Desiccation and Germination Studies of Cocoa (*Theobroma cacao* L.) Seeds - A Physiological and Biochemical Approach

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

By
Abis V. Cherussery

Division of Plant Physiology and Biochemistry
Department of Botany
University of Calicut
Kerala

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Department of Botany

UNIVERSITY OF CALICUT

Dr. K.M. JAYARAM, Ph.D.
Lecturer

Division of Plant Physiology
and Biochemistry
Department of Botany
University of Calicut
Kerala, India – 673 635
Phone: 0494-2401144 * 406, 407
Fax : 0494 – 2400269
Grams: UNICAL
e-mail: jayaramkm@yahoo.co.in

10·08·2007

CERTIFICATE

This is to certify that the thesis entitled **"Desiccation and Germination Studies of Cocoa (*Theobroma cacao* L.) Seeds – A Physiological and Biochemical Approach"** submitted by **Mr. Abis V. Cherussery** in partial fulfillment of the requirement for the degree of **Doctor of Philosophy** in Botany, University of Calicut, is a bonafide record of research work undertaken by him in this Department under my supervision during the period 2001-2007 and that no part there of has been presented before for any other degree or diploma.

Dr. K.M. Jayaram

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Declaration

I hereby declare that the thesis entitled **"Desiccation and Germination Studies of Cocoa (*Theobroma cacao* L.) Seeds - A Physiological and Biochemical Approach"** 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.

Place : Calicut University Campus
Date: 10.08.2007.

Abis V. Cherussery

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INTRODUCTION

Based on their response to desiccation, seeds are classified into orthodox and recalcitrant (Roberts, 1973). The orthodox seeds undergo a period of desiccation before being shed from the tree and are able to withstand severe desiccation afterwards, and their longevity increases in a predictable way when the moisture content and temperature are reduced (Ellis and Roberts, 1980; Dickie *et al.*, 1990; Ellis *et al.,* 1990; Gee *et al.*, 1994; Connor *et al.*, 1998). The recalcitrant seeds on the other hand, do not undergo maturation drying and are shed at relatively high moisture content. They are extremely sensitive to desiccation and low temperatures and rapidly loss viability if they are dried below some critical moisture content (King and Roberts, 1979; Berjak *et al.*, 1984; Nautiyal and Purohit, 1985a; Chin, 1988; Fu *et al.,* 1990; Hong and Ellis, 1996; Nedeva and Nikolova, 1997; Pammenter and Berjak, 1999; Le Tam *et al.,* 2004). Since recalcitrant seeds are highly sensitive to desiccation and chilling, their storage necessitates relatively high humidity conditions and ambient temperatures. Even under such conditions, seed storage is possible only for relatively short periods of time and therefore pose a significant challenge for *ex situ* conservation (Mumford and Brett, 1982; Akoroda, 1986; Fu *et al.*, 1990; Oliveira and Valio, 1992; Finch-Savage, 1998; Pritchard and Manger, 1998; Anilkumar *et al.*, 2000; Berjak *et al.,* 2004).

Cocoa (*Theobroma cacao* L.) is well known for the recalcitrant nature of its seeds (King and Roberts, 1982; Hor *et al.,* 1984; Farrant *et al.,* 1988). It is a small tree belonging to the family Sterculiaceae, 4.0 to 8.0 meters tall with a straight stem, light wood and thin brownish bark. The fruits are usually considered drupes but are referred to as pods. They are indehiscent and are borne on the trunk or the branches. The pods are 10-32 cm long, pointed or blunt, smooth or warty, with or without 5-10 furrows. They are usually green, ripening to yellow. There are 20-60 seeds per pod, arranged in five rows. The seeds are 1.5 to 3.0 cm long, 1.2 to 2.0 cm broad, elliptical or ovoid and are covered with a sweet white pulp. The cotyledons are deep purple and are convoluted. The cocoa seeds consist of the seed coat or testa and the kernel or cotyledons. Analysis of unfermented cocoa beans revealed that they contain moisture, fat, proteins, starch, glucose, pectins, theobromine, caffeine, mucilage and gums, tannins, acetic and oxalic acids, and minerals. Trace elements like Mn, Al, Mo, Pb, and S were also found to be associated with the organic molecules (Anonymous, 1976).

Because of relatively high moisture content and lack of quiescent stage in the seeds, the long term storage of cocoa seeds is unsuccessful, and viability could not be maintained for more than a few weeks (Evans, 1950). Swarbrick (1965) made a number of attempts for the safe storage of cocoa seeds and observed that the seeds rapidly lose their viability at moisture contents below 27.5%. Hunter (1959) recommended a moisture content of approximately 33.8% and a temperature range of 19°C to 30°C for the safe storage of cocoa seeds. King and Roberts (1982) reported that cocoa seeds stored under controlled relative humidity conditions or in solutions of polyethylene glycol at 20°C and 98% relative humidity could survive for eight months. According to Hor *et al.* (1984), germinability of cocoa seeds could be maintained at more than 67% for at least 70 days if they were pre-dried, treated with fungicides and stored within the temperature range of 13°C to 30°C with a moisture content of 27% to 35%. Mumford and Brett (1982) maintained the viability of cocoa seeds for several weeks by maintaining the moisture content constant in a dilute solution of polyethylene glycol, but by the end of the storage period the rate of germination was found to be declined. According to Farrant *et al.* (1988), the seeds of cocoa do not tolerate much water loss and germinate relatively faster than other recalcitrant seeds.

However, in the absence of additional water, germination is slow enough to enable maintenance of viability for several weeks if the moisture content is kept high.

Chandel *et al.* (1995) studied the desiccation and freezing sensitivity of excised cocoa seeds, dehydrated in a laminar air chamber, along with those of tea and jackfruit and showed that decline in viability is associated with increased leachate conductivity, lipid peroxidation products and soluble carbohydrates. Ruhl (1995) elucidated a few ultrastructural changes associated with the desiccation intolerance of the cocoa seeds and generalized that the enveloping surfaces and membranes in cocoa seeds are destabilized during desiccation and this causes the loss of germinability in the seeds. Li and sun (1999) observed a decrease in the activity of free radical scavenging enzymes like superoxide dismutase and peroxidases in isolated tissues of cocoa embryonic axis and cotyledon dehydrated in a laminar airflow chamber. Liang and Sun (2000) investigated the optimal drying rate for desiccation tolerance in cocoa embryonic axis and suggested that such a rate represents a situation where combined damages from mechanical and metabolic stresses become minimal.

A thorough perusal of the literature has shown that only scant information is available on the biochemical causes and consequences of desiccation sensitivity on intact cocoa seeds under desiccation. Almost all reports on recalcitrant seeds have been done in isolated embryonic tissues dehydrated *in vitro* in simulated conditions. The author feels that analysis of desiccation intolerance in entire seeds under natural and common storage conditions will be more appropriate and essential to understand the phenomenon of recalcitrance in cocoa seeds.

The present investigation is an attempt to make a comprehensive study of various aspects of desiccation intolerance in cocoa seeds. Metabolism associated with the germination is initiated at the onset of storage and desiccation. The nature of physico-chemical events occurring during the storage of cocoa seeds in different dehydration regimes is being assayed in the present investigation. The deranged metabolism during desiccation is compared with the metabolic events of the normal germination process.

The effect of desiccation on a recalcitrant seed is reflected immediately on the physical attributes of that seed. So, the changes in the physical behaviour such as seed vigour index and leachate conductivity of cocoa seeds during desiccation at different storage conditions have been analysed in the present study.

Biochemical aspects of desiccation sensitivity in seeds are elucidated in terms of carbohydrate metabolism in general and sucrose and monosaccharides in particular (Koster and Leopold, 1988; Steadman *et al.*, 1996; Crowe *et al.*, 1998; Pammenter and Berjak 1999; Buitink and Leprince, 2004). Hence special emphasis is given in the present study to analyse the role of starch, soluble sugars and reducing sugars in the recalcitrant behaviour of cocoa seeds. Impact of desiccation on other biomolecules like proteins, free amino acids, phenolics and total lipids has also been investigated.

In orthodox seeds, a balance is being maintained between the generation and scavenging of free radicals and peroxides during desiccation due to their tolerant nature. But during desiccation of recalcitrant seeds, the control over the free radical generation may be lost and the scavenging enzymes may fail to function properly (Li and Sun, 1999; Greggains *et al.*, 2001; Varghese and Naithani, 2002; Nkang *et al.*, 2003). So, one of the important objective of the present investigation is the assay of lipid peroxidation and the estimation of the activities of some of the free radical scavenging enzymes.

The cellular and metabolite changes taking place in the seed tissues during desiccation are being examined through histochemical studies to facilitate the localization of metabolites like insoluble polysaccharides and proteins.

Even though the recalcitrant seeds are characterized by germination-associated changes during storage (Berjak *et al.*, 1984; Pammenter and Berjak, 1999), the metabolism of natural germination and reserve mobilization are not well documented. In the present investigation, the mobilization of important reserve materials like soluble sugars, starch, lipids and proteins during cocoa seed germination and seedling development is assayed though biochemical and histochemical means. Establishment of a correlation between the metabolic events occurring during desiccation and germination is also attempted.

The biochemical and physiological causes responsible for the recalcitrant behaviour of seeds have not been fully understood. A thorough understanding of the mechanics behind the behaviour of seeds in storage may enable the farmer and the breeder to device storage strategies, which may help to alleviate the problems associated with the long-term maintenance of the recalcitrant seeds.

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REVIEW OF LITERATURE

Extensive investigations have been done regarding the nature and factors responsible for the differential behaviour of seeds in different storage conditions, on the means and ways to prolong the longevity of the seeds in storage and on the processes associated with the mobilization of reserve materials during germination.

Based on the behaviour during storage, Roberts (1973) categorized seeds into orthodox and recalcitrant. Orthodox seeds are long lived and can be dried to moisture contents as low as 5% without injury and are able to tolerate freezing temperatures. Recalcitrant seeds cannot be dried to moisture contents below 30% without injury and are unable to tolerate freezing. They can live for a short duration only and are difficult to store successfully because of the high moisture content.

Using *Coffea arabica* as a model, Ellis *et al.* (1990) defined an intermediate category of seeds with respect to their level of tolerance to desiccation and storage behaviour. Ellis *et al.* (1991) described intermediate storage behaviour in *Carica papaya* seeds also. Seeds of this category were able to withstand considerably greater drying than recalcitrant seeds but could not tolerate extreme water loss as the orthodox seeds. In contrast to the orthodox seeds, lowering the storage temperature in intermediate seeds decreased seed longevity at low water contents. Medium-term storage is feasible in intermediate seeds whereas in recalcitrant seeds viability can be maintained only for a short duration. However orthodox seed viability can be maintained in long-term storage. Furthermore, the most striking feature of the intermediate species is that in dry seeds low temperature reduces longevity.

Generally recalcitrant seeds are heavier due to their high moisture content ranging from 30% to 70% and also due to their large size (Chin *et al.*, 1984). Large seeds lose water at a slower rate which may be of benefit to recalcitrant seeds. The authors also observed that some of the recalcitrant species like *Citrus* sp., *Coffea* sp. and *Elaeis guineensis* were killed at intermediate moisture levels of 18% to 25% while others like those of *Artocarpus heterophyllus* were killed on drying to higher level of 43% moisture content. The critical moisture content of the seeds of *Theobroma cacao* was found to be less than 20%.

The difference in dehydration response of recalcitrant and orthodox seeds was investigated by Berjak *et al.* (1984) and suggested that the seeds of orthodox species terminated their development on the parent plant after completing the maturation drying phase. The seeds were generally shed at a moisture content of 10 to 15% which was in equilibrium with ambient humidity. In contrast, the recalcitrant seeds did not undergo maturation drying on the parent plant and were shed at relatively high moisture content. In most recalcitrant species even relatively small measures of subsequent dehydration led to total lose of viability.

Corbineau and Côme (1988) studied the effects of storage conditions on germinabilty and viability of the recalcitrant species of *Shorea roxburghii, Hopea odorata, Symphonia globulifera* and *Mangifera indica.* The authors observed that the seeds lost their viability as they dried, though sensitivity to dehydration depended on the species. Total loss of viability was found to occur when the moisture content fell to about 17% in *S. roxburghii* and *H. odorata*, 30% in *M. indica* and 37% in *S. globulifera*. It was found that wet storage of these seeds was difficult since the temperature must be low enough to prevent germination or to reduce seedling growth rate. But this introduced the risk of chilling injury, which can lead to the death of the seeds or the seedlings.

The newly shed recalcitrant seeds were observed to have high moisture content and were metabolically quiescent with a high proportion of water existing as bulk water (Farrant *et al.*, 1988). Moderately recalcitrant species like *Theobroma cacao* and *Hevea brasiliensis* were found to germinate slightly faster than the minimally recalcitrant seeds like *Quercus* species and *Podocarpus henkelii* and were intolerant to rapid water loss. However, in the absence of additional water, germination was slow enough to maintain seed viability for several weeks if the moisture content was retained at optimal levels.

Anilkumar *et al.* (2000) observed in *Syzigium aromaticum* that the seed pulp retarded the percentage of germination by encouraging insect and microbial infections. Fresh seeds with 48.9% moisture content registered 100% germination in 5 days. The authors found a correlation between the decrease in moisture content and the percentage of germination. Pulpy seeds of *S. aromaticum* retained viability for a week at open room conditions. In all the other storage conditions, pulpy seeds remained viable for 15 days while de-pulped seeds lost their viability in 4 days. Seeds stored in polyethylene bags at room temperatures retained viability up to one month. Both pulpy and de-pulped seeds lost their viability in a day when stored at 0°C. The critical moisture content of *S. aromaticum* was found to be about 40%.

From the variation in moisture content due to desiccation in two species, *Acer saccharinum* and *Aesculus pavia*, Connor and Bonner (2001) hypothesized that positioning in the seed might have an effect on moisture loss from the embryo and that seed morphology might strongly influence the uneven drying rates detected in recalcitrant seeds. In both the species, the embryo was found encased by the cotyledons and might be acting as moisture sink. Thus the moisture content of the embryo remained high as long as the seed coat remained intact. However, once expansion of the embryo had begun and the seed coat was broken, rapid and often fatal moisture loss might occur. According to the authors, varying biochemical, physiological and morphological features observed in recalcitrant seeds lacked commonality.

When seeds of *Vitellaria paradoxa* were subjected to desiccation to various levels of moisture contents, smaller seeds in the population dried most rapidly (Daws *et al.,* 2004). Consequently, a significant linear relationship was noted between whole-seed water content and seed mass during the drying process suggesting that the larger seeds survived, not as a consequence of great relative desiccation tolerance, but as a result of taking longer time to desiccate.

Changes in germination and desiccation sensitivity were measured throughout the seed expansion phase of development in fruits of *Quercus robur* (Finch-Savage, 1992). The onset of a reduction in sensitivity to desiccation during development of seeds coincided with acquisition of the capacity for seed germination on moist sand substrate. Tolerance to desiccation then increased throughout development to shedding but viability was still lost at relatively high moisture content. Therefore, the seeds did not pass through a fully desiccation tolerant phase, suggesting that desiccation sensitivity in *Q .robur* might have resulted from the premature termination of development.

The changes in water status and desiccation tolerance of the seeds of *Aesculus hippocastanum* was studied by Tompsett and Pritchard (1993). The desiccation sensitivity and viability of seeds in storage was found to depend on the decrease in moisture content during development, which was accompanied by increase in desiccation tolerance and germinability. In developing orthodox seeds the acquisition of the ability to be dried rapidly without damage at low moisture contents coincided with the attainment of maximum dry weight.

The cellular and metabolic damages induced by desiccation in recalcitrant *Araucaria* seeds were studied by Espindola *et al.* (1994). The authors found that the seeds of *A. angustifolia* showed no dormancy, germinated easily at temperatures ranging from 10°C to 30°C and were recalcitrant. At harvest, the mean moisture content of the seeds was about 120% on a dry weight basis and the viability was reported to be lost completely when the moisture content fell to about 30%. The cotyledons were more sensitive to dehydration induced deterioration of cell membranes than the radicle as indicated by a higher increase in leakage of solutes. Desiccation resulted in a rapid decrease in the respiratory activity and in the rate of protein synthesis; 50% inhibition of protein synthesis was observed after 30-60 minutes of desiccation in both the axis and the cotyledons.

To test recalcitrant nature of *Quercus nigra* acorns, Bonner (1996) subjected the seeds to fast to moderate rates of drying at 27°C. It was found that drying for 10 days was suitable for a test of recalcitrance for *Q. nigra* acorns and the viability loss was independent of desiccation method or rate. The critical moisture content of intact acorns was found to be 10-12% at 27°C. When the lethal minimum moisture content was approached, rehydration within a few hours could revive the seeds and prevent total loss of viability. According to the author, this might be due to the ability of the seed coat to restrict movement of moisture to or from the axes.

Chien and Lin (1997) investigated the variation in moisture content and germinability in response to dry and wet storage of desiccation sensitive seeds of *Machilus kusanoi* and found that germination of the seeds decreased throughout the period of desiccation. The decline in germination was faster in mature seeds than in maturing seeds. A decrease in water content of only 5%

or less reduced the germination percentage by 50%. The authors also noticed a decrease in respiration rate with the decrease in moisture content.

The cells of embryonic axes of seeds dried at different rates showed different ultrastructural responses to dehydration. Some of the damaging processes associated with dehydration are aqueous based. Slowly dried material will spend a long time at intermediate water contents where damages from the aqueous based processes can accumulate. However, if the material is dried rapidly, only a short time is spent at these intermediate water contents and hence little damage accumulates. Thus, faster the drying, lesser the damage that accumulates and the lower the water content that can be tolerated (Pammenter and Berjak, 1999).

Recalcitrant seed axes were reported to have survived lower water contents under fast-drying conditions. Rapid drying of *Theobroma cacao* seed axes at low relative humidity (RH) was found to increase the electrolyte leakage with an accompanying decrease in the viability (Liang and Sun, 2000). The effect of drying rate on desiccation tolerance was associated with regulation of metabolism and also with physical processes of dehydration itself. Under very slow drying conditions, seed axes may be damaged by various deleterious processes that take place during the period of prolonged dehydration, ranging from disruption of metabolic regulation to the failure of antioxidant systems.

Wesley-Smith *et al.* (2001) studying the effects of two drying rates on the desiccation tolerance in the embryonic axis of jack fruit (*Artocarpus heterophyllus*), observed that rapid drying of whole seeds allowed lower water content to be attained whilst still retaining viability. Even then, the lower limit to desiccation tolerance persisted and the exposure to such low water contents resulted in desiccation damage. The authors suggested that normal functions may be perturbed at water contents intermediate between full hydration and the lower limit of survival during slow drying, resulting in deleterious reactions due to unregulated metabolism. Accordingly, reducing the time of exposure to intermediate hydration levels is likely to minimize desiccation damage.

While investigating the influence of rehydration techniques on the response of recalcitrant seed embryos to desiccation, Peran *et al.* (2004) observed considerable leakage of cell contents when dry seed tissues were plunged directly into water. They formulated the concept of 'imbibitional damge'. Slow drying of desiccation sensitive seeds or excised embryonic axes resulted in damage at high water contents. Excised embryonic tissues of *Artocarpus heterophyllus*, *Podocarpus henkelii* and *Ekbergia capensis* were rapidly dried to levels of water contents at which viability was lost during drying, and subsequently re-imbibed rapidly or slowly. In all the cases, direct re-imbibition of water resulted in higher survival than those from slow rehydration techniques. The authors ascribed this to the increased aqueous based deleterious processes occurring during slow rehydration.

Akoroda (1986) observed that abundance of moisture content in mesocarpic pulps of recalcitrant seeds of *Telfairia occidentalis* coupled with a porous seed coat provided favourable conditions for the easy germination of seeds inside the pod and after extraction. The critical moisture content was found to be attained when *Telfairia* seeds were air-dried for 5-8 days implying that the seeds can recover completely only when they contained about 40-60% of moisture. The seeds stored for 17 days in water proof bags maintained a significantly higher viability than those air dried for 8 days.

Seeds of *Theobroma cacao* dried for three hours suffered a noticeable decline in viability compared with the fresh seeds (King and Roberts, 1982). The authors suggested that some cellular disruption had occurred and that such desiccation injuries could have been avoided if the seeds were maintained at a slightly higher moisture content and relative humidity.

Hor *et al.* (1984) stored cocoa seeds in polyethylene bags at different moisture and temperature regimes. They observed that the seeds stored at a moisture content of 32% and a temperature of 32°C or 22°C retained viability for the longest period than any other storage conditions. In contrast, the germination of seeds stored at 27% moisture content showed highly reduced viability irrespective of the temperature at which they were maintained.

The relationship between moisture content and germination of cocoa seeds was studied by Mumford and Brett (1982). They reported that viability of the seeds can be extended from 2 to 25 weeks by maintaining the moisture content constant in a dilute solution of polyethylene glycol. However, by the end of the storage period, the rate of germination was found declined. This implied that germination had proceeded far enough such that the water content of the seeds became limiting, thus resulting in some metabolic disruptions.

The effect of moisture content and temperature on the viability of seeds of three recalcitrant species, *Mangifera indica*, *Litchi chinensis* and *Euphoria longan* in wet storage and during dehydration was studied by Fu *et al.* (1990). Seeds of all the three species had very high moisture content at the time of harvest. The authors noticed that if the seeds were desiccated continuously they would die at a moisture content called critical moisture content. The critical moisture content was found to be 40% for *M. indica*, 33% for *L. chinensis* and 25% for *E. longan*. As desiccation progressed the seeds deteriorated and the soluble sugar content, UV-absorption substances and conductivity of the seed soak solution were found to increase in close correlation with a decline in moisture content and viability. The darkening value greatly increased during deterioration of mango seeds representing damage to the membranes and oxidation of phenolic compounds. It was also observed that if the seeds were dried slowly, viability can be maintained at a higher level than that from natural drying for the same period.

Storage of *Hancornia speciosa* seeds in polyethylene bags maintained their moisture content at high levels, and high percentage of germination was obtained after 9 weeks, irrespective of the storage temperature. After 9 weeks, the germination percentage was found decreased considerably, even when seeds were stored at high moisture contents. When seeds were stored in paper bags, there was a rapid drop in the moisture content and consequently the seeds failed to germinate. One week of storage in paper bags at room temperature was enough to reduce the moisture content to a level at which the seeds were no longer capable of germination (Oliveira and Valio, 1992).

Berjak *et al.* (2004) developed strategies for field collection and storage of seeds of some tropical recalcitrant species and suggested that storage of such seeds must be carried out under conditions that do not permit their dehydration and at the lowest non-injurious temperature, which varied with the species. The seeds of *Quercus* sp. tolerated cold storage but those of *Theobroma cacao* and *Trichilia dregeana* did not survive storage at $\leq 10^{\circ}C$ and $6^{\circ}C$ respectively. The authors suggested that recalcitrant seeds were hydrated and actively metabolic when shed or harvested. During hydrated storage, the physiological status of the seeds was found to change as a consequence of their ongoing development which progressed into germination. The germinated seeds lost their vigour, viability and consequently the storage potential.

The performance capability of seeds in general and soybean seeds in particular was found to decline during storage as evidenced by delayed germination and emergence, slower growth, increased susceptibility to environmental stresses and ultimately a decline in germinability (Parish and

Leopold, 1978). The accelerated ageing treatments caused a marked lowering of early respiration in isolated cotyledons, increased the initial leakage of electrolytes and enhanced the dry weight loss. The increased leakage associated with ageing might be the result of more permeable membranes. The direct result could be the loss of metabolites, inability to maintain electrical, chemical or pH gradients and a mixing of the normally separated cellular constituents. The indirect result would be the loss of vigour.

The analysis of electrolyte leakage in recalcitrant seeds of *Acer saccharinum* and *Chrysalidocarpus lutescens* (Becwar *et al.*, 1982), *Pisum sativum, Zea mays* and *Glycine max* (Koster and Leopold, 1988), *Hancornia speciosa* (Oliveira and Valio, 1992) and *Camellia sinensis* (Berjak *et al.*, 1993) showed a negative correlation between the moisture content of the seed and the efflux of electrolytes from it. The seeds with higher moisture content showed a higher percentage of germination and released fewer electrolytes and organic substances to the medium. Dehydration of the seeds reduced germination and increased the efflux of electrolytes and organic substances. Electrolyte leakage from tissues could be used to indicate the effectiveness of membranes as barriers to solute diffusion. Relatively low levels of leakage occurred when the cellular membranes remained semi permeable and high levels of leakage resulted from damage to the membranes in response to dehydration stress.

Leprince *et al.* (1993) reviewed the mechanisms of desiccation tolerance in developing seeds and suggested that an early indication of desiccation-induced damage to membranes was leakage of various cytoplasmic solutes like ions, sugars and proteins that occurred upon rehydration of desiccated seed tissues. The rate and extent of leakage was found to be positively correlated with the degree of desiccation sensitivity. The leakage reflected a partial loss of membrane semi-permeability suggesting that desiccation injury was closely associated with membrane disruption.

Nautiyal and Purohit, (1985b) found in *Shorea robusta* that one of the earlier events associated with the loss of seed viability in recalcitrant seeds was the loss of membrane integrity as indicated by an enhanced concentration of solutes in seed leachates. The authors found that below specific moisture content of about 5%, the monomolecular layer of water that surrounds the macromolecules in sal seeds ceased to be continuous, facilitating the destruction of macromolecules such as enzymes and membrane proteins. As the seeds proceeded towards a non viable state, a decline in soluble carbohydrates, starch, proteins and acid phosphatase activity was observed. In contrast, phenolic content was found to increase.

Berjak *et al.* (1994) examined the ultrastructural features associated with viability characteristics in seeds of *Zizania palustris* dehydrated at various temperatures. The authors found that correlated with the loss of viability of *Z. palustris* with the dehydration regime is a deranged metabolism, including abnormal electron transport and electron dysfunction. It also resulted in extensive intracellular degradation of the membrane system. The cell organelles and the nuclear envelope were found considerably distorted. The authors also cited a phase transition of membrane lipids from liquid crystalline to gel. This membrane phase transition ultimately resulted in leakage of cellular contents and electrolytes at high hydration levels.

It has been observed that accumulation of a critical level of complex reserves might limit the mechanical disruption caused by drying and so would allow tolerance to desiccation. The embryonic tissues of *Avicennia marina* accumulated predominantly soluble reserves and remained highly vacuolated throughout the development, contributing to the high degree of desiccation sensitivity in the seeds (Farrant *et al.,* 1993). In seeds with simple soluble reserves, these could be mobilized rapidly, promoting swift germination and thus resulting in a short post-shedding life span. Seeds with complex, insoluble storage products were characterized by slow reserve mobilization and slow germination and hence a longer post-shedding life span. The authors suggested that in recalcitrant seeds which stored complex, insoluble reserves within the embryo, the cells were less vacuolated, and hence were more desiccation-tolerant than seeds with soluble reserves.

According to Black *et al.* (1996) the deposition of starch was found to begin when the embryo entered its major phase of growth and reached a plateau at the time when it attained maximum fresh weight. The authors found that during maturation the starch content decreased to a very low value when the growth of the embryo was completed. The decrease in starch content was not found associated with the generation of reducing sugars or sucrose. There was an initial increase in the raffinose to sucrose ratio which later remained constant.

A six fold increase in soluble carbohydrates was noticed during the desiccation of tea and cocoa axes. According to Chandel *et al.* (1995), this increase could have been caused by the degradation of starch into simple sugars

Nkang *et al.* (2003) found that limited desiccation enhanced germination in *Telfairia occidentalis*. Seeds dried at lower temperatures maintained viability for longer periods during the desiccation treatments, probably due to slower rate of moisture loss. Increased carbohydrate and lipid contents at the developmental stage indicated a continuous flow of nutrients from the fruit to the developing seeds. Sugar and lipid levels increased during desiccation, as starch levels decreased, suggesting that starch might be utilized in the biosynthesis of lipids, the major storage reserves.

Lipids were also suggested to function as cotyledonary sinks thereby avoiding the build up of starch hydrolysis.

Koster and Leopold (1988) found in the seeds of *Pisum sativum*, *Zea mays* and *Glycine max* that the loss of desiccation tolerance was coincident with the loss of non-sucrose oligosaccharides. The disappearance of oligosaccharides at the time when desiccation tolerance was lost supported the idea that oligosaccharides might be necessary for desiccation tolerance in seeds.

The membrane system was found to be the most vulnerable cellular component to damages upon withdrawal of water. As water is withdrawn, the membranes were suggested to lose their liquid crystal structure unless some protection was provided. Caffrey *et al.* (1988) suggested a 'water replacement theory' in which a sugar such as sucrose formed a substitute for water at the membrane surface. However, sucrose in nearly dry conditions crystallized, thereby limiting its availability. The oligosaccharide, raffinose was found to serve as an effective inhibitor of sucrose crystallization, thus contributing to desiccation tolerance in angiosperm seeds. From the ability of sucrose to provide protection to a membrane upon drying and the enhancement of this effect with the presence of raffinose, the authors suggested an interaction between phospholipids and sugars with a direct relevance to the viability of dry seeds.

Bruni and Leopold (1991) correlated desiccation tolerance with cytoplasmic glass formation. The ability of the disaccharides to protect membranes and proteins against dehydration was connected with their ability to induce glass formation at physiological temperatures. The glass formation slows down all chemical reactions requiring molecular diffusion and thus impart to the seeds quiescence and stability over time.

A negative correlation between the amount of sucrose present and storability in maize seeds was established by Bernal-Lugo and Leopold (1992; 1995). The cultivar with the highest sucrose content showed the poorest storability. Similarly, a positive correlation was observed between raffinose fraction of the sugar mass and storability. They suggested that the ratio of sucrose to raffinose was critical for the survival of seeds in storage. A progressive decline in the sucrose-raffinose ratio was associated with an increase in viability during storage. The decline in soluble sugar content in seeds under storage might also result in limited availability of respiratory substrates for germination and could contribute to the decline in vigour and germinability of such seeds.

Bochicchio *et al.* (1994) opined that acquisition of desiccation tolerance in maize was only temporarily related to an increase in the ratio of raffinose to sucrose. Incubation of maize embryos in conditions of desiccation resulted in a decrease in sucrose content. Even though raffinose was detected during the drying process, its ratio to sucrose was always very low and far from the value of 0.3 which was required for optimum preservation of dry model membranes and to prevent sucrose crystallization. In fact, rapid drying caused an abrupt loss of water, which presumably was not favourable to enzyme reactions including those responsible for an increase in the raffinose concentration.

Finch-Savage and Blake (1994) argued that moisture content at the time of seed shedding was more critical in the determination of desiccation sensitivity than the accumulation of LEA proteins or soluble sugars. In the seeds of *Quercus robur*, sucrose, glucose, fructose and raffinose were found present in the cotyledons at shedding and the ratio of sucrose to raffinose was of similar order as those noted for desiccation tolerant types. However, the seeds remained recalcitrant. A negative correlation was observed between seed moisture content and seed viability; the lower the moisture content at harvest or shedding, the greater its tolerance to desiccation.

Steadman *et al.* (1996) studied the sugar composition of seed tissues of 18 species and found that the total sugar content and sucrose level of the embryo were highly variable among different species and that no simple association with seed storage physiology was evident. Monosaccharide level was found to be low in most seeds studied, even in those of the recalcitrant category. Sucrosyl oligosaccharides, raffinose and stachyose were noticed to be lower in recalcitrant seeds as compared to orthodox seeds. In general, orthodox and recalcitrant seeds had tissues with sucrosyl-oligosaccharide to sucrose mass ratio 1:7 and 1:12 respectively. They concluded that the ratio of sucrosyl-oligosaccharide to sucrose in seed tissues is a good indicator of seed storage category.

The gradual acquisition of desiccation tolerance during maize seed development was found to occur only after an accumulation of raffinose and with the sucrose-to-raffinose mass ratio remaining less than 20:1 (Brenac *et al.,* 1997). Full desiccation tolerance was associated with a sucrose-to-raffinose mass ratio of less than 10:1. Sucrose was the prominent sugar accumulated in maize embryos and its accumulation preceded or was coincident with the accumulation of raffinose. Mixtures of sucrose and raffinose enhanced the stability of lipids of the model system during desiccation.

Desiccation tolerant seeds of mature yellow lupine (*Lupinus luteus*) contained 10.9% oligosaccharides and 1.5% sucrose (Górecki *et al.,* 1997). Accumulation of oligosaccharides during maturation of seeds was observed to be associated with the acquisition of desiccation tolerance and storability. It also was found to prevent accumulation of reducing sugars. Elimination of galactosyl oligosaccharides may be resulting in reduced desiccation tolerance and storability.

According to Crowe *et al.* (1998), upon dehydration irreversible changes occur in the structural and functional integrity of the cell membranes and proteins. Certain sugars were found to replace the water around polar residues in membrane phospholipids and proteins, thereby maintaining their integrity in the absence of water. An alternative concept was that the sugars involved in stabilizing anhydrobiotic organisms do so by virtue of their ability to form glasses. Vitrification was found required for stabilization of biomolecules and for the preservation of the labile components, but in itself was insufficient to accomplish these. Both vitrification and water replacement hypothesis were suggested to be required for desiccation tolerance.

Lin *et al.* (1998) studied the variation in oligosaccharide content during hydration and dehydration in five different species and concluded that monosaccharides increased slightly or were maintained at a constant level before the seeds lost desiccation tolerance. Sucrose content also was constant or showed an increase in seeds having abundant oligosaccharides which may in turn be converted into sucrose. A great decrease in mole ratio of oligosaccharides/sucrose from the original value was noticed with the disappearance of desiccation tolerance. Among the sugars only the oligosaccharide quantity and the oligosaccharides/sucrose ratio showed significant correlation with desiccation tolerance. Sucrose was ruled out as a key element in this process as the correlation between sucrose mass and desiccation tolerance was found to be ambiguous.

According to Li and Sun (1999), the embryo axis of cocoa seeds contained more than 20% soluble sugars on dry weight basis and traces of reducing monosaccharides. The mass ratio between sucrose and oligosaccharides was 4.5:1 which was similar to that of the orthodox seeds. Sucrose and oligosaccharides stabilize membranes and macromolecules during desiccation, possibly through several mechanisms such as direct hydrogen bonding and glass formation.

Pammenter and Berjak (1999) reviewing the recalcitrant physiology suggested that on dehydration, specific sugars replace the water normally associated with membrane surfaces, thereby maintaining the correct lipid head-group spacing and preventing liquid crystalline - gel phase transitions in the lipid bilayer. Moreover, on desiccation, sucrose and certain oligosaccharides form amorphous, super saturated solutions of high viscosity. The presence of glasses, because of their high viscosity was found to impose a stasis on intracellular activity, reducing the deleterious effects of deranged metabolism, protecting macromolecules against denaturation and preventing or minimizing liquid crystalline to gel phase transitions in the membrane lipid bilayer. Vitrification conferred desiccation tolerance and maintained viability for extended periods in the dry state.

Large amounts of soluble carbohydrates have been suggested to be involved in the acquisition of desiccation tolerance in the somatic embryos of carrot. Wolkers *et al.* (1999) found that sugars functioned as protectants of proteins and membranes in dehydrating seeds and formed a glassy state, immobilizing cytoplasmic components and slowing down all chemical reactions including damaging free radical reactions. Sucrose was the major soluble carbohydrate after fast drying. Accumulation of oligosaccharides in seeds was linked with their long-term survival in the dry state. The ratio of sucrose to oligosaccharides was suggested to be more important in this respect than sucrose and oligosaccharides in their absolute amounts. Even small amounts of oligosaccharides prevent the crystallization of sucrose, thus improving the survival in the dry state.

Buitink *et al.* (2000) studied in *Impatiens wallerina* and *Capsicum annum,* whether oligosaccharides extend seed longevity by increasing the intracellular glass stability. They observed that glass formation and longevity of the cytoplasm was a function of the relative seed water content and mobility. Osmo-priming of the seeds resulted in considerable decrease in seed longevity and oligosaccharide content, while the sucrose content increased. No difference in the glass transition temperature was found between control and primed *Impatiens* seeds at the same temperature and water content. Therefore, the authors concluded that oligosaccharides in seeds did not affect the stability of the intracellular glassy state, and that the reduced longevity after priming was not the result of increased molecular mobility in the cytoplasm. They also refuted the suggestion that the oligosaccharides prevent crystallization of sucrose during storage.

While studying the induction of desiccation tolerance in plant somatic embryos, Hoekstra *et al.* (2001a) observed a correlation between the monosaccharide contents in the somatic embryos and desiccation sensitivity. High contents of glucose and fructose were found to occur in desiccation-sensitive carrot pro-embryogenic masses. The desiccation stress was found to result in destabilization of membranes, proteins and cytoplasmic glasses of low molecular density and partitioning of cytoplasmic amphiphiles into membranes. The fluidization resulting from the partitioning destabilized the membrane structure and function. The low desiccation tolerance in the presence of ample sugars was suggested to be caused by an inadequate response to the effects of dehydration induced partitioning of cytoplasmic amphiphiles into membranes.

Intracellular glass formation is the key factor involved in the desiccation tolerance of anhydrobiotes. In such organisms, glasses could be formed from cell solutes like sugars that provide protection from denaturation of large molecules and formation of molecular aggregates (Buitink and Leprince, 2004). It was also proposed that glasses might fill spaces in a tissue during dehydration and that their high viscosity might stop all chemical reactions that require molecular diffusion. The glass formation was suggested to involve non-reducing sugars and proteins which in turn interact with additional cytoplasmic molecules such as amino acids, ions and salts.

Soluble sugars, especially sucrose, glucose and fructose play an obviously central role in plant structure and metabolism at the cellular level (Couee *et al.,* 2006). Various metabolic reactions and regulations directly link soluble sugars with the production rates of reactive oxygen species such as mitochondrial respiration or photosynthetic regulation and conversely with antioxidant processes such as oxidative pentose phosphate pathway. All these links place soluble carbohydrates in a pivotal role in pro-oxidant and antioxidant balance.

The role of Amadori and Maillard reactions in the degeneration of seeds was established by Wettlaufer and Leopold (1991). An increase in the amount of reducing sugars like glucose, fructose and galactose could threaten the viability of the seeds through the Amadori and Maillard reactions. According to the authors, the orthodox seeds are characterized by the occurrence of reducing sugars in trace amounts. In seeds where reducing sugars may increase with ageing, a proportional loss of vigour and viability was observed. Similarly high hydration levels would enhance the Amadori Maillard reactions in the seeds.

Hoekstra *et al.* (1994) found that in cauliflower seeds, more than 90% of the dry mass and almost the whole of the sugar content was found in the cotyledons than in other seed parts. Desiccation damage of the cotyledon coincided more closely with changes in sugar in the whole seeds. The content of the most prominent sugar, sucrose, was still high when the damage occurred. The loss of desiccation tolerance of the seed coincided well with an increase in the contents of the reducing monosaccharides like glucose and fructose which were associated with browning reaction. High content of glucose and fructose during imbibition was suggested to indicate a raised rate of metabolism and respiration which was likely to depend on water content. As respiration rates before drying represent the amount of damaging free radicals generated in the tissues, the loss of desiccation tolerance is attributed to free radical damage rather than to changes in sugar contents.

The role of reducing sugars in desiccation sensitivity was examined by van der Toorn and McKersie (1995). Lipid peroxidation initiated by hydroxyl radicals of the membrane system was observed as a prime cause of desiccation sensitivity. Autoxidation of reducing sugars and increased respiration correlated with the reducing sugar content during seed germination were considered as the sources of hydroxyl radicals. The colour of desiccated radicles changed from opaque white to brown upon reimbibition indicating the involvement of Maillard reaction which in turn was initiated by Amadori reaction between aldehydes and amino groups. The authors suggested the reducing sugars as the source of primary aldehydes involved.

A correlation between increase in the content of glucose, lipid peroxidation, Amadori and Maillard reaction and seed deterioration was established by Murthy and Sun (2000). The content of glucose in seed axis increased during storage through a gradual hydrolysis of disaccharides and oligosaccharides. The presence of reducing sugars such as fructose, galactose and glucose was the primary driving force of Amadori and Maillard reaction. The authors observed that sugar hydrolysis during storage might contribute to seed deterioration through the formation of reducing sugars which in turn initiated Amadori and Maillard reactions, the products of which damaged the DNA and proteins beyond repair, leading to seed deterioration. The secondary products of lipid peroxidation might also participate in non-enzymatic protein and DNA degradation through the Amadori and Maillard reactions.

High contents of glucose and fructose were found to occur in desiccation-sensitive carrot proembryogenic masses upon slow drying. Hoekstra *et al.* (2001b), studying the protective role of sugars in the induction of desiccation tolerance in plant somatic embryos, suggested that although monosaccharides are known to react with cytoplasmic proteins, elevated monosaccharide contents might be a marker of a highly active metabolic state rather than being detrimental *per se*. This view was supported by the observation that treatments that suppress metabolic rates of germinating seeds before drying also reduce oxidative damage and improve desiccation tolerance.

Murthy *et al.* (2003) studied the involvement of lipid peroxidation and non-enzymatic protein glycosylation with reducing sugars in seeds ageing under different seed water contents and storage temperatures. The authors found that the viability loss in *Vigna radiata* seeds during storage was associated with Maillard reactions and that its contribution to seed deterioration varied under different storage conditions. Under long term storage conditions, seeds were likely to be in the glassy state because of the cool storage environment and low seed water content. The extremely high viscosity and low molecular mobility of the seed cytoplasm could prevent or inhibit many deleterious processes. With increasing temperature or seed water content, the glass state might soften into liquid state since the glass transition temperature would fall below the storage temperature. The low viscosity and enhanced molecular mobility in the liquid state would permit certain deleterious reactions to proceed rapidly, which were otherwise retarded in the glassy state.

Variation in the protein profile of seed tissues of *Shorea robusta* was studied at specific intervals after maturation and an overall decline in the number of soluble proteins was observed as the seeds proceeded towards a non-viable state (Nautiyal *et al.,* 1985). It was observed that as the seeds aged, proteins of high mobility got denatured as expressed in the disappearance of certain bands in PAGE gel. The substantial deteriorative changes in proteins during ageing of seed parts were evident by the presence of broad diffused protein bands in non-viable seeds as compared to sharp clearly defined peaks of protein in viable seeds. The authors opined that denaturation of proteins led to structural changes in cellular membranes, increasing their permeability.

Among the protective components, suggested by Blackman *et al.* (1992), in the acquisition of desiccation tolerance in soybean were proteins and soluble sugars. A group of proteins called Late Embryogenesis Abundant or LEA were found accumulating during the maturation drying phase of soybean seed development, correlating with the ability of the seeds to progress into seedling growth and with desiccation tolerance. During the slow drying process, the sucrose and stachyose content was observed to increase in the axes. It was suggested by the authors that maturation proteins might be an essential part of an early response system that protects against the stresses imposed at the onset of desiccation before oligosaccharides reach high levels or they might work in concert with oligosaccharides to function in the development of desiccation tolerance.

Farrant *et al.* (1992) observed an increase in the protein content and rates of protein synthesis in the seeds of *Avicennia marina* during histodifferentiation. Thereafter, the protein metabolism remained qualitatively the same in axis and cotyledons which was equivalent to basal metabolism typical of vegetative tissue. Synthesis of storage protein was conspicuous by its absence. The sensitivity of the seeds to dehydration was attributed to the absence of desiccation related LEA proteins. In *A. marina,* maturation drying was not found to occur and the mature seeds remained metabolically active on shedding. However, the activity was found to increase after shedding, suggesting an amplification of metabolism associated with germination.

Many labile proteins lost their functional as well as structural integrity when they were desiccated (Crowe *et al.,* 1998). Interaction of the proteins with water was critical for the formation of the native folded protein. The level of hydration achieved during air-drying of seeds was low enough that the hydration shell of proteins was completely removed, i.e. almost all interactions with water were lost. It was shown that most of the unprotected proteins were unfolded in the dried condition. It was documented with several proteins that the ability of stabilizers to inhibit aggregation and to increase recovery of activity after rehydration correlated directly with their capacity to retain the native structure.

Wolkers *et al.* (1999) investigated the changes in the properties of the cytoplasmic matrix associated with desiccation and observed that rapidly dried carrot somatic embryos have decreased phospholipid contents, elevated free amino acid contents and irreversible protein aggregates in their plasma membrane which was leaky. The authors also observed that, even though slow drying of the embryos led to a greater relative proportion of α-helical structures, the overall protein secondary structures of the slowly and rapidly dried somatic embryos resembled one another. However, in some of the rapidly dried out embryos, signs of protein breakdown were observed.

Chaitanya *et al.* (2000) observed a gradual loss of protein content in the embryonic axis and cotyledon of the desiccating seeds of *Shorea robusta,* correlating with the rate of dehydration and the consequent loss of viability.

The authors suggested that, net loss of proteins in deteriorating seeds was perhaps the most basic of all ageing related events as these changes underlie all other aspects of metabolic decline. Seeds undergoing ageing were found to have enhanced protease activity, which accounted for the loss of total protein content. Alternatively, changes in the protein synthesizing capacity might be instrumental for the differential rate of protein loss in embryonic axes and cotyledons.

Different mechanisms of protection were suggested to act at different stages of water loss during desiccation (Hoekstra *et al.,* 2001b). The survival strategy during early dehydration was to avoid protein unfolding and to restrict membrane disturbance by preferential dehydration. Upon further removal of water from the hydrated cells, sugar molecules replaced water at hydrogen bonding sites to preserve the native protein structure and spacing between phospholipids. Meanwhile, curtailing the production of reactive oxygen species (ROS) was an important mechanism of survival. In the study of plant desiccation tolerance, it was often found that, one specific mechanism was not sufficient to confer tolerance on its own, but that an interaction of several mechanisms simultaneously was essential.

Dodd *et al.* (1989) studied the levels of free amino acids in the seeds of *Podocarpus henkelii* and noticed only slight change in the level of free amino acids during storage. After 9 days of storage the total levels of amino acids increased in the seed components and this increase was linked to the decline in protein levels.

In *Machilus thumbergii* seeds, the proline content was found to increase approximately two folds during desiccation and to reach a maximum at 57% relative humidity in the initial phases of development (Lin and Chen, 1995). The monosaccharides, glucose and fructose were found decreasing during late stages of seed development. Sucrose showed accumulation in later stages, although it had declined during the early stages. The induced sucrose and proline accumulation and a noticed decrease in abscissic acid at desiccation over a period of several days indicated the activation of some maturation specific metabolic activities. The authors suggested this phenomenon as common to both recalcitrant and orthodox seeds.

In many plants, free proline accumulates in response to the imposition of a wide range of biotic and abiotic stresses (Andarwulan, 1999). Proline has the ability to mediate osmotic adjustments, stabilize subcellular structures and scavenge free radicals. During germination, free proline content in the seeds increased. High levels of proline synthesis might be involved in the maintenance of NAD (P^+) / NAD (P) H ratio, enhancing the activity of the oxidative pentose phosphate pathway. The increase in proline linked stimulation of the pentose phosphate pathway may make available $NADPH_2$ and phosphorylated sugars for the phenylpropanoid pathway (phenolic antioxidants and lignin) and other anabolic pathways.

The level of free proline was found to increase up to 50 fold in many living organisms under various stressful conditions. This led to the suggestion that proline accumulation might be involved in the protection of these organisms against singlet oxygen induced damages (Alia *et al.,* 2001). As a cyclic secondary amine, proline has a low ionization potential and can therefore act as a quencher of singlet oxygen, either chemically by forming products as superoxide or peroxide or physically by inter-system crossing via spin-orbit coupling.

Phenolics or phenolic acids play many important roles in plant cells, tissues and organs and were also known to be involved in growth regulation, differentiation and organogenesis. During seed germination the phenolic content increased, corresponding to the increased antioxidant activity (Andarwulan *et al.,* 1999). The increased phenolic content indicated that the plant produced precursors for the potential synthesis of lignin. The antioxidant activity of the phenolic extract showed that phenolics in seeds function as antioxidants when oxygen demand during germination was high. The antioxidant might be protecting the cell from potential oxidation induced deterioration.

Based on the antioxidant assays carried out on the beans of *Theobroma cacao*, Othman *et al.* (2007) suggested that phenolic compounds in the beans have strong free radical scavenging ability. The authors also suggested that, besides phenolic compounds, methyl xanthines like theobromine and caffeine and anthocyanins in cocoa beans might influence the antioxidant capacity.

Pence *et al.* (1981) found that maturation of the embryo of *Theobroma cacao* was characterized by the accumulation of large amounts of storage lipids collectively known as cocoa butter. The fatty acid composition of these lipids became more saturated during development such that, lipid of the mature embryo is composed predominantly of triglycerides of palmitic, stearic and oleic acids.

Studies on the effect of desiccation on lipids, proteins and carbohydrates of the embryonic axis and cotyledons of *Quercus alba* revealed that membrane lipid structure initially exhibited reversible shifts between gel and liquid crystalline phases in response to drying and rehydration (Connor and Sowa, 2003). However, reversibility declined as viability was lost. Sucrose concentration in the embryonic axis was found to increase rapidly upon drying and became significantly greater than that in the cotyledons. It was hypothesized that, increased concentration of sucrose did not prevent the loss of viability in drying acorns, but acted as a glycoprotectant against cell collapse and cell wall membrane damage, as water stress increased. The most sensitive indicator of desiccation damage was the irreversible change in protein secondary structure in embryonic axes and cotyledon tissues.

Lipid peroxidation was cited as one of the major contributors of deterioration in ageing seeds (Wilson and McDonald, 1986; Hendry, 1993). Seed storage subjects lipids to a slow consistent attack by oxygen, forming hydroperoxides, other oxygenated fatty acids and free radicals. The free radicals may react with and damage nearby molecules. Hydroperoxide lyase becomes active and breaks down oxygenated fatty acids. This reaction may damage the seeds further by producing an increase in free radicals and by forming toxic secondary products like ethane and unsaturated aldehydes. The associated processes involve biomembrane degradation, protein denaturation, interference with DNA and protein synthesis, accumulation of toxic materials and destruction of the electron transport system of oxidative phosphorylation.

In *Shorea robusta,* Chaitanya and Naithani (1994) observed a consistent increase in the lipid peroxidation product (MDA) during desiccation. They suggested that, the rapid loss of viability in *Shorea* seeds in the initial phases of desiccation might be due to the cumulative effect of enormous peroxidation products of stored polyunsaturated fatty acids, peroxidation of membrane lipids and the damaging effect in the disorganization of metabolism.

According to Chandel *et al.* (1995), the significant decline in viability associated with desiccation and freezing in cocoa and freezing alone in jackfruit was accompanied by an increase in electrical conductivity and lipid peroxidation. Cocoa seeds showed a very high increase in lipid peroxidation over the fresh seeds after desiccation as well as freezing. The jack fruit axes showed a two-fold increase in lipid peroxidation products after freezing and thawing. The inter-relationship between seed germination, ultrastructure, leakage and lipid peroxidation during desiccation and freezing confirmed the close association between membrane integrity and viability. The authors suggested that disruptions to the cell organelles such as the nucleus and mitochondria could also be contributing to the decline in the viability of embryonic axes during desiccation and freezing. They considered cocoa as a highly recalcitrant seed.

Studying the ageing of soybean seeds, Sung (1996) found that ageing, both natural and accelerated, led to lipid peroxidation. This resulted in membrane perturbation leading to electrolyte leakage during seed imbibition. According to the author, a balance between generation and scavenging of free radicals and peroxides must be maintained within the stored seeds.

Activities of three free radical scavenging enzymes – ascorbate peroxidase, peroxidase and superoxide dismutase (SOD) – were monitored during desiccation of *Theobroma cacao* embryonic axes (Li and sun, 1999). Activities of all these enzymes decreased as water content in axes decreased to the critical level for desiccation sensitivity. A decrease in enzymic protection was probably associated with enhanced lipid peroxidation in cocoa seed tissues, which produced highly reactive free radical intermediates that could damage membranes, proteins and nucleic acids. The increased electrolyte leakage and loss of seed viability probably resulted from the increased oxidative damage.

Greggains *et al.* (2001) found that in *Avicennia marina* seeds, oxygen radicals were formed during normal oxidative metabolism and respiratory electron transport. During desiccation, however, controls of these processes may become impaired leading to increased reactive oxygen species formation and associated lipid peroxidation, membrane damage and eventual cell death. The enzymatic mechanisms to quench the free radicals - the tocopherol, the activities of superoxide dismutase and guaiacol peroxidase - showed variation in different seed tissues at different hydration levels. The viability of *A. marina* propagules was lost at high moisture contents during drying. Lipid peroxidation increased in root primordia and cotyledons as propagules dried to 57% moisture content, suggesting that they were experiencing oxidative stress. However, the major reduction in viability occurred in a narrow moisture content range between 57 and 54%. In this range, lipid peroxidation was found decreased. The result was interpreted as the propagules initially reacting to oxidative stress during drying, but then overtaken by more catastrophic events.

Mittler (2002) opined that reactive oxygen intermediates (ROI), the partially reduced forms of atmospheric oxygen, are capable of unrestricted oxidation of various cellular components and can lead to the oxidative destruction of the cell. The ROI induced cell death can result from oxidative processes such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage. The major ROI scavenging mechanisms of plants include superoxide dismutase, ascorbate peroxidase and catalase. The balance between superoxide dismutase and the other two enzymes is crucial for determining the steady state level of superoxide radicals and hydrogen peroxide.

The desiccation-induced loss of viability was closely associated with over accumulation of superoxide anion and lipid peroxidation products both in the embryonic axes and cotyledons. Varghese and Naithani (2002) observed that, freshly harvested mature neem seeds with 42.2% seed moisture content and 100% viability deteriorated when naturally desiccated to below 10.9%. The authors found that the levels of superoxide anion and lipid peroxidation products were higher in axes compared to cotyledons. Superoxide dismutase activity was not much affected, both in the axes and cotyledons of 100% viable seeds during desiccation from 42.2% to 10.9% seed moisture content. Steep rise in its activity was observed during drying below lowest safe moisture content (LSMC). Activities of catalase and peroxidase exhibited substantially higher levels in the 100% viable seeds dehydrated up to LSMC. Their activities declined sharply in seeds with water content below LSMC. Impairment of catalase and peroxidase activities possibly leads to enhanced accumulation of reactive oxygen species.

The role of active oxygen species and the antioxidant systems in the acquisition of desiccation tolerance was reviewed by Bailly (2004). The ability of developing seeds to withstand desiccation depended on the drying rate. Active oxygen species generation was known to occur during dehydration of various plant tissues and in recalcitrant seeds. It might result from metabolic imbalances leading to leakage of high energy intermediates from plastids and mitochondria. In bean seeds, acquisition of drying tolerance appeared to be associated with a reorientation of the enzymatic antioxidant defence system.

Pukacka and Ratajczak (2005) studied the loss of germination capacity and viability of beech (*Fagus sylvatica*) seeds during storage under different temperatures and humidity levels. The accumulation of reactive oxygen species was cited as the prime cause of seed deterioration as they initiated reactions with polyunsaturated fatty acids, leading to lipid peroxidation and destruction of cellular membranes. To counteract the toxic events evoked through ROS, antioxidative defence systems are present in plant tissues. These protective systems are composed of low molecular and enzymatic scavengers, such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione oxidase. The intensity of ROS production proved to depend on storage conditions, and was strongly correlated with seed germinability.

Seed deterioration in sunflower as related to moisture content during ageing, energy metabolism and active oxygen species scavenging was investigated by Kibinza *et al.* (2006). The authors observed that lipid peroxidation and oxidative stress were the major causes of deterioration of oil seeds during ageing. The loss of vigour and seed viability in sunflower during deterioration were attributed to an impairment of enzymatic antioxidant systems like catalases and superoxide dismutase which resulted in an accumulation of hydrogen peroxide and an increase in lipid peroxidation.

Becker *et al.* (1978) observed that the cotyledonary metabolism during cucumber germination is characterized initially by gluconeogenic utilization of stored fat through the glyoxylate cycle. Concomitant with fat metabolism, protein reserves of the cotyledons are also mobilized and degraded gradually to polypeptides and amino acids.

Starch reserves in legume seeds are normally degraded during germination. Briarty and Pearce (1982) reported the formation of a new family of starch granules during the germination of *Vicia faba* and *Phaseolus vulgaris.* In contrast to the granules formed during seed development, these were very much smaller. Such synthesis appeared to be a fairly common phenomenon in geminating legume cotyledons, and the production of these small granules may represent a mechanism for secondary carbohydrate storage – perhaps more significant in epigeal species where the cotyledons take on a leaf like role.

Bhandari and Chitralekha (1984) carried out histochemical investigations on dry and germinating seeds of *Brassica campestris* to study the degradation of protein bodies with globoidal inclusions. During germination, the protein bodies of the seeds were broken down to provide free amino acids to the growing seedlings. In *B. campestris*, the embryonic cells of the mature seed contain large amounts of reserve materials in the form of protein bodies and lipid droplets. Carbohydrate reserve material is lacking. The authors found that it is the axis which controls the protein body digestion in the cotyledons. The axis acts as a sink, accepting the products of hydrolysis from the site of the action, thereby ensuring the continued activity of the enzyme.

An increase in the starch content in the seeds of *Podocarpus henkelii* was noticed by the 6th day of germination (Dodd *et al.,* 1989). This was not accompanied by a marked decrease in the levels of soluble sugars. This increase may be accounted for by lipid catabolism which would supplement the pool of free sugars, allowing starch synthesis. It was also suggested that when catabolic activity exceeded the load capability of the transport system, or the sink, excess carbohydrate may be channeled into starch as a temporary reserve

Studies on the storage products in the seeds of *Lupinus luteus* carried out by Asghar and DeMason (1990) showed that the reserve products of the species consisted of proteins, lipids and wall polysaccharides. The authors noticed degradation of these compounds during germination and early seedling growth and suggested that the degradation occurred for the mobilization to the growing points.

Changes in structural and storage lipids and fatty acid composition during seed germination have been studied in *Medicago sativa* (Huang and Grunwald, 1990) and in *Corylus avellana* (Li and Ross, 1990b). During germination, storage lipids were mobilized to supply the necessary energy and got depleted. Structural lipids on the other hand increased, reflecting membrane formation and transformation during germination and seedling development. The total lipid content did not change significantly during the first four days of germination. The simple lipids however started to decrease from the first day of germination. At the same time, the amount of complex lipids increased. The decrease in simple lipids might be due to their use as respiratory substrate. The increase of complex lipids reflected membrane formation and transformation during germination and seedling development.

Lipids and proteins were reported as the major reserve constituents in hazel nut seeds with only trace amounts of carbohydrates present (Li and Ross 1990a). The unimbibed embryonic axes contained starch approximately 3.8% of total dry matter. For the seeds incubated at 20°C, there was a rapid decline in starch content of about 20% during the first week of imbibition, following which the level remained constant. This decrease during the initial week was partially attributed to the respiratory demand at a higher temperature, leading to hydrolysis of starch immediately following imbibtion. Histochemical study of starch had confirmed this.

In *Tagetes minuta,* the starch levels did not change significantly during germination at 25°C, relative to the dry seeds (Drewes and Van Staden, 1991). At 35°C a significant transient increase was observed after the first 25 hours of incubation after which the levels declined. Sucrose was found to be the dominant sugar present, accounting for between 65 and 90% of the free sugars under different conditions. Changes in the sugar complement were more marked than the slight fluctuations in starch levels. Level of sucrose was found to decline by about 20% after 48 hours at 25°C.

Giorgini and Campos (1992) observed that the starch content in the cotyledons of the dark grown seedlings of *Coffea arabica* increased during the first 40 days of germination, remained constant for a period and declined thereafter. The soluble sugar content decreased from the initial high levels to the 50th day of germination and showed a small increase, coinciding with the decline in the starch level. The soluble sugar content of the axis was high at the 10th day and decreased rapidly during the first 40 days. Close correlation was observed between the increase in the starch content and number of starch granules in the cotyledons as well in the root plus hypocotyls.

Storage proteins synthesized during seed maturation are degraded during germination to small peptides or amino acids that are subsequently transported to the growing seedlings (Callis, 1995). Typically, storage proteins are first cleaved by specific endoproteinases, the resulting peptides are then hydrolysed to free amino acids by the action of multiple, less specific exopeptidases or endopeptidases or both.

According to Baleroni *et al.* (1997) there was a decrease in the lipid content in the cotyledons during germination of *Brassica napus* seeds. Carbohydrates showed an initial decrease and an increase later on to reach a higher value. During germination, while the concentration of sucrose decreased, the concentration of glucose and fructose increased. Therefore, sucrose was suggested as supplying fructose and glucose through invertase action. The increase in concentration of glucose and fructose is accompanied by an increase in isocitrate lyase activity. Thus, the authors opined that monosaccharides could either be generated from sucrose degradation or through invertase action or from malate produced by glyoxylate cycle through gluconeogenesis.

Suda and Giorgini (2000) investigated seed composition and reserve mobilization in *Euphorbia heterophylla* and found that lipids constituted about 60%, proteins about 20% and soluble sugars about 3.6% of the dry mass. Sucrose was found to be the predominant sugar while starch was not detected. Lipid depletion started after initial imbibition and was completed between 72 and 96 hours. Soluble sugars increased in the embryo with no concomitant decrease in the endosperm suggesting that sugars were mostly originated from the catabolism of lipids. A decline in total sugar was observed after 72 hours. Reducing sugars showed rapid increase up to 48 hours and declined sharply after 72 hours. Glucose and fructose occurred in higher amounts in the embryos after 48 hours of germination. The correlation between the increase in reducing sugars and the appearance of glucose and fructose indicated that the hydrolysis of sucrose may be intense in the embryo over that period. The total sugar level in the endosperm did not decrease when embryo sugar levels were increasing. Probably, in the endosperm the conversion of stored lipids to carbohydrates occurred, maintaining the sugar levels. Another source of sugar in the endosperm may be from amino acids derived from the breakdown of protein occurring at the same time.

Desiccation and Germination Studies of Cocoa (*Theobroma cacao* L.) Seeds - A Physiological and Biochemical Approach

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

By
Abis V. Cherussery

Division of Plant Physiology and Biochemistry
Department of Botany
University of Calicut
Kerala

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MATERIALS AND METHODS

1. Collection of Materials

Pods of cocoa (*Theobroma cacao* L.) were collected from a selected group of plants in a plantation at Thamarassery, Kozhikode district. The ripe pods 150±7 days after anthesis were selected for the study. Seeds isolated from fresh pods were de-pulped and cleaned. Seeds were selected randomly from different pods, pooled together and were used for storage and germination studies.

2. Storage Studies

For the present study, the cocoa seeds were stored under three different conditions. The de-pulped, cleaned and dried seeds were divided into three equal lots. One of the seed lots was spread uniformly in open trays and kept at room temperature (30±3°C). The second lot was stored in clean, air-filled polyethylene bags and kept at room temperature. The third lot was stored in air-filled polyethylene bags and kept in a refrigerator at an average temperature of 4±2° C.

2.1. Sampling

From the seeds stored under different conditions, samples for various studies were drawn daily up to four days and thereafter at regular intervals of two days till the viability of the seeds were found lost.

2.2. Percentage of Moisture Content

For the determination of moisture content of seeds desiccated under different storage conditions, 10 seeds each in triplicate were drawn from the seed lots in each of the storage condition at regular intervals. The seeds were weighed accurately and kept in a hot-air oven at 100±3°C for 1 hour. Then the temperature was adjusted to 60°C. After 24 hours, the weight of the seeds was determined. The seeds were again kept in the oven. The process was repeated till the values became constant. From this the percentage of moisture content of cocoa seeds was estimated.

2.3. Critical Moisture Content

For the determination of critical moisture content, beyond which the recalcitrant seeds fail to germinate, samples of ten seeds each in triplicate were drawn at regular intervals. The seeds were weighed accurately at the time of sampling and placed in petri plates lined with moist Whatman No.1 filter paper to allow germination. The difference in fresh weight of seeds of the control and that of seeds at the time of sampling at different intervals was noted. The emergence of the radicle to a length of 5.0 mm was taken as a criterion for germination (Pritchard *et al.,* 1995; Chien and Lin, 1997). The moisture content at that stage of storage where the seeds fail to germinate was taken as the critical moisture content (Bonner, 1996).

2.4. Percentage of Germination

Ten seeds each in triplicate were drawn at regular intervals from the seeds stored in different storage conditions such as open at room temperature, polyethylene bags at room temperature, and polyethylene bags at 4°C. The seeds were placed in petri dishes lined with moist Whatman No.1 filter paper to allow germination. The seeds were moistened regularly. The number of seeds germinated in each sample at specific interval was noted and the percentage of germination was calculated.

2.5. Seed Vigour Index

Ten seeds each in triplicate were drawn from the seeds stored under different storage conditions such as open at room temperature, polyethylene bag at room temperature and polyethylene bag at 4°C in the refrigerator, at specific intervals. The samples thus collected were sown in garden pots filled with clean sand in the net house of the Department of Botany, University of Calicut. The number of seeds germinated on each day was noted and the seed vigour index was calculated.

Seed vigour index (SVI) was calculated according to the formula suggested by Copeland and McDonald (1995).

$$\mathrm{SVI} = \frac{\text{No. of seeds germinated on first count}}{\text{Days of first count}} + \cdots + \cdots + \cdots + \frac{\text{No. of seeds germinated on last count}}{\text{Days of last count}}$$

2.6. Conductivity Studies

The electrolyte leakage from the seeds of *Theobroma cacao* was measured as an indicator of membrane damage occurring during the process of desiccation and storage. The conductivity of leachate was estimated using the method suggested by of Mullet and Wilkinson (1979). From the seeds stored under different conditions, five seeds each were drawn in six replicates daily, weighed and soaked in 25 ml of distilled water taken in numbered containers. After 24 hours, the leachate was collected and conductivity measurements were made using Toshinwale TCM 15 Autoranging conductivity and TDS meter. The conductivity of distilled water was subtracted from the measurements made for the leachates.

2.7. Biochemical Studies

Biochemical analysis of seeds stored under different storage conditions were carried out at a regular interval of 24 hours up to 4 days and then at an interval of two days till the total loss of viability of the seeds was found to occur. The seeds were de-coated and the entire seed tissue was cut into small pieces and pooled. Samples for biochemical studies were drawn from the pooled tissue. The biochemical components of the seed tissues, like starch, total soluble sugars, reducing sugars, proteins, free amino acids, proline, total phenolics and lipids were extracted and estimated according to standard procedures. Estimation of lipid peroxidation and the activity of the enzymes such as superoxide dismutase (SOD) guaiacol peroxidase and catalase were also carried out.

2.7.1. Dry Weight Percentage of Tissues

A known quantity of tissues of fresh seeds and seeds desiccated under different storage conditions was taken, weighed and kept in a hot air oven at 100°C for one hour and then at 60°C till the dry weight of the tissues became constant. The percentage of dry weight was calculated.

2.7.2. Estimation of Starch

The starch content of the seeds stored under different conditions was determined at various intervals of desiccation using the method of Pucher *et al.* (1948).

2.7.2.1. Extraction

Two hundred mg of the seed tissue was weighed and homogenized in diethyl ether to remove the lipids that may interfere with the processes of extraction and purification of starch. Then the diethyl ether was decanted off and the residue was ground in 30% (v/v) perchloric acid for the extraction of starch. The homogenate was centrifuged and the supernatant was collected. The residue was again homogenised in 30% (v/v) perchloric acid, centrifuged and starch was re-extracted. The processes of homogenisation, centrifugation and extraction were repeated till it was ensured that the entire starch content of the tissue was extracted. Volume of the combined supernatant was noted. A known volume of the aliquot was taken from the combined supernatant and an equal volume of freshly prepared iodine–potassium iodide reagent (7.5% w/v) was added to the tube and mixed well using a vortex shaker. The mixture was then kept undisturbed for 10-20 minutes and centrifuged at 4000 x g for 10 minutes. The supernatant was decanted off. The excess iodine reagent present in the residue was removed by washing with alcoholic sodium chloride followed by centrifugation. After centrifugation, the coloured residue was treated with alcoholic sodium hydroxide till the blue colour was found disappeared. The residue was again washed with alcoholic sodium chloride. It was then dissolved in a known volume of 10% (v/v) sulphuric acid by heating in a boiling water bath. After cooling, the supernatant was collected and used for the estimation of starch.

2.7.2.2. Estimation

Estimation of starch was done according to Montgomery (1957). A known volume of aliquot was made up to 1.0 ml and 0.1 ml of 80% (w/v) phenol was added to it and mixed well. Five ml of concentrated sulphuric acid was quickly added to the mixture from a burette and allowed to cool. The optical density of the resultant solution was measured at 540 nm using a colorimeter. Soluble starch was used as the standard.

2.7.3. Estimation of Total Soluble Sugars

For the estimation of soluble sugars the method proposed by Giorgini and Campos (1992) was adopted.

2.7.3.1. Extraction

Two hundred mg of the tissues of cocoa seeds stored under different conditions and desiccated for different intervals were weighed and homogenised in 10.0 ml of 80% (v/v) ethyl alcohol using a clean glass mortar and pestle. The homogenate was transferred to centrifuge tubes and heated for 10 minutes in a boiling water bath. After cooling, it was centrifuged at 4000 x g for 10 minutes and the supernatant was collected. The residue was again ground in 10 ml of alcohol and then boiled for 10 minutes. After cooling and centrifugation, the supernatant was added to the homogenate collected earlier. Volume of the combined extract was noted and then evaporated to dryness in a clean china dish kept in a hot air oven at 100°C.

2.7.3.2. Estimation

The total soluble sugar was estimated using the method proposed by Montgomery (1957). The dry residue left in the china dish was dissolved in distilled water and the volume was noted. From this, a known volume of aliquot was taken in a test tube and made up to 1.0 ml. To this, 0.1ml of 80% (w/v) phenol was added and mixed well. Five ml of concentrated sulphuric acid was added to the tube quickly from a burette. After cooling, the optical density of the resultant solution was measured at 490 nm in a colorimeter. D-Glucose was used as the standard.

2.7.4. HPLC Analysis of Sugars

Individual sugars in the seed tissues of *Theobroma cacao* were analysed using HPLC. The sample was prepared according to the protocol of Gorecki *et al.* (1997). One gram of seed tissue was homogenised in a known volume of 80% (v/v) ethanol and refluxed for 2 hours. It was then centrifuged at 16000×g for 20 minutes. The supernatant was collected and the pellet was re-extracted in 80% (v/v) alcohol, centrifuged and the supernatant was collected again. The combined supernatant was evaporated to dryness in a hot air oven. The residue was dissolved in 2.0 ml of distilled water and filtered through a column containing Dowex 50 W- X 8 cation exchanger. The sugar fraction was eluted with 10 ml of distilled water. The eluate was subjected to HPLC analysis.

Twenty μl aliquot was injected into the HPLC system consisting of Waters u Bondapak –NH2 column, Waters 600 pump and Rheodyne 7725 injector. The mobile phase was acetonitrile-water (70:30) at a flow rate of 1.0 ml / minute. The sugars were detected using Waters 2414 refractive index detector and quantified by comparison of the peak areas of the sample with those of standard solutions.

2.7.5. Estimation of Total Reducing Sugars

For the estimation of total reducing sugars of cocoa seeds desiccated under different conditions for specific periods, an alcoholic extract of the seed tissues was prepared.

2.7.5.1. Alcoholic Extraction

One gram of cocoa seed tissue was homogenised in 80% (v/v) ethyl alcohol using a glass mortar and pestle. The homogenate was carefully transferred to a round-bottomed flask fitted with a water condenser and refluxed over a steam bath for 4 hours. The flask was cooled and the extract was centrifuged at 4000 x g for 10 minutes. The supernatant was collected in a boiling tube. The residue was homogenized again in 80% (v/v) ethanol and refluxed for 1 hour. The extract was centrifuged, the supernatant was collected and combined with the original. Final volume of the combined supernatant was noted.

2.7.5.2. Ion Exchange Chromatography

A known quantity of the alcoholic extract was transferred to a china dish and dried over a steam bath. The dried matter was dissolved in a known volume of distilled water and was subjected to ion exchange chromatographic procedure using a Dowex 50W-X-8 cation exchanger. The ion exchanger was activated by treating with 0.1N HCl, and washed with distilled water repeatedly till all traces of HCl were removed. The activated ion exchanger was filled to a height of 10 cm in a glass column with sintered filter. Two ml of the sample was loaded on the column. The column was then washed with 60 ml of distilled water to remove neutral and anionic molecules, which contain the reducing sugars. The aqueous fraction thus obtained was dried in an evaporating dish.

The column was again washed with 30 ml of 5% (w/v) ammonium hydroxide solution. The washings were collected, dried and were used for the determination of total free amino acids and total free proline.

The aqueous fraction obtained from the ion-exchange chromatography was dried and dissolved in known volume of distilled water. This was used for the estimation of total reducing sugars according to the Nelson-Somogyi's method (Nelson, 1944; Somogyi, 1952).

2.7.5.3. Preparation of Somogyi's Copper Reagent

This reagent was prepared by dissolving 24 g of anhydrous sodium carbonate and 12 g of sodium potassium tartrate in about 250 ml of distilled water. To this, 4.0 g of copper sulphate as a 10% (w /v) solution was added and mixed followed by the addition of 16 g of sodium bicarbonate. Then 180 g of sodium sulphate was dissolved in about 500 ml of distilled water and boiled to expel air. After cooling, the two solutions were mixed and the volume was made up to 1000 ml (Somogyi, 1952).

2.7.5.4. Preparation of Nelson's Arsenomolybdate Reagent

Nelson's arsenomolybdate reagent was prepared by dissolving 25 g of Ammonium heptamolybdate in 450 ml of water, to which 21 ml of sulphuric acid was added and vortexed. To the mixture, 3.0 g of disodium hydrogen arsenate, dissolved in 25 ml of distilled water was added. The solution was mixed well and incubated for 24 hours at 37°C (Nelson, 1944).

2.7.5.5. Estimation

From the sample a known volume of aliquot was pipetted out and was made up to 1.0 ml using distilled water. To this, 1.0 ml of Somogyi's copper reagent was added and mixed. The mixture was placed in a bath of boiling water and heated for 20 minutes. After cooling under tap water, 1.0 ml of Nelson's arsenomolybdate reagent was added with immediate mixing till the effervescence ceased. The intensity of colour development was measured at 540 nm using a Photochem Digital Colorimeter. D-Glucose was used as the standard.

2.7.6. Protein Analysis

2.7.6.1. Estimation of Total Proteins

Total protein content of the tissues of cocoa seeds stored under different conditions and desiccated for various intervals was determined using the method of Lowry *et al.* (1951).

Two hundred mg of seed tissue was homogenised using a chilled glass mortar and pestle in a medium containing 50 mM phosphate buffer (pH 7.5) and 50 mM 2-mercaptoethanol. The homogenate was centrifuged at 4000 x g for 10 minutes. The supernatant was collected and the volume noted. From the supernatant, an aliquot of 2.0 ml was pipetted out into a centrifuge tube and an equal volume of 10% (w/v) trichloroacetic acid was added and mixed well. This was kept in an ice bath for one hour for flocculation. The mixture was centrifuged for 10 minutes and the supernatant was decanted off. The precipitate was washed with 2.0% (w/v) trichloroacetic acid and again centrifuged. The washing and centrifugation of the precipitate was repeated twice with 15% (v/v) perchloric acid and thrice with diethyl ether. The brown coloured precipitate thus obtained was treated repeatedly with anhydrous acetone to remove the pigments. Finally the precipitate was washed with 80% (v/v) acetone.

The dry pellet obtained after centrifugation was digested in a known volume of 0.1 N sodium hydroxide by heating in a bath of boiling water for 10 minutes. The digest was centrifuged and the supernatant was collected. From the supernatant, aliquots of known volume were pipetted out in triplicate and made up to 1.0 ml with distilled water. To this, 5.0 ml of alkaline copper reagent was added and mixed well. After 10 minutes, 0.5 ml of 1N Folin and Ciocalteu's phenol reagent was added and shaken well immediately. The tubes were kept for 30 minutes for colour development.

The optical density was measured at 700 nm using Shimadzu UV-1601 spectrophotometer. Bovine serum albumin-fraction V was used as the standard.

2.7.6.2. Electrophoresis for the Determination of Protein Profile

SDS Poly acrylamide gel electrophoresis was carried out according to the method of Gaal *et al.* (1980). Two hundred mg of the tissue of cocoa seeds stored under different conditions was homogenised using a chilled mortar and pestle in 50 mM phosphate buffer in presence of polyvinyl polypyrrolidone (PVPP) as phenolic binder and 10% sodium dodecyl sulphate (SDS). The 10% homogenate was centrifuged at 16,000×g for 20 minutes using a Kubota KR 20000 T refrigerated centrifuge at 4°C and the supernatant was collected.

2.7.6.2.1. Preparation of Gels

The resolving gel was prepared by mixing 3.3 ml of acrylamide / bisacrylamide (30% T and 2.67% C), 5 ml of 1.0 M resolving gel buffer (pH 8.8), 50 μl of 10% ammonium persulphate, 50 μl of 10% SDS and 5.0 μl TEMED. The mixture was made up to 10 ml with deionised water.

The stacking gel was prepared by mixing 0.99 ml of acrylamide / bisacrylamide (30% T and 2.67% C), 3 ml of 0.5 M resolving gel buffer (pH 6.8), 30 μl of 10% ammonium persulphate, 30 μl of 10% SDS and 5.0 μl TEMED. The mixture was made up to 6 ml with deionised water.

2.7.6.2.2. Gel Casting

The gel was cast in a Genie mini vertical gel casting unit. The glass plates, the comb and the spacers of the casting unit were wiped clean with alcohol using tissue paper. The glass plates were wiped with acetone. The dried glass plates were clamped on the casting unit with the spacers placed in between them. The resolving gel was poured into the casting unit and the top was layered with a small volume of deionized water to avoid contact with air. After the completion of polymerization, the water was removed with strips of filter paper. Then the comb was placed and the stacking gel was poured carefully. The gel was topped with deionised water. After polymerization, the comb was removed carefully and the wells were cleaned thoroughly.

2.7.6.2.3. Electrophoresis

40 μl of the extract containing 20% sucrose was added to each well. Bromophenol blue was used as the tracking dye. Low molecular weight marker (Biorad) was loaded in one of the wells. Electrophoresis was carried out using the electrophoretic reservoir buffer, Tris-glycine, pH 8.4. Initially the gels were maintained at a voltage of 80 V. Once the stacking has taken place, the voltage was raised to 120 V and was maintained there till the electrophoretic run was completed. At the end of the run, the gel was carefully removed and was stained with 0.2% (w/v) coomassie brilliant blue R 250 in methanol-acetic acid mixture. After 3 hours of staining, the gels were destained in methanol-acetic acid mixture and stored in 7% (v/v) acetic acid.

The gels were analysed in a Biorad Geldoc and molecular weight of the bands was determined using the software, Quantity-One.

2.7.7. Estimation of Total Amino Acids

The amino acid content of the extract was quantitatively estimated using the procedure of Lee and Takahashi (1966).

The ammonia fraction of the eluate obtained in the ion-exchange chromatography was evaporated to dryness at 45°C in an evaporating dish in a hot-air oven. The residue was dissolved in a minimum quantity of 10% (v/v) isopropyl alcohol and made up to 3.0 ml.

2.7.7.1. Preparation of Ninhydrin-Citrate-Glycerol Reagent

One ml of 1% (w/v) ninhydrin solution in 0.5 M citrate buffer (pH 5.5) was mixed thoroughly with 2.4 ml of glycerol and 0.4 ml of 0.5 M citrate buffer (pH 5.5).

To 0.2 ml of aliquot 3.8 ml of ninhydrin-citrate-glycerol reagent was added. After shaking well, the mixture was kept in a bath of boiling water for 12 minutes. It was then cooled in tap water and brought down to room temperature. The optical density of the resultant solution was measured at 570 nm within one hour using a Photochem colorimeter. The reagent blank contained 0.2 ml distilled water and 3.8 ml ninhydrin-citrate-glycerol mixture. An equimolar mixture of four amino acids namely, DL-alanine, DL-tryptophan, DL-histidine and DL-aspartic acid was used as the standard.

2.7.8. Estimation of Proline

Total proline content of cocoa seed tissue under desiccation in different conditions for different periods was estimated according to the method of Bates (1973).

2.7.8.1. Preparation of Acid Ninhydrin

Acid ninhydrin was prepared by warming 1.25 g of ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid with agitation till dissolved.

2.7.8.2. Estimation

The samples prepared for the estimation of total amino acids were used for the estimation of proline also. Two ml of the sample was heated with 2.0 ml of acid ninhydrin and 2.0 ml of glacial acetic acid in test tubes for one hour. The reaction was terminated in an ice bath. The reaction mixture was extracted with 4.0 ml toluene. The mixture was stirred vigorously for 15-20 seconds. The chromatophore containing toluene was aspirated from its aqueous phase, warmed to room temperature and its absorbance was read at 520 nm using a Photochem digital colorimeter. Proline was used as the standard.

2.7.9. Estimation of Total Phenolics

Phenolic content of the cocoa seeds desiccated under different storage conditions was determined colorimetrically according to the method of Folin and Denis (1915).

2.7.9.1. Preparation of Folin-Denis Reagent

The Folin-Denis reagent was prepared by dissolving 100 g sodium tungstate, 20 g phosphomolybdic acid and 50 ml orthophosphoric acid in 750 ml of distilled water. The mixture was refluxed for 2 hours, cooled and made up to 1000 ml. Normality of the reagent was determined by titrating it against an alkali like NaOH with phenolphthalein as an indicator.

2.7.9.2. Estimation

A known volume of the alcoholic extract of cocoa seed tissues was taken in a test tube and was made up to 2.0 ml with distilled water. To this 2.0 ml of Folin-Denis reagent was added, mixed thoroughly and 2.0 ml of 1.0 N sodium carbonate was added. Following mixing, the tubes were kept for an hour for colour development. Absorbance was measured at 725 nm using a Photochem digital colorimeter. Tannic acid was used as the standard.

2.7.10. Estimation of Total Lipids

Total lipid content of the seed tissues of cocoa desiccated under various storage conditions was determined gravimetrically using the modified form of the Folch method (Folch *et al.,* 1957; Christie, 1993). One gram of seed tissue was homogenized thoroughly in chilled diethyl ether using a clean glass mortar and pestle. The homogenate was centrifuged at 4000 x g for 10 minutes. The supernatant was collected in a pre-weighed china dish. The sediment was homogenized again with chilled diethyl ether and the process was repeated several times and the supernatants were added to the bulk in the china dish. The china dish containing the combined supernatant was kept in a hot air oven at 60°C for 24 hours. The china dish along with the contents left after evaporation was weighed again and the difference between the initial weight and the final weight was found out.

2.7.11. Estimation of Lipid Peroxidation

The lipid peroxidation occurring in cocoa seeds desiccated for specific intervals at different storage conditions was estimated according the procedure of Heath and Packer (1968). The assay estimated the amount of malondialdehyde (MDA) which is the direct product of lipid peroxidation in the tissues. The reaction mixture consisted of 0.25% (w/v) thiobarbituric acid in 10% trichloroacetic acid (w/v) (TBA-TCA reagent). Two hundred mg of seed tissue was homogenized in 100 mM phosphate buffer (pH 7.5) using a pre-chilled glass mortar and pestle. The homogenate was transferred to centrifuge tubes and centrifuged at 5000 x g for 1 minute. The supernatant was collected and an aliquot of 0.2 ml was added to 2.0 ml of the TBA-TCA reagent and incubated at 95°C for 30 minutes. The sample was then cooled and centrifuged at 16000 × g for 10 minutes in a Kubota KR 20000 T refrigerated centrifuge at 4°C. The absorbance of the supernatant was measured at 532 nm. Samples incubated with 10% TCA without TBA were used as control. Results were expressed as micrograms of MDA g^{-1} dry mass of tissue.

2.7.12. Estimation of Superoxide Dismutase Activity

For the estimation of superoxide dismutase (SOD) activity the protocol of Giannopolitis and Ries (1977) was adopted. Two hundred mg of cocoa seed tissues at various stages of desiccation under different storage conditions were thoroughly homogenised using a chilled glass mortar and pestle in a medium consisting of 50 mM phosphate buffer (pH 7.8) and 100 mg of polyvinyl polypyrrolidone (PVPP) as phenolic binder. The homogenate was then centrifuged at 16000 x g for 15 minutes in a Kubota KR 20000 T refrigerated centrifuge at 4°C. Part of the supernatant collected was used for the assay of SOD activity and the remaining part was used for the estimation of proteins.

2.7.12.1. Enzyme Assay

The SOD activity was assayed photochemically. Three sets of assay systems were prepared separately; one for the assay, one for the dark-control and one for the light-control. The reaction mixture consisted of 0.1 ml of 1.5 M Sodium carbonate, 0.3 ml of 0.13 M methionine, 0.3 ml of 10 mM EDTA, 0.3 ml of 13 μM riboflavin, and 0.3 ml of 0.63 mM nitroblue tetrazolium. The nitroblue tetrazolium was withheld in the light-control system. To the reaction system, 0.1 ml of the enzyme extract was added. No extract was added to the blank and to the light-control system. The reaction mixture was made up to 3.0 ml using 50 mM phosphate buffer (pH 7.8). The tubes with dark-control samples were kept in a dark chamber and the tubes of the light-control and the assay systems were kept under a fluorescent lamp. After 30 minutes of incubation the optical density of the solutions of the assay, the dark-control and the light-control systems were measured at 560 nm using Shimadzu UV-1601 spectrophotometer. Results were expressed as units SOD mg^{-1} protein. One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of nitroblue tetrazolium to blue formazan by 50%.

2.7.12.2. Estimation of Soluble Protein

The amount of protein in the enzyme extract was determined according to the procedure of Lowry *et al.* (1951) as described earlier.

2.7.13. Estimation of Guaiacol Peroxidase Activity

Two hundred mg of seed tissue of cocoa was homogenised using a pre-chilled glass mortar and pestle. The medium for homogenization was prepared by mixing 50 mM Tris-HCl of pH 7.5 and 200 mg polyvinyl polypyrrolidone (PVPP) as phenolic binder. The extract was filtered through two layers of muslin cloth and the filtrate was made up to 10.0 ml using the Tris–HCl buffer. The filtrate was transferred to centrifuge tubes and centrifuged at 16000 x g for 15 minutes in a Kubota KR 20000 T refrigerated centrifuge at 4°C. The supernatant was transferred to a clean test tube and kept in an ice bath. A part of the extract was used for enzyme assay and the other part was used for the estimation of soluble protein.

2.7.13.1. Enzyme assay

The assay system was patterned after Abeles and Biles (1991) with minor modifications.

The reaction mixture consisted of 1.5 ml of 100 mM potassium phosphate buffer at pH 7.0, 0.3 ml of 10 mM guaiacol, 0.3 ml of 10 mM hydrogen peroxide, 0.3 ml of enzyme extract and 0.6 ml of distilled water, together making up to 3.0 ml.

All the ingredients of the reaction mixture except hydrogen peroxide were added and mixed well in a test tube. The hydrogen peroxide was added at the end to initiate the enzyme activity. The enzyme activity was measured immediately after the addition of hydrogen peroxide at 470 nm by direct spectrophotometry using a Shimadzu UV-1601 spectrophotometer for 3 minutes. A total of 6 measurements were taken at an interval of 30 seconds. The enzyme activity was measured in terms of the amount of enzyme required to decompose 1.0 µmol of H_2O_2 and is expressed as µmol of H_2O_2 mg^{-1} protein min^{-1}.

2.7.13.2. Estimation of Soluble Protein

The quantity of protein in the enzyme extract was determined according to the procedure of Lowry *et al.* (1951) as described earlier.

2.7.14. Estimation of Catalase Activity

Two hundred mg of the seed tissues of cocoa at various stages of desiccation under different storage conditions were homogenised using a chilled glass mortar and pestle in a medium consisting of 50 mM phosphate buffer (pH 7.0) and 100 mg of polyvinyl polypyrrolidone (PVPP) as phenolic binder. The homogenate was filtered through two layers of muslin cloth and was made up to 10.0 ml using the phosphate buffer. The filtrate was then centrifuged at 16000 x g for 15 minutes in Kubota KR 20000 T refrigerated centrifuge at 4°C. A part of the extract was used for enzyme assay and the remaining part was used for the estimation of protein.

2.7.14.1. Enzyme Assay

The catalase assay system consisted of 1.0 ml of 50 mM phosphate buffer pH 7.0, 2.0 ml of the enzyme extract and 1.0 ml of 30 mM hydrogen peroxide (Kar and Mishra, 1976). The phosphate buffer and the enzyme extract were pipetted out and mixed well in a test tube. To this, hydrogen peroxide was added to initiate the enzyme activity. Immediately after the addition of hydrogen peroxide, enzyme activity was measured at 240 nm for 90 seconds at 15 seconds interval using a Shimadzu UV-1601 spectrophotometer.

2.7.14.2. Estimation of Soluble Protein

The amount of protein in the enzyme extract was determined according to the procedure of Lowry *et al.* (1951) as described earlier.

2.8. Histochemical Studies

Histochemical studies were carried out to localize metabolites like insoluble polysaccharides and proteins of the cotyledonary tissues of cocoa seeds during desiccation under room temperature. Samples were collected from the seed tissues at a regular interval of 24 hours. The samples fixed in FAA were dehydrated through alcohol–TBA series, infiltered and embedded in paraffin wax (Johansen, 1940).

2.8.1. Sectioning

Sections of the materials embedded in the paraffin wax were cut at 10 μm using a Leica (RM2125RT) Rotary Microtome. The sections were mounted on clean microslides using Mayer's adhesive. The sections were de-paraffinised, hydrated and stained for the localization of insoluble polysaccharides and proteins.

2.8.2. Localization of Insoluble Polysaccharides

For the localization of the insoluble polysaccharides, periodic acid-Schiff's reagent was used (Berlyn and Miksche, 1976).

2.8.2.1. Preparation of Periodic Acid - Schiff's Reagent

Periodic acid was prepared by dissolving 0.5 g periodic acid in distilled water and making up to 100 ml.

For the preparation of Schiff's reagent 2.0 g of basic fuchsine and 3.8 g of potassium metabisulfite were dissolved in 200 ml of 0.15 N HCl in a conical flask. It was shaken for 2 hours using a magnetic stirrer. One gram of activated charcoal was added to the mixture and stirred constantly for one hour for decolorizing the solution. The mixture was filtered and stored in a refrigerator at 4°C.

2.8.2.2. Staining

The serial sections prepared as described earlier were deparaffinised and brought down gradually to water. The hydrated sections were then treated with aqueous periodic acid at 23°C for 15 minutes. Excess periodic acid was removed by washing the slides in running water for 10 minutes. The slides were then immersed in Schiff's reagent for 10 minutes at 4±1°C and washed thereafter for 20-30 seconds in tap water. The stained sections were treated with 2.0% (w/v) sodium bisulfite for 2 minutes and washed again for 4-5 minutes. The sections were then dehydrated in alcohol-TBA series and mounted in DPX.

2.8.3. Localization of Proteins

The total protein content of the seed tissues was localized using the mercuric bromophenol blue method (Mazia *et al.*, 1953; Chapman, 1975).

2.8.3.1 Preparation of Mercuric Bromophenol Blue

10 g of mercuric chloride was dissolved in 100 ml of distilled water and 100 mg of bromophenol blue was dissolved in it. The stain was filtered and kept in a refrigerator.

2.8.3.2. Staining

Sections were de-paraffinised and brought down to water through the alcohol series. The sections were stained with mercuric bromophenol blue for 15 minutes and washed in 0.5% (v/v) acetic acid for 20 minutes to remove excess dye. The sections were then washed in tap water for 15 minutes and then dehydrated through alcohol - TBA series and finally mounted in DPX.

3. Germination studies

The behaviour of cocoa seeds during germination and the mobilization of reserve food materials were studied by allowing the seeds to germinate in petri plates. The seeds after de-pulping and cleaning were kept in petri plates in between moist filter paper and kept in a dark chamber. The filter paper was moistened regularly with distilled water. Samples for biochemical and histochemical studies were drawn from the seed lot on the second and third days of incubation, i.e. stages of imbibition and initiation of germination with the emergence of the radicle and thereafter at intervals of five days each, till the seedlings showed symptoms of wilting and death. Cotyledons and embryonic axis were collected separately from the samples, randomized, stored and used for various physiological and biochemical assays. Dry weight percentage of the cotyledon and axis tissues of various stages of germination was also determined.

3.1. Determination of Moisture Content

Samples of cotyledonary and axial tissue were drawn at specified intervals mentioned above and weighed accurately and kept in a hot-air oven at 100±3°C for 1 hour. Then the temperature was adjusted to 60°C. After 24 hours, the weight of the tissues was determined. The tissues were again kept in the oven. The process was repeated till the values became constant. From this the percentage of moisture content of cocoa tissues was estimated.

3.2. Biochemical Characterization of Seeds during Germination

The cotyledon and axis tissues of known weight were collected from the seeds of cocoa, germinating *in vitro* at the intervals specified above and was analyzed for, total soluble sugars, starch, total lipids, proteins, and free amino acids as per the procedures mentioned under desiccation.

3.3. Histochemical Studies

Histochemical studies were carried out to localize metabolites like insoluble polysaccharides and proteins of cotyledonary tissues of cocoa seeds at different stages of germination.

4. Microphotography

The stained and mounted sections were observed under a Nikon Eclipse 400 microscope and microphotographs were taken at 40 X using a Nikon DXM 1200 F digital camera.

5. Statistical Analysis

All the analyses described were carried out in 6-8 replicates and the values expressed as mean ± standard deviation and standard error. The statistical significance was tested using the t-test. Values of $P < 0.05$ were taken as statistically significant.

Desiccation and Germination Studies of Cocoa (*Theobroma cacao* L.) Seeds - A Physiological and Biochemical Approach

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

By
Abis V. Cherussery

Division of Plant Physiology and Biochemistry
Department of Botany
University of Calicut
Kerala

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RESULTS

1. Desiccation Studies

1.1. Moisture Content

The percentage of moisture content (MC) of fresh seeds of cocoa (*Theobroma cacao* L.) at the time of harvest was very high but was found to decrease as the period of storage and desiccation progressed (Table 1, Fig. 1A). Seeds stored in open conditions at room temperature (RT) showed a rapid and significant decrease in MC throughout the period of storage. But such a change in MC was not observed in seeds stored in polyethylene bags, either at RT or at 4°C in a refrigerator. The moisture content of seeds kept in polyethylene bags at RT was found to decrease throughout the period of storage, but the decrease was negligible up to 4 days of desiccation. Thereafter the decrease in MC was found to be rapid and significant. Seeds stored in polyethylene bags at 4°C showed a more rapid and steady loss of moisture content than that of seeds in polyethylene bags at RT. The decrease in moisture content was rapid and significant up to 6 days of storage and then became gradual and insignificant.

1.2. Percentage of Germination

Cent percent germination was found to occur in fresh seeds of cocoa upon incubation (Table 2, Fig. 1B). Seeds stored open at room temperature (RT) did not show any reduction in germination percentage after one day of desiccation. A slight decline in the value was noted on the second day. The germination percentage was found to decrease sharply in the subsequent stages of desiccation and by the 4th day it was reduced to zero.

Table 1:Effect of desiccation under different storage conditions on percentage of moisture content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Percentage of moisture content									
Open at RT	36.98 ± 1.02	29.63 ± 2.01	24.87 ± 1.25	20.48 ± 1.09	15.00 ± 0.99	12.64 ± 0.71	11.56 ± 1.01	10.47 ± 0.84	9.65 ± 0.91	8.02 ± 0.51
Polyethylene bags at RT	36.98 ± 1.02	36.41 ± 1.96	35.96 ± 2.16	35.85 ± 1.14	34.41 ± 1.24	32.32 ± 1.04	28.00 ± 1.11	26.64 ± 1.05	23.21 ± 0.81	20.57 ± 0.88
Polyethylene bags at 4°C	36.98 ± 1.02	34.66 ± 1.21	31.61 ± 2.52	28.86 ± 1.65	26.54 ± 1.35	22.01 ± 1.14	21.54 ± 1.88	20.51 ± 1.55	19.47 ± 0.75	19.01 ± 0.98

Table 2: Effect of desiccation under different storage conditions on percentage of germination in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Percentage of germination									
Open at RT	100 ± 0.00	100 ± 0.00	85 ± 3.24	30 ± 1.76	0	0	0	0	0	0
Polyethylene bags at RT	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	80 ± 2.85	64 ± 2.14	30 ± 1.96	0
Polyethylene bags at 4°C	100 ± 0.00	100 ± 0.00	100 ± 0.00	75 ± 2.33	35 ± 2.01	10 ± 0.98	0	0	0	0

In seeds stored in polyethylene bags at RT, 100 % germination was found to be maintained for 6 days. Thereafter a rapid reduction in the germination percentage was observed up to the 12th day of storage. No germination was noticed in seeds stored for 14 days.

Seeds stored in polyethylene bags at 4°C in refrigerator showed 100 % germination for 2 days only. From the 3rd day of storage onwards, the germination percentage was found to decrease rapidly. By the eighth day, the germination percentage was reduced to zero.

Of the seed lots maintained in the three different storage conditions, those stored in polyethylene bags at room temperature exhibited longer period of viability than the others.

1.3. Seed Vigour Index

Seed vigour index (SVI) of cocoa seeds was found to vary according to the storage conditions (Table 3, Fig. 1C). The SVI of seeds stored open at RT decreased slowly in the initial two stages. Thereafter, a rapid reduction in the SVI was found to occur and was reduced to zero in seeds desiccated for 4 days.

Seeds maintained in polyethylene bags at RT exhibited a gradual and insignificant decrease in SVI up to three days of storage. Beyond this, the decline in the value was more pronounced and on the 14th day of storage it was reduced to zero.

Seeds stored in polyethylene bags at 4°C showed only a gradual decrease in SVI for the first 2 days. The value declined rapidly in the subsequent stages of storage, reaching zero value by the 8th day.

Table 3: Effect of desiccation under different storage conditions on seed vigour index of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Seed Vigour Index									
Open at RT	3.78 ± 0.16	3.33 ± 0.14	2.28 ± 0.13	0.91 ± 0.02	0	0	0	0	0	0
Polyethylene bags at RT	3.78 ± 0.16	3.68 ± 0.09	3.43 ± 0.14	3.29 ± 0.24	3.11 ± 0.16	3.09 ± 0.21	2.85 ± 0.15	1.8 ± 0.05	1.05 ± 0.02	0
Polyethylene bags at 4°C	3.78 ± 0.16	3.70 ± 0.19	3.38 ± 0.21	2.65 ± 0.12	1.15 ± 0.06	0.32 ± 0.01	0	0	0	0

Table 4: Effect of desiccation under different storage conditions on electrical conductivity of leachates of the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Conductivity of leachate (mho cm^{-1} g^{-1})									
Open at RT	10.73 ± 0.45	21.54 ± 1.05	26.81 ± 1.21	34.25 ± 1.89	38.57 ± 1.56	NE	NE	NE	NE	NE
Polyethylene bags at RT	10.73 ± 0.45	12.71 ± 0.82	16.21 ± 0.78	19.19 ± 1.12	22.64 ± 0.96	25.54 ± 1.23	32.52 ± 1.08	46.73 ± 1.80	47.56 ± 1.30	46.27 ± 2.36
Polyethylene bags at 4°C	10.73 ± 0.45	16.45 ± 1.01	20.33 ± 1.06	26.97 ± 1.32	30.08 ± 1.95	34.25 ± 1.64	38.12 ± 2.15	41.65 ± 2.12	42.03 ± 2.07	42.51 ± 1.88

NE-Not Estimated

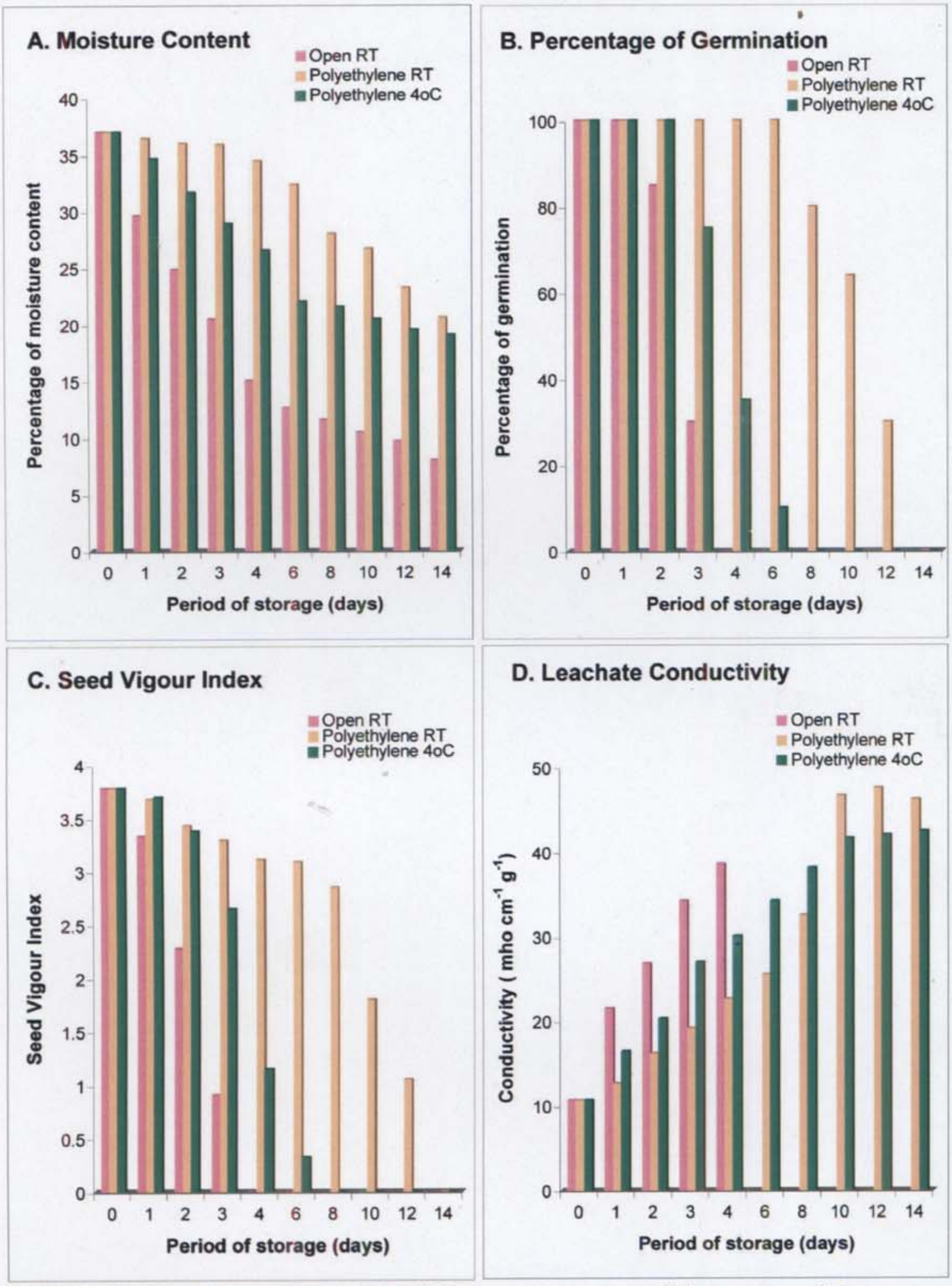


Fig 1: Effect of desiccation under different storage conditions on moisture content, germination percentage, seed vigour index and leachate conductivity of the seeds of *Theobroma cacao*

Among the three different storage conditions employed in the present study, high SVI values were found to be maintained for a longer period in seeds stored in polyethylene bags at room temperature.

1.4. Leachate Conductivity

A general increase in electrical conductivity was observed in the leachates of cocoa seeds kept under different storage conditions (Table 4, Fig. 1D). In seeds stored open at room temperature, the increase in conductivity was rapid and on the 4th day of storage it reached nearly four times the value of the fresh seeds. Leachates of seeds stored in polyethylene bags at RT showed a slow increase in conductivity in the initial stages. From the 6th day onwards it became rapid. However, the value became gradual and insignificant after 10 days of storage. A slight decline in the conductivity was noticed on the last day of desiccation. In seeds stored in polyethylene bags at 4°C, the increase in leachate conductivity was gradual and more or less uniform throughout the period of desiccation except towards the latter stages where the values showed only a very slow increase.

1.5. Biochemical Studies of Seeds under Desiccation

1.5.1. Total Starch Content

The total starch content in fresh seeds of cocoa was found to decrease on storage and desiccation (Table 5, Fig. 2A). Seeds stored open at room temperature showed a rapid and significant decrease ($p<0.05$) in starch content through out the period of desiccation. The starch content in seeds desiccated for 4 days was found to be one-third of the value of fresh seeds. Seeds stored in polyethylene bags at RT and at 4°C showed a more gradual decrease in starch content in the initial stages as compared to that of the seeds kept open at RT. The decrease in starch content was found to occur

Table 5: Effect of desiccation under different storage conditions on starch content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Starch (mg g^{-1} dw)									
Open at RT	62.73 ± 3.03	55.25 ± 2.12	46.82 ± 1.26	26.59 ± 1.94	21.09 ± 1.34	NE	NE	NE	NE	NE
Polyethylene bags at RT	62.73 ± 3.03	57.51 ± 2.01	50.49 ± 1.65	47.06 ± 1.20	44.22 ± 1.21	40.15 ± 1.96	37.76 ± 1.23	37.54 ± 1.25	33.43 ± 1.32	31.54 ± 1.51
Polyethylene bags at 4°C	62.73 ± 3.03	61.54 ± 2.21	55.30 ± 2.18	51.21 ± 2.10	45.86 ± 1.99	43.16 ± 2.00	42.71 ± 2.05	42.08 ± 1.51	40.61 ± 1.51	38.54 ± 1.32

NE: Not Estimated

Table 6: Effect of desiccation under different storage conditions on total soluble sugar content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Total soluble sugar (mg g^{-1} dw)									
Open at RT	72.50 ± 2.34	79.54 ± 3.16	86.72 ± 3.26	64.37 ± 3.09	58.41 ± 2.41	NE	NE	NE	NE	NE
Polyethylene bags at RT	72.50 ± 2.34	77.84 ± 3.02	79.65 ± 3.22	81.85 ± 3.19	84.83 ± 4.14	88.57 ± 3.26	82.64 ± 2.06	66.51 ± 2.87	54.32 ± 3.94	49.57 ± 1.76
Polyethylene bags at 4°C	72.50 ± 2.34	77.96 ± 3.21	81.35 ± 3.45	83.00 ± 2.81	83.66 ± 3.59	69.88 ± 2.01	57.3 ± 2.85	43.63 ± 3.00	39.96 ± 1.99	36.41 ± 2.68

NE: Not Estimated

significantly up to 6 days of storage and more gradually thereafter. In seeds stored in polyethylene bags at 4°C, the decrease in starch content was very insignificant after desiccation for one day. Subsequently, the decrease in the value became more pronounced and significant up to 6 days of storage. Later on, the decrease in starch content became gradual and insignificant.

1.5.2. Total Soluble Sugar Content

An insignificant increase in the total soluble sugar content was observed in cocoa seeds in the early stages of storage. In seeds stored open at RT, the total sugar content reached the maximum value after desiccation for 2 days (Table 6, Fig. 2B). In the subsequent stages, the value was found to decline significantly ($p<0.05$). The increase in total sugar content was gradual and insignificant in seeds stored in polyethylene bags at RT. The value was observed to increase for 6 days and to decrease significantly thereafter. A similar trend was observed in the total sugar content of seeds stored in polyethylene bags at 4°C. But the increase was observed only up to the 4th day of storage. A sharp decrease in the total sugar content was noticed thereafter.

1.5.3. Sucrose Content

The sucrose content in cocoa seeds was found to increase in the initial stages of desiccation under different storage conditions (Table 7, Fig. 2C). In seeds kept open at RT, a two-fold increase in the sucrose content was found to occur when desiccated for two days. The sucrose content continued to increase up to the 4th day of desiccation. The increase was slower in seeds kept in polyethylene bags at RT. The value continued to increase up to the 8th day of desiccation and declined slightly thereafter. A similar pattern of change in sucrose content was observed in seeds stored in polyethylene bags

Table 7: Effect of desiccation under different storage conditions on sucrose content in the seeds of *Theobroma cacao*

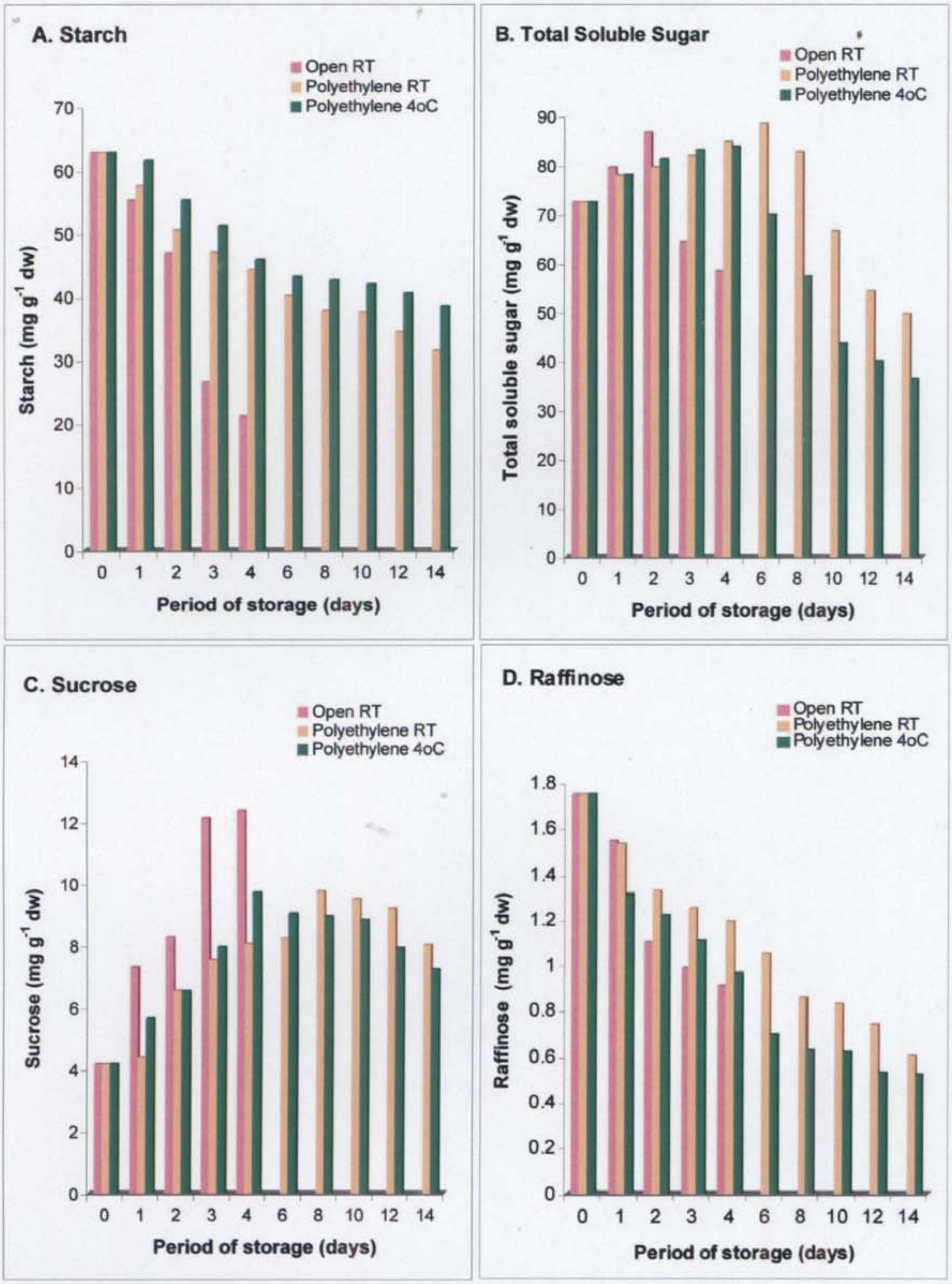
Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Sucrose (mg g^{-1} dw)									
Open at RT	4.17	7.29	8.27	12.12	12.36	NE	NE	NE	NE	NE
Polyethylene bags at RT	4.17	4.39	6.54	7.54	8.06	8.24	9.76	9.51	9.19	8.02
Polyethylene bags at 4°C	4.17	5.65	6.52	8.21	9.71	9.03	8.95	8.83	7.92	7.23

NE: Not Estimated

Table 8: Effect of desiccation under different storage conditions on raffinose content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Raffinose (mg g^{-1} dw)									
Open at RT	1.75	1.55	1.10	0.99	0.91	NE	NE	NE	NE	NE
Polyethylene bags at RT	1.75	1.53	1.33	1.25	1.19	1.05	0.86	0.83	0.74	0.61
Polyethylene bags at 4°C	1.75	1.31	1.22	1.02	0.97	0.70	0.63	0.62	0.53	0.51

NE: Not Estimated



A. Starch

B. Total Soluble Sugar

C. Sucrose

D. Raffinose

Fig 2: Effect of desiccation under different storage conditions on starch, total soluble sugar, sucrose and raffinose contents in the seeds of *Theobroma cacao*

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Table 9: Comparison of percentage of germination and sucrose:raffinose ratio in the seeds of *Theobroma cacao* under storage

Storage condition		Period of desiccation (days)									
		0	1	2	3	4	6	8	10	12	14
Open RT	Germination %	100	100	85	30	0	NE	NE	NE	NE	NE
	Sucrose: Raffinose	2.38	4.7	7.52	12.24	13.58	NE	NE	NE	NE	NE
Polyethylene RT	Germination %	100	100	100	100	100	100	80	64	30	0
	Sucrose: Raffinose	2.38	2.87	4.92	6.03	6.72	7.85	11.35	11.46	12.42	13.15
Polyethylene 4°C	Germination %	100	100	100	75	35	10	0	0	0	0
	Sucrose: Raffinose	2.38	4.30	5.34	8.05	10.01	12.72	13.98	14.24	14.94	14.18

NE: Not estimated

at 4°C also, with the value peaking on the 4th day of storage and declining subsequently.

1.5.4. Raffinose

A marked decrease in the amount of raffinose was noticed in the seeds of cocoa in all the three storage conditions employed in the present study (Table 8, Fig. 2D). In seeds desiccated in open at RT, approximately 50 % reduction in raffinose content was observed by the 4th day. Those kept in polyethylene bags at RT showed a sharp decline in the value and the raffinose content was reduced approximately to one-half of the initial value on the 8th day of desiccation. Thereafter the value was found to decline gradually up to the 14th day. A more pronounced decrease in raffinose content was observed in seeds kept in polyethylene bags at 4°C and the value on the 14th day of desiccation was less than one-third of the raffinose content of the fresh seeds.

1.5.5. Reducing Sugar Content

Reducing sugar content in fresh cocoa seeds was comparatively low and showed an increase during the initial stages of desiccation under all the three storage conditions (Table 10, Fig. 3A). The increase was rapid and significant ($p<0.05$) throughout the period of desiccation in seeds kept open at RT. A three-fold increase in reducing sugar content was noticed in seeds after desiccation for 4 days. Seeds stored in polyethylene bags at RT and at 4°C exhibited an initial increase in the reducing sugar content, followed by a decrease. The rate of increase in seeds stored in polyethylene bags was less than that of seeds stored open at RT. The value of reducing sugar content in cocoa seeds on the 8th day of storage was three times greater than that of the fresh seeds. Thereafter a sharp and significant ($p<0.05$) reduction in the value was noticed. Seeds maintained in polyethylene bags at 4°C showed a gradual increase in reducing sugar content up to 3 days of storage. The increase

Table 10: Effect of desiccation under different storage conditions on reducing sugar content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Reducing sugars (mg g^{-1} dw)									
Open at RT	11.55 ± 0.47	15.97 ± 0.75	17.35 ± 0.83	28.54 ± 1.49	32.00 ± 1.01	NE	NE	NE	NE	NE
Polyethylene bags at RT	11.55 ± 0.47	15.51 ± 0.98	17.96 ± 0.98	20.03 ± 1.11	22.45 ± 0.85	24.38 ± 1.16	33.29 ± 1.32	29.43 ± 0.88	21.62 ± 0.97	18.74 ± 0.65
Polyethylene bags at 4°C	11.55 ± 0.47	12.01 ± 0.95	12.37 ± 0.68	14.55 ± 1.07	18.44 ± 0.94	25.61 ± 1.21	23.99 ± 0.91	19.35 ± 0.84	12.17 ± 0.44	12.25 ± 0.67

NE: Not Estimated

Table 11: Effect of desiccation under different storage conditions on glucose content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Glucose (mg g^{-1} dw)									
Open at RT	1.63	2.21	3.22	3.37	2.93	NE	NE	NE	NE	NE
Polyethylene bags at RT	1.63	1.92	2.06	4.36	5.82	5.53	3.92	3.45	3.12	3.03
Polyethylene bags at 4°C	1.63	2.01	2.44	3.72	4.72	4.00	2.35	2.31	1.99	1.59

NE: Not Estimated

became rapid and significant subsequently. Maximum value was found in seeds stored for 6 days. A significant decline in the amount of reducing sugar was observed in the later stages of storage.

1.5.6. Glucose

The glucose content was found to increase gradually in the initial phases of desiccation in cocoa seeds maintained in all the storage conditions (Table 11, Fig. 3B). In seeds stored open at RT, the glucose content reached the highest value on the 3rd day of desiccation. The value was found to decline in the next stage. In seeds kept in polyethylene bags both at RT and at 4°C, the glucose content was found to increase till the 4th day of storage and to decline subsequently.

1.5.7. Fructose

Cocoa seeds were found to contain fructose in very low amounts (Table 12, Fig. 3C). The value was found to increase rapidly in seeds kept open at RT and a three-fold increase was observed in seeds desiccated for four days. A sharp increase in the value was observed in the initial stages of desiccation in seeds stored in polyethylene bags at RT with the value reaching approximately 4 times the value of the fresh seeds on the 3rd day. A gradual decline was observed in the subsequent stages. In seeds kept in polyethylene bags at 4°C, fructose content peaked on the 4th day of storage and then decreased gradually to the last stage of storage.

1.5.8. Galactose

An increase in galactose content was found to occur in the initial stages of desiccation in seeds maintained in all the storage conditions (Table 13, Fig. 3D). By the 4th day of desiccation in open at RT, a three-fold

Table 12: Effect of desiccation under different storage conditions on fructose content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Fructose (mg g^{-1} dw)									
Open at RT	0.71	0.77	1.45	1.85	2.10	NE	NE	NE	NE	NE
Polyethylene bags at RT	0.71	0.81	1.74	2.79	1.82	1.44	1.23	0.73	0.71	0.52
Polyethylene bags at 4°C	0.71	0.86	1.35	2.54	2.63	2.01	1.77	1.01	0.99	0.77

NE: Not Estimated

Table 13: Effect of desiccation under different storage conditions on galactose content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Galactose (mg g^{-1} dw)									
Open at RT	0.49	0.80	1.15	1.46	1.49	NE	NE	NE	NE	NE
Polyethylene bags at RT	0.49	0.51	0.84	1.10	1.48	1.71	1.40	1.22	1.02	0.95
Polyethylene bags at 4°C	0.49	0.62	1.04	1.21	1.24	1.02	0.95	0.95	0.90	0.75

NE: Not Estimated

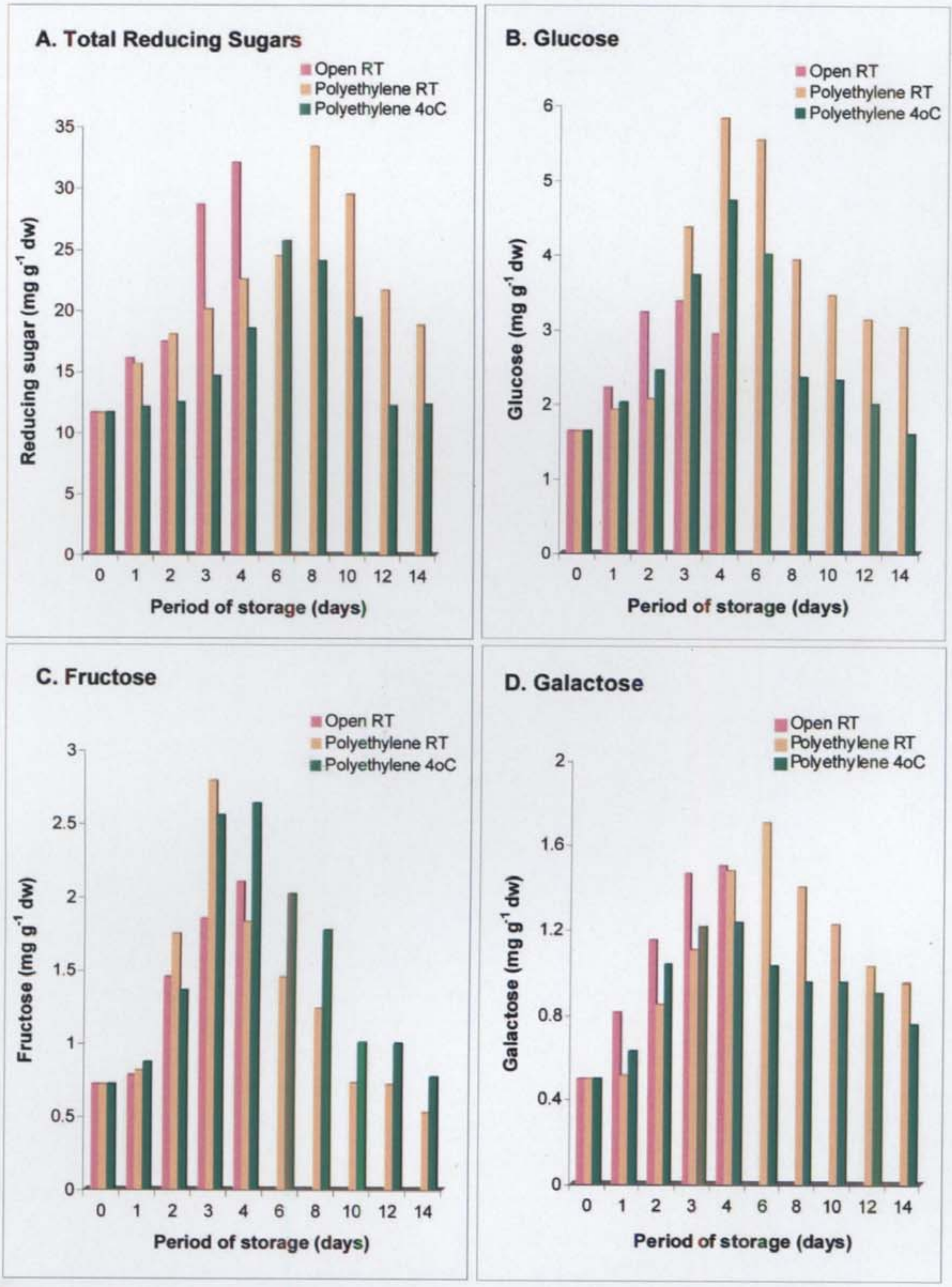

Fig 3: Effect of desiccation under different storage conditions on total reducing sugar, glucose, fructose and galactose contents in the seeds of *Theobroma cacao*

increase in the amount of galactose was observed. A similar increase was noticed in seeds stored in polyethylene bags at RT and at 4°C, the values peaking on the 6th day and 4th day respectively and declining subsequently.

1.5.9. Total Protein Content

High protein content was observed in fresh seeds of cocoa immediately after harvesting. In all the three storage conditions, the seeds exhibited a steady decrease in proteins throughout the period of storage and desiccation (Table 14, Fig. 4A). The total protein content of seeds stored open at RT was found to decrease rapidly and significantly ($p<0.05$) after desiccation for one day. The decrease in protein content was gradual and insignificant in the subsequent stages of desiccation and attained the minimum level in seeds desiccated for 4 days. Seeds kept in polyethylene bags at RT exhibited a gradual and insignificant decrease in the protein content up to the fourth day of desiccation and a more rapid and significant decline thereafter. The protein content of seeds stored in polyethylene bags at 4°C was found to decrease more rapidly than that of the seeds stored in polyethylene bags at RT. Of the three storage conditions, seeds stored in polyethylene bags at RT exhibited a relatively higher content of protein throughout the period of desiccation than those stored under the other two conditions.

1.5.10. Protein Profile

One dimensional SDS Polyacrylamide Gel Electrophoresis showed considerable changes in the protein profile of cocoa seeds desiccated open at RT. The gel of fresh seeds exhibited 10 bands ranging from 11.13 KDa to 110.87 KDa. The bands of 49.47, 30.56, 20.68 and 11.13 KDa were deeply stained whereas others were feebly stained (Plate 1, A). As desiccation progressed, a considerable change in the profile of the polypeptides was

Table 14: Effect of desiccation under different storage conditions on total protein content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Protein (mg g^{-1} dw)									
Open at RT	169.32 ± 5.65	135.82 ± 3.17	125.75 ± 4.19	113.94 ± 4.90	111.26 ± 3.47	NE	NE	NE	NE	NE
Polyethylene bags at RT	169.32 ± 5.65	169.08 ± 4.51	168.54 ± 4.03	167.25 ± 3.98	165.26 ± 4.36	159.32 ± 5.41	154.35 ± 2.64	145.32 ± 4.96	137.64 ± 5.76	123.21 ± 4.51
Polyethylene bags at 4°C	169.32 ± 5.65	162.51 ± 3.68	161.04 ± 6.51	150.01 ± 3.12	143.75 ± 5.12	141.26 ± 3.45	129.31 ± 4.41	121.06 ± 3.36	117.52 ± 3.15	109.63 ± 4.29

NE: Not Estimated

Table 15: Effect of desiccation under different storage conditions on total free amino acid content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Free amino acids (µg g^{-1} dw)									
Open at RT	198.59 ± 4.85	366.22 ± 4.58	476.57 ± 8.14	673.00 ± 9.12	724.25 ± 10.47	NE	NE	NE	NE	NE
Polyethylene bags at RT	198.59 ± 4.85	240.55 ± 4.15	265.43 ± 5.62	277.11 ± 4.56	297.58 ± 6.45	425.43 ± 12.87	564.21 ± 11.35	618.55 ± 10.35	719.43 ± 15.45	744.66 ± 11.00
Polyethylene bags at 4°C	198.59 ± 4.85	220.64 ± 5.12	248.49 ± 8.02	274.67 ± 4.15	311.54 ± 6.85	474.58 ± 11.52	511.53 ± 8.30	631.47 ± 13.25	634.06 ± 12.66	637.21 ± 11.31

NE: Not Estimated

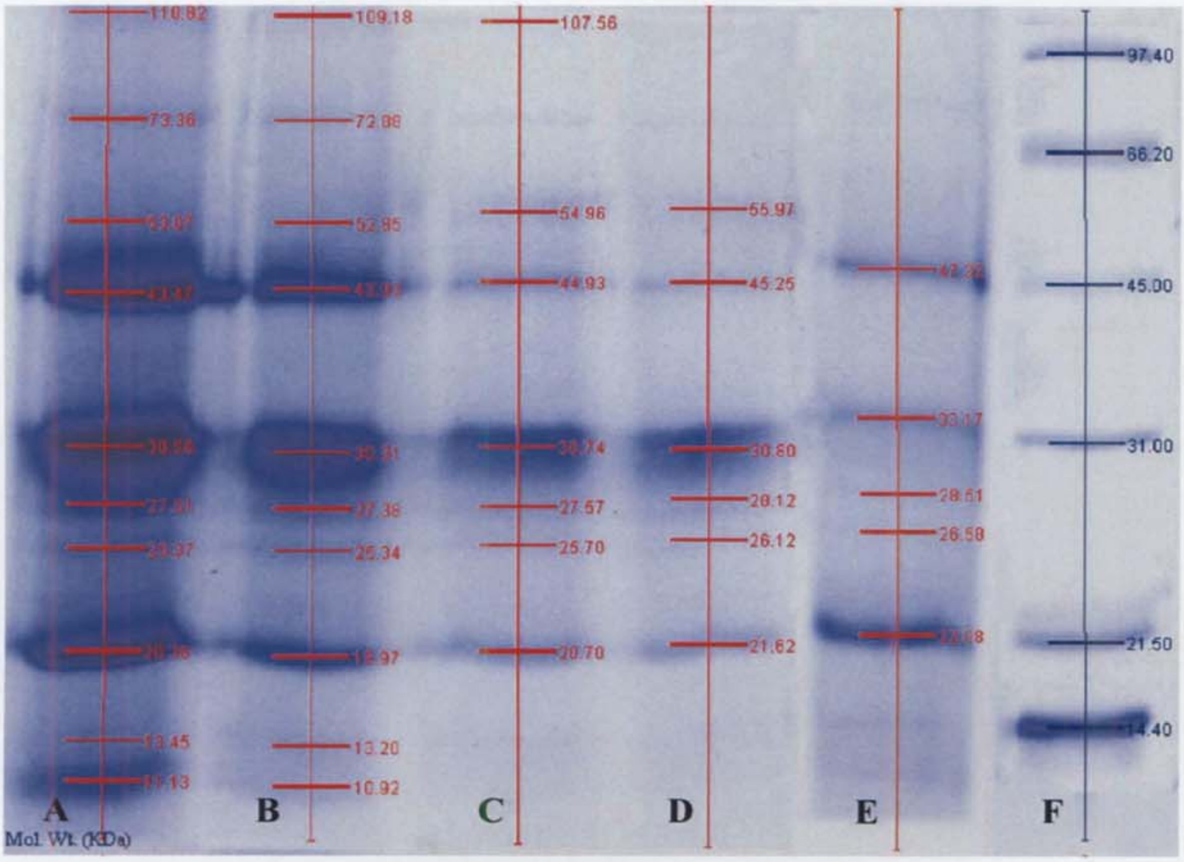

Plate 1: SDS PAGE protein profile of the seeds of *Theobroma cacao* during desiccation.

A. Fresh seeds; B. After 1 day; C. After 2 days; D. After 3 days; E. After 4 days; F. Marker proteins.

noticed. For seeds desiccated for one day, no noticeable change was found to occur in the position of the bands (Plate 1, B). Some bands were stained less intensely at this stage of desiccation. By the second day of desiccation, many bands on the gel were found disappeared. The bands around 73 KDa, 13 KDa and 11 KDa could not be detected in the gel of seeds desiccated for 2 days (Plate 1, C). On the 3rd day of desiccation, the band at 107 KDa disappeared. The other bands remained more or less the same as in the previous stages of desiccation but staining only faintly (Plate 1, D). For seeds desiccated for 4 days, only five of the original bands remained and most of them were very feeble (Plate 1, E).

1.5.11. Free Amino Acids

Free amino acid content in fresh seeds of cocoa was very low, but was found to increase during desiccation (Table 15, Fig. 4B). The increase in the free amino acid content was rapid and significant ($p<0.05$) in seeds stored open at RT during desiccation, with the maximum value being reached on the 4th day. Seeds stored in polyethylene bags at room temperature exhibited a gradual and significant increase in free amino acid content up to 4 days of storage. From the 5th day onwards, an enhanced increase in free amino acid content was noticed till the 14th day of desiccation. Seeds stored in polyethylene bags at 4°C showed a rapid increase in free amino acid content up to the 10th day of desiccation. Thereafter, the rate of increase became very low and insignificant. The highest rate of increase in free amino acid content was noticed in seeds stored open at RT.

1.5.12. Proline

Proline was found to occur in low amounts in fresh seeds of cocoa. It was found to increase during desiccation under different storage conditions (Table 16, Fig. 4C). Seeds stored open at RT showed a rapid and significant

Table 16: Effect of desiccation under different storage conditions on proline content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Proline (µg g^{-1} dw)									
Open at RT	24.62 ± 0.99	32.18 ± 0.65	41.38 ± 1.54	44.70 ± 1.03	51.96 ± 2.51	NE	NE	NE	NE	NE
Polyethylene bags at RT	24.62 ± 0.99	27.00 ± 2.27	29.65 ± 1.07	38.55 ± 1.13	46.93 ± 1.62	49.86 ± 1.94	53.79 ± 1.19	54.36 ± 2.26	55.71 ± 2.91	57.42 ± 1.51
Polyethylene bags at 4°C	24.62 ± 0.99	23.01 ± 0.85	25.95 ± 1.04	28.15 ± 0.79	31.31 ± 1.94	36.43 ± 1.46	41.57 ± 1.65	49.54 ± 2.02	49.72 ± 2.10	52.65 ± 2.49

NE-Not Estimated

Table 17: Effect of desiccation under different storage conditions on total phenolic content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Total phenolics (mg g^{-1} dw)									
Open at RT	30.81 ± 0.75	37.15 ± 1.83	41.42 ± 1.29	55.00 ± 1.39	57.47 ± 2.50	NE	NE	NE	NE	NE
Polyethylene bags at RT	30.81 ± 0.75	31.22 ± 1.16	34.68 ± 1.06	36.89 ± 1.87	40.13 ± 1.49	44.87 ± 2.02	50.23 ± 1.70	52.12 ± 2.89	48.21 ± 1.36	46.22 ± 2.11
Polyethylene bags at 4°C	30.81 ± 0.75	32.01 ± 1.02	33.88 ± 0.96	37.09 ± 1.68	43.06 ± 1.35	49.23 ± 2.01	47.21 ± 1.98	45.00 ± 2.24	43.22 ± 1.99	41.98 ± 1.24

NE-Not Estimated

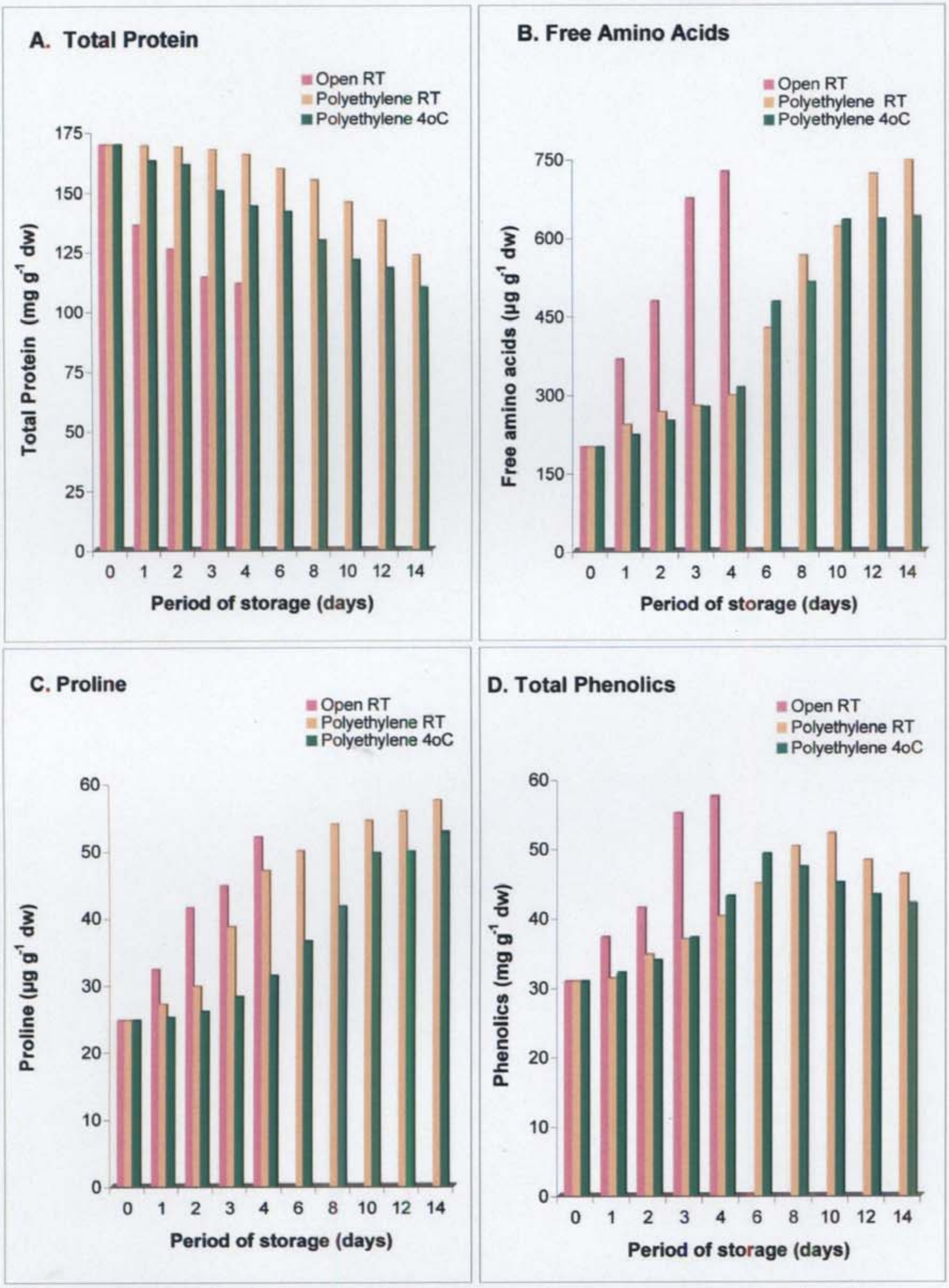

Fig 4: Effect of desiccation under different storage conditions on total protein, total free amino acid, proline and phenolic contents in the seeds of *Theobroma cacao*

increase in proline content throughout the period of desiccation and after 4 days of storage the value was found to increase to double the value of fresh seeds. Seeds stored in the other two conditions such as polyethylene at RT and polyethylene at 4°C also exhibited an increase in proline content during desiccation. Seeds stored in both the conditions exhibited a gradual increase in the initial stages of desiccation which became rapid later on. Among the three storage conditions, the seeds stored in polyethylene bags at RT exhibited maximum amount of proline during storage and desiccation.

1.5.13. Total Phenolic Content

Significant amount of phenolics was found to be present in the fresh seeds of cocoa. Generally, the total phenolic content showed an increase during the desiccation in seeds maintained in all the three storage conditions (Table 17, Fig. 4D). A rapid and significant increase in total phenolic content was observed in seeds stored open at RT ($p<0.05$). A two-fold increase was noticed in seeds desiccated for 4 days. In seeds kept in polyethylene bags at RT, the increase in the phenolic content was less rapid and the maximum value was reached on the 10th day of desiccation. The values declined thereafter. A similar pattern of change in the phenolic content was observed in seeds stored in polyethylene bags at 4°C. The maximum phenolic content was reached on the 6th day of storage and the value was found to decline in the subsequent stages of storage.

1.5.14. Total Lipid Content

Total lipid content in fresh seeds of cocoa was found to be about 43% of the dry weight (Table 18, Fig. 5). Seeds stored open at RT showed gradual increase in the lipid content up to 2 days of desiccation and a decrease subsequently. A similar trend in total lipid content was found in seeds stored in polyethylene bags at RT and at 4°C also. In seeds stored in polyethylene

Table 18: Effect of desiccation under different storage conditions on lipid content in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Lipids (mg g^{-1} dw)									
Open at RT	434 ± 9.26	471 ± 8.21	508 ± 6.23	516 ± 8.55	497 ± 11.23	NE	NE	NE	NE	NE
Polyethylene bags at RT	434 ± 9.26	458 ± 7.51	476 ± 6.53	511 ± 5.24	517 ± 8.22	521 ± 7.63	516 ± 5.14	504 ± 7.23	491 ± 9.55	482 ± 5.11
Polyethylene bags at 4°C	434 ± 9.26	466 ± 8.33	485 ± 6.11	497 ± 9.20	522 ± 7.21	510 ± 8.45	484 ± 5.21	482 ± 8.23	471 ± 4.51	455 ± 6.23

NE: Not Estimated

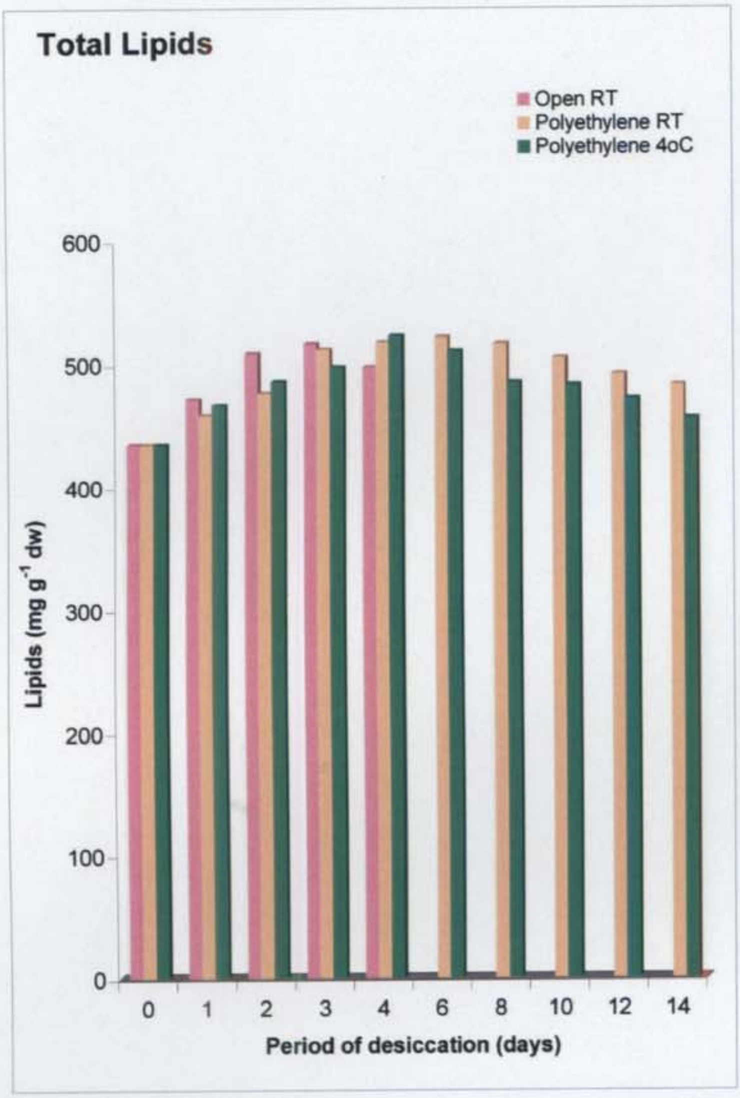

Fig. 5: Effect of desiccation under different storage conditions on total lipid content in the seeds of *Theobroma cacao*

bags at RT, the increase was gradual and insignificant, extending up to 6 days of desiccation. The value decreased afterwards. Lipid content of seeds stored in polyethylene bags at 4°C was found to increase slowly during the initial stages of storage and the maximum value was observed in seeds stored for 3 days. Thereafter a gradual decrease was found to occur.

1.5.15. Lipid Peroxidation

Lipid peroxidation is estimated in terms of the thiobarbituric acid (TBA) reactive products, mainly malondialdehyde (MDA). MDA was found to occur in cocoa seeds in very low amounts (Table, 19, Fig. 6A). In seeds kept open at RT, a rapid and significant ($p<0.05$) increase in MDA content was observed. A three-fold increase in the value was noticed on the 4th day of desiccation. In seeds stored in polyethylene bags, either at RT or at 4°C, the increase in MDA content was gradual and significant. In cocoa seeds maintained in polyethylene bags at RT, the MDA content reached the maximum value on the 10th day of storage, and the amount was approximately four times the MDA content in fresh seeds. Thereafter, a slight decrease in the value was observed. A similar trend was observed regarding the change in MDA content in seeds stored in polyethylene bags at 4°C. The maximum value was reached on the 10th day of storage. The value declined slightly and became constant for the remaining period of storage.

1.5.16. Superoxide Dismutase

Superoxide dismutase (SOD) activity in the fresh seeds of cocoa was noticed to be very high. The enzyme activity was found to decrease drastically during the process of desiccation (Table 20, Fig. 6B). In seeds kept open at RT, this decrease was highly significant ($p<0.05$) and the activity was reduced to one-tenth of the initial value after desiccation for one day. Thereafter, the SOD activity was found to decline further. The decrease in

Table 19: Effect of desiccation under different storage conditions on lipid peroxidation (MDA content) in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Malondialdehyde (µg g^{-1} dw)									
Open at RT	0.49 ± 0.02	0.64 ± 0.04	0.83 ± 0.05	1.01 ± 0.05	1.72 ± 0.06	NE	NE	NE	NE	NE
Polyethylene bags at RT	0.49 ± 0.02	0.51 ± 0.06	0.57 ± 0.04	0.63 ± 0.024	0.79 ± 0.04	0.87 ± 0.084	1.24 ± 0.124	1.91 ± 0.09	1.84 ± 0.10	1.75 ± 0.07
Polyethylene bags at 4°C	0.49 ± 0.02	0.53 ± 0.02	0.6 ± 0.03	0.74 ± 0.03	0.92 ± 0.05	1.11 ± 0.04	1.78 ± 0.04	1.84 ± 0.05	1.81 ± 0.07	1.81 ± 0.08

NE: Not Estimated

Table 20: Effect of desiccation under different storage conditions on superoxide dismutase activity in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Superoxide dismutase (Units mg^{-1} protein)									
Open at RT	115.55 ± 3.25	12.79 ± 0.52	1.9 ± 0.10	1.43 ± 0.09	1.34 ± 0.04	NE	NE	NE	NE	NE
Polyethylene bags at RT	115.55 ± 3.25	86.35 ± 2.98	62.13 ± 2.44	41.55 ± 2.22	38.52 ± 1.06	22.18 ± 1.38	8.66 ± 0.49	4.55 ± 0.18	2.94 ± 0.15	2.51 ± 0.10
Polyethylene bags at 4°C	115.55 ± 3.25	74.30 ± 3.09	33.73 ± 1.98	11.55 ± 0.75	7.54 ± 0.84	6.32 ± 0.29	2.36 ± 0.13	2.01 ± 0.09	1.85 ± 0.07	1.65 ± 0.09

NE: Not Estimated

enzyme activity in seeds stored in polyethylene bags at RT and at 4°C was not as drastic as found in the seeds stored in open at RT. Seeds stored in polyethylene bags at RT exhibited a rapid and significant decrease up to the 12th day of storage. The value on the 14th day remained more or less the same as that of the 12th day. Similar pattern of SOD activity was observed in seeds stored in polyethylene bags at 4°C also. A rapid and significant reduction in the activity was observed up to the 8th day of desiccation ($p<0.05$). The values showed a highly insignificant decline thereafter.

1.5.17. Guaiacol Peroxidase

The activity of guaiacol peroxidase showed a sharp decline in seeds stored under different conditions (Table 21, Fig. 6C). The decrease in peroxidase activity was very rapid in seeds stored open at RT. Even one day of desiccation reduced the peroxidase activity to nearly one-fourth of that of fresh seeds. The value remained low for the remaining period. Seeds stored in polyethylene bags at RT showed a gradual reduction in the activity of the enzyme throughout the period of desiccation while those stored in polyethylene bags at 4°C showed only a slight decline in the activity after desiccation for one day. But in subsequent stages of desiccation the value declined rapidly and significantly ($p<0.05$). In seeds stored for more than 8 days, the peroxidase activity remained very low. Of the three different storage conditions, seeds stored in the polyethylene bags at RT exhibited a more or less uniform pattern of reduction in the activity of peroxidase.

1.5.18. Catalase

The activity of the enzyme catalase was found to increase in the fresh seeds of cocoa in the initial stages of storage (Table 22, Fig. 6D). Seeds desiccated in open at RT showed an increase in catalase activity on the first day of desiccation. The activity was found to decrease in the subsequent

Table 21: Effect of desiccation under different storage conditions on guaiacol peroxidase activity in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Guaiacol peroxidase (µmol mg^{-1} protein min^{-1})									
Open at RT	1.4114 ± 0.0471	0.4040 ± 0.0157	0.2353 ± 0.0122	0.2035 ± 0.0163	0.1515 ± 0.0068	NE	NE	NE	NE	NE
Polyethylene bags at RT	1.4114 ± 0.0471	1.2034 ± 0.0521	1.1450 ± 0.0433	0.9654 ± 0.0420	0.7828 ± 0.0301	0.7069 ± 0.0409	0.4301 ± 0.0198	0.2817 ± 0.0078	0.1921 ± 0.0086	0.1765 ± 0.0045
Polyethylene bags at 4°C	1.4114 ± 0.0471	1.1838 ± 0.0322	0.5596 ± 0.0298	0.3370 ± 0.0152	0.2777 ± 0.0142	0.2505 ± 0.0101	0.1995 ± 0.018	0.1756 ± 0.0084	0.1652 ± 0.0052	0.1432 ± 0.0065

NE: Not estimated

Table 22: Effect of desiccation under different storage conditions on catalase activity in the seeds of *Theobroma cacao*

Storage condition	Period of storage (days)									
	0	1	2	3	4	6	8	10	12	14
	Catalase (µmol mg^{-1} protein min^{-1})									
Open at RT	0.2641 ± 0.0156	0.2804 ± 0.0142	0.1864 ± 0.0190	0.1073 ± 0.0053	0.0923 ± 0.0062	NE	NE	NE	NE	NE
Polyethylene bags at RT	0.2641 ± 0.0156	0.2709 ± 0.0127	0.2835 ± 0.0101	0.2400 ± 0.0122	0.2011 ± 0.0097	0.1809 ± 0.0104	0.1217 ± 0.0085	0.1001 ± 0.0071	0.0852 ± 0.0083	0.0821 ± 0.0074
Polyethylene bags at 4°C	0.2641 ± 0.0156	0.2698 ± 0.0161	0.2754 ± 0.0156	0.2265 ± 0.0130	0.1654 ± 0.0126	0.1422 ± 0.0092	0.1011 ± 0.0071	0.0951 ± 0.0064	0.0942 ± 0.0072	0.0891 ± 0.0051

NE: Not estimated

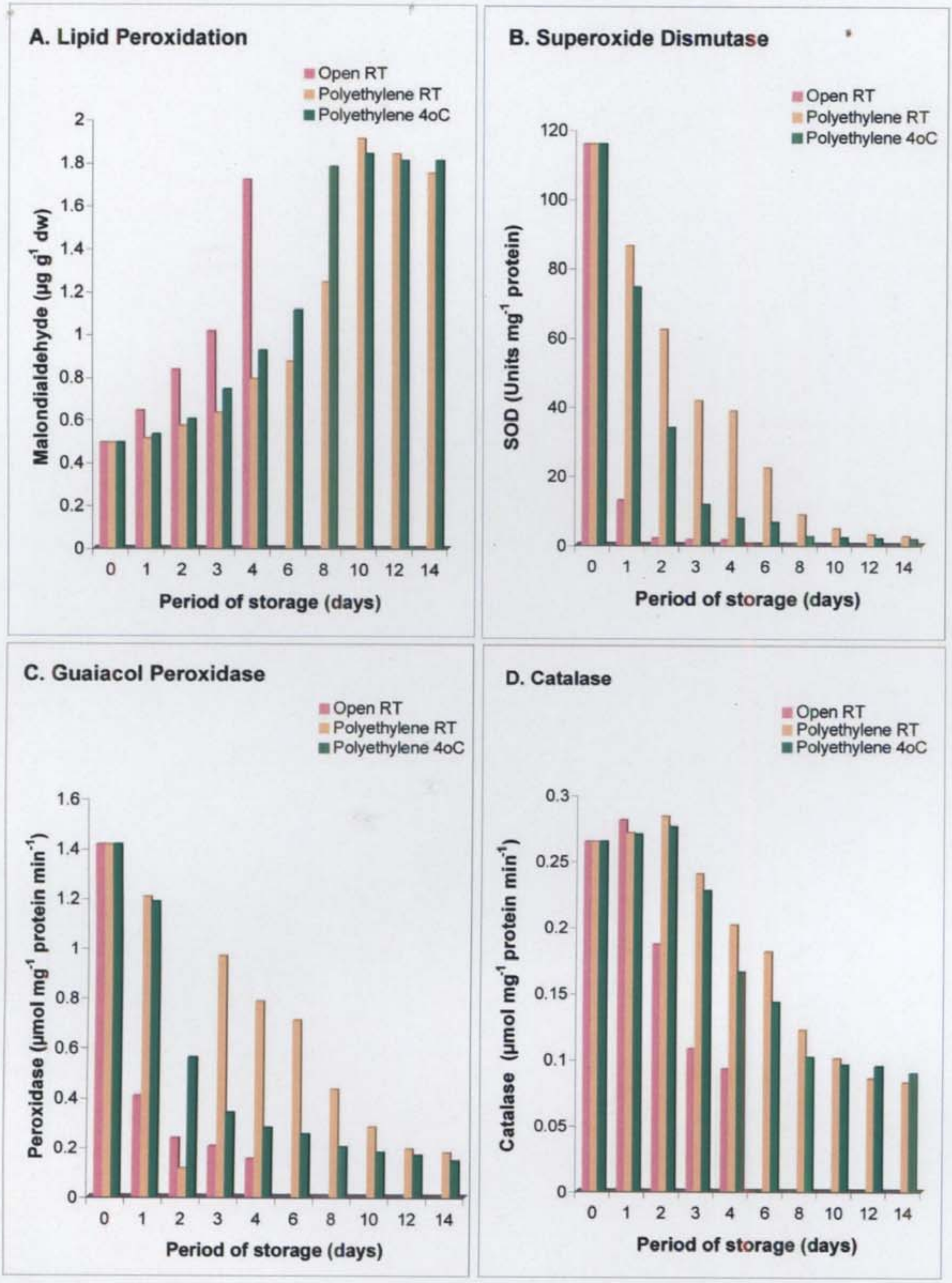

Fig 6: Effect of desiccation under different storage conditions on lipid peroxidation and on the activities of superoxide dismutase, guaiacol peroxidase and catalase in *Theobroma cacao*

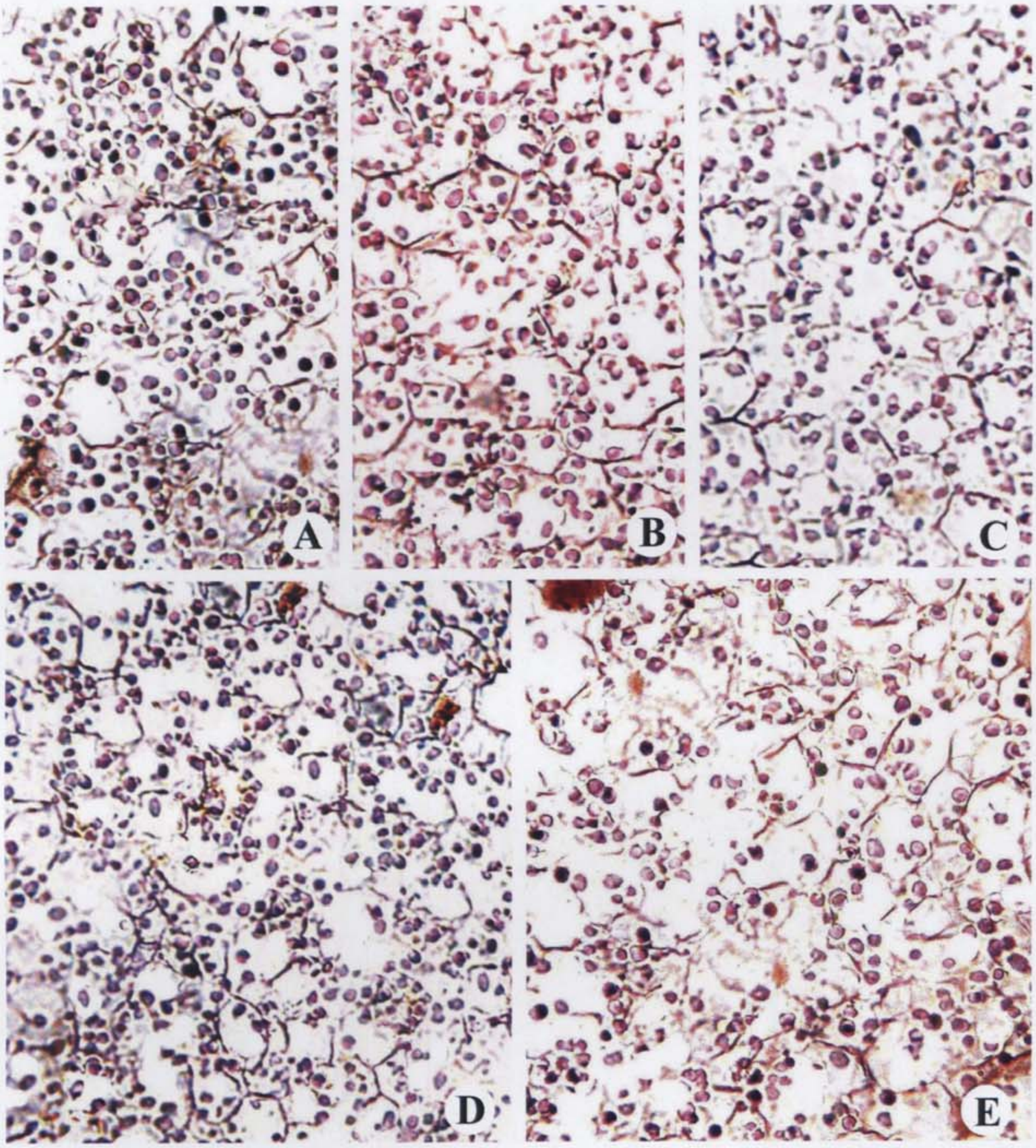

Plate 2: Effect of desiccation on the localization of starch in the seeds of *Theobroma cacao*.
A. Fresh seeds; B. After 1 day; C. After 2 days; D. After 3 days; E. After 4 days.

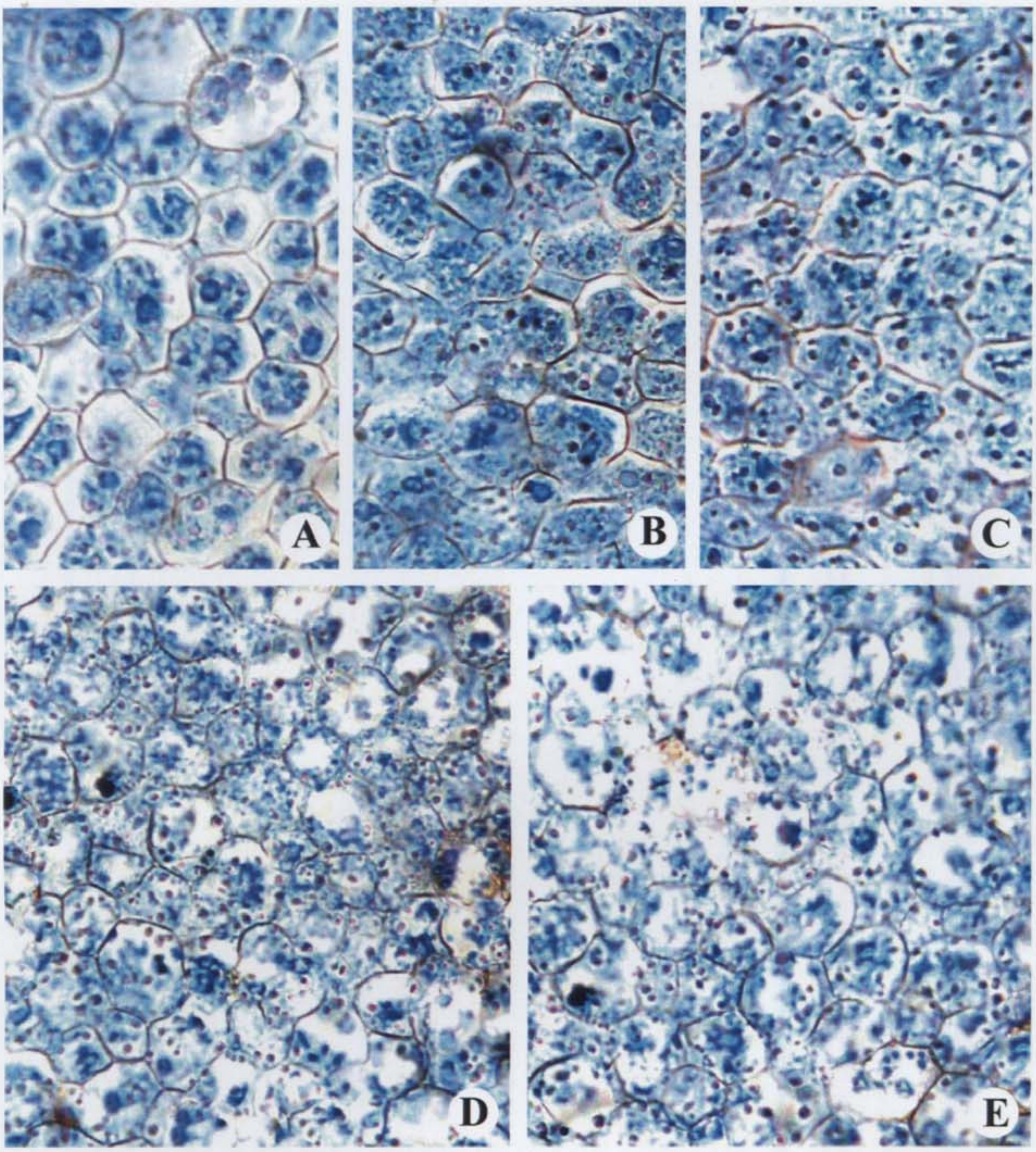

Plate 3: Effect of desiccation on the localization of protein in the seeds of *Theobroma cacao.*
A. Fresh seeds; B. After 1 day; C. After 2 days; D. After 3 days; E. After 4 days.

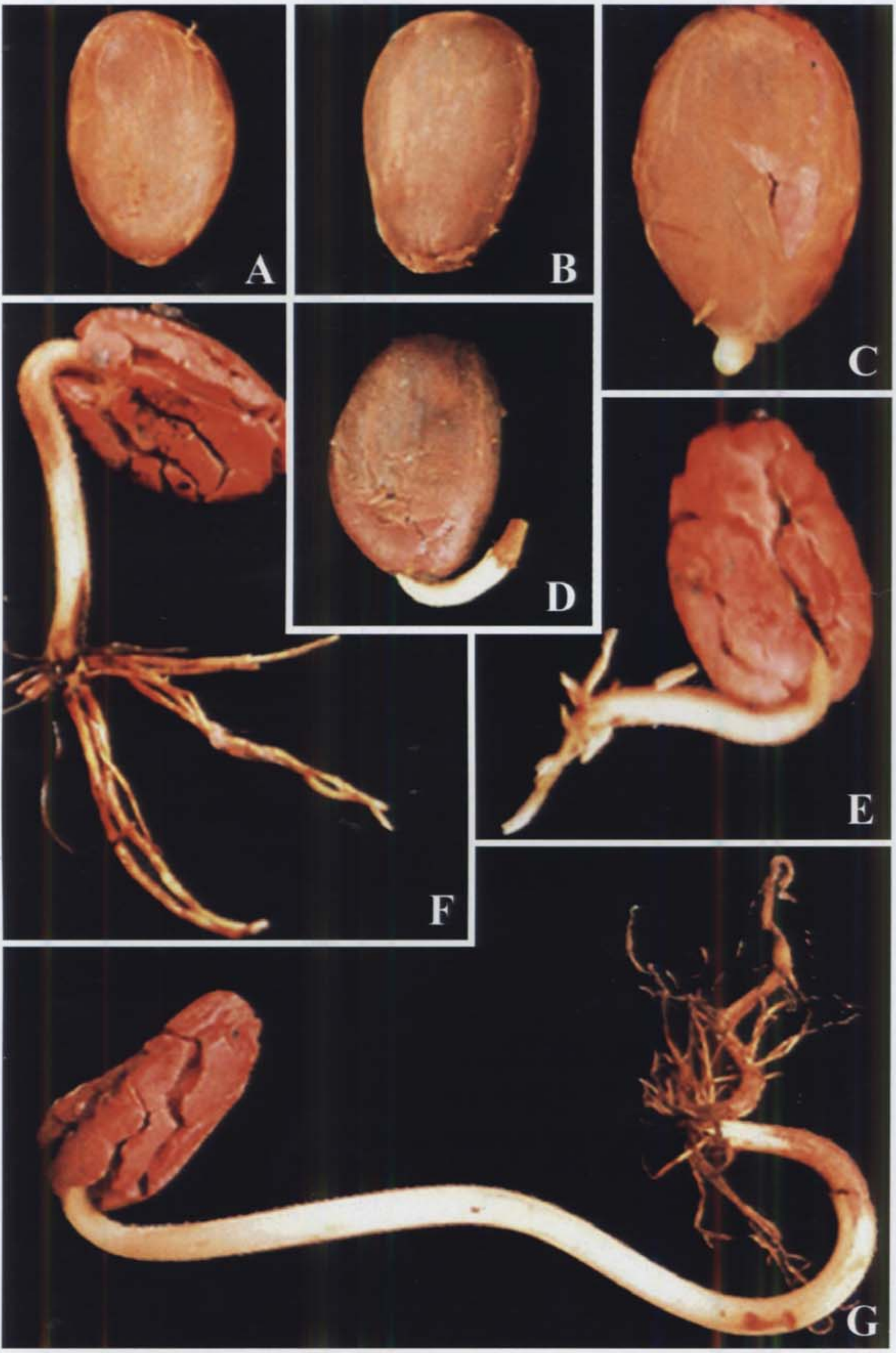

Plate 4: Stages of germination in the seeds of *Theobroma cacao*.
A. Fresh seeds; B. After 1 day; C. After 2 days; D. After 7 days; E. After 12 days; F. After 17 days; G. After 22 days.

stages. Seeds stored in polyethylene bags at RT showed a gradual increase up to the 2nd day. The activity was found to decrease rapidly from the 3rd day of storage up to the 8th day and more gradually thereafter. A similar trend was observed in seeds maintained in polyethylene bags at 4°C, the maximum catalase activity being observed in seeds desiccated for 2 days. The values showed a decline in the later stages.

1.6. Histochemical Studies

1.6.1. Localization of Starch

Transverse sections of seeds of *Theobroma cacao* stained with Periodic acid–Schiff's reagent showed deeply stained spherical to ovoid bodies within the cell. The cell wall was moderately stained and remained intact up to the second day of desiccation. The fresh seeds were found to have plenty of deeply stained starch grains filling the lumen of the cell (Plate 2, Fig. A). The distribution of the grains was found to be more or less uniform through out the cotyledonary tissue. The starch grains were rather small. During desiccation, the number of starch grains was found to decline gradually in the cells of the cotyledon. In seeds desiccated for one day, no considerable change in the number or size of the starch grains was found to occur. (Plate 2, Fig. B). From the second day onwards, a gradual decrease in the number of starch grains was observed (Plate 2, Fig. C to E). The starch grains in the centre of the lumen of the cells were found to disappear in the beginning. The decline in the starch grains was more pronounced in the cotyledonary tissues of seeds desiccated for 3 days. The number of starch grains was found to be reduced to about half that of fresh seeds. The cell walls of the cotyledonary tissues were found ruptured in seeds desiccated for more than 3 days (Plate 2, Fig. D and E).

1.6.2. Localization of Proteins

Sections of cotyledons of cocoa seeds stained with mercuric bromophenol blue showed the presence of deeply stained protein mass within the cells. In the cotyledons of fresh seeds, the protein mass was found to fill the cell cavity (Plate 3, Fig. A). A gradual decline in the protein content was observed as desiccation progressed. The intensity of the blue colour and the prominence of the granules decreased with successive stages of desiccation (Plate 3, Fig. B-E). The protein granules in the cells of the cotyledon of seeds desiccated for 4 days showed the lowest intensity of blue colour and the smallest size.

2. Germination Studies

The seeds kept in the dark chamber for germination showed a slight swelling after 1 day due to imbibition (Plate 4, Fig. B). Radicle was found to emerge after two days (Plate 4, Fig. C). The testa was found to be detached from the seed from the 7th day of germination onwards. The seedlings elongated in the subsequent stages of germination (Plate 4, Fig D-G). After 22 days, the seedlings wilted and withered away.

2.1. Change in Dry Weight Percentage

Change in the dry weight percentage of both the cotyledon and embryonic axis of the seeds of *Theobroma cacao* through successive stages of germination was estimated (Table 23, Fig. 7A). The cotyledons of the fresh seeds were found to have a higher dry weight percentage than that of the axis. In the cotyledons, the dry weight percentage was found to decrease steadily and significantly from the initial stages to the final stages of germination. On the 22nd day of germination, the dry weight percentage was found reduced approximately to one half of the initial dry weight. The embryonic axis also

Table 23: Change in dry weight percentage in the seeds of *Theobroma cacao* during germination

Sample	Period of germination (days)						
	0	1	2	7	12	17	22
	Dry weight percentage						
Cotyledon	65.46 ± 3.15	63.98 ± 2.54	60.66 ± 2.74	53.56 ± 2.01	42.25 ± 1.37	38.77 ± 1.41	36.03 ± 1.98
Axis	27.97 ± 1.01	23.79 ± 1.15	20.485 ± 0.99	11.4 ± 0.70	9.24 ± 0.31	10.49 ± 0.44	14.69 ± 0.51

Table 24: Change in soluble sugar content in the seeds of *Theobroma cacao* during germination

Sample	Period of germination (days)						
	0	1	2	7	12	17	22
	Total soluble sugar (mg g^{-1} dw)						
Cotyledon	70.19 ± 1.71	61.24 ± 2.25	57.19 ± 1.60	30.73 ± 1.54	60.59 ± 3.58	64.63 ± 2.24	72.53 ± 2.85
Axis	94.65 ± 3.24	90.32 ± 4.06	92.36 ± 2.04	101.36 ± 3.22	116.31 ± 4.85	121.02 ± 3.14	130.25 ± 3.00

showed a decrease in the dry weight which was reduced to one-third of the control value by the 12th day of germination. From 17th day onwards, the dry weight percentage of the axis was found to increase gradually and significantly ($p<0.05$).

2.2. Biochemical Studies

2.2.1. Change in Soluble Sugar Content

In cocoa seeds, the total sugar content showed an initial decrease and a subsequent increase in the cotyledon as well as in the axis during germination and seedling development (Table 24, Fig. 7B). In the cotyledon, the soluble sugar content was found to decline up to the 7th day of germination. From the 12th day onwards, a rapid and significant increase was noticed till the final stage. In the axial tissue, there was a slight decline in the amount of soluble sugar on the 1st day. From 2nd day onwards, the soluble sugar content increased steadily and significantly till it reached the maximum value on the 22nd day of germination.

2.2.2. Change in Starch Content

The total starch content in the cotyledon and the embryonic axis of cocoa seeds showed a very high degree of depletion during germination (Table 25, Fig. 7C). The starch content in the cotyledon was found to decline rapidly and significantly ($p<0.05$) through out the period of germination. In the final stage of germination, the cotyledon was found to have only one-twentieth of the starch content present in the cotyledons of fresh cocoa seeds. In the embryonic axis, the starch content was showing only a marginal decline on the 1st day of germination. From the 2nd day onwards, the decrease became highly noticeable up to the 12th day. A gradual increase in the starch content was observed thereafter.

Table 25: Change in starch content in the seeds of *Theobroma cacao* during germination

Sample	Period of germination (days)						
	0	1	2	7	12	17	22
	Starch (mg g^{-1} dw)						
Cotyledon	88.35 ± 3.73	80.24 ± 2.02	71.38 ± 3.03	53.14 ± 2.74	21.77 ± 1.09	6.11 ± 0.35	4.45 ± 0.21
Axis	166.01 ± 5.14	162.07 ± 4.90	123.72 ± 4.24	73.25 ± 2.50	48.85 ± 2.13	50.23 ± 2.20	55.70 ± 1.81

Table 26: Change in the total lipid content in the seeds of *Theobroma cacao* during germination

Sample	Period of germination (days)						
	0	1	2	7	12	17	22
	Total lipids (mg g^{-1} dw)						
Cotyledon	462 ± 11.21	444 ± 12.42	437 ± 11.81	392 ± 9.58	314 ± 7.56	280 ± 4.34	244 ± 4.58
Axis	202 ± 5.32	191 ± 4.21	177 ± 5.16	162 ± 3.22	145 ± 2.15	121 ± 2.56	112 ± 2.65

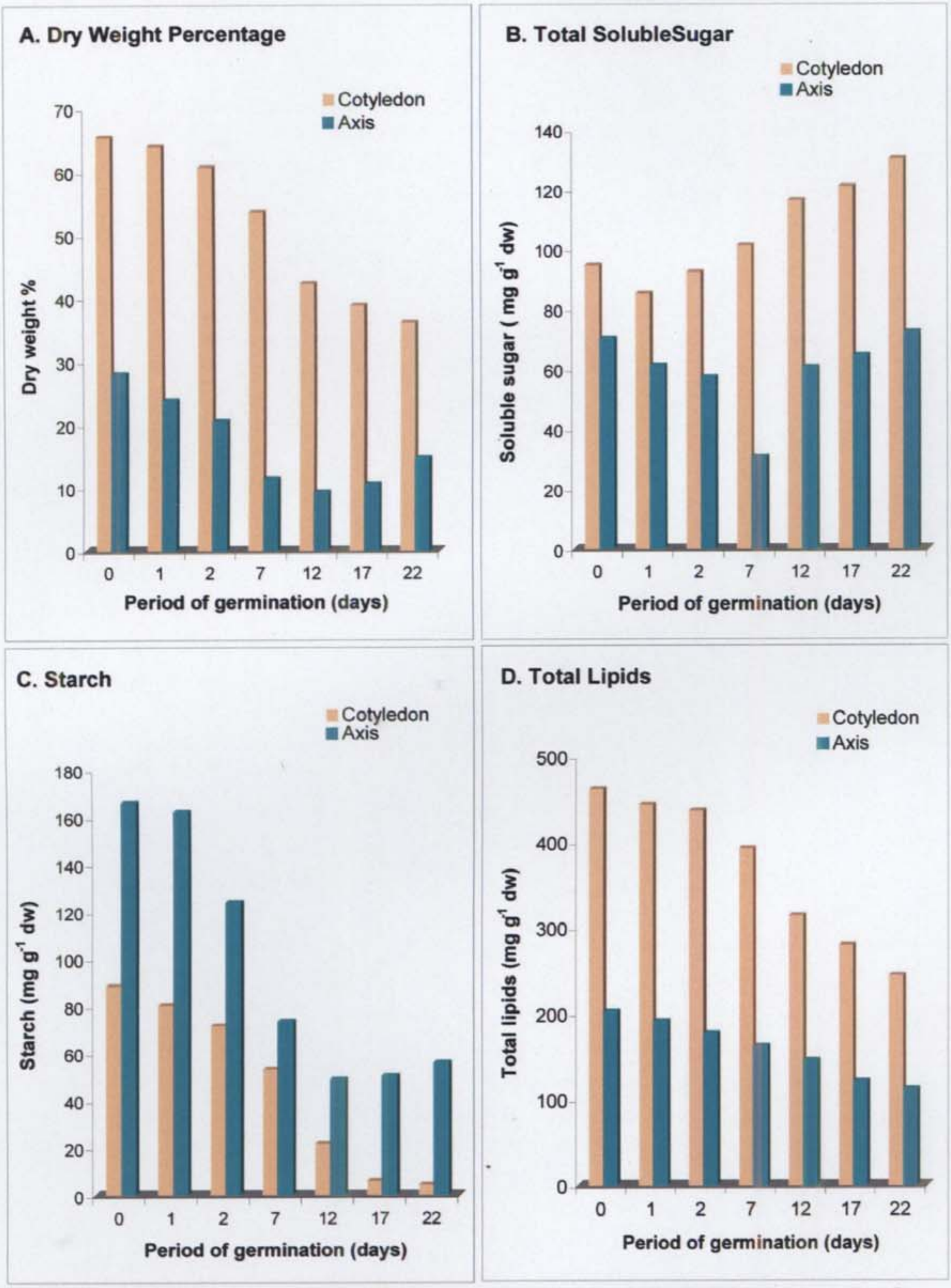

Fig 7: Change in the dry weight percentage and total soluble sugar, starch and total lipid contents in the seeds of *Theobroma cacao* during germination

2.2.3. Change in Lipid Content

A gradual and progressive reduction in the lipid content was found to occur in the cotyledons of cocoa seeds throughout the process of germination and seedling development (Table 26, Fig. 7D). During the initial stages of germination, the rate of reduction was slow but became rapid and significant from the 7th day of germination onwards. In the axis, the amount of lipid was considerably lower than that of the cotyledons and was found to decline gradually in the initial stages of germination. Later, the rate of lipid depletion was found enhanced.

2.2.4. Change in Protein Content

Both the cotyledonary and axial tissues in *Theobroma cacao* showed a decrease in the total protein content during the process of germination and seedling development (Table 27, Fig. 8A). The decrease in the total protein content was gradual in the cotyledonary tissues in the initial stages of germination. From the 2nd day of germination onwards, a rapid decrease in the value was observed. On the final stage of germination, the total protein content was found to be reduced to approximately one-third of the initial value. The axial tissue was found to have a higher amount of protein than the cotyledons on a dry weight basis. A steep decline in the amount of protein present was noticed in the axis. The magnitude of reduction in total protein in the axial tissue was far greater than that of the cotyledons, the value being reduced to about 50 % by the 12th day of germination. An increase in the total protein content was observed in the axis in the subsequent stages.

2.2.5. Change in Free Amino Acid Content

The free amino acid content in the embryonic axis of cocoa seeds was found to be greater on a per gram dry weight basis. In the cotyledons, the free amino acid content showed an initial decrease in amount up to the second day of germination and a rapid and significant ($p<0.05$) increase thereafter (Table

Table 27: Change in the total protein content in the seeds of *Theobroma cacao* during germination

Sample	Period of germination (days)						
	0	1	2	7	12	17	22
	Protein (mg g^{-1} dw)						
Cotyledon	184.30 ± 4.92	174.98 ± 5.44	156.28 ± 3.08	131.19 ± 4.69	106.79 ± 3.89	72.64 ± 2.26	58.83 ± 2.46
Axis	425.94 ± 7.98	379.51 ± 8.24	292.97 ± 10.30	253.12 ± 8.15	205.25 ± 4.45	227.37 ± 5.84	264.52 ± 6.29

Table 28: Change in the total free amino acid content in the seeds of *Theobroma cacao* during germination

Sample	Period of germination (days)						
	0	1	2	7	12	17	22
	Free amino acids (mg g^{-1} dw)						
Cotyledon	0.36 ± 0.012	0.27 ± 0.008	0.24 ± 0.011	0.28 ± 0.011	0.39 ± 0.10	0.52 ± 0.018	0.74 ± 0.043
Axis	0.96 ± 0.046	1.06 ± 0.065	1.17 ± 0.009	1.85 ± 0.072	2.27 ± 0.13	2.54 ± 0.096	3.03 ± 0.140

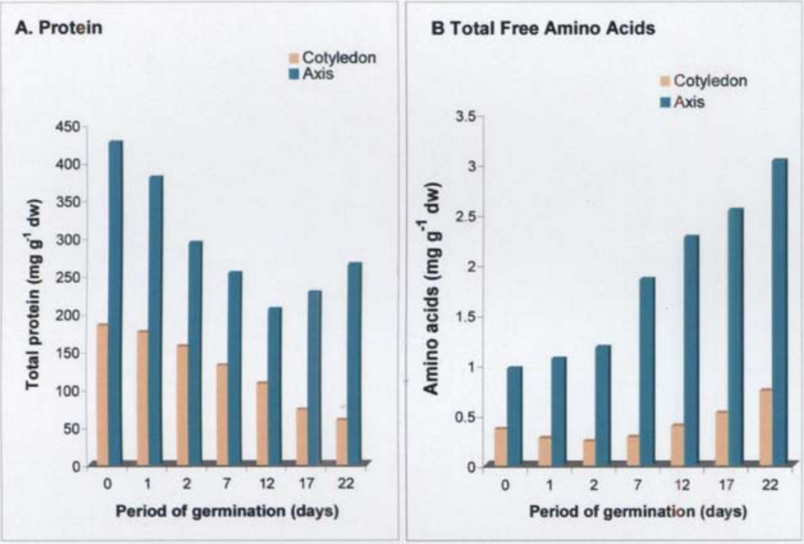

Fig. 8: Change in the total protein and free amino acid contents in the seeds of *Theobroma cacao* during germination

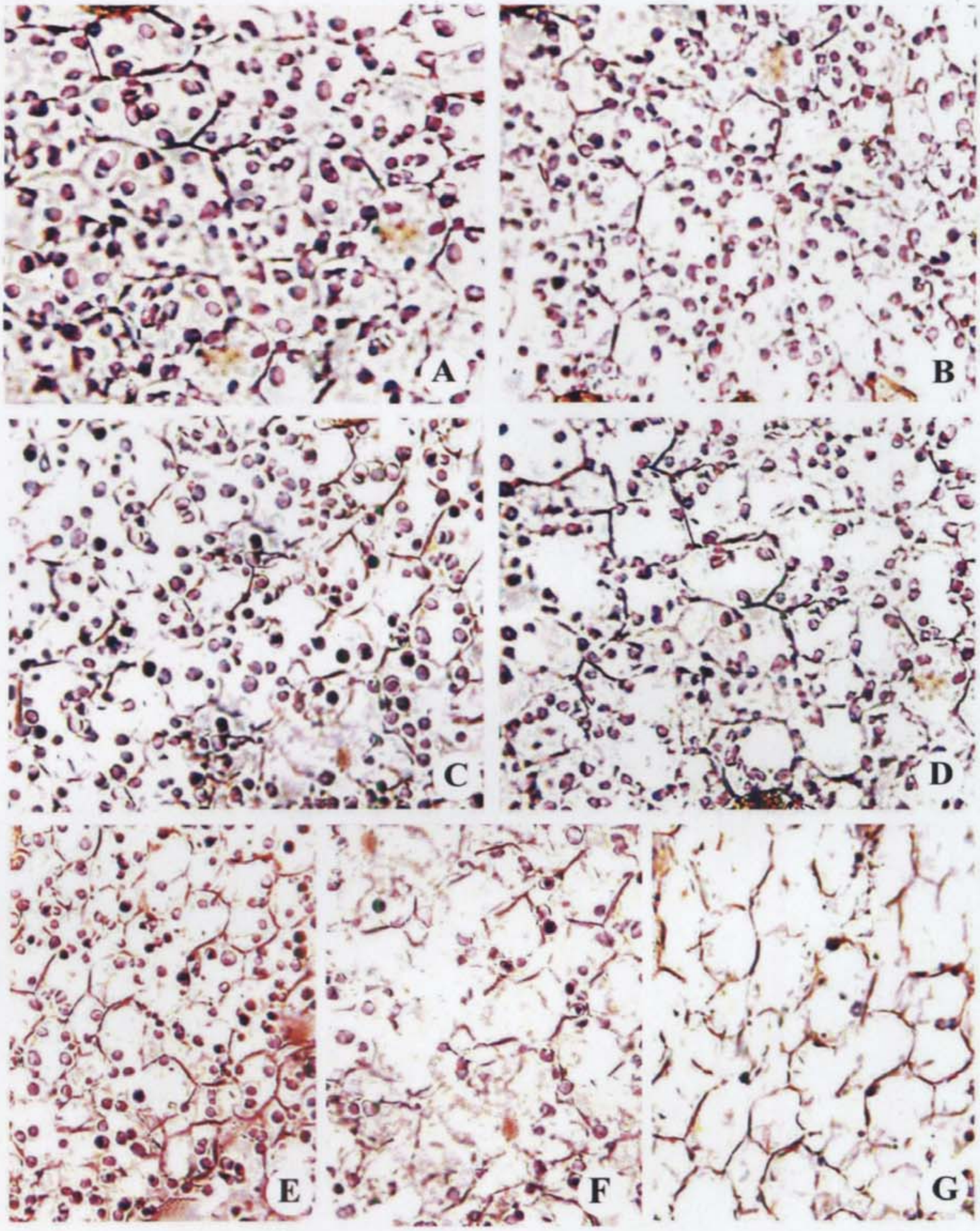

Plate 5: Localization of starch in different stages of germination in the seeds of of *Theobroma cacao*.

A. Fresh seeds; B. After 1 day; C. After 2 days; D. After 7 days; E. After 12 days; F. After 17 days; G. After 22 days.

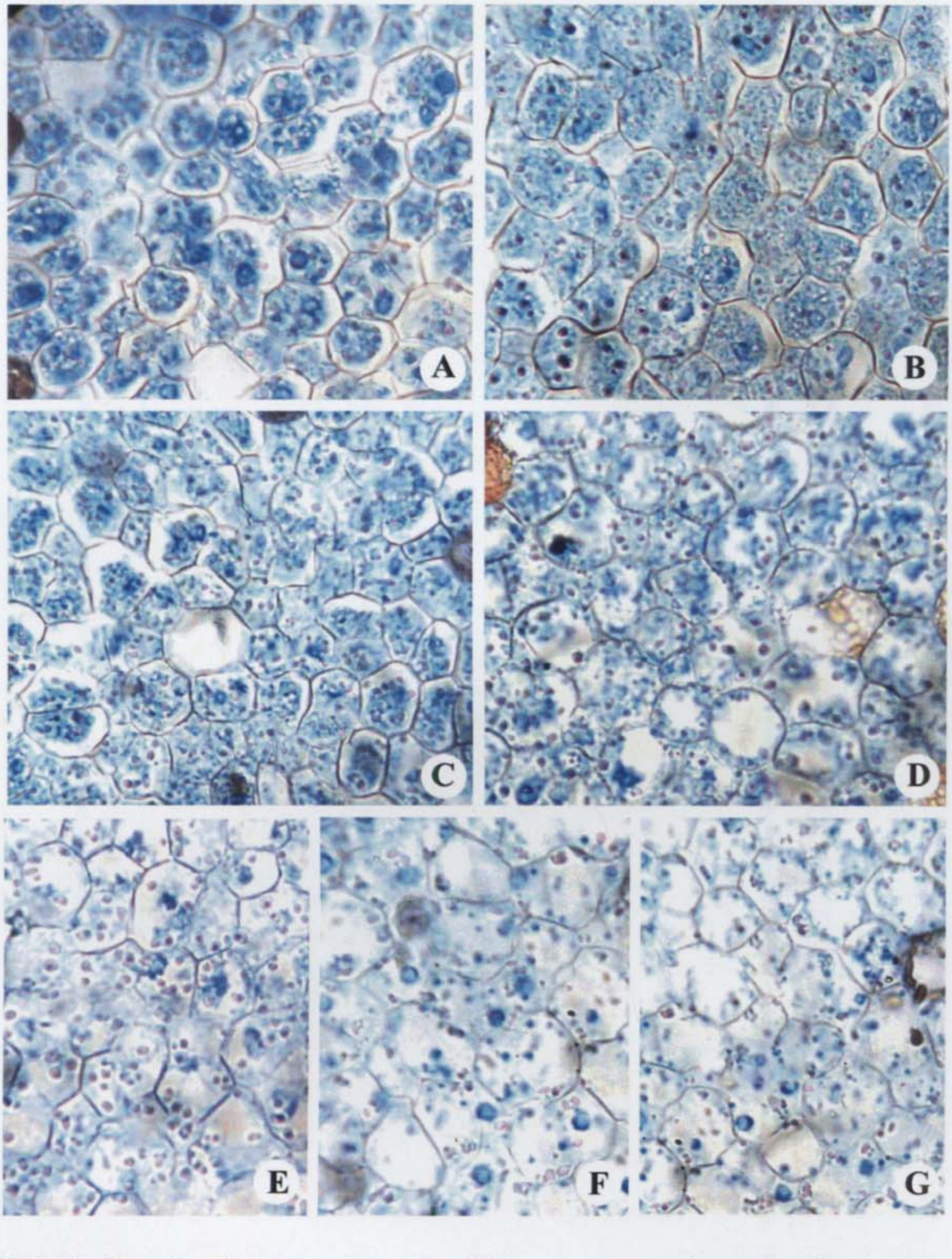

Plate 6 : Localization of proteins in different stages of germination in the seeds of *Theobroma cacao*.
A. Fresh seeds; B. After 1 day; C. After 2 days; D. After 7 days; E. After 12 days; F. After 17 days; G. After 22 days.

28, Fig. 8B). In the axis, the amount of free amino acids showed a progressive and steady increase from the control seeds to the final stage of germination. The increase was slight and insignificant in the initial stages, but became rapid and significant later on.

2.3. Histochemical Studies

2.3.1. Localization of Starch

A rapid decrease in the deeply stained starch granules was observed in the cotyledons of cocoa seeds during germination. A large number of starch granules were found in the cotyledons of fresh seeds, filling the lumen of the cells (Plate 5, Fig. A). A slight decrease in the number of starch grains was observed on the first day of germination (Plate 5, Fig. B). A gradual but noticeable reduction in the number of starch grains was found to occur in the subsequent stages of germination. The starch grains towards the centre of the lumen were the first to disappear (Plate 5, Fig. C to E). On the 17th day of germination, most of the starch grains were found to have disappeared. On the 22nd day of germination, the cells had an apparently empty appearance. The cell walls appeared to be collapsed and broken (Plate 5, Fig. F and G).

2.3.2. Localization of Proteins

The cotyledonary tissues of cocoa seeds stained with mercuric bromophenol blue showed a gradual decrease in the density of the protein granules as well as in the intensity of their colouration. The cotyledonary cells of fresh seeds were filled with deeply stained dense granules of protein. (Plate 6, Fig A). The protein content showed a steep decline as germination progressed. The decrease was slight, both in density and in colour intensity, after one day of germination (Plate 6, Fig B). The decrease became more noticeable in the subsequent stages (Plate 6, Fig. C-F). On the 22nd day of germination, the cells were found to have only very sparse amount of proteins (Plate 6, Fig. G).

Desiccation and Germination Studies of Cocoa (*Theobroma cacao* L.) Seeds - A Physiological and Biochemical Approach

Thesis submitted to the University of Calicut in partial
fulfillment of the requirement for the degree of
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By
Abis V. Cherussery

Division of Plant Physiology and Biochemistry
Department of Botany
University of Calicut
Kerala

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DISCUSSION

Recalcitrant seeds are generally heavy due to their large size and high moisture content, which may range from 30% to 70% of the fresh weight (King and Roberts, 1979; Chin *et al.,* 1984; von Teichman and van Wyk, 1994). The large size may benefit the recalcitrant seeds as they lose water at a slower rate. The seeds of cocoa are rather large and fleshy, having an average fresh weight of 1.61±0.12 g. The embryonic axis, however, is very small with an average fresh weight of 0.022±0.001 g, occupying only about 1.35% of the seed mass.

King and Roberts (1979) and Chin *et al.* (1984) suggested that, under certain circumstances large seed size may actually contribute to recalcitrant behaviour as such seeds face more internal translocation problems than smaller seeds. According to Chin (1988) and Pritchard *et al.* (1995), the rate of moisture loss from the embryonic tissues is highly dependent on embryo size and drying conditions, with larger embryos losing moisture content at a slower rate. Daws *et al.* (2004) identified a significant linear relationship between whole-seed water content and seed mass during the drying process implying that the larger seeds survive, not because of great relative desiccation tolerance, but as a result of taking longer time to desiccate. However, in cocoa, even though the seeds are rather large with high initial moisture content (37%), the rate of dehydration is rapid under natural conditions and hence the seeds remain viable only for a short period of 2 days. Thus, it can be concluded that the size of the seed is not a critical factor in the loss of moisture content and hence in maintaining the viability of cocoa seeds.

Fresh seeds of cocoa possess a moisture content of 36.98±1.02%. The high moisture content is a characteristic feature of the recalcitrant seeds (Berjak *et al.,* 1984; Fu *et al.,* 1990; Farrant *et al.,* 1993b). The fresh seeds show 100% germination, which decreases significantly with a decrease in moisture content (Table 1-2, Fig. 1A-B).

In cocoa seeds stored open at room temperature, a decrease in moisture content from approximately 37% to 20% results in 70% decline in viability on the 3rd day of desiccation. Further reduction in moisture content results in total loss of viability. This phenomenon is characteristic of recalcitrant seeds which are shed at high moisture content and in which even small measures of subsequent dehydration lead to loss of viability (Berjak *et al.,* 1994). Corbineau and Côme (1988) observed in *Shorea roxburghii* and *Hopea odorata* that the seeds lose total viability when their moisture content fall to about 17%. Fu *et al.* (1990) made a similar observation in *Litchi chinensis* in which viability was completely lost after four days of drying to a moisture content of 21-22%. Anilkumar *et al.* (2000) found in *Syzygium aromaticum* that fresh seeds with 48.9% moisture content registered 100% germination, and a reduction of 3% moisture content brought down germination to 6%. Many identical instances have been reported in recalcitrant seeds in which rapid loss of moisture content results in a significant reduction in germination. Dickie *et al.* (1991) observed a rapid loss of moisture content in the embryos of *Acer pseudoplatanus, which* was reduced from 55-60% to less than 10% in one day with severe reduction in seed viability. The desiccation of seeds of *Vitellaria paradoxa* from the moisture content of 30% to 20-26% resulted in 50% viability loss (Pritchard *et al*., 1997; Danthu *et al*., 2000).

The loss of moisture content of the seeds stored in polyethylene bags at room temperature is gradual and insignificant and is reduced to about 32% on the 6th day of desiccation. The seeds show 100% germination up to this level of moisture content (Table 1). According to Akoroda (1986), seeds of *Telfairia* stored in waterproof bags maintain a significantly higher viability than the air-dried seeds. Hor *et al.* (1984) found that, of the cocoa seeds stored in polyethylene bags at different moisture and temperature regimes, those stored at 30°C or 22°C retain viability for a longer period than any other storage conditions. Storage of *Hancornia speciosa* seeds in polyethylene bags maintained their moisture content at high levels and high percentage of germination was observed after 9 weeks irrespective of the storage temperature (Oliveira and Valio, 1992). Similarly, cocoa seeds stored in polyethylene bags at room temperature show the maintenance of high moisture content throughout the period of storage and consequently exhibit extended period of seed viability compared to those in other storage conditions (Table 1).

The loss of moisture content and hence, the loss of viability in cocoa seeds stored in polyethylene bags at 4°C is more pronounced and rapid than those stored in polyethylene bags at room temperature (Table 1-2, Fig. 1: A-B). Corbineau and Côme (1988) found a lower percentage of germination in the recalcitrant seeds of species like *Hopea odorata* and *Shorea roxburghii* when stored at the chilling temperature of 5°C. Seed germination is maintained cent percent up to 3 days, after which it decreased rapidly and total loss of viability is found to occur after 6 days of storage. The lowering of seed viability in seeds stored in polyethylene bags at 4°C may be due to an additional stress imposed by the low temperature, as suggested by Vertucci and Roos (1993). They suggested that in coffee seeds, lowering the temperature caused a reduction in the chemical potential of the water, which in turn may induce an additional stress. The reduced viability of cocoa seeds stored in polyethylene bags at 4°C is also attributed to the loss of moisture content up to 4 days.

It is a common behaviour of many recalcitrant seeds that they lose viability quickly when their moisture content fall below some critical level (King and Roberts; 1979; Becwar *et al.*; 1982; Berjak *et al.*, 1984; Akoroda, 1986; Corbineau and Côme, 1988, Fu *et al.*, 1990; Chandel *et al.*, 1995). The critical moisture content of the seeds has been defined variously as the water content corresponding to the onset of viability loss (Pritchard *et al.*,1995; Sun and Liang, 2001), 50% viability loss (Dussert *et al.,* 1999) and complete viability loss (Bonner, 1996). The present author has chosen the moisture content at which complete loss of viability occurs as the critical moisture content as proposed by Bonner (1996). In cocoa, the seeds are shed at a moisture content of 36.98%. The seeds show cent percent germination only up to a moisture level of 29-32% during desiccation irrespective of the period of storage in all the storage conditions employed in the present study. The percentage of seed germination is decreased rapidly as further reduction in moisture content occurred. Therefore, cocoa seeds come under the category of highly recalcitrant seeds. Total loss of viability is observed when moisture content was reduced to less than 21%, which can be considered as the critical moisture content of cocoa seeds. This observation is in agreement with the views of Chin *et al.* (1984) who reported 20% moisture content as the critical moisture content in the seeds of cocoa.

A linear relationship between germination percentage and moisture content is evident in cocoa seeds in all storage conditions. After the loss of viability, such a correlation is not found to exist because in nonviable seeds moisture content gets continuously reduced in all storage conditions. Direct relationship between moisture content and viability has been reported in many recalcitrant seeds such as *Aesculus hypocastanum* (Tompsett and Pritchard, 1993*), Machilus thumbergii* (Lin and Chen, 1995), *Quercus nigra* (Bonner, 1996), *Shorea robusta* (Chaitanya *et al.,* 2000*) Avicennia marina* (Greggains *et al.,* 2001) and *Garcinia* (Malik *et al.,* 2005).

The desiccation tolerance of cocoa seeds is observed to vary with the storage conditions and the rate of dehydration. In seeds stored at room temperature, germination, even though at a reduced percentage continued up to a moisture content of 20.48% whereas seeds stored in polyethylene bags at room temperature and at 4°C failed to germinate beyond 23.21% and 22.01% respectively.

According to Farrant *et al.* (1985), Pammenter *et al.* (1991) and Pammenter and Berjak (1999) rapidly dried recalcitrant seeds can tolerate greater moisture loss because water content is reduced to levels that inhibit both metabolic and degenerative processes before they can occur. Slowly dried material will spend a long time at the intermediate water contents where damages from aqueous based processes can accumulate (Farrant *et al.,* 1993a; Pammenter and Berjak, 2000; Peran *et al.,* 2004). Thus, the faster the drying, the lesser the damage that accumulates and the lower the water content that is tolerated. During slow drying at water contents intermediate between full hydration and the lower limit of survival, deleterious reactions may result due to unregulated metabolism including the failure of antioxidant systems (Liang and Sun, 2000; Wesley-Smith *et al.,* 2001). Accordingly, reducing the time of exposure to intermediate hydration levels is likely to minimize desiccation damage. In the present investigation, the tolerance to desiccation to a comparatively lower level of moisture content in seeds stored open at room temperature may also be due to reduced deteriorative processes.

To account for the loss of viability accompanying the dehydration regime in recalcitrant seeds, Farrant *et al.* (1988) proposed that loss of water during desiccation would result in disruption of certain metabolic pathways, accompanied eventually by a loss of stability of sub-cellular structures including membranes. Loss of stability of the membranes would lead to loss of integrity of the plasmalemma, the tonoplast and other membranes and ultimately to the loss of viability of the seeds. Pammenter and Berjak (1999) envisaged three types of damages in desiccation sensitive seeds upon the withdrawal of water. They were, mechanical damage associated with the reduction in cell volume; loss of coordination of metabolism leading to aqueous based degradative processes probably mediated by free radicals and compounded by the failure of antioxidant systems and the loss of structural integrity of cellular macromolecules.

A close correlation is observed between the moisture content, percentage of germination and seed vigour index (SVI) in the seeds of cocoa during desiccation in all the conditions. During hydrated storage, the physiological status of the seeds changes as a consequence of their ongoing development which progresses into germination. Germinated seeds no longer have storage potential, as first their vigour declines and ultimately lose viability (Berjak *et al.*, 2004). The SVI is at its maximum in fresh seeds and any type of storage decreased the values. Even though cent percent germination is observed in seeds desiccated for one day in open at room temperature, 6 days in polyethylene bags at room temperature and 2 days in polyethylene bags at 4°C, the SVI is found decreased all the while (Table 3, Fig 1C). Desiccation led to a decrease in SVI as the number of seeds germinated is diminished and the time taken for radicle emergence increased. The decline in seed vigour may be due to structural changes of intracellular membrane systems with the accompanying inability on the part of the cells to maintain electrical, chemical or pH gradients and a mixing of the normally separated cellular constituents (Parish and Leopold, 1978). Bernal-Lugo and Leopold (1992) suggested that changes in soluble carbohydrate content in maize seeds result in the loss of seed vigour and subsequently in the loss of viability.

In cocoa seeds, the decline in SVI is closely correlated with the loss of moisture content and the corresponding degradative processes as desiccation progresses. As the moisture content is reduced to approximately 30% in all the three storage conditions, the SVI also declines and reaches a critical value of about 3.0. Up to this stage of desiccation, the seeds exhibited cent percent germination. Further loss of moisture content results in a reduction in SVI with an accompanying decline in germination percentage. Eventually the SVI and the germination percentage are brought down to zero when the moisture content reached a value of around 20%. This observation is in conformity with the view of Fu *et al.* (1994) who suggested that seed vigour index is an accurate measure for testing seed quality. Those authors found in *Clausena lansium* and *Litchi chinensis* that desiccation damage was a progressive deteriorative process. When desiccation was slight, vigour index decreased first. Later, when deterioration became more serious, the germination percentage also dwindled.

A significant increase in electrical conductivity of leachates is observed in seeds of cocoa desiccated under different storage conditions (Table 4, Fig. 1D). Electrical conductivity of leachates from seeds stored open at room temperature shows a rapid increase during the initial phases of desiccation, reaching the maximum value on the 4th day. For the seeds stored in polyethylene bags either at room temperature or at 4°C, the increase is gradual.

One of the earlier events associated with the loss of viability in recalcitrant seeds is the loss of membrane integrity as indicated by an enhanced concentration of solutes in seed leachates (Nautiyal and Purohit, 1985c). A negative correlation has been drawn between the seed moisture content and the efflux of electrolytes from the seeds (Parish and Leopold, 1978; Becwar, *et al.*, 1982; Koster and Leopold, 1988; Leprince *et al.*, 1993).

Electrolyte leakage from tissues could be used to indicate the effectiveness of membranes as barriers to solute diffusion (Berjak *et al.,* 1993; Reisdorph and Koster, 1999). Irreversible solute leakage is correlated with loss of viability in desiccation-sensitive seeds in response to dehydration. Whereas relatively low levels of leakage indicated that cellular membranes are semi-permeable, high levels of leakage indicated damage to the membranes in response to dehydration stress. The desiccation of cocoa seeds in polyethylene bags at room temperature showed only a gradual increase in leachate conductivity up to 6 days probably due to less damage to cell membranes. After 6 days, the germination percentage decreases with a concomitant increase in leachate conductivity. This may be due to the loss of membrane integrity as a result of desiccation for longer periods. A similar behaviour is observed in cocoa seeds stored in polyethylene bags at 4°C also. Almost similar results were obtained in *Hancornia speciosa* (Oliveira and Valio, 1992), *Araucaria angustifolia* (Espindola *et al.,* 1994) and in tea, cocoa and jackfruit (Chandel *et al.,* 1995).

According to Parish and Leopold (1978) and Senaratna and McKersie (1983), the increase in electrolyte leakage is associated with a more permeable membrane system, the direct result of which can be the loss of metabolites, inability to maintain electrical, chemical or pH gradients and a mixing of the normally separated cellular constituents. The indirect result would be the loss of vigour and viability. The seeds with higher moisture content show a higher percentage of germination and release fewer electrolytes and organic substances to the medium. Stewart and Bewley (1980) opined that, biochemical consequences of membrane deterioration include loss of solute control and the dispersal of the highly ordered system of membrane-associated enzymes.

The significant increase in leachate conductivity in cocoa seeds indicates the loss of membrane integrity during desiccation and the accompanying loss of moisture content, resulting in an irreversible solute leakage. So, a negative correlation is observed between moisture content and leachate conductivity. The present study shows that in cocoa seeds, the rate of increase in electrolyte leakage varies with the storage conditions. Thus, the role of seed moisture content in membrane integrity and solute control is established.

Recalcitrant seeds are hydrated and are actively metabolic when they are shed or harvested (Berjak *et al.,* 1993). According to Tompsett and Pritchard (1993), desiccation intolerance in recalcitrant seeds is related to the initiation of germination at or soon after shedding. Berjak *et al.* (1984) observed that in the recalcitrant situation, metabolic events suggestive of germination changes accompany the early stages of dehydration. In cocoa seeds, biochemical events taking place during initial phases of desiccation are analogous to those of germinative metabolism.

The total starch content in cocoa seeds is comparatively very low and is found to decrease in seeds maintained in all the three conditions of storage (Table 5, Fig. 2A). A drastic reduction is observed in seeds kept open at room temperature and the value is reduced to one-third within four days of desiccation. Even though the seeds are nonviable after 2 days, drastic reduction of starch is indicative of respiratory metabolism occurring at the ambient room temperature.

Chandel *et al.* (1995) observed a six fold increase in soluble carbohydrates during desiccation of tea and cocoa axes which could be due to degradation of starch into simple sugars. According to Nkang *et al.* (2003), starch level decreased and sugar and lipid levels increased during the desiccation of *Telfairia occidentalis* seeds, implying the utilization of starch for the biosynthesis of lipids, the major storage reserve. Lipids were also suggested to function as cotyledonary sinks thereby avoiding the build up of the products of starch hydrolysis. In cocoa seeds stored in polyethylene bags at room temperature, the reduction in starch content is gradual and less pronounced. Desiccation sensitive seeds like cocoa are shed with high moisture content and are metabolically active (Farrant *et al.,* 1993b, Pammenter and Berjak, 1999). According to Leprince *et al.* (2000) during hydrated storage, the physiological status of the seeds changes because of their ongoing development which progresses into germination. Starch initially present in the cocoa seeds may have been hydrolyzed as an energy source in the ensuing respiratory metabolism and hence resulting in only negligible increase in soluble sugar content (Table 6). In seeds stored at 4°C, starch depletion is very slow and not directly related to desiccation stress. However, after viability loss insignificant reduction in starch content is observed, presumably due to slow respiration at low temperature. The rate of starch degradation is directly related to the rate of desiccation of cocoa seeds according to the storage conditions.

Histochemical localization of insoluble polysaccharides revealed that starch content in terms of grain size and number decreased during desiccation (Plate 2, Fig. A-E). During the later stages of desiccation, the staining intensity of starch grains was found to decrease due to the initiation of grain degradation. Shrinkage of cells during desiccation may also contribute to cell wall structural and textural changes resulting in increased staining intensity.

Changes in the soluble sugar content in seeds have been identified with changes in the ability to survive desiccation (Koster and Leopold, 1988;

Leprince *et al.*, 1993; Blackman *et al.*, 1992). Hoekstra *et al.* (1994) found that in cauliflower seeds, more than 90 % of the dry mass and almost the whole of the sugar content was present in the cotyledons than in other seed parts. According to these authors, desiccation damage of the whole seeds coincided more closely with changes in sugar in the cotyledons.

The total soluble sugar content in cocoa seeds, show an insignificant increase in the initial stages of desiccation in all the storage conditions but decline in the later stages (Table 6, Fig. 2B). The increase in soluble sugar content, though insignificant, may be the consequence of starch degradation observed in cocoa seeds under desiccation (Table 5). In cocoa seeds, when the seed viability is reduced to less than 80%, a significant decline in the total sugar content is found to occur, probably due to active utilization in the early respiratory metabolism. This decline results in the limited availability of respiratory substrate in the later stages of desiccation leading to decreased seed vigour and viability as opined by Bernal-Lugo and Leopold (1992; 1995). According to Leprince *et al.* (1993) the protective effects of sugars during desiccation also include protein stabilization. However, such stabilization may not be occurring in cocoa seeds as protein degradation occurs in seeds desiccating naturally at room temperature, even at stages where the soluble sugar content is on the increase.

A three-fold increase in sucrose content is observed on the 3rd day of desiccation in cocoa seeds stored open at room temperature (Table 7, Fig. 2C). A rapid increase in the sucrose content is observed in seeds stored in polyethylene bags at room temperature as well as at 4°C. Even though the sucrose content is found to decline later on, the values always remain much higher than the control value. The increase in sucrose content in the seeds is envisaged to occur through the conversion of the oligosaccharides into sucrose or by the hydrolysis of starch into soluble sugars (Lin *et al.*, 1998).

This concept is supported by the significant decline in starch content observed in desiccating cocoa seeds in the present study. According to Beevers (1980), sucrose is the product of lipid degradation in the cotyledons of most oil seeds and is transported eventually to the axis.

The membrane system is the cellular component most vulnerable to damages, upon withdrawal of water. The membranes are suggested to lose their liquid crystal structure during dehydration, unless some protection is provided. According to the 'water replacement theory' of Caffrey *et al.* (1988) a sugar such as sucrose formed a substitute for water at the membrane surface during desiccation. The hydroxyl groups of sucrose may replace water by hydrogen-binding to the phospholipid head groups of the membrane (Koster and Leopold, 1988).

Involvement of sucrose as a factor contributing to desiccation tolerance has been widely reported (Caffrey *et al.,* 1988; Koster and Leopold, 1988; Bernal-Lugo and Leopold, 1992; Crowe *et al.,* 1998; Lin *et al.,* 1998; Buitink *et al.,* 2000, Halperin and Koster, 2006). However, the high amount of sucrose did not always prevent the desiccation induced loss of viability in the seeds. The main limitation of sucrose is that it crystallizes upon slow drying, and therefore the hydroxyls will be unavailable for water replacement in biomembranes as suggested by Caffrey *et al.* (1988). Hoekstra *et al.* (1994) suggested that loss of desiccation tolerance may have other causes than loss of sucrose as drying of seeds caused an increase in sucrose content which need not always result in desiccation tolerance. In cocoa seeds, the high amount of sucrose formed during desiccation is not found to prevent the loss of viability as dehydration advances. In seeds stored in all the conditions, the viability is noticed to decline rapidly even though the sucrose content remains higher than that of the fresh seeds. Thus it may be concluded that sucrose alone is having only a limited role in conferring desiccation tolerance in cocoa seeds.

According to Lin *et al.* (1998) and Buitink *et al.* (2000), sucrose as such might not be the key element in conferring desiccation tolerance, since ambiguous correlations exist between sucrose mass and desiccation sensitivity.

Leprince *et al.* (1993) and Buitink *et al.* (2000) observed that oligosaccharides like raffinose prevent crystallization of sucrose during desiccation, thus contributing to desiccation tolerance in angiosperm seeds. The ability of sucrose to provide protection to membranes upon drying and the enhancement of this effect in the presence of raffinose, led the authors to suggest an interaction between phospholipids and sugars with a direct relevance to the viability of dry seeds. In the present study, raffinose is found to occur in low amount in cocoa seeds and the content is found to decline steadily throughout the period of desiccation irrespective of the storage condition (Table 8, Fig. 2D). A significant reduction in raffinose content occurs only when the seed viability is decreased below 100%. Hence the enhancement of membrane protection by sucrose in the presence of raffinose is manifested in cocoa seeds. Bochicchio *et al.* (1994) implied that abrupt loss of water in maize seeds as a result of rapid drying might have impaired enzyme reactions including those responsible for an increase in raffinose concentration. The declining raffinose content may also be due to its hydrolysis into monosaccharides.

According to Lin *et al.* (1998), the oligosaccharides such as raffinose were consistently present during the tolerant stage and the desiccation tolerance disappeared as the oligosaccharides were decreased to a low level. A great decrease in the mole ratio of oligosaccharides/sucrose from the original value was noticed with the disappearance of desiccation tolerance.

Steadman *et al.* (1996) opined that the ratio of sucrosyl-oligosaccharide to sucrose in seed tissues is a good indicator of seed storage category. In general, orthodox and recalcitrant seeds had tissues with sucrosyl-oligosaccharide: sucrose mass ratio 1:7 and. 1:12 respectively. The authors suggested that the amount of sucrosyl-oligosaccharides, raffinose and stachyose was lower in recalcitrant seeds as compared to orthodox seeds. A similar result was obtained in cocoa seeds in the present study. The seeds exhibited cent percent germination in all the three storage conditions when the sucrose to raffinose ratio was less than 8:1(Table 9). An increase in the ratio results in an increase in the loss of viability. Total loss of viability is found to occur when the ratio exceeds about 13:1 in seeds stored in all the conditions. It is thus obvious that seed viability in cocoa is maintained in the initial stages of desiccation by the sucrose fraction in conjunction with the raffinose content. As the raffinose content declined below a critical value and the optimal sucrose:raffinose ratio could not be maintained, the deleterious effects of desiccation set in, culminating in the loss of vigour and viability.

It is also suggested that Sucrose and raffinose impart desiccation tolerance through their involvement in vitrification - the formation of high viscosity, super saturated solutions called amorphous glass during drying (Koster and Leopold, 1988; Bruni and Leopold, 1991; Crowe *et al.*, 1998; Pammenter and Berjak 1999; Buitink and Leprince, 2004). Because of its high viscosity, the glassy state of cytoplasm is found to impose a stasis on intracellular activity, reducing the deleterious effects of deranged metabolism, including damaging free radical reactions and protecting macromolecules against denaturation. According to Wolkers *et al.* (1999), the ratio of sucrose to oligosaccharides is more important in vitrification than sucrose and oligosaccharides in their absolute amounts. Sucrose is the major soluble carbohydrate occurring in seeds after fast drying. Raffinose serves to amplify substantially the magnitude of the glass signal. The decline in the stability of seeds is correlated with a decrease in raffinose content (Bernal-Lugo and Leopold, 1995). This implies that, it is the sucrose to oligosaccharide ratio that is responsible for vitrification in the initial stages of desiccation in cocoa seeds, as suggested by Wolkers *et al.* (1999). However, the role of vitrification in extending the period of cocoa seed viability is uncertain. This is in agreement with the view of Li and Sun (1999) who suggested that the recalcitrance of cocoa axes is not caused by the lack of such a mechanism of vitrification.

The total reducing sugar content in cocoa seeds stored in different conditions is found to increase in the initial phases of desiccation and to decline later on (Table 10, Fig. 3A). An increase of similar magnitude was observed in the initial stages of desiccation in seeds stored in polyethylene bags at room temperature and at 4°C. According to Chandel *et al.* (1995) and Nkang *et al.* (2003), the increase in reducing sugars might be due to hydrolysis of starch. Bernal-Lugo and Leopold (1992) suggested that hydrolysis of raffinose during storage resulted in a substantial increase in galactose and sucrose or an increase in galactose, glucose and fructose, leading to an increase in reducing sugars. The source of the monosaccharides in cocoa seeds may be any of these, as the amount of starch as well as raffinose showed a gradual decline during desiccation (Tables 5 & 8).

Analysis of the monosaccharides of cocoa seeds using HPLC showed an initial increase and a subsequent decrease of glucose, fructose and galactose (Table 11-13, Fig. 3B-D). According to Bernal-Lugo and Leopold (1992) and Li and Sun (1999), the reducing sugars / monosaccharides may initiate seed deterioration through Amadori and Maillard reactions, the products of which damaged the DNA and proteins beyond repair. The Amadori and Maillard reactions involve a series of complex processes in which the non-enzymic condensation of an aldehyde or ketose with a free amino group of proteins or nucleic acids occur to form a glycosylamine which is rearranged in to the Amadori product. The Maillard reaction represents the subsequent complex interactions between glycated Amadori products to form polymeric brown coloured products (Wettlaufer and Leopold, 1991; Murthy and Sun, 2000). These non-enzymatic reactions could also be expected to result in lowered activity of enzyme component of the embryo.

Reducing sugars are suggested to be the source of primary aldehydes involved in the Amadori reaction (van der Toorn and McKersie, 1995). The presence of reducing sugars such as glucose, fructose and galactose is a primary driving force of Amadori Maillard reaction and galactose is the most reactive of the hexose sugars in this regard (Bernal-Lugo and Leopold, 1992). Dry seeds are apparently unable to exercise repair and therefore protein or DNA damages by Amadori and Maillard reactions would accumulate over time and eventually contribute to seed death (McDonald, 1999). The initial increase in the reducing sugar content / monosaccharides including galactose, presumably the most reactive of the hexoses, may have led to the Amadori Maillard reactions in cocoa seeds (Table 10-13). Participation in the Amadori and Maillard reaction can be the reason for the significant decline in the reducing sugars and monosaccharides during the later stages of desiccation as suggested by Bernal-Lugo and Leopold (1992). The change in the colour of radicles of desiccated cocoa seed from opaque white to brown upon re-imbibtion, observed in the present study, indicates the involvement of Amadori Maillard reactions in the loss of seed viability in agreement with the views of van der Toorn and McKersie (1995).

Leprince *et al.* (1992) found a significant correlation between increased concentrations of monosaccharides and increase in respiration rates in germinating maize radicles and suggested that the former could regulate the latter. According to Hoekstra *et al.* (1994) high content of glucose and fructose during imbibition was suggested to indicate a raised rate of metabolism and respiration which was likely to depend on water content. This correlation might have some significance in the loss of desiccation tolerance, since a desiccation induced electron leakage from respiratory chains could initiate lethal peroxidative damages. As the enhanced respiration rates before drying represent the amount of damaging free radicals generated in the tissues, the loss of desiccation tolerance was attributed to free radical damage rather than to changes in sugar contents. According to van der Toorn and McKersie (1995), autoxidation of reducing sugars and increased respiration correlated with the reducing sugar content were the sources of hydroxyl radicals which initiated lipid peroxidation, cited as a prime cause of desiccation damage.

Protein, constituting about 17% of the dry weight of fresh cocoa seeds, was found decreased throughout the period of desiccation (Table 14, Fig. 4A). A gradual decline in the total protein content is observed in cocoa seeds undergoing desiccation at various storage conditions. The rate of protein depletion is closely correlated with the rate of dehydration, which varies with the storage condition. The loss of viability accompanying the moisture loss can be correlated with the loss of proteins also. According to Chaitanya *et al.* (2000), the rapid loss of viability in desiccation sensitive *Shorea* seeds, when desiccated naturally during storage, is due to a gradual loss of total protein. The net loss of proteins in deteriorating seeds is perhaps the most basic of all ageing-related events as these changes could underlie all other aspects of metabolic decline. Declining amount of proteins during desiccation may be due to either decreased synthesis or degradation through enhanced proteolytic activity which may account for the loss of total protein content in these tissues. Alternatively, the relatively faster rates of desiccation in cotyledons might damage the protein synthesizing system resulting in reduced protein level. In *Shorea robusta* (Nautiyal *et al.,* 1985; Chaitanya *et al.,* 2000) and *Mangifera indica* (Mathew, 2006), the decrease in protein content is closely correlated with the rate of dehydration. However, in cocoa seeds only an insignificant decline in the protein content is observed up to the stage of critical moisture content, when the loss of viability is initiated. In the subsequent stages of rapid viability loss also, only a gradual decrease in the protein content is noticed. Therefore, the loss of viability in cocoa seeds may not be solely due to the degradation of proteins. Loss of structural integrity of the proteins due to continued dehydration may also contribute to the desiccation induced viability loss as suggested by Crowe *et al.* (1998). According to these authors, interaction with water is critical for the formation of the native folded protein and many labile proteins lose their functional as well as structural integrity when they are desiccated. The level of hydration achieved during air-drying of seeds is low enough that the hydration shell of proteins is completely removed, *i.e.* almost all interactions with water are lost. It is shown that most of the unprotected proteins are unfolded in the dried condition. Thus, both protein degradation and denaturation appear to be involved in the loss of viability of cocoa seeds during desiccation.

The PAGE studies showed the disappearance of certain bands of protein in the gels of seeds desiccated in open at room temperature (Plate 1). This disappearance of bands is attributed to degradation of proteins during desiccation resulting in structural and functional changes in the seed tissues including altered membrane permeability leading to loss of viability.

Histochemical localization of proteins by bromophenol blue method also shows a gradual decrease in the staining intensity during desiccation (Plate 3, Fig. A-E). These observations are in agreement with the reduction of protein content observed in biochemical estimation of proteins and the disappearance of certain bands of proteins on the gel of PAGE.

Corresponding to the decrease in protein content, an increase in free amino acid content was observed in the seeds of cocoa during desiccation in all the storage conditions, (Table.15, Fig. 4B). Identical results were obtained in the seeds of *Podocarpus henkelii* (Dodd *et al.,* 1989) and *Mangifera indica* (Mathew, 2006). Seeds stored open at room temperature exhibited a two-fold increase in free amino acids and almost the same trend was followed up to the 4th day. The increase in the free amino acid content in the seeds of cocoa indicates that the depletion of cellular proteins is due to an enzyme-mediated proteolysis and not due to a decreased synthesis as suggested by Chaitanya *et al.* (2000) who reported increased proteolytic activity in *Shorea robusta* during desiccation. Wolkers *et al.* (1999) and Ramakrishna and Rao (2005) reported the accumulation of amino acids in rapidly dried embryos corresponding with rapid proteolysis.

The rapid increase in free amino acid content in the seeds stored in open at room temperature and gradual increase observed in seeds stored in polyethylene bags at room temperature and at 4°C may be due to the difference in the activity of the proteolytic enzymes. According to Chaitanya *et al.* (2000) the declining amounts of proteins, in the embryonic axis and the cotyledon are closely related with the rate of dehydration of these tissues. In the present study, the decrease in the protein content with the corresponding increase in free amino acids in seeds stored in different conditions and the rate of decrease in moisture content during desiccation show that the breakdown of proteins and release of amino acids are regulated by the seed moisture content.

Proline content in the seeds of cocoa showed a steady increase during desiccation (Table 16, Fig. 4C). Lin and Chen (1995) observed approximately a two-fold increase in proline content in the seeds of *Machilus thumbergii* during desiccation. According to these authors, accumulation of sucrose and proline and a decrease in abscissic acid during desiccation over a period of several days indicated the activation of some maturation specific metabolic activities. Andarwulan, (1999) found that free proline accumulates in many plants in response to the imposition of a wide range of biotic and abiotic stresses. Alia *et al.* (2001) suggested that proline accumulation might be involved in the protection of the organisms against singlet oxygen induced damages.

Fresh seeds of cocoa possess about 25 $\mu g\ g^{-1}$ dw of proline which constitutes about 12.39% of the total free amino acids. In seeds desiccated in open at room temperature, more than 100% increase in the proline content was observed by the fourth day. However, it constitutes only 7.17% of the total free amino acid content of this stage. In seeds stored in polyethylene bags at room temperature and at 4°C also, a steady and progressive increase in the proline content is found to occur. However, of the total free amino acid content of this stage, these values come to only about 7.71 and 8.26% respectively. It is obvious that the increase in proline content in the desiccating seeds of cocoa is associated with the increase in the total free amino acid content resulting from proteolysis and may not be related to any stress relieving mechanism as mentioned above.

Cocoa seeds exhibit a progressive increase in the total phenolic content during the initial period of desiccation in all the three storage conditions (Table 17, Fig. 4D). The increase is significant in seeds kept open at room temperature compared to those kept in the other two conditions. A decline in phenolic content is observed in the later stages of desiccation. It is obvious that the increase in phenolic content in the desiccating seeds of cocoa depends on the rate of moisture loss. This is in agreement with the observation of Schulz-Schomburgk and Rodriguez (1998) that in cocoa seeds there is quantitative increase in phenolics for the first seven days of desiccation and a decline thereafter. Nautiyal and Purohit (1985b) had also observed an increase in the phenolic content in the desiccating seeds of *Shorea robusta.* Sgherri *et al.* (2004) suggested that phenolic accumulation may be due to new synthesis during dehydration.

A negative correlation is observed between viability and phenolic accumulation in cocoa seeds. Therefore the opinion of Sgherri *et al.* (2004) that the accumulation of phenolics may constitute a reserve which allows the plant to tolerate oxidative damage during dehydration is not relevant in the present situation as viability of cocoa seeds is found to be decreasing, even with an increase in the phenolic content.

Polyphenolics affect plant growth by interacting with one or another of the major classes of plant hormones such as the auxins. Harborne (1980) suggested that many of the phenolics have a potentially inhibiting effect on growth as they are capable of auxin destruction by augmenting indole acetic acid oxidase. Phenolics may also interact with other hormones by synergism or inhibition. Besides their action through the hormones, they may also have indirect influence on the physiology of the cell through more non-specific effects on intermediary metabolism. Many phenolics are capable of inhibiting ATP synthesis in mitochondria and uncoupling respiration. The effect of phenolics on cocoa seed viability may be due to their inhibitory effect on phytohormones and/or on intermediary metabolism.

The lipids form the major storage components in the seed tissues of *Theobroma cacao* constituting approximately 43% of the dry mass of the fresh seeds. On desiccation, a quantitative increase is observed at the initial stages (Table 18, Fig. 5). The values declined subsequently in all the storage conditions. Nkang (2003) observed an increase in sugar and lipid content during desiccation of *Telfairia* seeds with a decrease in starch and suggested that starch might be utilized in the biosynthesis of lipids, the major storage reserves. Lipids could also be functioning as cotyledonary sinks thereby avoiding the build up of the products of starch hydrolysis. In the present study also lipid content was found increased with increase in sugar content and decrease in starch. The reduction of starch may be due to its utilization in the lipid biosynthesis.

Desiccation tolerant seeds are characterized by their ability to avoid oxidative damage that may accumulate during drying (Leprince *et al.,* 1994). Plant tissue dehydration is known to be associated with an increased production of reactive oxygen species (ROS) which can react together and trigger numerous deleterious oxidative processes (Leprince *et al.,* 1993; Smirnoff, 1993). Many ROS sources have been identified in plants, and any transfer or transport chain of electrons towards oxygen can potentially generate ROS. The mitochondrial respiratory chain is one of the major sources of ROS and the amount of H_2O_2 produced is thus directly proportional to respiratory activity.

In recalcitrant species, the seeds do not undergo maturation drying and are shed at very high moisture content (Farrant *et al.,* 1988). The seeds are metabolically very active immediately after shedding and hence there would be enormous generation of superoxides. It might result from metabolic imbalances leading to leakage of high energy intermediates from plastids and mitochondria, redox states of the electron carriers inside mitochondria and absence of effective antioxidant systems (Leprince *et al.,* 2000; Bailly, 2004). In normal conditions, approximately 2-3% of the oxygen used by the mitochondria can be converted into superoxide and H_2O_2 (Puntarulo *et al.*, 1988). The superoxides will lead to H_2O_2 formation through enzymatic or non enzymatic processes. H_2O_2 has a longer life span than that of superoxides. The Haber-Weiss and Fenton reactions involving superoxide radicals and H_2O_2 may lead to the formation of hydroxyl radicals which is the most aggressive form of the oxygenated derivatives (Bailly, 2004; Navrot *et al.,* 2007). According to van der Toorn and McKersie, (1995) the source of hydroxyl radicals may be the autoxidation of reducing sugars or the increased respiration correlated with the reducing sugar content during the germination process. Among the many harmful effects of hydroxyl radicals on cellular macromolecules, the most conspicuous is lipid peroxidation (Bailly, 2004).

A gradual but significant increase in the thiobarbituric acid (TBA) reactive product of malondialdehyde (MDA) is observed in cocoa seeds maintained in all the three storage conditions (Table 19, Fig. 6A). Seeds kept open at room temperature showed more than two-fold increase of MDA by the 3rd day whereas those kept in polyethylene bags at room temperature and at 4°C showed an almost four-fold increase on the 10th day of storage. The distinct increase in MDA content suggests that lipid peroxidation is taking place at a remarkable rate in cocoa seeds leading eventually to the loss of viability. Chandel *et al.* (1995) observed that a significant decline in viability associated with desiccation in cocoa and jackfruit seeds were accompanied by an increase in lipid peroxidation. These authors suggested that disruptions to the cell organelles such as the nucleus and mitochondria could also be contributing to the decline in the viability of embryonic axes during desiccation. According to Sung (1996), Greggains *et al.* (2001) and Pukacka and Ratajczak (2005), increased reactive oxygen species (ROS) formation during desiccation, the associated lipid peroxidation, reduction in antioxidant defenses, consequent membrane damage, electrolyte leakage and eventual cell death are the prime causes of deterioration of recalcitrant seeds in storage. Kibinza *et al.* (2006) opined that lipid peroxidation and oxidative stress are the major causes of deterioration of oil seeds like sunflower during ageing. Lipid peroxidation is cited as one of the major contributors of deterioration in ageing and this process was found to produce highly reactive free radical intermediates that can damage membranes, proteins and nucleic acids and was observed to precede the loss of viability (Wilson and McDonald, 1986; Hendry, 1993; Finch-Savage *et al.,* 1996). According to Chaitanya and Naithani (1994), the rapid loss of viability in *Shorea* seeds during desiccation may be due to the cumulative effect of the peroxidation products of stored polyunsaturated fatty acids, peroxidation of the membrane lipids and the damaging effect in the disorganization of metabolism.

It is also presumed that secondary products of lipid peroxidation might participate in non-enzymic degradation of proteins and DNA through Amadori and Maillard reactions (Murthy and Sun, 2000). Lipid peroxidation generates a variety of lipid hydroperoxides which can further be degraded into reactive ketones, aldehydes and alcohols. They may react with terminal groups of amino acids in proteins. These authors found that the content of Amadori products in seeds was increased by 400% during storage and showed a strong correlation with the content of TBA reactive products supporting the hypothesis that a possible coupling existed between lipid peroxidation and the initiation of Amadori reactions.

The increase in the reducing sugar content of cocoa seeds, observed in the initial stages of desiccation, might have contributed to lipid peroxidation through autoxidation and the consequent formation of hydroxyl radicals, as suggested by van der Toorn and McKersie (1995). The deteriorative changes in the membrane system of cocoa seeds due to lipid peroxidation are indicated by the increase in the electrical conductivity of leachates during storage. The increased peroxidation of lipids during storage might have resulted in deteriorative changes in the membrane systems of cocoa seeds as was indicated by the increase in electrolyte conductivity. Macromolecular damage and perturbed metabolism could also be ascribed to the enhanced lipid peroxidation leading to the loss of viability and germinability of cocoa seeds in storage.

The accumulation of ROS in seed tissues plays an important role in the loss of seed viability during storage as they initiate reactions with polyunsaturated fatty acids, leading to lipid peroxidation and destruction of cellular membranes. The break down in metabolic co-ordination in cells may initiate uncontrolled free radical attack and decreased enzymic and non-enzymic protection against such oxidative damage (Hendry *et al.,* 1992). To counteract the toxic events evoked through ROS, antioxidative defence systems are present in plant tissues (Scandalios, 1993; Pukacka and Ratajczak, 2005). These protective systems are composed of enzymatic scavengers, such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione oxidase. A balance between generation and scavenging of free radicals and peroxides must be maintained within the stored seeds; otherwise, the longevity of the seeds would be shortened (Ponquett *et al.,* 1992; Sung and Jeng, 1994; Sung, 1996; Farrant *et al.,* 2004)

The behaviour of three free radical scavenging enzymes - guaiacol peroxidase, catalase and superoxide dismutase (SOD) - during desiccation and storage of *Theobroma cacao* seeds under different conditions was studied. All the three showed, in general, a drastic reduction in activity during desiccation (Tables 20-22).

Of the free radical scavenging enzymes in cocoa seeds, the activity of SOD is the most pronounced (Table 20, Fig. 6B). The fresh seeds show SOD activity of about 115.55±3.25 units mg^{-1} protein. In seeds desiccating in open at room temperature, a very sharp decline in the value is observed. The decline in SOD content is less sharp in seeds stored in polyethylene bags either at room temperature or at 4°C.

Li and Sun (1999) found that activities of free radical scavenging enzymes like ascorbate peroxidase, peroxidase and SOD decreased in the embryonic axis of cocoa seeds with water content decreased to the critical level for desiccation sensitivity. In the cotyledon, peroxidase and SOD activities decreased sharply while ascorbate peroxidase activity remained low. Greggains *et al.* (2001) noted that the activities of superoxide dismutase and guaiacol peroxidase showed variation in different seed tissues of *Avicennia marina* at different hydration levels. In the present investigation cocoa seeds stored in polyethylene bags at room temperature showed a slow decline in moisture content and a comparatively slow but significant reduction of SOD activity.

According to Nkang *et al.* (2003) the major enzymic protective processes against damage by products of peroxidation include the main enzymes of hydroperoxide metabolism like SOD, catalase and guaiacol type peroxidase. Kibinza *et al.* (2006) observed in sunflower that glutathione reductase and superoxide dismutase activities declined faster than that of catalase enzymes. The drastic reduction in the SOD activity in cocoa seeds during desiccation is in correlation with the rapid rate of dehydration and the corresponding loss of viability observed. The primary consequence of this sudden drop in SOD activity would be the failure to quench the superoxide radicals generated during the deranged metabolism and respiratory activity early in the germinative events associated with the storage of cocoa seeds. The consequent free radical attack eventually leads to membrane perturbation, loss of vigour and seed viability.

The peroxidase activity in cocoa seeds is found rapidly decreasing during desiccation of seeds stored open at room temperature and in polyethylene bags at 4°C (Table 21, Fig. 6C). The decrease is relatively slower and gradual in seeds stored in polyethylene bags at room temperature up to 6 days. In both cases, the decrease in peroxidase activity becomes rapid and significant once the seeds have desiccated beyond the critical moisture content. The rapid decrease in peroxidase activity after the onset of viability loss in the seeds of cocoa during storage and desiccation implies a loss in the ability of the enzyme to eliminate the free radicals and H_2O_2. Leprince *et al.* (1992) observed that loss of desiccation tolerance of maize embryonic axis during germination is associated with decrease in SOD, glutathione reductase and peroxidase activities. Peroxidase, which has been reported in many biological systems as possessing a parallel polyphenoloxidase activity, may be involved in reducing high contents of auxin and phenolics (Okpuzor and Omidiji, 1988; Nkang, 2001). Nkang *et al.* (2003) observed that in *Telfairia*, seeds are having high initial peroxidase levels, conferring high capacity to eliminate harmful free radicals and decreased peroxidase and polyphenoloxidase activity during desiccation with increased likelihood of free-radical attack and decreased germination responses. In cocoa seeds, the rapidly decreased peroxidase activity could account for the decrease in germination percentage during desiccation in different storage conditions.

The specific activity of the enzyme catalase is found to be rather low in the seeds of cocoa. This is in agreement with the observation of Hendry *et al.* (1992) who detected only low activity of catalase in the cotyledons of *Quercus robur*. The specific activity of catalase in cocoa seeds is found to remain more or less unchanged as long as the seed viability is retained, but is found to decline subsequently (Table 22, Fig. 6 D). In seeds stored open at room temperature, enzyme activity shows a sharp decline from the second day of desiccation onwards. In seeds stored in polyethylene bags at room temperature and at 4°C, the catalase activity is observed to remain relatively unchanged up to the 4th day of desiccation. A gradual decline is observed thereafter. Varghese and Naithani (2002) observed in neem seeds that activities of catalase and peroxidase exhibited substantially higher levels of activity in 100% viable seeds dehydrated up to critical moisture content and that their activities declined sharply in seeds with water content below the critical level.

Results of analysis of free radical scavenging enzymes are in agreement with the observations of Li and Sun (1999) that a decrease in the activities of the free radical scavenging enzymes and an enhanced occurrence of lipid peroxidation resulted in the loss of seed viability. The extent of lipid peroxidation and the activity of free radical scavenging enzymes in drying seeds are inversely correlated. Li *et al.* (2007) found in *Limonium aureum* seeds that an increase in the activities of enzymes like catalase, peroxidase and glutathione reductase resulted in a reduction in the occurrence of lipid peroxidation there by prolonging the viability of the seeds in storage. A steady increase in lipid peroxidation in cocoa seeds in the present study is indicative of the higher levels of superoxide and hydroxyl radicals accumulating during desiccation. This is apparently the consequence of the rapid decline in the activities of free radical scavenging enzymes like superoxide dismutase, guaiacol peroxidase and catalase, which become pronounced once the moisture content of the desiccating seeds has passed the threshold of critical moisture content and viability loss has been initiated. Varghese and Naithani (2002) observed an enhanced incidence of lipid peroxidation and reduction in the activities of enzymes like catalase and peroxidase in neem seeds desiccated to lethal moisture contents below the critical level. The extent of decline in the activity of the free radical scavenging enzymes in cocoa seeds is found to depend on the storage conditions. The rate of reduction in the activities of peroxidase and SOD was faster than that of catalase in conformation with the observation of Kibinza *et al.* (2006). The decline in the activities of these enzymes in cocoa seeds may make them more liable to free radical attack. The decrease in SOD activity results in reduced scavenging of superoxides and the concomitant accumulation of hydrogen peroxides and hydroxyl radicals leading to enhanced lipid peroxidation. The highly reactive products of lipid peroxidation possibly damage membranes, proteins and nucleic acids, eventually imparting desiccation intolerance to desiccating cocoa seeds. This concept is supported by the enhanced electrical conductivity observed in cocoa seeds in the present investigation (Table 4). According to Sung (1996), stimulated lipid peroxidation and the depressed ability of the metabolic system to limit the damage from free radicals and peroxides result in seed deterioration. The enhanced lipid peroxidation and depressed free radical and peroxide scavenging activity may partially explain the loss of viability and vigour during natural and accelerated ageing of recalcitrant seeds under storage.

During seed germination, the principal reserve materials in the cotyledons like starch, proteins and lipids are degraded and the products released are translocated to the developing axis. The major mobilization of stored reserves in the tissues of the seeds commences after radicle elongation (Mayer and Shain, 1974; Garcia-Agustin and Primo-Millo, 1989; Mayer and Poljakoff-Mayber 1989; Bewley and Black, 1994).

The dry weight percentage of both the cotyledon and the embryonic axis of the seeds of *Theobroma cacao* is found to change during the period of germination and seedling growth (Table 23, Fig. 7A). Cotyledons of fresh seeds have a very high dry weight percentage than that of the axis. In cotyledons, the dry weight percentage is found to decrease steadily and significantly through out the period of study. The embryonic axis also shows a decrease in the dry weight percentage at the initial stages but an increase in the later stages. Similar results were obtained by Adams *et al.* (1980) and Harris *et al.* (1986) in the cotyledons of soybean. They found that the dry weight of the cotyledon declines during germination due to utilization of the reserves for the growing axis. A decrease in dry weight of the cotyledons in germinating cocoa seeds is an indication that food reserves are being mobilized to the growing embryonic axis. The reserve mobilization in cocoa seeds is slow and gradual in the initial stages, as is substantiated by the gradual decrease in dry weight percentage. The decrease in dry weight percentage becomes rapid and significant from the 2nd day onwards, after the emergence of the radicle, indicating the commencement of active mobilization of the major reserve materials. The increase in dry weight of the axis during seedling growth may be due to the synthesis and incorporation of cellular materials as a part of growth and differentiation.

The total soluble sugar content in both the cotyledon and axis of cocoa seeds shows a decrease in the initial stages of germination and an increase in the subsequent stages of seedling growth. (Table 24, Fig.7B). More or less similar results were obtained in seeds of *Citrus* (Garcia-Agustin and Primo-Millo, 1989), coffee (Giorgini and Campos, 1992) watermelon and sunflower (Bewley and Black, 1994). The significant reduction in the soluble sugar content in the seed tissues of cocoa up to the 7th day of germination, without any substantial reduction in starch content indicates that these sugars form the primary sources of reserve material mobilized / utilized in the initial states of germination and seedling growth.

Giorgini and Campos (1992) and Baleroni *et al.* (1997) noticed an increase in sugar content concomitant with a decrease of starch in the cotyledons of germinating coffee seeds. The increase in the sugar content may be due to the hydrolysis of starch or gluconeogenesis from lipids. In the present study also, an increase in the total sugar content is noticed in the cotyledons from the 12th day of germination/seedling growth onwards, coincident with a sharp decrease in starch indicating that the degradation of starch is much faster during later stages of seedling growth contributing to the total sugar content in the cotyledons and axes. The excessive production of soluble sugars at these stages may be due to the increased rate of gluconeogenesis from lipids as suggested by Bewley and Black (1994). According to the authors, lipids are largely converted to hexoses/sucrose. Suda and Giorgini (2000) also noticed large quantities of soluble sugars during germination of *Euphorbia* seeds and opined that it was mainly due to the catabolism of lipids.

The continuous increase of soluble sugars in the embryonic axis of cocoa seeds is due to the hydrolysis of starch whose content is significantly reduced during this period (Table 25). Lipid degradation also contributes to the sugar accumulation in the axis. As a growing organ, the embryonic axis requires more energy for growth, for which storage compounds must be hydrolyzed or converted to soluble sugars in the cotyledon and translocated to the growing embryo (Copeland and McDonald, 1995). In support of this view, the starch and lipid contents in the cotyledon are found to decrease continuously during germination of cocoa seeds with a concomitant increase in soluble sugars in the embryonic axis.

The starch content in the cotyledon and the embryonic axis of cocoa seeds is found to decrease during germination (Table 25, Fig. 7C). In the axis however, after a period of decline, an increase in starch content was found to occur. According to Briarty and Pearce (1982), Rosenberg and Rinnie (1987), and Bewley and Black, (1994) degradation of starch occur in the cotyledons during germination as a result of mobilization of reserve materials to the growing region. Rosenberg and Rinnie (1987) noted a gradual depletion of both starch and soluble sugars in soybean cotyledons in the initial stages of germination and a more abrupt depletion of starch with the onset of seedling elongation. Similarly, *Corylus* seeds incubated at 20°C showed a rapid decline in starch to about 20% of the initial content during the first week of germination (Li and Ross, 1990b). The authors also opined that the decrease might be due to the respiratory demand of the seeds leading to hydrolysis of starch immediately following imbibition. More or less similar results are obtained in the present investigation also. The degraded starch may be utilized to meet the respiratory demand of the seeds during germination and seedling development.

Even though the reduction in starch content in the cotyledon is noticeable at imbibition stage, rapid depletion becomes apparent only after the radicle has emerged from the seeds, *i.e.*, 2 days after germination. This indicates the requirement of more energy for the active growth of the embryonic axis resulting in the mobilization of more reserve materials.

Histochemical localization of insoluble polysaccharides in the cotyledons of cocoa seeds during germination shows decrease in staining intensity and number and size of starch granules (Plate 5, Fig A-G). At later stages, the cotyledonary cells become almost devoid of starch grains. This is due to the hydrolysis of starch as a result of mobilization during seedling growth .

Mobilization of lipid reserves provides chemical energy and carbon skeleton for embryonic growth during germination in oil seeds. Baleroni *et al.* (1997) reported a rapid decline in lipid content during germination, indicating the use of these reserves during germination. According to Li and

Ross (1990a), mobilization of storage lipids is initiated prior to germination, although a greater part of the hydrolysis takes place during post-germinative growth of *Corylus* seeds.

In cocoa seeds, lipids form the major reserve material constituting about 46% of the dry weight of the fresh seeds. The lipid content in the cotyledon of cocoa seeds is found to decrease gradually in the initial phases of germination (Table 26, Fig.7D). After 22 days of seedling growth, about 50% of the initial lipid content in the cotyledon is found to be mobilized. The amount of lipids present in the axis is considerably less than that of the cotyledons and is found to decline gradually through out the seedling growth. Similar results have been reported in *Citrus* (Garcia-Agustin and Primo-Millo, 1989), *Brassica* (Baleroni *et al.,* 1997) and *Euphorbia* (Suda and Giorgini, 2000). According to these authors, a rapid disappearance of the oil reserves was observed within 20 days of germination after which the mobilization became gradual. However, Muto and Beevers (1974), Becker *et al.* (1978) and Lin *et al.* (1983), opined that the total lipid content remained more or less unchanged during early periods of germination but declined quickly thereafter.

Slack *et al.* (1977) observed a close correlation between axis growth, lipid depletion and the decline in dry weight of the cotyledon in *Cucumis*. According to the authors, the onset of lipid breakdown in intact germinated seeds coincides with the automatic removal of the testa which is necessary for oxygen consumption and for full development of enzyme activity and lipid breakdown. In the present study, the decrease in dry weight percentage of the cotyledons in cocoa more or less coincides with the growth of the axis and mobilization of lipids. The lipid mobilization is slow in the initial stages of germination and the rate increases rapidly from the 7th day onwards when a partial separation of the testa from the cotyledon is observed. The increase in the rate of lipid mobilization in the cotyledons after the 7th day of germination may be due to the partial removal of testa from the cotyledons (Plate 4, Fig. D) which promotes the uptake of oxygen and enhanced breakdown of lipids as suggested by Slack *et al.* (1977).

Rosenberg and Rinne (1987) observed that gluconeogenesis from the oil reserves could lead to the synthesis of new starch. However, Mayer and Poljakoff-Mayber (1989) was of the opinion that lipids are not always converted to carbohydrates during germination. According to Bewly and Black (1994), the products of lipid hydrolysis are largely converted to hexoses and finally to a soluble sugar like sucrose. Suda and Giorgini (2000) observed that the increase in the soluble sugar content in the *Euphorbia* embryo is mainly due to the catabolism of lipids. This may explain the increase in the starch content in the embryonic axis of cocoa seeds in the later stages of germination. The increase in the total soluble sugar content and starch in the desiccating seeds of cocoa support the views of these authors regarding the gluconeogenetic origin of carbohydrates.

Degradation of storage proteins, activation of pre existing enzymes and *de novo* synthesis of enzymes are apparently a chain of events leading to the transport of metabolites to the growing axis for the synthesis of new proteins and other nitrogenous compounds (Bewley and Black, 1994; Callis, 1995; Shewry *et al.,* 1995).

The embryonic axis of cocoa seeds is found to have a high protein content than the cotyledons on a gram dry weight basis. A steep decline in the protein content is noticed during the early stages of germination in the axis as compared to the cotyledons (Table 27, Fig.8A). In the cotyledon, the protein content is continuously decreased throughout the period of study. A similar pattern of decrease in protein content in the cotyledons during germination was also reported in lima bean (Heywood and Gainer, 1974), horse gram (Karunagaran and Ramakrishna Rao, 1990), *Vicia sativa* (Misra and Kar, 1990), lupine (Nandi *et al.,* 1995) and *Vigna mungo* (Taneyama *et al.,* 1996). In the present study, about 50% of the protein content of the axial tissue is found lost by the 12th day of seedling growth and increased in the later stages. Yomo and Varner, (1972) observed a loss of storage protein in the cotyledons and a gain in the axis during germination of the pea seeds, resulting in an increase in the dry weight of the axis. Likewise, in the present study, the protein content in the axis is found to increase in the later stages of germination, during which the protein content in the cotyledons is very low.

The total free amino acid content in the cotyledon and axis behave differently during germination and seedling development of cocoa seeds. The free amino acid content of the cotyledons shows a rapid decrease up to the 7th day of germination and an increase in the subsequent stages. The free amino acid content of the axis is found to increase throughout the period of germination and seedling development (Table 28, Fig.8 B). The initial decrease in free amino acid content in the cotyledon suggests the translocation to the growing axis resulting in an increase there or utilization in the cotyledon itself as an early energy source. Similar results were obtained in *Podocarpus henkelii* (Dodd *et al.,* 1989) and in *Dolichos lablab* (Ramakrishna and Rao, 2005). According to Yomo and Varner (1972), the increased activity of proteolytic enzymes may lead to decrease in storage protein. In the present study also the storage proteins declined in the cotyledons continuously, probably due to the increased activity of the proteolytic enzymes. The histochemical localization of proteins in the cotyledons of cocoa seeds during germination also shows a decrease in the staining intensity (Plate 6; Fig. A-G). These observations are in agreement with the biochemical estimation of proteins during germination.

A close correlation is observed in the metabolic events occurring in the seeds of *Theobroma cacao* during desiccation and those occurring during germination. The recalcitrant seeds are shed at relatively high moisture content, with the metabolic activities occurring at a high rate for a limited period and slowing down subsequently. They are metabolically active and show germination associated changes in storage (Pammenter *et al.,* 1994; Berjak *et al.,* 1990; Pammenter and Berjak, 1999). According to Tompsett and Pritchard (1993), desiccation intolerance in recalcitrant seeds is related to the initiation of germination at or soon after shedding. Germinated seeds no longer have the storage potential and desiccation tolerance (Berjak *et al.,* 2004). Throughout pre and post-shedding development, desiccation sensitivity changes parallel with metabolic rate. Retardation of the metabolic activities in desiccating seeds usually correspond with the stage at which the seeds are rendered non viable, usually corresponding with the critical moisture content. During germination, presence of external water provides continued opportunity to extend the metabolic activities and to complete the process of germination (Finch-Savage, 1992; Finch-Savage and Clay, 1994). Pammenter *et al.* (1994) proposed that stored hydrated recalcitrant seeds lose viability because of water stress of long duration. This stress arises because germinative metabolism in storage generates a requirement for extra water. Differences in storage lifespan are related to differences in rates of germinative metabolism and possibly to the relationship among temperature and water content. The nature of the damage is similar to that occurring as a result of dehydration stress, but during hydrated storage, it takes longer to reach lethal levels. Differences in storage lifespan are related to differences in rates of germinative metabolism and possibly to the relationship among temperature and water content. The nature of the damage is similar to that occurring as a result of dehydration stress, but during hydrated storage, it takes longer to reach lethal levels. As a result of water loss, such ongoing metabolism is suggested to become restricted in a disorganized manner causing derangement of the cytoplasm and organellar aqueous phase, with a consequent loss of stability of subcellular structures (Leprince, 2000). Clatterbuck and Bonner (1985) and Farrant *et al.* (1993b) opined that the usual conditions of storage for recalcitrant seeds allow breakdown of food reserves into soluble and more translocatable forms and the transport of these forms to the site of metabolic activity promoting swift germination and thus resulting in a short post-shedding life span.

The seeds of cocoa are shed at a high moisture content of 37%, a feature characteristic of the recalcitrant seeds. The fresh seeds germinate rapidly and radicle emergence occurs within 2 days of storage / desiccation, indicating active metabolism in the seeds in continuum with that of the developmental stages, a fact supported by analysis of biochemical events in fresh seeds and in seeds desiccating under different storage conditions. Comparison of the metabolic activities taking place in cocoa seeds during desiccation and germination supports the germinative nature of the biochemical events occurring in fresh seeds and a similar pattern of metabolic events are exhibited by seeds desiccating towards a non-viable state.

A rapid decrease in the starch content observed in desiccating seeds, accompanied by an insignificant increase in soluble sugars is indicative of active respiratory metabolism especially in the early stages of desiccation. Similar behaviour of starch during germination corroborates this concept. A rapid decline in the starch and sugar contents in the later stages of desiccation probably deprives the seeds of respiratory substrate leading to loss of viability. The increasing amount of sucrose in the drying seeds suggests a possible role in imparting desiccation tolerance to cocoa seeds but found to exert its effect only in conjunction with the raffinose content. The raffinose content on the other hand declines steadily during desiccation and hence the optimal sucrose to raffinose ratio could be maintained only to a limited period in desiccating seeds, corresponding to their short life span. The availability of these biomolecules varies with the storage conditions and with the rate of moisture loss. An enhanced reducing sugar / monosaccharide content in the initial phases of desiccation, attributed to starch and raffinose hydrolysis, is assumed to contribute to desiccation intolerance through Amadori and Maillard reactions.

The protein content in cocoa seeds desiccating in different storage conditions show proteolysis as is evident from a corresponding increase in free amino acid content. This process is similar to the mobilization of proteins taking place during germination. The active depletion of proteins results in the loss of structural and enzymic proteins leading to loss of viability when the seeds have desiccated beyond the critical moisture content.

The active metabolism early in the desiccation of cocoa seeds results in the generation of free radicals such as superoxides, and hydroxyl radicals. The failure to quench the free radicals due to the sharp decline in the free radical scavenging enzymes like superoxide dismutase, peroxidase and catalase during desiccation in cocoa seeds leads to degradative processes the prominent of which is lipid peroxidation. Lipid peroxidation manifested in the form of enhanced malondialdehyde content causes degeneration of membrane lipids and cellular macromolecules, resulting eventually in altered membrane permeability and enhanced electrolyte leakage. An enigmatic behaviour observed in cocoa seeds is the pronounced increase in the lipid content in the seeds during the initial stages of desiccation. Role of starch and sugars in lipid biogenesis is illuminated in numerous botanical literatures. In cocoa seeds also, the author suggests such a possibility but more attention is required in the matter.

The present study has expounded the physiological and biochemical factors leading to the rapid loss of viability in cocoa seeds in storage / desiccation and has established that the metabolic activities taking place during storage in continuum to the developmental metabolism is germiative in nature. When seeds are harvested / shed, the high moisture content enable the seeds to switch the metabolism associated with seed development into the germinative pathway. As the seeds desiccate, the water available in the seed tissues become limiting and the metabolism is inevitably interrupted and disorganized. This metabolic disarray eventually results in the loss of viability. The turning point in many of the biochemical processes occurring in the desiccating seeds is found to be around the point of critical moisture content. When the fresh seeds are placed for germination in moist conditions, the external water facilitates the completion of germinative metabolism resulting in the emergence of viable cocoa seedlings. Thus, the absence of maturation drying, switching of the developmental metabolism into germinative pathway, the disruption of the germination process due to restricted availability of water in the seed tissues and the consequent cellular and metabolic disarray and are the factors responsible for the recalcitrant nature of cocoa seeds.

Desiccation and Germination Studies of Cocoa (*Theobroma cacao* L.) Seeds - A Physiological and Biochemical Approach

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

By
Abis V. Cherussery

Division of Plant Physiology and Biochemistry
Department of Botany
University of Calicut
Kerala

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SUMMARY AND CONCLUSIONS

The present study attempts to determine the physiological and biochemical changes of cocoa seeds (*Theobroma cacao* L.), known for the recalcitrant nature, in different dehydration regimes. The seeds were extracted from the fresh ripe pods, and stored in three different conditions *viz* open trays at room temperature, polyethylene bags at room temperature and polyethylene bags at 4°C in a refrigerator. Samples from seeds desiccated under different conditions were drawn daily up to 4 days and then at regular intervals of two days until the viability was found to be lost completely. The seeds were used for the estimation of moisture content, germination percentage, seed vigour index and electrical conductivity of the leachates.

The biochemical components of seed tissues of cocoa such as starch, total soluble sugars, reducing sugars, free amino acids, proline, proteins, lipids and total phenolics were extracted and estimated according to standard procedures. Analysis of sugars was also made using HPLC. Lipid peroxidation, and the activities of free radical scavenging enzymes like superoxide dismutase, peroxidase and catalase were assayed. Polyacrylamide gel electrophoresis was used for protein profiling of the seeds.

To determine the mobilization of the reserve materials, cocoa seeds germinated in dark were sampled at specific intervals and metabolites such as total soluble sugars, starch, lipids, proteins and total free amino acids were estimated.

Histochemical localization of important cellular macromolecules like starch and proteins was also carried out during desiccation and seed germination.

The following observations and conclusions were made from the present investigation.

Recalcitrant nature of cocoa seeds is established due to the following characters.

1. The seeds are fleshy, heavier; contain high moisture content (37%) and loss viability when desiccate beyond 20% MC.
2. Rapid germination of seeds immediately after shedding.
3. Short life span of seeds and loss of viability within two days.
4. Germination-associated metabolic changes in seeds under desiccation.

A close correlation is observed between moisture content, viability and seed vigour index during desiccation of cocoa seeds in different storage conditions.

Rapid increase in electrical conductivity of leachates of the seeds under desiccation indicates progressive membrane perturbation under storage and desiccation. The seed vigour index decreases gradually during desiccation, indicating a decline in vigour before the loss of viability.

A steady decrease in starch content during the early stages of desiccation shows the cocoa seeds are in a metabolically active state in the initial phases of desiccation. Further decline coincides with a loss of viability.

The decline in the total soluble sugar content in the later stages of desiccation is ascribed to its utilization during active metabolism. This may result in limited availability of respiratory substrate later on, leading to loss of seed vigour and viability.

The continued loss of seed viability in spite of an increase in sucrose content during desiccation indicates that sucrose alone is not a critical factor in imparting desiccation tolerance to cocoa seeds.

A steady decline in the raffinose content correlates with the desiccation induced loss of seed viability implying that sucrose imparts desiccation tolerance in conjunction with raffinose and that an optimal sucrose to raffinose ratio is critical for it.

An increase in the reducing sugar content is suggested to initiate the Amadori-Maillard reactions in desiccating seeds leading to the damage of cellular macromolecules like DNA and proteins beyond repair.

A decline in protein content with a corresponding increase in free amino acid content indicates progressive proteolysis in desiccating seeds, which results among other things in structural changes of cellular membranes, leading to increased permeability and eventually to the loss of seed viability.

The proline content, even though showing an increase during desiccation is found to be not related to any stress relieving mechanism in desiccating seeds.

A negative correlation can be drawn between seed viability and increasing phenolic content in cocoa seeds. The declining seed viability is attributed to inhibitory effects of increasing phenolic content on phytohormones and primary metabolism.

Lipids constitute 43 % of the dry mass of seeds of cocoa. An increase in the lipid content in desiccating cocoa seeds remains as an enigma. Though a decrease in starch content implies the utilization of starch in lipid biosynthesis, further studies are warranted.

A substantial increase in malondialdehyde content is observed in cocoa seeds as desiccation progresses, indicating the extensive occurrence of lipid peroxidation with moisture loss. The enhanced lipid peroxidation leads to macromolecular damage and membrane perturbation, closely correlated with enhanced loss of seed vigour and viability.

A drastic decline in the activities of free radical scavenging enzymes like superoxide dismutase, peroxidase and catalase results in the failure to quench the superoxides, peroxides and hydroxyl radicals generated during the deranged metabolism of cocoa seeds during storage. This increases the likelihood of free radical attack resulting in reduced seed viability.

Even though germination in cocoa seeds starts immediately, as a continuum of development due to its recalcitrant nature, reserve mobilization is similar to that of orthodox seeds.

The dry weight of the cotyledons was found to decline drastically during germination and seedling growth coinciding with a depletion of soluble sugars, starch and lipids implying active mobilization of metabolites to the growing axis.

A significant reduction in protein content in the cotyledons of germinating seeds is found correlated with an increase in the amount of free amino acids implying active protein degradation and its mobilization to the growing axis.

Histochemical studies corroborate the rapid mobilization of starch and proteins in cocoa seeds during germination and seedling growth.

A parallelism is found to exist between the metabolic events occurring during earlier period of desiccation and germination indicating that germination-associated changes are occurring during desiccation. The processes associated with desiccation are prematurely terminated and viability is lost when water becomes limiting. The unrestricted availability of water during germination permits the germination and reserve mobilization processes to proceed further.

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Division of Plant Physiology and Biochemistry
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