Asn)	10	0
Glutamine (Gln)	10	0
Lysine (Lys)	10	0

Amino acids were incorporated in the assay system at a final concentration of 10 mM. Pooled active fractions of Sephadex G-200 gel filtrate was used as the source of enzyme. Details are as explained in **Materials and Methods**.

The results of a typical experiment are recorded in Table 15.

Table 15

3.6.8. Effect of dithiothreitol and glutathione (reduced)

At a final concentration of 10 mM, dithiothreitol registered an inhibition of 28 % followed by glutathione (26 %).

The results of a typical experiment are recorded in Table 16.

Table 16

3.6.9. Effect of selected nucleotides and nucleotide derivatives

NADPH, NADH and cAMP were tested at a final concentration of 0.1 mM and ATP and NAD^+ were tested at 0.2 mM final concentration. The effects were tested in the presence and absence of Mg^{2+} in the assay system. NADPH recorded 42 % inhibition on the activity of glucose-6-phosphate dehydrogenase in the absence of Mg^{2+} and 37 % inhibition in the presence of Mg^{2+}. Mg^{2+} reduced the inhibition caused by NADPH by 5%. NADPH was found to give the maximum inhibition in the absence or presence of Mg^{2+}. Other compounds tested, showed either no inhibition or marginal inhibition in the absence of Mg^{2+}. However Mg^{2+} had an enhancing influence on the inhibition by these compounds in contrast to the effect of NADPH.

Table 15

Effect of selected anions on the activity of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum.*

Compound (anion)	Concentration, mM	Inhibition, %
Na_2SO_3 (SO_3^{2-})	10	41
NaF (F^-)	10	32
$Na_2S_2O_3$ ($S_2O_3^{2-}$)	10	31
NaNO3 (NO_3^-)	10	23
NaNO2 (NO_2^-)	10	19

The pooled active fractions from Sephadex G-200 column chromatography step were used as the source of enzyme. The supplements were neutralized and prepared in 500 mM Tris-HCl buffer, pH 8.1 and added to the assay system. Details are as explained in **Materials and Methods**.

Table 16

The effect of dithiothreitol and glutathione (reduced) on the activity of glucose-6-phosphate dehydrogenase from *P. nigrum*

Compound	Concentration, mM	Inhibition, %
Dithiothreitol	10	28
Glutathione (reduced)	10	26

Pooled active fractions of Sephadex G-200 gel filtration step was used as the source of enzyme. All the additives were prepared in 500m M Tris-HCl buffer pH 8.1 and incorporated to the assay system. The values are expressed as % of enzyme inhibition of the control (in the absence of additive). Other details are as explained in **Materials and Methods**

The results of a typical experiment are recorded in Table 17.

Table 17

3.7. Identification of the isozymes of glucose-6-phosphate dehydrogenase

The 0-90 % ammonium sulphate fraction contained six isoenzymes of glucose-6-phosphate dehydrogenase as indicated by the dehydrogenase reaction with nitroblue tetrazolium. However only five isoenzymes out of this six could be detected clearly in the 30-70 % ammonium sulphate fraction. Corresponding protein bands (five in all) were also detected by silver staining in the 30-70 % fraction. Protein staining by Coomassie brilliant blue for the 0-90 % saturation hardly revealed detectable bands corresponding to glucose-6-phosphate dehydrogenase isoenzymes. Silver nitrate staining resulted in heavy dark bands by the non-enzymic proteins (figure not included).

The electrophoretic patterns obtained from typical experiment are represented in Fig. 11.

Fig. 11

Table 17

Effect of selected nucleotides and nucleotide derivatives on the activity of glucose-6-phosphate dehydrogenase from the leaves of *P. nigrum.*

Compound	Concentration, mM	Inhibition, %	
		In the absence of Mg^{2+}	In the presence of Mg^{2+} (10 mM)
NADPH	0.1	42	37
NADH	0.1	7	16
cAMP	0.1	0	25
ATP	2.0	14	25
NAD^+	0.2	4	20

Pooled active fractions of Sephadex G-200 gel filtration step was used as the enzyme source. The additives were prepared in Tris-Hcl buffer pH 8.1 and incorporated to the assay system (with and without $MgCl_2$). The values are expressed as percentage activities of the control. Other details are as explained in **Materials and Methods.**

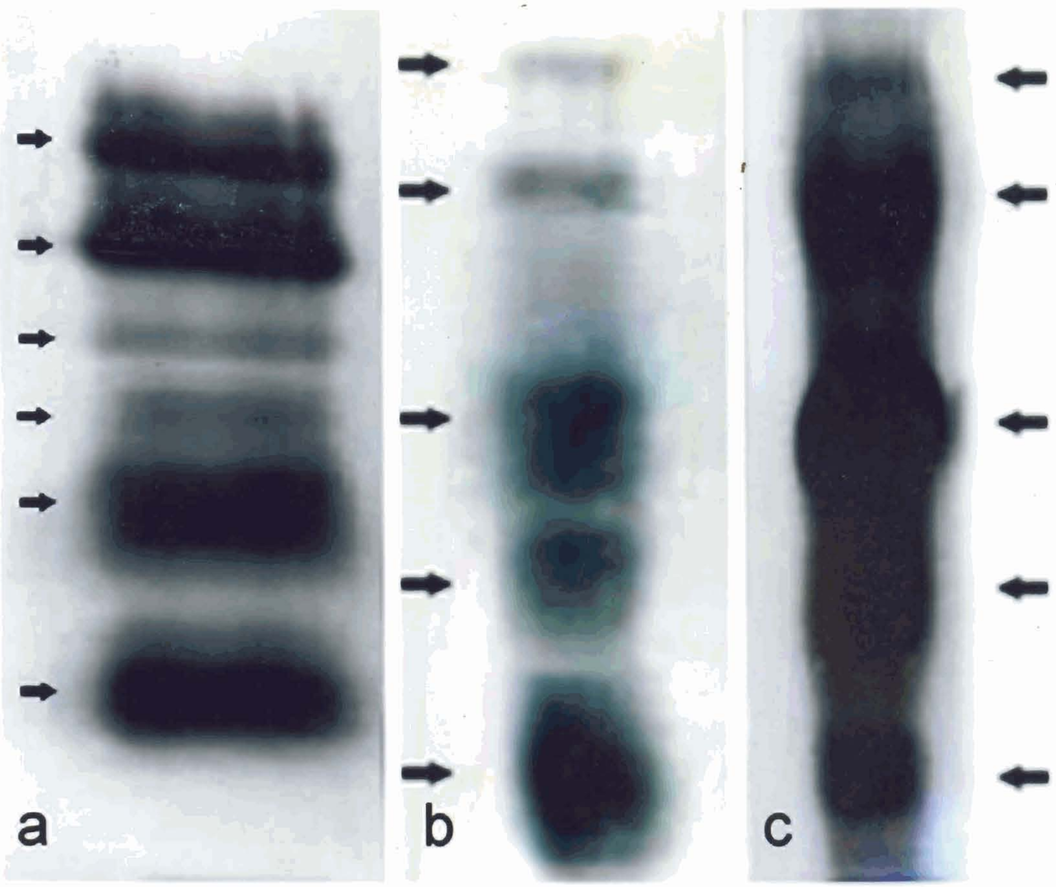

Fig. 11 Native poly acrylamide gel electrophoresis of the isoforms of glucose-6-phosphate dehydrogenase in the leaves of *P. nigrum*.

a. 0-90 % ammonium sulphate fraction
b. 30-70 % ammonium sulphate fraction
c. Silver staining of the protein bands of b.

3.8. Biochemical studies in the leaves of control and P. capsici – infected *P. nigrum*

3.8.1. Determination of dry weight percentage

The control leaf tissues had an average dry weight percentage of about 15 %. A decrease in the dry weight percentage was noted in all the cases of infected plants. 24 hours after infection, the leaves registered a marginal decrease of about 10 % in dry weight ($p < 0.124$). In 48 hours there was a decrease of 15.5 % ($p < 0.038$), and in 72 hours a decrease of only 8.48 % ($p < 0.4$) in the dry weight percentages was observed

The results indicated that infection by *Phytophthora capsici* did not bring about a drastic loss of dry matter in the leaves of *Piper nigrum.*

The data are presented in Table 18.

Table 18

3.8.2. Estimation of total phenolics

The total phenolic content was calculated both on fresh tissue and dry tissue equivalents. A statistically significant increase in total phenolic content was noted in the 24 and 48 hours- infected leaves in comparison to the controls. In leaves 24 hours after inoculation there was an increase of 52.72 % phenolic content ($p < 0.015$) on a fresh tissue basis and an increase of 72.39 % ($p < 0.015$) phenolic content on a dry tissue basis. The magnitude of increase showed a downward trend in 48 hours and the

Table 18

Percentage of dry weight during different periods of infection by *P. capsici* in the leaves of *P.nigrum*

Dry weight, %						
Sl. No of Expt.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1.	14.57	13.61	18.97	15.75	19.67	17.69
2.	17.19	16.40	15.83	11.75	12.10	11.73
3.	16.59	12.52	14.90	13.10	14.22	13.37
4.	13.22	12.44	13.60	12.02	12.85	12.31
5.	16.74	15.22	15.94	12.63	15.94	12.63
6.	14.37	13.20	13.59	13.16	13.59	13.16
Mean ±	15.45	13.90	15.47	13.07	14.73	13.48
S.D	1.61	1.59	2.00	1.43	2.75	2.14
p <	0.124		0.038		0.402	

HAI - hours after inoculation

increase in phenolics content was only 40.40 % ($p < 0.04$) on a fresh tissue basis and an increase of 66.44 % ($p < 0.001$) on a dry tissue basis. 72 hours after inoculation, the increases were only 13.16 % and were statistically not significant ($p < 0.58$) on a fresh tissue basis and 20.16 % ($p < 0.24$) on a dry tissue basis.

In general the infection resulted in an increase in phenolics content; however, this was prominent only in the early phase of infection.

The results are recorded in Tables 19 and 20.

Table 19

Table 20

3.8.3. Estimation of total free amino acids

The total amino acid content was calculated both on fresh tissue and dry tissue equivalents. The amino acid content increased significantly in plants 24 HAI. The rate of increase during the 48-hour period was marginal when compared to the 24 HAI plants; a further increase was noted again during the 72-hour period.

In the plants 24 HAI, there was an increase of 36.03 % ($p < 0.043$) on a fresh tissue basis and 53.31 % ($p < 0.022$) on a dry tissue basis. In the plants 48 HAI, the increase in amino acid content was only 13.26 % ($p < 0.189$) and 32.28 % ($p< 0.024$) on a fresh tissue and dry tissue basis respectively. In the plants 72 HAI, the increase was 27.80 % ($p < 0.080$) on a

Table 19

Changes in the phenolic content during different stages of infection by *P. capsici* on the leaves of *P. nigrum*

Total Phenolics, mg / g fresh tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	4.56	5.60	3.32	3.67	6.01	7.6
2	3.16	3.64	2.07	3.80	3.72	4.06
3	2.48	5.57	3.61	3.71	3.97	4.06
4	2.19	6.04	3.14	4.36	2.58	2.98
5	3.28	4.45	3.15	4.29	4.21	4.14
6	3.13	3.39	2.52	5.16	2.33	2.99
Mean ±	3.13	4.78	2.97	4.17	3.80	4.30
S.D	0.82	1.12	0.59	0.57	1.33	1.71
p <	0.015		0.004		0.58	

Table 20

Changes in the phenolic content during different stages of infection by *P. capsici* on the leaves of *P. nigrum*

Total Phenolics, mg / g dry tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	31.30	41.15	17.50	23.30	30.55	42.96
2	18.38	22.20	13.08	32.34	30.74	34.61
3	14.95	44.5	24.22	28.32	27.92	30.37
4	16.57	48.55	23.09	36.27	19.92	20.05
5	19.60	29.24	19.76	33.97	26.41	32.78
6	21.78	25.68	18.54	39.21	17.14	22.72
Mean ±	20.43	35.22	19.37	32.24	25.45	30.58
S.D	5.83	10.91	4.03	5.71	5.67	5.69
p <	0.015		0.001		0.240	

The values for phenolics on dry tissue equivalence were calculated using the data from **Table 18** for percentage of dry weight.

fresh tissue equivalent and 40.57 % ($p < 0.039$) on a dry tissue equivalent. There was a general increase in the total amino acid content in all the infected plants (24 HAI, 48 HAI, and 72 HAI) when compared to the controls.

The results are recorded in Tables 21 and 22.

Table 21

Table 22

3.8.4. Estimation of total proline

When compared to the corresponding controls an increase in total proline content in all the infected plants was noted. In plants 24 HAI, the increase was not significant both on fresh tissue basis and dry tissue basis. The increase was 23.08 % ($p < 0.44$) on a fresh tissue basis and 35.37 % ($p < 0.228$) on a dry tissue basis. The plants 48 HAI registered a further increase in proline content of 25 % ($p < 0.278$) on a fresh tissue basis and an increase of 54.05 % ($p < 0.086$) on a dry tissue basis. In plants 72 HAI, the increase was 60 % ($p < 0.091$) on a fresh tissue basis and 79.80 % ($p < 0.05$) on a dry tissue basis. In contrast to the changes of phenolic content, the increase in proline content was significant towards the later advanced phase of infection. A tendency of increase in the total proline content was noted as the process of infection progressed and this has culminated in plants 72 HAI.

Table 21

Changes in the total amino acids during different periods of infection by *P. capsici* on the leaves of *P. nigrum*

Total amino acids μ mole /g fresh tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	15.91	24.12	11.20	13.30	16.32	18.21
2	10.91	13.38	9.08	10.69	10.92	14.75
3	16.32	18.21	11.16	11.45	1077	11.10
4	11.47	20.13	12.54	12.83	13.15	23.28
5	13.65	17.74	7.26	9.41	12.85	16.06
6	10.04	12.94	10.75	12.54	13.67	15.87
Mean ±	13.05	17.75	10.33	11.70	12.95	16.55
S.D	2.66	4.21	1.87	1.47	2.04	4.04
p <	0.043		0.189		0.080	

Table 22

Changes in the total amino acids during different periods of infection by *P. capsici* on the leaves of *P. nigrum*

Total amino acids, μ mole / g dry tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	109.20	177.22	59.04	84.44	82.97	102.94
2	63.47	81.59	57.36	90.98	90.25	125.74
3	98.37	145.45	74.89	87.40	75.74	83.02
4	86.76	161.82	92.21	106.74	102.33	189.11
5	81.54	116.58	45.55	74.51	80.61	127.16
6	69.87	98.03	79.10	95.29	100.59	120.59
Mean ±	84.87	130.11	68.03	89.89	88.75	124.76
S.D	17.16	37.49	17.05	10.84	10.92	35.73
p <	0.022		0.024		0.039	

The values for amino acids on dry tissue equivalence was calculated using the data from **Table 18** for dry weights.

The results are recorded in Tables 23 and 24.

Table 23

Table 24.

3.8.5. Estimation of total proteins

The protein content of the infected leaves at different periods of infection was determined along with that of the controls. There was a general decrease in the protein content during infection compared to the controls. The decrease in total proteins in infected plants was significant on a fresh tissue equivalent even though the decrease was not significant on a dry tissue equivalent during the different periods of infection. The plants 24 HAI, recorded a decrease in protein content of 14.47 % ($p < 0.017$) on a fresh tissue equivalent and 4.27 % ($p < 0.518$) on a dry tissue equivalent. The 48 HAI plants recorded a decrease in protein content of 14.19 % ($p < 0.042$) on a fresh tissue equivalent and 0.93 % ($p < 0.93$) on a dry tissue equivalent. In the 72 HAI plants, the decrease in protein content was 18.05 % ($p > 0.008$) on a fresh tissue equivalent and 11.67 % ($p > 0.34$) on a dry tissue equivalent.

The results are recorded in Tables 25 and 26.

Table 25

Table 26

Table 23

Changes in the total proline content during different periods of infection by *P. capsici* on the leaves of *P. nigrum*

Total Proline, µ mole /g fresh tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	0.17	0.25	0.16	0.13	0.24	0.38
2	0.22	0.22	0.17	0.23	0.19	0.38
3	0.11	0.08	0.08	0.09	0.11	0.17
4	0.08	0.12	0.08	0.17	0.12	0.15
5	0.07	0.12	0.09	0.11	0.11	0.18
6	0.11	0.14	0.11	0.14	0.11	0.18
Mean ±	0.13	0.16	0.12	0.15	0.15	0.24
S.D	0.06	0.07	0.04	0.05	0.06	0.108
p <	0.444		0.278		0.091	

Table 24

Changes in the total proline content during different periods of infection by *P. capsici* on the leaves of *P. nigrum*

Total Proline, µ mole / g dry tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	1.17	1.84	0.84	0.83	1.22	2.15
2	1.28	1.34	1.07	1.96	1.57	3.24
3	0.66	0.64	0.54	0.69	0.77	1.27
4	0.61	0.96	0.59	1.41	0.93	1.22
5	0.42	0.79	0.56	0.87	0.69	1.43
6	0.77	1.06	0.81	1.06	0.81	1.37
Mean ±	0.82	1.11	0.74	1.14	0.99	1.78
S.D	0.34	0.43	0.21	0.473	0.34	0.79
p <	0.228		0.086		0.050	

The values for proline on dry tissue equivalence was calculated using the data from **Table 18** for dry weights.

Table 25

Changes in the total proteins during different periods of infection by *P. capsici* on the leaves of *P. nigrum*

Total proteins, mg / g fresh tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	6.40	6.11	7.82	6.22	8.49	5.57
2	7.48	6.31	8.57	5.48	7.99	6.77
3	8.53	6.37	6.78	6.66	8.57	6.20
4	7.34	6.72	8.24	7.59	7.30	5.43
5	8.84	6.82	7.85	7.60	8.90	5.74
6	7.43	7.01	6.80	6.00	7.65	7.06
Mean ±	7.67	6.56	7.68	6.59	7.48	6.13
S.D	0.89	0.35	0.74	0.87	0.75	0.67
p <	0.017		0.042		0.008	

Table 26

Changes in the total proteins during different periods of infection by *P. capsici* on the leaves of *P. nigrum*

Total proteins, mg /g dry tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	43.93	44.89	41.22	39.49	32.99	31.49
2	43.51	38.48	54.14	46.62	66.03	57.72
3	51.51	50.88	45.50	50.84	60.27	46.37
4	55.52	54.02	68.59	63.14	56.81	44.11
5	52.81	44.81	49.25	60.17	43.29	45.45
6	51.70	53.11	50.04	45.59	56.29	53.65
Mean ±	49.83	47.70	51.46	50.98	52.61	46.47
S.D	4.95	6.01	8.46	9.08	12.18	9.05
p <	0.518		0.930		0.344	

The values for total proteins on dry tissue equivalence was calculated using the data from **Table 18** for dry weights.

3.9. Changes in the activity of glucose-6-phosphate dehydrogenase

3.9.1. Total enzyme activity

The total enzyme activity during different periods of infection was expressed both on fresh tissue equivalent and dry tissue equivalent. The 24 HAI plants showed an increase in total enzyme activity by 78.88 % ($p < 0.0163$) on a fresh tissue basis and 102.18 %($p < 0.018$) on a dry tissue basis. The 48 HAI plants showed the highest increase in total enzyme activity. The increase in total enzyme activity in these plants was over 112.02 % ($p < 0.007$) on a fresh tissue equivalent and 147.24 % ($p < 0.007$) on a dry tissue equivalent. There was also an increase in total enzyme activity in the 72 HAI plants. The increase was nearly comparable with that of the 48 HAI plants. These plants have recorded an increase in total enzyme activity of 117.10 % ($p < 1.38365$ E- 06) on a fresh tissue equivalent and 134.15 % ($p < 3.36549$ E- 05) on a dry tissue equivalent.

As the infection progressed, the glucose-6-phosphate dehydrogenase showed an increase in enzyme activity from 24 hours to 72 hours after inoculation when compared to the controls.

The results are recorded in Tables 27and 28.

Table 27

Table 28

Table 27

Changes in the activity of glucose-6-phosphosphate dehydrogenase during different periods of infection by *P. capsici* in the leaves of *P. nigrum.*

Total enzyme activity, units / g fresh tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	23.18	45.93	20.14	44.25	26.94	70.68
2	24.00	28.27	26.92	32.91	21.99	70.42
3	36.98	63.83	27.42	68.26	33.50	59.97
4	33.89	74.05	38.91	90.29	38.29	62.77
5	26.75	60.58	21.90	45.21	30.71	74.50
6	25.98	32.82	24.44	57.72	38.78	74.57
Mean ±	28.46	50.91	26.62	56.44	31.70	68.82
S.D	5.64	18.22	6.65	20.57	6.55	6.10
p <	0.0163		0.007		1.38365 E-06	

Table 28

Changes in the activity of glucose-6-phosphosphate dehydrogenase during different periods of infection by *P. capsici* in the leaves of *P. nigrum.*

Total enzyme activity, units / g dry tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	159.09	337.47	106.17	280.95	136.96	399.55
2	139.62	172.38	170.06	280.01	181.74	600.34
3	222.91	509.82	184.03	521.07	235.58	448.54
4	256.35	595.26	286.10	751.02	297.98	509.91
5	159.80	398.03	137.39	357.96	192.66	589.87
6	180.79	248.64	179.84	438.60	285.36	566.64
Mean ±	186.43	376.93	177.27	438.29	221.71	519.14
S.D	44.50	158.51	60.98	179.44	62.75	81.52
p <	0.018		0.007		3.36549 E-05	

The values for total enzyme activity of glucose-6-phosphosphate dehydrogenase during different periods of infection by *P. capsici* in the leaves of *P. nigrum* was calculated using the data from **Table 18** for percentage of dry weight.

3.9.2. Specific activity

All the infected plants showed statistically significant increase in specific activity. The 24 HAI plants have registered an increase in specific activity of 97.58 % ($p < 0.003$). The 48 HAI plants showed a similar trend of increase in specific activity. These plants recorded an increase in specific activity of 143.39 % ($p < 0.0008$). The increase in specific activity in the 72 HAI plants also showed a similar tendency of increase as that of the 48 HAI plants. They have registered an increase of 165.49 % ($p < 8.25628$ E- 07).

As a result of infection, the glucose-6-phosphate dehydrogenase showed progressive increase in specific activity from 24 hours to 72 hours after inoculation when compared to the controls.

The results are recorded in Table 29.

Table 29

3.9. Changes in the activity of 6-phosphogluconate dehydrogenase in the leaves of control and *P. capsici* infected *P. nigrum*

3.10.1. Total enzyme activity

The total enzyme activity during different periods of infection was expressed both on fresh tissue equivalent and dry tissue equivalent. The 24 HAI plants showed an increase in total enzyme activity of 30.7 % ($p < 0.025$) on a fresh tissue basis and 37.39 % ($p < 0.032$) on a dry tissue basis. The 48 HAI plants registered the highest total enzyme activity. The increase in

Table 29

Changes in the specific activity of glucose-6-phosphosphate dehydrogenase during different periods of infection by *P. capsici* in the leaves of *P. nigrum.*

Specific activity, units / mg protein						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	3.62	7.52	2.58	7.11	4.15	12.69
2	3.20	4.48	3.14	6.01	2.75	10.4
3	4.34	10.02	4.04	10.25	3.91	9.67
4	4.62	8.49	4.72	11.90	5.25	11.56
5	3.03	8.88	2.79	5.95	4.45	12.98
6	3.50	4.68	3.59	9.62	5.07	10.56
Mean ±	3.72	7.35	3.48	8.47	4.26	11.31
S.D	0.63	2.29	0.81	2.47	0.90	1.33
p <	0.003		0.0008		8.25628 E-07	

The values for specific activity of glucose-6-phosphosphate dehydrogenase during different periods of infection by *P. capsici* in the leaves of *P. nigrum* were calculated using the data from **Table 25** for total proteins on fresh tissue equivalence total enzyme activity in these plants was 81.83 % ($p < 0.0004$) on a fresh tissue equivalent and 116.55 % ($p < 0.0008$) on a dry tissue equivalent. There was hardly any further increase in enzyme activity in 72 HAI plants and was comparable with that of the 48 HAI plants but slightly lower. They have recorded an increase in total enzyme activity of 84.14 % ($p < 1.66166$ E- 05) on a fresh equivalent and 95.48 % ($p < 0.0007$) on a dry tissue equivalent.

As a result of infection the 6-phosphogluconate dehydrogenase showed progressive increase in enzyme activity from 24 hours to 72 hours after inoculation when compared to the controls.

The results are recorded in Tables 30 and 31.

Table 30

Table 31

3.10.2. Specific activity

The changes in the levels activity of the enzyme 6-phodphogluconate dehydrogenase during different periods of infection were studied in plants 24 HAI, 48 HAI and 72 HAI and compared with that of the controls. All the infected plants showed very significant increase in specific activity. The 24 HAI plants registered an increase in specific activity of 51.34 % ($p < 0.006$). The 48 HAI plants showed a similar trend of increase in specific activity. These plants recorded an increase in specific activity of 115.90 % (p <

Table 30

Changes in the activity of 6-phosphogluconate dehydrogenase during different periods of infection by *P. capsici* in the leaves of *P. nigrum.*

Total enzyme activity, units / g fresh tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	23.44	24.97	19.42	26.78	18.22	31.78
2	27.56	33.50	21.86	38.26	17.25	45.71
3	21.93	31.16	16.05	36.82	22.22	39.26
4	21.96	38.14	23.73	41.32	23.47	34.85
5	20.32	23.12	14.15	28.36	20.03	34.50
6	19.95	25.82	15.40	29.61	20.96	38.84
Mean ±	22.53	29.45	18.44	33.53	20.36	37.49
S.D	2.77	5.80	3.85	6.03	2.36	4.92
p <	0.025		0.0004		1.66166E-05	

Table 31

Changes in the activity of 6-phosphogluconate dehydrogenase during different periods of infection by *P. capsici* in the leaves of *P. nigrum.*

Total enzyme activity, units / g dry tissue						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	160.88	183.47	102.37	169.4	115.68	179.65
2	160.33	204.27	138.09	325.62	142.56	389.68
3	132.19	248.88	107.72	281.07	156.26	293.64
4	166.11	306.59	174.49	343.76	182.65	283.10
5	181.12	151.91	88.77	224.54	125.66	273.16
6	138.83	195.61	113.32	225.00	154.23	295.14
Mean ±	156.58	215.12	120.79	261.57	146.17	285.73
S.D	18.09	54.78	30.90	67.00	23.92	66.89
p <	0.032		0.0008		0.0007	

The values for total enzyme activity on dry tissue equivalence was calculated using the data from **Table 18** for percentage of dry weight.

0.0002). The specific activity in the 72 HAI plants showed a greater increase with an increase of 133.08 % ($p < 6.04294$ E- 08).

As a result of infection the 6-phosphogluconate dehydrogenase showed progressive increase in specific activity from 24 hours to 72 hours after inoculation when compared to the controls.

The results are recorded in Table 32.

Table 32

3.11. Identification of isoenzymes of glucose-6-phosphate dehydrogenase in the leaves of control and *P. capsici* - infected *P. nigrum*

Electrophoresis of glucose-6-phosphate dehydrogenase isoenzymes in control and disease -induced plants was carried out as explained earlier (see section **2.2.6.1**) using the enzyme preparation obtained from the 48 HAI plants. Six identical isoforms were clearly visible in both control and infected plants. There was no increase in the number of isozymic forms of glucose-6-phosphate dehydrogenase as a result of infection but all the isoforms in the infected plants exhibited stronger dehydrogenase reaction than that of the controls as indicated by the reaction with nitroblue tetrazolium. The data on total enzyme activity presented in Tables 27 and 28 also support this.

The results of a typical experiment are shown in Figure 12.

Figure 12

Table 32

Changes in the specific activity of 6-phosphogluconate dehydrogenase during different periods of infection by *P. capsici* in the leaves of *P. nigrum.*

Specific activity, units / mg protein						
Expt. No.	24 HAI		48 HAI		72 HAI	
	Control	Infected	Control	Infected	Control	Infected
1	3.66	4.09	2.48	4.31	2.81	5.74
2	3.68	5.31	2.55	6.98	2.16	6.75
3	2.57	4.89	2.37	5.53	2.59	6.33
4	2.99	5.68	2.88	5.44	3.22	6.42
5	2.29	3.39	1.80	3.73	2.25	6.01
6	2.69	3.68	2.26	4.94	2.73	5.50
Mean ±	2.98	4.51	2.39	5.16	2.63	6.13
S.D	0.58	0.92	0.36	1.13	0.39	0.46
p <	0.006		0.0002		6.04294E-08	

The values for specific activity of 6-phosphogluconate dehydrogenase during different periods of infection by *P. capsici* in the leaves of *P. nigrum* was calculated using the data from **Table 25** for total proteins on a fresh tissue equivalence

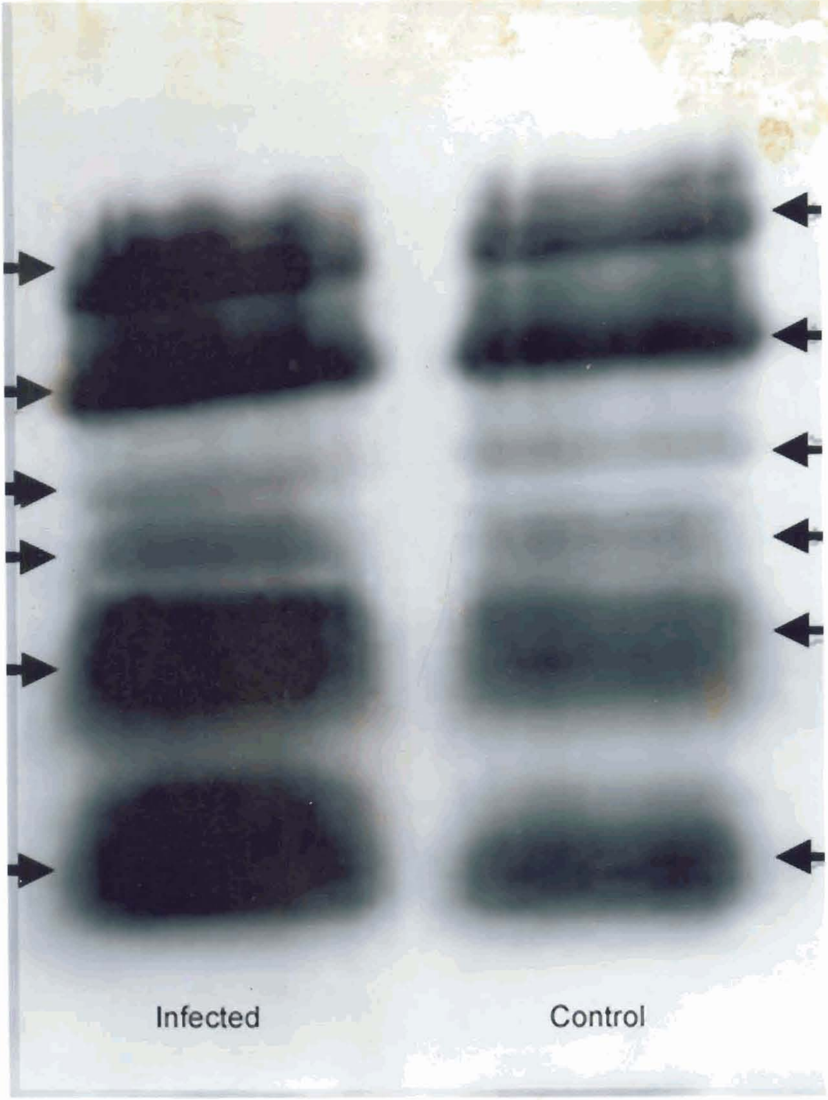

Fig. 12 Native polyacrylamide gel electrophoresis of the isoforms of glucose-6-phosphate dehydrogenase in the *Phytophthora capsici* infected and control leaves of *Piper nigrum*

3.12. Identification of the isoenzymes of 6-phosphogluconate dehydrogenase in the leaves of control and *P. capsici* - infected *P. nigrum*

Electrophoresis of the isoenzymes of 6-phosphogluconate dehydrogenase in control and disease - induced plants was carried out as explained earler (see section **2.2.6.1**) using the enzyme preparation obtained from the 48 HAI plants. As in the case of glucose-6-phosphate dehydrogenase, six similar isoforms of 6-phosphogluconate dehydrogenase were clearly visible in both control and infected plants. They were electrophoretically identical. There was no increase in the number of isoenzymes of as a result of infection but all the isoforms in the infected plants exhibited stronger dehydrogenase reaction than that of the controls as indicated by the reaction with nitro blue tetrazolium.

The data on total enzyme activity presented in Tables 30 and 31 also support this.

The results of a typical experiment are shown in Figure 13.

Figure 13

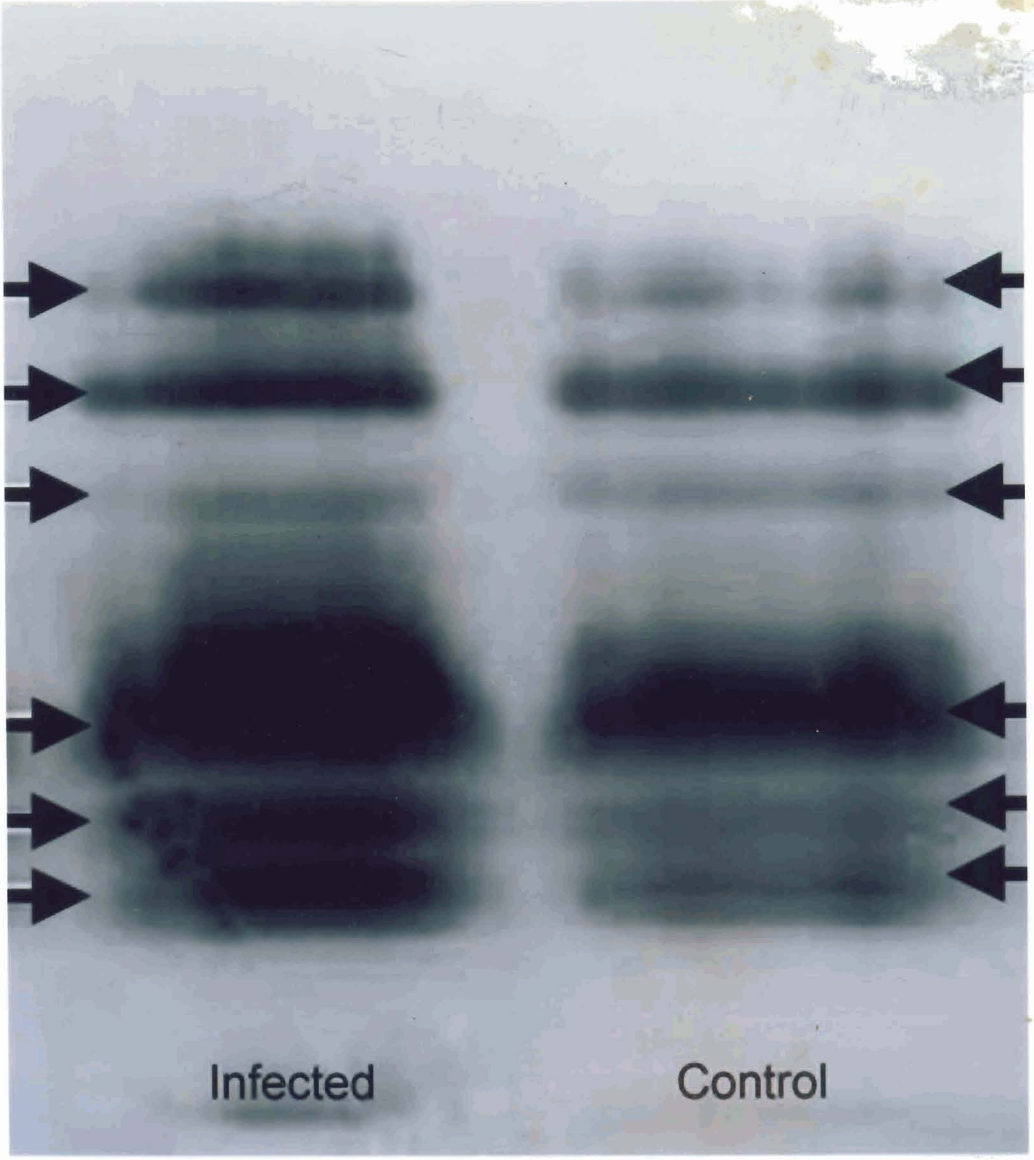

Fig. 13 Native polyacrylamide gel electrophoresis of the isoforms of 6-phosphogluconate dehydrogenase in the *Phytophthora capsici* infected and control leaves of *Piper nigrum*

DISCUSSION

4

DISCUSSION

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4.1. Relevance of the study

4.2. Glucose-6-phosphate dehydrogenase.

The importance of pentose phosphate pathway in plant metabolism has been highlighted in the **Introduction**. Glucose-6-phosphate dehydrogenase is the first enzyme of the pentose phosphate pathway. This enzyme and the second enzyme, 6-phosphogluconate dehydrogenase have been suggested as the controlling enzymes of the pathway. The regulation of the flux of carbon through the oxidative pentose phosphate pathway is an especially important process in metabolism. There is a strong competition for glucose-6-phosphate between oxidative pentose phosphate pathway and glycolysis. The pentose phosphate pathway produces precursors of nucleic acids and phenylpropanoids; ribose-5-phosphate is required for nucleotide biosynthesis and erythrose-4-phosphate is used for the synthesis of phenolic compounds *via* the shikimate pathway.

The NADPH formed during the pathway serves as the readily available reducing equivalent for the reductive biosynthetic reactions in the cytosol such as fatty acid biosynthesis. The pentose phosphate pathway is activated according to the metabolic status of the organism. In general, this pathway and especially the glucose-6-phosphate dehydrogenase are activated in response to pathogenic infection and mechanical wounding. It has been proposed that this pathway may have a greater role in the metabolism of carbohydrates in nodules of ureide- exporting legumes such as soybeans, than in amide exporters (Hong and Copeland, 1990).

The studies on kinetics, the influence of effectors, as well as the isoenzymes of glucose-6-phosphate dehydrogenase are important since the metabolism of glucose-6-phosphate in the pentose phosphate pathway is multi-functional. The occurrence of isozymic forms of the enzyme in chloroplast and cytosol is another means of metabolic control.

The key enzyme, glucose-6-phosphate dehydrogenase, has been extensively investigated by various workers in a variety of plants. The present author's main interest was to study in detail some properties of the enzyme and compare them with those reported for the enzyme from other plant tissues. Also of interest was to follow the changes in the activities of the shunt dehydrogenases and relate these to infection by *Phytophthora capsici.* Being the key enzyme of a multifunctional metabolic pathway, it was important to know how the pathogenic infection in *Piper nigrum* affected the host plant. Supplementary data were also collected in the form of biochemical estimations of total phenolics, total proteins, total amino acids and total proline to correlate pathogenicity with metabolic events.

The results are expected to give a better understanding of the characteristic properties of the enzyme in *P. nigrum*; the influence of the pathogen in the metabolism of the host plant could also be assessed.

The present work may be mainly divided into three phases. The first phase is the preliminary experiments such as propagation of experimental plants, and the standardization experiments for the measurements of enzyme activities. The experimental details of these have already been given in **Materials and Methods.**

The second phase encompasses the purification and isolation of glucose-6-phosphate dehydrogenase and study of the properties. In the third phase of the work the author carried out studies on the effect of infection caused by *P.capsici* on the activities of the shunt dehydrogenases. This phase also includes the various biochemical estimations.

4.3. Investigating a plant enzyme

Researchers often encounter a number of problems associated with the isolation and study of plant enzymes. Toughness of the tissue in many cases, the presence of chlorophyll and other pigments and acidity of the tissue are some of the factors to be taken into account. But a more serious problem encountered is with respect to the high phenolic content of many tissues. Keeping in mind the above, the author carried out several preliminary experiments. The rationale behind these is briefly discussed below.

The inhibition of enzyme activities by phenolics was observed by several earlier workers. (Loomis and Battalle, 1966; Hulme and Jones, 1963; Anderson, 1968). Tannin was shown to be inhibitory to banana aldolase (Young 1965), and polyphenol oxidase (Badran and Jones, 1965). Khanna *et al.*, (1971), showed that the inhibition of glucan phosphorylase in the leaves of *Dendrophthoe falcata* could be considerably reduced by incorporating cysteine and EDTA in the homogenizing medium. A detailed review on enzyme extraction from plant tissues rich in phenols / phenolases was made by Krishnan *et al.*, (1969). While studying the activities of a number of enzymes from the leaf, fruit, pseudostem and root tissues of banana, Baijal *et al.*, (1972) gave a detailed account of the effect of supplementing the homogenizing medium with PVPP and Triton. Haising and Schipper (1975) reported that PVPP and 2-mercaptoethanol alone could stabilize malate dehydrogenase from many plants. Guttenberger *et al.*, (1994) could minimize the effect of interferences by the phenolic compounds on glucose-6-phospate dehydrogenase and other dehydrogenases in *Picea abies* roots by incorporating PVPP during extraction. There are many other reports, which stress the need for the proper formulation of the homogenizing medium by incorporating reducing agents and chelating agents such as EDTA.

The systematic assessment of the effect of additives such as 2-mercaptoethanol, EDTA and PVPP by the present author resulted in obtaining a suitable homogenization medium with minimum interference from the endogenous phenolics.

Apart from the inhibitory effects of phenolics on enzyme activities is the serious interference by phenolics in the colorimetric estimation of proteins. Quinones readily enter into covalent bonding with sulfhydryl groups (Mason, 1955). Stable covalent linkages between proteins and polymerized quinones can also occur (Gustavson, 1956). Presence of phenolics gives high colorimetric readings with the Folin-Ciocalteu phenol reagent for protein estimation by Lowry *et al.*, method.

Khanna *et al.*, (1969) made a comparison of different extraction procedures for determining proteins from the homogenates of *Dendrophthoe falcata* leaves. Based on the results, they recommended a reducing medium of extraction and also acetonic washing of the TCA precipitated protein for minimizing phenolic interferences. Further investigations in this direction were made by Mattoo (1969).

The present author took into consideration the above points while standardizing the extraction conditions for measurements of enzyme activity and protein determination.

4.4. Standardization of enzyme assay

Quantitative measurement of *in vitro* enzyme activity is subject to various factors such as pH, concentration of substrate(s), concentration of enzyme, temperature and incubation period. Unless conditions are standardized, comparisons become erroneous and meaningless. This prompted the author to carry out several preliminary experiments to standardize the assay conditions.

4.4.1. pH optimum for glucose-6-phosphate dehydrogenase

Available literature point to wide variation in pH optima for glucose-6-phosphate dehydrogenase from plant tissues. Ashihara and Komamine (1976) in their studies on the characterization and regulatory properties of glucose-6-phosphate dehydrogenase from black gram (*Phaseolus mungo*) reported a pH optimum of 7.0 (imidazole-HCl). Hoover *et al.*, (1977) studied the effect of phenolic compounds on glucose-6-phosphate dehydrogenase isoenzymes at a pH optimum of 8.1 (Tris-HCl). They also identified two isoenzymes of glucose-6-phosphate dehydrogenase from tobacco cells. These isoenzymes showed different pH optima. The pH optimum for glucose-6-phosphate dehydrogenase I (G6PDH I) was 9.0 and that of G6PDH IV was between 8.0 - 8.3. Lendzian (1980) studied the modulation of G6PDH by NADPH, NADP$^+$ and dithiothreitol at variable NADPH / NADP$^+$ ratios in an illuminated reconstituted spinach (*Spinacia oleracea* L.) chloroplast system at a pH 8.0. Valenti *et al.,* (1984) assayed the isoenzymes of glucose-6-phosphate dehydrogenase from maize leaves at pH 7.5 (Tris-HCl). Gomez-Rodrigues *et al.,* (1987) in their studies on the evolution of G6PDH and 6-phosphogluconate dehydrogenase (6-PGDH) activities and the o-diphenolic content of sunflower leaves cultivated under different boron treatments assayed the enzyme at pH 8.0 (glycylglycine buffer). Hong and Copeland (1991) reported pH optima of 8.5 and 8.1(Tris-HCl) for the isoenzymes of glucose-6-phosphate dehydrogenase in the plant fraction of soybean nodules. Huppe and Turpin (1996) assayed the isoforms of glucose-6-phosphate dehydrogenase in *Chlamydomonas reinhardtii* during growth on nitrate at a pH 7.9 (Tris-HCl). Esposito *et al.*, (1998) also assayed glucose-6-phosphate dehydrogenase at a pH 7.9 (Tris-HCl) in their study on ammonium metabolism stimulation of glucose-6-phosphate dehydrogenase and phosphoenolpyruvate carboxylase in young barley roots. Robinson (2000) assayed G6PDH at pH 8.0 (Tricine buffer) in his study on the dark and day light activity of G6PDH and 6-PGDH in the leaves of nitrogen limited spinach and soybean plants. Esposito *et al.,* (2001) in their studies on the kinetic properties and localization of the isoenzymes of G6PDH in barley roots showed different pH optima of 8.5 for G6PDH I and pH 7.9 (Tris-HCl) for G6PDH II. The plant enzyme is thus optimally active over a pH range of 7.0 to 9.0.

The present author found that the G6PDH in *P. nigrum* leaves showed a pH optimum of 8.1 using Tris-HCl buffer. The pH optimum is comparable to many other reports mentioned above.

4.4.2. Substrate saturation

Experimental measurements of enzyme activities, as far as possible, are to be made at V_{max} so that changes in activities are directly related to the amount of enzyme present in the sample. The author has separately determined the saturating concentration of the substrates - $NADP^+$ and glucose-6-phosphate - and employed these saturating concentrations for all enzyme activity measurements. The saturating concentrations of $NADP^+$ and glucose-6-phosphate were found to be 0.2 and 2.0 mM respectively in the present investigation.

4.4.3. Enzyme concentration and incubation period

Experiments were carried out as described in **Materials and Methods** (see sections **2.2.2.4** and **2.2.2.5)** to ensure that the enzyme velocity measurements are linear with respect to concentration of enzyme and time of incubation. The author strictly maintained these conditions through out the course of the experiments.

The standard assay system described by the author is expected to be the most suitable and valid one for quantitative measurements of enzyme activities carried out in the present studies.

4.4.4. Mg^{2+} as an activator

Mg^{2+}, though not an obligatory requirement for the activity of glucose-6-phosphate dehydrogenase is known to be an activator (Kornberg and Horecker, 1955; De Moss, 1955; Ashihara and Komamine, 1976). It is customary, therefore, to incorporate Mg^{2+} in the assay system for glucose-6-phosphate dehydrogenase. The present author found that 10 mM Mg^{2+} was optimal for glucose-6-phosphate dehydrogenase in the leaf tissues of *P. nigrum.*

4.5. Properties of the purified glucose-6-phosphate dehydrogenase

4.5.1. Specificity towards $NADP^+$

Based on the nucleotide specificity, glucose-6-phosphate dehydrogenase can be classified into three groups (Olive *et al.*, 1971). Yeast and *E. coli* enzyme reacts exclusively with $NADP^+$ while the enzyme from animal sources is mainly $NADP^+$ dependent with weak response towards NAD^+. The enzyme from microorganisms is equally active with $NADP^+$ and NAD^+.

In the present study it was found that the *P. nigrum* leaf glucose-6-phosphate dehydrogenase is very specific for NADP$^+$. This is in agreement with the reports on bamboo (Higuchi and Shimada, 1967), sweet potato (Muto *et al.*, 1969) and lettuce (Speer, 1974). The present result also is in conformity with NADP$^+$- specificity reported for a number of glucose-6-phosphate dehydrogenases from plants.

4.5.2. Km value for glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase catalyzes a bisubstrate reaction. Attempts were made to determine the Km values for both the substrates - NADP$^+$ and glucose-6-phosphate in the present study.

4.5.2.1. Km value for NADP$^+$

Ashihara and Komamine (1976) in their studies on the characterization and regulatory properties of glucose-6-phosphate dehydrogenase from black gram reported a Km value of 14 x 10^{-6} M for NADP$^+$.

In their studies on the isoenzymes of glucose-6-phosphate dehydrogenase from tobacco cells, Hoover *et al.*, (1977) reported different Km values for the two isoenzymes-G6PDH I and G6PDH IV. The Km value for NADP$^+$ of G6PDH I was 6.0 X 10^{-5} M. They also reported negative cooperativity with NADP$^+$ in the case of G6PDH IV. The two Km values for high and low NADP$^+$ concentrations were 6.0 X 10^{-5} M and 1.5 X 10^{-5} M respectively. Hong and Copeland (1991) in their studies on the isoenzymes of glucose-6-phosphate dehydrogenase from the plant fraction of soybean nodules identified two isoenzymes. Both the isoforms showed Km values of approximately 10 X 10^{-6} M for $NADP^+$.

Studies of Esposito *et al.,* (2001) on the kinetic properties of glucose-6-phosphate dehydrogenase in barley roots led to the purification of two different isoforms of glucose-6-phosphate dehydrogenase. G6PDH I exhibited a Km value of 9.4 ±1.9 X 10^{-6} M for $NADP^+$, while that of G6PDH II was 7.0 ± 2.0 X 10^{-6} M.

In the present study, glucose-6-phosphate dehydrogenase of *P.ni*grum leaves showed a Km value of 6.08 X 10^{-5} M for $NADP^+$. This is comparable to the value reported by Hoover *et al.*, (1977) for tobacco cells. Both the black gram enzyme (Ashihara and Komamine, 1976) and barley root enzyme (Esposito *et al.*, 2001) showed significantly lower Km values. The high Km value observed in the present case is quite likely due to the high concentrations of $NADP^+$ employed.

4.5.2.2. Km value for glucose-6-phosphate

In the present study glucose-6-phosphate dehydrogenase showed a Km value of 2.31 x 10^{-4} M for glucose-6-phosphate.

In their studies on the characterization and regulatory properties of glucose-6-phosphate dehydrogenase from black gram Ashihara and Komamine (1976) reported Km values of 1.6 x 10^{-4} M and 6.3 x 10^{-4} M at low and high concentrations of the substrate (1.0 - 6.0 x 10^{-4} M and 6.0 - 30.0 x 10^{-4} M) respectively for glucose-6-phosphate.

In their studies on the isoenzymes of glucose-6-phosphate dehydrogenase from tobacco cells, Hoover *et al.*, (1977) reported different Km values for the two isoenzymes -G6PDH I and G6PDH IV. The reported Km values for G6PDH I and G6PDH IV were 2.2 x 10^{-4} M and 3.1 x 10^{-4} M respectively for glucose-6-phosphate.

Hong and Copeland (1991) in their studies on the isoenzymes of glucose-6-phosphate dehydrogenase from the plant fraction of soybean nodules identified two isoenzymes. Both the isoforms showed Km values of approximately 0.7-1.0 x 10^{-4} M for glucose-6-phosphate. These values are markedly lower when compared to the Km, reported in the present work

Studies of Esposito *et al.,* (2001) on the kinetic properties of glucose-6-phosphate dehydrogenase in barley roots led to the purification of two different isoforms of glucose-6-phosphate dehydrogenase. The two isoforms showed different affinities for glucose-6-phosphate. G6PDH I exhibited a higher Km for glucose-6-phosphate, 1.03 ± 0.1 x 10^{-3} M compared to G6PDH -II, which showed a Km of 0.5 ± 0.06 x10^{-3} M.

4.5.3. Stabilizing effect of NADP$^+$

One of the characteristic properties of purified preparations of glucose-6-phosphate dehydrogenase from various plant tissues is the instability of the enzyme. Valenti *et al.,* (1984) incorporated NADP$^+$ (24 μM) at various stages of purification of glucose-6-phosphate dehydrogenase with a view at stabilization.

Hong and Copeland (1991) in their studies on isoenzymes of glucose-6-phosphate dehydrogenase from the plant fraction of soybean nodules observed that both the isoenzymes-G6PDH I and G6PDH II were very labile in the absence of $NADP^+$, losing 50 % of their activity after 2 hours at 4 ° C in 20 mM Tris-HCl buffer pH 8.0 and 90 % after 12 hours. They could recover approximately 80 % of the lost activity after 12 hours when the enzyme preparation was supplemented with 10 µM $NADP^+$. The stabilizing effect of $NADP^+$ during the various stages of purification of the isoforms of glucose-6-phosphate dehydrogenase from roots of barley was also reported by Esposito *et al.,* (2001).

That the *P. nigrum* leaf glucose-6-phosphate dehydrogenase could be stabilized to some extent by 1.0×10^{-5} M $NADP^+$ was evident from the present experiments. Preliminary experiments were carried out in the G-25 gel filtrate within 2-3 h after homogenization of the tissue and, therefore, $NADP^+$ was not supplemented to stabilize the enzyme. As shown in table-6, 50 % of the activity was retained by 20000 x g supernatant even after 24 h storage However, for electrophoretic studies of G6PDH the procedure was protracted, involving several steps such as ammonium sulphate fractionation, Sephadex G-200 gel filtration and concentration by reverse dialysis (usually after 60 h). Therefore, $NADP^+$ (1.0×10^{-5} M final concentration) was incorporated successfully to stabilize the enzyme.

As was mentioned in the **Introduction**, the function of $NADP^+$ is possibly to facilitate the association of the dimeric forms to tetramer at low concentration of the co-enzyme. The *P. nigrum* enzyme is also expected to behave in a similar manner with respect to the association patterns.

4.5.4. Phenolic compounds and glucose-6-phosphate dehydrogenase

The regulation of pentose phosphate pathway and related phenylpropanoid metabolism is best exemplified by the known inhibition of glucose-6-phosphate dehydrogenase by L-phenylalanine.

The phenylpropanoids, biosynthesized through the shikimate pathway requires erythrose-4-phosphate provided by the operation of pentose phosphate pathway.

Important amino acids such as phenylalanine and tyrosine also take their biosynthetic route from the phenylpropanoids. p-Coumaric acid, chlorogenic acid, caffeic acid and a variety of other phenolic compounds are synthesised using the pentose phosphate pathway intermediates. The role played by pentose phosphate pathway in the biosynthesis of a variety of secondary metabolites in plants is evident from the metabolic chart 1 below

Chart 1

It was, therefore, felt necessary to see whether phenolic compounds have any effect on glucose-6-phosphate dehydrogenase in the *p. nigrum*. Hoover *et al.,* (1977) made a detailed study on the effect of a number of phenolic compounds including ferulic acid, chlorogenic acid, p-coumaric

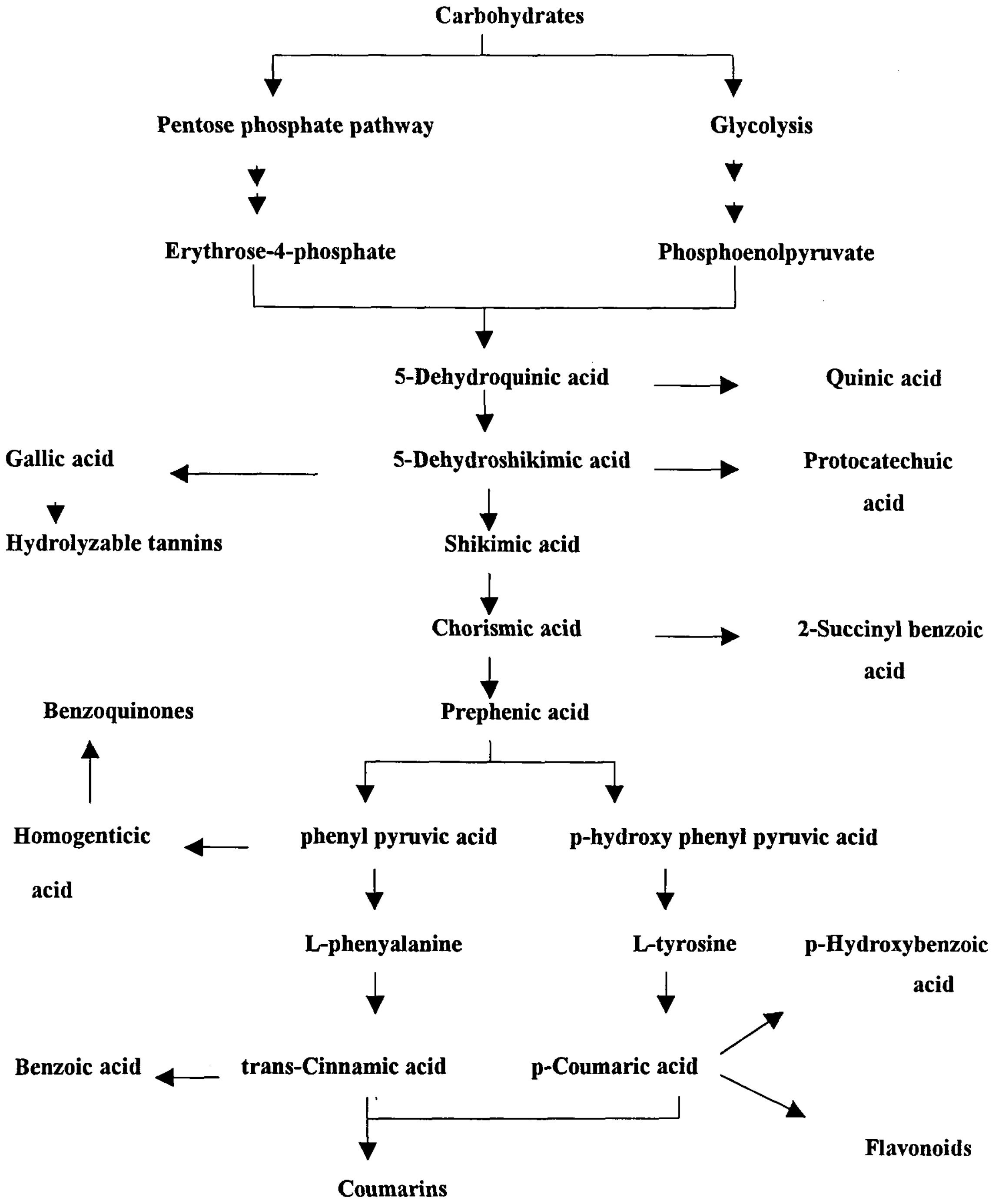

Chart 1. Biosynthetic pathway of phenylpropanoids / phenolics interrelationship with carbohydrate metabolism.

acid, scopoletin and esculetin on the isozymes of glucose-6-phosphate dehydrogenase in *Nicotiana tabaccum* suspension culture. Chlorogenic acid was found to be a potent inhibitor of both isoforms of the enzyme. In a similar study Al-Quadan *et al.*, (1981) found that chlorogenic acid was a very potent inhibitor of the isozymes of 6-phosphogluconate dehydrogenase. The other compounds tested had lower inhibitory effects including caffeic acid.

The present author found that unlike the above reports, chlorogenic acid was found to be less inhibitory. Tested at 0.1 mM concentration at saturating concentration of substrates, the highest inhibitory effect was shown by caffeic acid in the present study. Catechol and pyrogallol also showed marked inhibition. The powerful inhibition shown by chlorogenic acid in the study of Hoover *et al.*, (1977) may not be a general feature for all plant glucose-6-phosphate dehydrogenases. Caffeic acid being the simplest o-diphenol, this compound may have metabolic importance in *P. nigrum*. There is no available literature regarding the types of phenolic compounds *in P. nigrum*. The inhibition shown by caffeic acid may have regulatory significance in the biosynthesis of simple phenols. Further investigations in this direction are necessary with respect pentose phosphate pathway regulation. That the effects are due to the general inhibitory nature of phenolics on enzymes also cannot be ruled out. This was discussed in an earlier section (see section **4.3**).

4.5.5. Effect of cations

Mercury is known to be a very potent inhibitor of most enzymes. The phenomenon is attributed to Hg^{2+} complexing with the sulfhydryl groups-particularly catalytically important sulfhydryl groups of enzymes. Therefore, it is not surprising that this heavy metal ion was found to be the most potent inhibitor of the enzyme in *P. nigrum* (almost complete inhibition when tested at 10 µM). Both Zn^{2+} as well as Cu^{2+} ions elicited a high degree of inhibition at 0.5mM. However, at this non-physiological concentration the inhibitory effect may have little significance. The same may be the case with cadmium, nickel, molybdenum, which however, gave much lower inhibitory effects. Manganese can replace Mg^{2+} in enzymatic reactions where the latter is the preferred divalent cation. Ashihara and Komamine (1976) showed that Mn^{2+} was an effective activator of glucose-6-phosphate dehydrogenase though the activity was lower than that obtained by Mg^{2+}. In the present experiments on *P. nigrum* glucose-6-phosphate dehydrogenase, there was hardly any effect of Mn^{2+}. It may be noted that Mn^{2+} was incorporated in an assay system, which already contained optimum concentration of Mg^{2+}. Therefore, a further activation may not be expected to occur. Ashihara and Komamine (1976) reported 88 % activation by 10 mM Ca^{2+}. In contrast to this, in the present study 48 % inhibition was noted. In the absence of available literature on the effect of Ca^{2+} on plant glucose-6-phosphate dehydrogenase, it is not possible to make a conclusive inference on the inhibitory effect shown by Ca^{2+}.

4.5.6. Effect of sugar phosphates

As far as the author knows there are no literatures available on the effect of hexose sugar phosphates on the activity of pentose phosphate pathway, especially of glucose-6-phosphate dehydrogenase. Al-Quadan *et al.*, (1981) tested the effect of glucose 1,6- diphosphate on the purified isozymes of 6-phosphogluconate dehydrogenase from tobacco tissue cultures. They found that at low concentrations and at relatively higher substrate concentrations, there was hardly any effect. The effect of inhibitor was however, pronounced at $S_{0.25}$ (6-phosphogluconate concentration at 25 % V max). Isozymes I and II were inhibited 69 and 64 % respectively at 0.4 mM concentration of glucose-1,6- diphosphate.

The present author tested the effect of glucose-1-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate on the *P. nigrum* glucose-6-phosphate dehydrogenase. The latter two sugar phosphates are intermediates of the oxidative pentose phosphate pathway and possibly may have some effect on the pentose phosphate pathway. Glucose-1-phosphate, on the other hand, is primarily formed by the phosphorolytic breakdown of starches in plants. Following mutase reaction, glucose-6-phosphate can either enter into glycolytic sequence of reactions or can be diverted to the pentose phosphate pathway depending on the metabolic status of the organism.

Tested at saturating concentration of glucose-6-phosphate, the partially purified enzyme from the leaves of *P. nigrum* was uninfluenced by fructose-6-phosphate at 10 mM, whereas glucose-1-phosphate and fructose 1-6-diphosphate elicited 17 and 20 % inhibition respectively. The physiological function if any, of the observed inhibition requires further studies.

4.5.7. Effect of metabolic intermediates

Pyruvate, 2-oxoglutarate, acetate and citrate are intermediates in a number of intermediary metabolic pathways. The regulatory roles of these intermediates in several enzymatic reactions are well documented. Though the author has not come across any specific report on the effect of these compounds on glucose-6-phosphate dehydrogenase from plant systems, it was of academic interest to test for any possible effect. At 10 mM concentration, only pyruvate elicited notable inhibition (24 %). The other compounds had negligible inhibitory effects. It is to be pointed out that the concentration of pyruvate tested is likely to be much higher than the physiological concentration and as such the inhibitory effect may not be related to metabolic regulation, but may be nonspecific in nature.

4.5.8. Effect of plant growth regulators

The effect of auxin and gibberellin on the activity of glucose-6-phosphate dehydrogenase in wheat coleoptile cells was reported by Likholat and Levina (1973). They suggested that the plant growth regulators can act at the gene level or *via* allosteric influence at different stages of cell growth. Ashihara and Komamine (1976) observed little or no effect of indoleacetic acid and gibberellic acid on the activity of glucose-6-phosphate dehydrogenase from black gram. Based on the results, they suggested that indoleacetic acid and gibberellic acid do not function as allosteric effectors on black gram glucose-6-phosphate dehydrogenase .

In the present study, GA3, IAA and 2,4-D showed only marginal inhibition at 0.1 mM concentration. Though plant hormones are known to regulate primary metabolism in intact systems, their effect on isolated enzymes is not clear. Whether these compounds exert a modulatory effect on glucose-6-phosphate dehydrogenase and if so, the significance of this modulation merits further studies.

4.5.9. Effect of amino acids

The interdependence of carbohydrate metabolism and amino acid metabolism is universal in all living systems. However, specific pathways or enzymes of pathways can be regulated at the molecular level. This is a means of fine control of metabolic pathway.

In an isolated report (Esposito *et al.*, 2001) on the glucose-6-phosphate dehydrogenase isoforms in barley roots, Glu, Gln, Asp and Asn were found to be without any noticeable effects when tested at 2.0 mM concentration. Other amino acids were not tested by the above authors.

The results from the present study indicated that there was no uniform pattern with respect to the effect of amino acids on glucose-6-phosphate dehydrogenase. For example, in the case of aromatic amino acids, Phe and Trp showed appreciable inhibition and both have a common biosynthetic link with erythrose-4- phosphate, which is supplied by the pentose phosphate pathway. In the aspartate family of amino acids (Asp, Asn, Met, Thr, Lys, Ile) no common pattern could be visualized. While Asp, Asn, and Lys, were not inhibitory, Met and Thr showed some inhibition, while Ile showed very prominent inhibition. It is possible that a subtle mechanism may be operative in the regulation of glucose-6-phosphate dehydrogenase by isoleucine.

In the glutamate family of amino acids (Glu, Gln, Pro and Arg), except Gln, all others were inhibitory, the prominent being Pro. This might indicate that there is a possibility of pentose phosphate pathway being regulated by this family of amino acids at the glucose-6-phosphate dehydrogenase level itself.

Pyruvate acts as the precursor of Ala, Val and Leu. Pyruvate originates primarily from glycolysis; hence pentose phosphate pathway is not expected to be involved in the biosynthesis of these amino acids. But by diverting glucose-6-phosphate through the pentose phosphate pathway, the supply of carbon for pyruvate synthesis through glycolysis becomes limiting. In this context the inhibition by Ala, Val and Leu of glucose-6-phosphate dehydrogenase becomes relevant. Inhibition by Ser can also be interpreted in a similar manner since 3- phosphoglycerate is the precursor of Ser. In the case of His the inhibition can be directly correlated with the pentose phosphate pathway since phosphoribosyl pyrophosphate (PRPP) is necessary for histidine biosyntyesis and the pentose moiety is supplied by the pentose phosphate pathway.

Hydroxyproline, which is not a protein amino acid, was found to exert marginal inhibition on glucose-6-phosphate dehydrogenase in the present studies. Hydroxylation of proline is known to be a post-translational modification at the expense of NAD(P)H.

In the present case, independent mechanisms are likely to operate with respect to the inhibition of glucose-6-phosphate dehydrogenase by Pro and (OH) Pro.

4.5.10. Effect of fluoride and anionic radicals

Generally, the activation effect of anions on enzymes is not well understood. A well-known example of anion activation is of salivary amylase by chloride (Cl^-). Fluoride, on the other hand is known to be a non-specific inhibitor, particularly of divalent cation-dependent enymes. It is also observed that enzymes which require sulfhydryl groups for the catalytic activity can be inhibited by fluoride. The 32 % inhibition on glucose-6-phosphate dehydrogenase in the present study may be due either to the complexing of Mg^{2+} in the assay system by fluoride or due to blocking of sulfhydryl groups on the enzyme. 2-Mercaptoethanol supplemented in the enzyme preparations in the present studies was expected to protect the sulfhydryl groups of glucose-6-phosphate dehydrogenase and the inhibitory effects of fluoride could be also due to the reversal of this protection by fluoride ions.

A number of inorganic (anionic) radicals are known to influence the activity of a number of enzymes. However, reports on glucose-6-phosphate dehydrogenase are scarce. In the present study sulphite and metabisulphite strongly inhibited the activity of glucose-6-phosphate dehydrogenase at 10 mM concentration (41 and 31 % respectively). Sulphite and metabisulphite are strongly reducing agents. The effect could be due to the breaking of catalytically essential disulphide groups in the enzyme, thereby resulting in lowered catalytic activity. Sulphite is also known to be a carbonyl reacting reagent. Reaction with carbonyl groups of enzyme, substrate or the coenzyme also can result in an observed enzyme inhibition. Nitrate and nitrite also inhibited the enzyme in the present study. The exact role or the significance of this is unclear. As far as the author knows, reports of the effect of nitrate and nitrite on glucose-6-phosphate dehydrogenase are absent.

4.5.11. Effect of dithiothreitol and glutathione (reduced)

Dithiothreitol is a powerful reducing agent. It is proposed as a stereo selective reducing agent for disulphide linkages in complex molecules (polypeptides). Apart from its use in the homogenization of plant tissues rich in phenolics as a reducing agent, it is employed in enzyme studies to maintain the functional intactness of the sulfhydryl groups of enzymes.

The effect of dithiothreitol was tested on glucose-6-phosphate dehydrogenase from the leaf extracts and isolated chlorplasts of barley and spinach by Johnson (1972). While the cytosolic enzyme was unaffected by dithiothreitol, the chloroplastic enzyme was inhibited completely at 10 mM concentration. The inhibitory effect of dithiothreitol on the reconstituted spinach system was studied by Lendzian (1980). Dithiothreitol was found to inhibit the glucose-6-phosphate dehydrogenase. Dithiothreitol acts as non-competitive inhibitor as postulated by Lendzian .

In the present study 28 % inhibition was observed at 10 mM concentration of dithiothreitol. It may be noted that the enzyme preparation represents the whole leaf extract and the inhibition noted may be, in part, of the dehydrogenase of chloroplastic origin.

While discussing the pentose phosphate pathway in plants, Goodman *et al.*, (1986) proposed that the oxidation of NADPH could theoretically be considered to proceed through a pathway which is not coupled through cytochrome system.

NADPH → Glutathione → Ascorbate / dehydroascorbate → Ascorbic oxidase.

Alternatively a second scheme may be operative as shown below.

NADPH → Glutathione → Ascorbate → Quinones → Polyphenol oxidase → O_2.

The second scheme explains linkage of pentose phosphate pathway with phenolics, for the oxidation of NADPH.

If the ratio of NADPH / $NADP^+$ is very high, glucose-6-phosphate dehydrogenase would be completely inhibited. In the case of rat liver cytoplasm the inhibition could be effectively relieved only by oxidized glutathione (*c.f.* Turner and Turner, 1980). Similar metabolic regulation operates in plant systems as well.

In the present study reduced glutathione was found to exert 26 % inhibition at 10 mM concentration. As in the case of dithiothreitol, high reducing environment may be expected to have an inhibitory effect on the enzyme. The conversion of $NADP^+$ to NADPH is facilitated by creating a potentially oxidative environment. Glutathione (reduced) on the other hand contributes towards a reducing environment and hence the inhibition of the enzyme is likely.

4.5.12. Effect of nucleotides

Tested at 0.1 mM concentration, NADPH elicited inibition (37 %) in the presence of Mg^{2+} and a marginally higher percentage (42 %) in its absence. Inhibition of the dehydrogenase by NADPH is a means of regulation of pentose phosphate pathway, since one of the functions of the pathway is to generate NADPH for reductive biosynthesis.

Regulation of activity of glucose-6-phosphate dehydrogenase by NADPH was reported by Luzatto (1967); Yurowitzky and Milman (1968); Bonsignore and De Flora (1972); and Muto and Uritani (1970). Afolayan (1972) reported that NADPH is an allosteric effector as well as a competitive inhibitor. Lendzian (1980) in his studies on the modulation of glucose-6-phosphate dehydrogenase by varying the NADPH / NADP$^+$ ratios in an illuminated reconstituted spinach chloroplast system, reported that the addition of NADPH resulted in a decrease of the enzyme activity and that the inhibition was a competitive one. Hong and Copeland (1991) reported that NADPH acted as a potent inhibitor to both the isoforms of glucose-6-phosphate dehydrogenase from soybean nodules.

In soybean root nodules, the inhibition of the cytosolic glucose-6-phosphate dehydrogenase is identical to that measured for the plastid enzyme (Hong and Copeland, 1991). In pea leaves both isoforms showed a *Ki* for NADPH in the same range as the Km for NADP$^+$ (Fickenscher and Scheibe, 1986; Wenderoth *et al.*, 1997).

According to Esposito *et al.*, (2001), all plant glucose-6-phosphate dehydrogenases known to date are sensitive to high NADPH levels. The results presented by them from their studies on the two isoforms of glucose-6-phosphate dehydrogenase in barley roots confirmed that NADPH was the most effective inhibitor for both the isoforms of barley glucose-6-phosphate dehydrogenase. The glucose-6-phosphate dehydrogenase I isoform was highly influenced by the reducing power. NADPH / NADP$^+$ ratio of 1.0 caused 86 % inhibition of the enzyme. Similar results were also obtained for glucose-6-phosphate dehydrogenase purified from root plastids isolated from a different barley variety (Wright *et al.*, 1997). Glucose-6-phosphate dehydrogenase II was also affected by the NADPH / NADP$^+$ ratio, but to a lesser extent; a 30 % inhibition of the enzyme activity was measured when the NADPH / NADP$^+$ ratio was 1.0.

The ratio of NADPH / NADP$^+$ is critical in the expression of the inhibitory action by NADPH. This type of product inhibition is a means of fine regulation of the pathway. It should be noted that NADPH / NADP$^+$ ratio between 0.1 and 2.0 have been measured under physiological conditions, at least in green tissues (Lendzian and Bassham, 1975). This raises the question of whether glucose-6-phosphate dehydrogenases could support the increased requirement for reducing power during nitrogen assimilation *in vivo.* It has been postulated that in spite of this inhibition, residual glucose-6-phosphate dehydrogenase rates could be sufficient to support nitrite reduction in the roots *in vivo* (Wright *et al.*, 1997).

In the present study, NADPH at 0.1 mM concentration (ratio of NADPH / NADP$^+$ = 0.5) inhibited glucose-6-phosphate dehydrogenase of *P. nigrum* by 37 % in the presence of Mg^{2+}. This is in agreement with the report of Esposito *et al.*, (2001).

The various results support the allosteric regulation of glucose-6-phosphate dehydrogenase. The nucleotide specificity of plant glucose-6-phosphate dehydrogenase was already discussed. The present study reveals that NAD$^+$ could not substitute for NADP$^+$. As such NADH was not expected to exert an inhibition similar to the one shown by NADPH. The 16 % inhibition by NADH in the present study could be an artifact or it could also be due to a shift in the net ratio of reduced and oxidized forms of the nicotinamide adenine dinucleotides - (NADH / NADP$^+$). That NAD$^+$ also exerted the same level of inhibition as NADH (but at double the concentration) is surprising. A competition between NAD$^+$ and NADP$^+$ by the enzyme is not expected in the present case.

An interesting observation in the present study using nucleotide derivatives is the effect of cAMP. At 0.1 mM concentration, there was no effect when the assay system was without Mg^{2+}. A 25 % inhibition was noticed in the presence of Mg^{2+} (standard assay system).

The role of cAMP as a regulatory molecule in metabolism has been well documented (Brown and Newton, 1981; Assman, 1995). The possible mode of regulation of glucose-6-phosphate dehydrogenase by this cyclic mononucleotide is unclear. Available literature on glucose-6-phosphate dehydrogenase also does not indicate the involvement of cAMP as a regulator. The observation is certainly interesting in the light of the known regulatory role of cAMP in intermediary metabolism of plants.

In their studies on black gram glucose-6-phosphate dehydrogenase, Ashihara and Komamine (1976) reported inhibition of the enzyme by ATP and that the inhibition could be reversed to a large extent by Mg^{2+}.

Esposito *et al.*, (2001) in their studies on barley roots reported inhibition by ATP on the two isoforms of glucose-6-phosphate dehydrogenase I and II. ATP inhibited glucose-6-phosphate dehydrogenase I in the absence of Mg^{2+}. The inhibition of glucose-6-phosphate dehydrogenase II by ATP in the absence of Mg^{2+} was less evident. The presence of Mg^{2+} in the assay system completely reversed the behaviour of the isoforms of barley glucose-6-phosphate dehydrogenase.

According to Muto and Uritani (1972) and Graeve *et al.*, (1994) ATP is a competitive inhibitor of glucose-6-phosphate dehydrogenase activity. The effect could be reversed by Mg^{2+}· (Ashihara and Komamine, 1976; Valenti *et al.*, 1984; Mirfakhari and Auleb, 1989). Esposito *et al.*, (2001) explained the mechanism of enzyme inhibition postulating that ATP would compete with enzymic protein for the low Mg^{2+} levels, resulting in an inhibitory effect of glucose-6-phosphate dehydrogenase activity. At higher Mg^{2+} concentration the condition may be reversed.

The present data are not in agreement with the above reports of reversal of ATP inhibition by Mg^{2+}. It is possible that glucose-6-phosphate dehydrogenase in different tissues may show different responses towards ATP in the presence of Mg^{2+}.

4.6. Isozymicity of glucose-6-phosphate dehydrogenase

Within a single species many variants of an enzyme may often be present. This may be due to the occurrence of multiple gene loci coding for distinct versions of the enzymic protein or the existence of multiple alleles at a single gene locus. Such variation is a major explanation of the origin of isoenzymes. Isozymicity is a phenomenon of coarse control of metabolism in organisms.

The IUPAC-IUB Classification Commission on biochemical nomenclature suggested the following multiple forms of the enzymes.

1. Genetically independent proteins
2. Heteropolymers or hybrids of two or more polypeptide chains non-covalently bound
3. Allelic genetic variants
4. Proteins conjugated with other groups
5. Proteins derived from one polypeptide chain
6. Polymers of a single subunit
7. Conformationally different forms

Of these, the first three are the true isoenzymes (synthesis is controlled by distinct stretches of polydeoxyribonucleotides), whereas the remaining ones are considered to be secondary isoenzymes derived by post synthetic modification of a single gene product (*c.f.* Dixon and Web, 1979).

The distribution of different forms of isoenzymes of a given enzyme reflects four major factors.

1. The different metabolic patterns in different organs.
2. The different location and metabolic roles of a given enzyme with one type of cell.
3. The differentiation and development of adult tissues from their embryonic forms

4. The fine-tuning of metabolic rates through the different responses of isozymic forms to allosteric modulators

The pentose phosphate pathway being multifunctional in nature, isozymicity could form one of the bases of regulation of the pathway.

There are numerous reports of the occurrence of isozymic forms of glucose-6-phosphate dehydrogenase in various plant tissues. The chloroplastic and cytosolic isoforms and the different metabolic roles of these were discussed by Schnarrenberger *et al.*, (1973) in spinach leaves. According to Dennis *et al.*, (1997), the cytosolic enzyme functions in providing precursors for nucleic acid biosynthesis. On the other hand the isoform of plastidic origin is primarily important in the production of reducing power (NADPH) for fatty acid biosynthesis and nitrate reduction. It may be remembered that the site of fatty acid biosynthesis in green plants is chloroplasts. A major pathway of incorporation of nitrogen into organic form in plants is glutamine 2-oxoglutarate aminotransferase (GOGAT) which also requires NADPH (Bowsher *et al.*, 1992).

The present author was interested in testing the possibility of the existence of isozymes of glucose-6-phosphate dehydrogenase in the leaf tissues of *P. nigrum*. Tested in the 0-90 % (ammonium sulphate saturation) fraction, six dehydrogenase bands could be distinguished. Two of them had the fastest mobility and formed the major activity as indicated by the intensity of staining and bandwidth. The two minor bands with intermediate mobilities also existed followed by the two slow-moving bands, which were more or less comparable with the first two bands in both intensity and bandwidth.

Separate electrophoretic studies were carried out using the purified enzyme preparation following Sephadex G-200 gel filtration. This electrophoretogram revealed only 5 bands for dehydrogenase with an equal number of corresponding protein bands. This, inter alia, indicated the purity of the enzyme preparation as well. The missing isozyme in the purified preparations may be due to the very low levels of the enzyme (and the protein) resulting in quantities below detectable limits. It is also possible that a minor component / isoenzymic form got eliminated during fractionation.

In a detailed review on glucose-6-phosphate dehydrogenases in photoautotrophic organisms, Eichhorn and Corbus (1988) have listed the number of isoforms of glucose-6-phosphate dehydrogenase in a variety of plants. As many as 9 isoforms were reported in *Xanthium pensylvanicum* and *Chlorogonium elongatum*.

Attempts were not made by the present author to make a detailed study of the different properties of the multiple forms of the enzyme and to correlate them with the metabolic roles played by these isoforms. More detailed studies are needed in this direction. It goes without saying that the bands obtained represent isoforms of cytosolic origin and possibly of chloroplastic origin. Sub-cellular localization of glucose-6-phosphate dehydrogenase and the properties of the cytosolic and the chloroplastic isozymes can form an independent experimental approach, which did not come under the purview of the present study.

4.7. Biochemical studies under induced infection in *P. nigrum*

One of the most common pathological conditions in *P. nigrum* is the foot rot disease caused by *P. capsici.* Extensive studies have been carried out by several investigators in this direction. The IISR, Kozhikode, Kerala, over the past years have approached the problem from different aspects. Physiological, biochemical, pathological, genetics and crop improvement studies, molecular biology and biotechnological studies have contributed towards a better understanding of the disease in *P. nigrum.* (Anandaraj, 2000, Ravindran *et al.*, 2000)

Also of interest to the present author was the study of the pentose phosphate pathway under conditions of infection caused by *P. capsici* in *P. nigrum*. Apart from the changes in the levels of the key enzymes of pentose phosphate pathway the author evaluated other criteria such as changes in dry weight, soluble protein, total phenolics, total amino acids and total proline. The analyses of biochemical constituents were carried out to relate the gross biochemical changes associated with pathogenicity *per se* and in particular to relate the changes with the pentose phosphate pathway.

4.7.1. Dry weight

A decrease in dry matter content in the leaf tissue surrounding the necrotic region points to accelerated breakdown and utilization / mobilization of metabolites. The necrotic region functions as the sink and the surrounding regions function as the source under infected conditions.

The pattern of decrease in dry matter content in the surrounding leaf tissue of the necrotic region was indicative of the fact that the metabolite breakdown is accentuated during the process of infection. The results are in agreement with the reports on the enhanced rates of respiration and proteolysis caused by pathogenic infection (Goodman *et al.*, 1986). Respiratory substrates for the anaerobic and aerobic respiratory pathways are supplied under infected conditions by the endogenous breakdown of reserve materials. This is a general feature of infection in plant tissues irrespective of the causative organism.

In the present study maximum loss of dry weight occurred in the initial phase of infection *ie.*24 to 48 hours. This might indicate that the fungus- induced metabolic alteration is more pronounced during this phase.

4.7.2. Changes in activity of enzyme

Statistically significant increases in the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase throughout the course of infection points to the involvement of pentose phosphate pathway in the metabolism of leaves under infection. The increase was expected, as enhanced activity of pentose phosphate pathway is a general characteristic of tissues under various stress conditions

(Sindelar, 1986; Kabsch, 1988; Sindelarova *et al.*, 1997; Danson *et al.*, 1999)

Activation of glycolytic and pentose phosphate pathway by infection caused by sheath blight fungus in rice plants was reported by Danson *et al.*, (2000). Activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase progressively increased as the process of infection progressed. Over 100 % increase in activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were noted in 8 days.

A shift from the EMP pathway to the pentose phosphate pathway has been observed in many plant tissues infected by fungi, bacteria and viruses. An increase in pentose phosphate pathway reflects enhanced demand for NADPH whereas a similar increase in the rate of glycolysis indicates the need for ATP (linked to aerobic respiration). As will be discussed later, synthesis of phenolics and proline under conditions of infection depends on the magnitude of the pentose phosphate pathway for the supply of precursors as well as reducing equivalents. The increase in activities of the key enzymes of pentose phosphate pathway in the present study is in consonance with the above hypothesis.

A comparison of the specific activities of the two enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the present study shows that infection resulted in significant increases in specific activity in the infected plants throughout infection. Four distinct possibilities exist for the observed elevation of specific activity (i) enhanced *de novo* synthesis of specific enzymes (ii) preferential breakdown of non-enzymatic proteins (iii) both (i) and (ii) above, and (iv) fine control by allosteric regulation. The first possibility necessitates the activation of specific genes required for the synthesis of enzymic proteins. This may be a mechanism of the host to counter the infection. Pathogens are known to release many lytic enzymes either to facilitate the process of penetration to the host or to breakdown the metabolic reserves such as proteins and carbohydrates of the host. The breakdown products are utilized to meet the metabolic requirement of the pathogen as well.

Even if the enzyme synthesis is enhanced, it will not be reflected in colorimetrically estimated content of proteins, as the quantity of enzymic proteins is a negligible portion of total proteins present (usually the enzymic proteins constitute only a minor fraction of the total cellular proteins). However, a pronounced decrease in non-enzymic protein can result in increased specific activity. But the results by the present author preclude such a possibility (see tables 25 and 26). The decrease in proteins was not commensurate with the increase in specific activity. The obvious conclusion that can be drawn is that, there is a rapid synthesis of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as a result of infection by *P. capsici* in the leaves of *P. nigrum.* The activity measurements were made using Sephadex G-25 gel filtrate as the source of enzyme (that is, freed from small molecular weight compounds). The contribution by endogenously present positive modulators towards the measured activity was thus ruled out.

4.7.3. Infection and phenolics

It is generally accepted that synthesis of phenolic compounds is enhanced under various conditions of infection and stresses. Concomitantly, the pentose phosphate pathway is also enhanced. Synthesis of compounds such as dihydroxyphenols, tannins, quinones and aromatic amino acids also are enhanced (Lebeda *et al.*, 2001). The possible relationship between the activity of pentose phosphate pathway and enhanced synthesis of phenolic compounds was discussed by Farkas and Kiraly (1962). Patil *et al.*, (1966) showed that higher levels of phenolic compounds in resistant plants is a reflection of greater synthetic capacities for these substances.

The possible cause and effect relationship between synthesis and accumulation of various secondary metabolites such as phenolics, alkaloids, phytoalexins, cyanogenic glycosides etc and the tissue necrosis associated with hyper-sensitivity or resistance have attracted the attention of many investigators. The available data do not conclusively establish a causal relationship between enhanced aromatics biosynthesis and tissue necrosis or resistance. The importance of phenolic compounds and their role in disease resistance was extensively discussed by Nicholson and Hammerschmidt (1992). Antibiotic phenols have been formed in all plants investigated to date. Some occur constitutively and are thought to function as preformed inhibitors associated with non-host resistance (Millar and

Higgins, 1970, Schönbeck and Schlösser, 1976)) and others, which are formed in response to the ingress of pathogens, and their appearance is considered as part of an active defense response.

Infection by *P. capsici* resulted in an increased synthesis of phenolic compounds in *P. nigrum* leaves in the present investigations. This correlates with the increase in pentose phosphate pathway activity as discussed earlier. The severity of infection progressed with time, ultimately leading to the complete necrosis and abscission of the leaf. There is a concomitant increase in phenolics content, showing that increased synthesis of phenolics did not act as a defense mechanism. Therefore this may be considered as a general response of plants towards pathogens.

4.7.4. Changes in amino acids during infection

Two major pools of amino acids can be distinguished in relation to the partitioning of metabolites. Free amino acids can be utilized for the *de novo* synthesis of proteins depending on the metabolic status of the plant. Another function is the diversion of amino acid-carbon into respiration with accompanied release of ammonia. The ammonia is re-assimilated, stored and transported as high nitrogen compounds particularly amides. Specific amino acids are also utilized for the synthesis of other nitrogenous materials including nucleotides and alkaloids.

Under normal physiological conditions metabolic equilibrium is maintained with respect to the synthesis and utilization of amino acids.

Under pathological conditions the pathogen tends to shift this metabolic balance. For example, a number of proteolytic enzymes may be released by the invading organism to hydrolyze endogenous proteins. The amino acids thus made available can either be utilized by the pathogen for its respiratory need or for the synthesis of proteins.

The increase observed in the total extractable amino acids in the present investigation can be directly correlated with the decrease in protein content.

While studying the post infection changes in nitrogen metabolism in virus- infected *Carica papaya*, Johri (1975) showed that soluble proteins and total proteins decreased in the early stages of infection concomitant with an increase in proteolytic enzyme activities. Quantitative estimation of amino acids showed that the decrease in protein coincided with a corresponding increase in the total extractable amino acids. That this increase is not uniform was evident since Asp, Glu, Phe and Ile contents increased while Ala, Arg, Gly, Ser, Pro and Trp decreased.

Chopra and Jhootty (1974) reported that infection by the fungus *Alternaria cucumerina* on watermelon leaves resulted in an increase in some amino acids (Arg and Asp) while other amino acids decreased.

Raggi *et al.*, (1974) while studying the free amino acid fraction in tomato cultivars infected with *Fusarium oxysporum* reported an increase in the total free amino acids during the initial phases of infection. Comparison of individual amino acids however, showed that while some registered increases others decreased. These and other reports clearly show that the metabolic role of individual amino acids is critical during disease conditions. Some amino acids readily form respiratory substrates and therefore may show greater variation under altered metabolic situations as in the case of infection.

Detailed investigations on the changes in individual free amino acids could throw more light on the *P. capsici*-mediated metabolic changes in *P. nigrum* leaves, but this was beyond the scope of the present studies.

4.7.5. Proline in relation to infection

A number of biotic and abiotic stresses stimulate the accumulation of free proline in many plants. The adaptive significance of the stress induced proline accumulation is still a matter of debate. In a detailed review on the metabolic implications of stress-induced proline accumulation in plants, Hare and Cress (1997) discussed the various possibilities of this response of plants. Protecting enzymes, membranes and polyribosomes (Arakawa and Timasheff, 1985; Rudolph *et al.*, 1986; Kandpal and Rao, 1985) are some of the roles attributed to proline.

A biophysical effect associated with osmoregulation has also been proposed (Delauney and Verma 1993). In plants NADPH appears to be the preferred reductant in the reduction of pyrroline-5-carboxylate through a dehydrogenase step. Hare and Cress (1997) concluded that a small change in the intracellular $NADP^+$ / NADPH ratio mediated by enhanced proline biosynthesis is likely to have pronounced effect on the flux through a redox-sensitive pathway such as the oxidative pentose phosphate pathway.

A number of reports confirm the relationship between enhanced proline accumulations in plant tissues infected by various fungi (Chopra and Jhooty 1974; Raggi *et al.*, 1974; Rudgard and Wheelar 1985; Singh *et al.*, 1993; Zhou *et al.*, 1998).

In the present study *P. capsic*i, induced the accumulation of proline in the leaf tissues of *P. nigrum.* The enhancement was maximum at 72 hours after infection. The results are consistent with those of other workers. It should also be noted that the activity of pentose phosphate pathway also increased. Compared to the controls, the increase in proline content at 72 HAI was 60%. The corresponding increase in glucose-6-phosphate dehydrogenase activity was 117%. Small increase in proline biosynthesis might have a large impact on the level of reduction of the cellular $NADP^+$ pool. Thus, an increase in $NADP^+$ / NADPH ratio mediated by proline biosynthesis is likely to enhance oxidative pentose phosphate pathway. An increase in glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities under conditions of proline increase is, therefore, convincing.

4.8. Isozymes during infection

The importance of the existence of isozymes has already been discussed in an earlier section (see section **4.6**). As mentioned before, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities increased as a result of infection. It was felt necessary to see whether the enhancement of activities is at least in part related with isozymicity.

Change in isozymic patterns due to infection in apple fruits was reported by Naik and Powel (1973). They reported an additional band for glucose-6-phosphate dehydrogenase and acid phosphatase in infected plants.

In the present study six bands were obtained for glucose-6-phosphate dehydrogenase in the controls. A similar electrophoretic pattern was exhibited by the infected tissues. This indicated that the observed increase in the activity of glucose-6-phosophate dehydrogenase reported elsewhere (see section **4.7.2.**) was not related to changes in the isozyme profile. The intensities of bands were greater in the infected sample. This correlates with the enhanced activities of glucose-6-phosphate dehydrogenase under conditions of infection.

6-phosphogluconate dehydrogenase also gave six isozymic bands in both control and infected leaves. They were similar in mobility. As in the case of glucose-6-phosphate dehydrogenase the bands stained more intensely in the infected than in the control.

SUMMARY

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SUMMARY

Summary

The main objective of the present investigation by the author was to standardize the extraction and purification of glucose-6-phosphate dehydrogenase from *Piper nigrum* L. and to study the various properties of the enzyme-glucose-6-phosphate dehydrogenase, the first enzyme of pentose phosphate pathway, which has a multifunctional role in metabolism. The purification and the studies of various properties of the enzyme is relevant since glucose-6-phosphate dehydrogenase occupies an important position as far as the primary and secondary metabolism are concerned.

The effect of various additives like 2-mercaptoethanol, EDTA and PVPP were studied and the optimum concentrations of these compounds were determined and a standardized homogenizing medium was formulated which was used in all the subsequent experiments.

A standardized assay system was formulated taking into considerations of the optimum pH, substrate saturation, enzyme concentration and incubation period. The effect of Mg^{2+} as an activator was also determined.

Experiments were carried out to purify the enzyme by ammonium sulphate precipitation followed by Sephadex G-200 gel chromatography. The specificity of the enzyme towards $NADP^+$ was established. The Km values of both the substrates-$NADP^+$ and glucose-6-phosphate were determined which are in agreement with the previous reports. The stabilizing effect of

NADP$^+$ on glucose-6-phosphate dehydrogenase was also established. This rendered electrophoretic studies of the isoenzymes of glucose-6-phosphate dehydrogenase more reproducible.

The effect of various compounds like phenolic compounds, cations, sugar phosphates, metabolic intermediates, plant growth regulators, amino acids, fluoride, anionic radicals, dithiothreitol, glutathione (reduced) and nucleotides on the activity of purified glucose-6-phosphate dehydrogenase was studied and the inhibition / activation was determined.

The author also carried out electrophoretic studies to test the possibility of the existence of isozymic forms of glucose-6-phosphate dehydrogenase both in 0-90 % ammonium sulphate saturation fraction and 30-70 % ammonium sulphate saturation fraction. Six isozymic forms were detected in the 0-90 % as indicated by the nitro blue tetrazolium reaction while only five of the isozymes could be detected in the 30-70 % ammonium sulphate saturation fraction. The corresponding protein bands in the 30-70 % ammonium sulphate saturation fraction were also detected using silver staining method.

The activity of pentose phosphate pathway and especially glucose-6-phosphate dehydrogenase is known to be elevated under conditions of various biotic and abiotic stresses. The *P. nigrum* is highly susceptible to foot rot disease caused by *P. capsici*. The author was also interested to study the changes in the levels of activities of the first two enzymes of pentose phosphate pathway namely glucose-6-phosphate dehydrogenase and 6-

phosphogluconate dehydrogenase under pathogenic infection caused by *P. capsici* on the leaves of *P. nigrum.*

The leaves of the experimental plants were inoculated with *P. capsici* inoculum and changes in the levels of activities of the enzymes and also the specific activities during different periods of infection namely 24, 48 and 72 hours after inoculation. As reported by previous workers the activity of the two-pentose phosphate pathway enzymes were elevated. The total proteins also decreased as a result of infection.

The author also tested the changes in the dry weight percentages of the healthy and diseased plants. It was found that there was a decrease in dry weight in the initial phase of infection by *P. capsici.*

It was of interest to study the biochemical changes such as the total alcohol extractable phenolics, amino acids and proline during the process of infection. Infection by *P. capsici* resulted in an increase in the total phenolic content. A direct correlation between the increase in total free amino acids and decrease in buffer-extractable proteins was also observed, suggesting proteolysis and increased synthesis of amino acids as a result of infection. The proline content also increased as a result of infection. The increase was about 60 percent 72 hours after infection. This was consistent with the increase in activity of the two-pentose phosphate pathway enzymes-glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. This suggests the possible link between increased glucose-6-phosphate dehydrogenase activity and proline biosynthesis as a result of infection.

The author also studied the isozymic profiles of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in both the controls and the infected plants. Even though there was no increase in the number of bands of the two enzymes, the intensities of the bands were greater in the infected plants than the controls. This suggests a coarse control of the two enzymes during the process of infection.

The author has attempted to discuss the possible metabolic significance of the findings in relation to the pentose phosphate pathway in general and in relation to pathogenic infection in particular.

Some of the results offer promise for further investigations in this direction.

6

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