LIPID PEROXIDATION AND THE ANTIOXIDANT SYSTEMS IN SOYBEAN SEED MATURATION AND GERMINATION

By

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"...We will probably be unable to account satisfactorily for nonorthodox seed behavior, particularly that of truly recalcitrant seeds, until complete understanding is gained of the apparently numerous interacting factors that enable desiccation-tolerance to be achieved"

Berjak and Pammenter, 2001

ABSTRACT

The biochemical changes taking place during soybean seed development and germination, and some aspects of desiccation tolerance were assessed with reference to lipid peroxidation and antioxidant systems. During normal seed development, fresh weight and dry weight increased between 20 and 50 days after flowering (DAF), concomitant with the accumulation of triacylglycerols and sugar reserves, after which dry weight remained almost unchanged, and fresh weight decreased. Seed moisture content decreased rapidly during the last stages of development. High levels of lipid peroxidation were evident between 20 and 45 DAF, and decreased thereafter. An examination of antioxidant systems revealed that whereas total glutathione levels accumulated continuously throughout the 80 days of seed development, both dehydroascorbic acid (DHA) reductase and ascorbate free radical (AFR) reductase increased concurrently with the increase in total ascorbate content, and the overall levels did not decrease markedly during maturation drying. Ascorbate peroxidase (ASC POD) activity was high during the period of greatest ascorbate accumulation. Both catalase (CAT) and superoxide dismutase (SOD) activities increased progressively during early seed development (20-40 DAF), but showed variable patterns of change during maturational drying, in marked contrast to ASC POD which declined from 40 DAF to undetectable levels at 70 DAF.

An assessment of the relationship between the antioxidant systems and lipid peroxidation was made during imbibition and germination, as it has been suggested that controlling free radicals was a critical event in early imbibition. Unexpectedly, lipid peroxidation increased progressively in both seeds and isolated axes, and were eight-fold higher at 48 hours of imbibition compared to dry tissues. A progressive, and co-ordinated, increase in CAT, total glutathione, total ascorbate pool, guaiacol POD, ASC POD, and SOD appeared to parallel the rise in lipid peroxidation in both whole seeds and axes. Variable responses were evident between seeds and axes for the enzymes AFR reductase and DHA reductase

In order to gain a further insight into the dynamics of desiccation-tolerance and desiccation-sensitivity, imbibing seeds were subjected to an unscheduled dehydration treatment, and then rehydrated for up to 24 hours. During these hydration-dehydration-rehydration (H-D-R) treatments, changes in lipid peroxidation and antioxidant systems were measured. Concurrent with the loss of viability in the axes of seeds dehydrated after 24 and 36 hours of imbibition, there were increases in both lipid peroxidation and solute leakage. Unscheduled drying was seen to be a critical stage, as intolerant axes showed four- to eight-fold increases in lipid peroxidation, which were only partially reduced on subsequent rehydration. Tolerant axes, on the other hand, were able to maintain low, basal levels of lipid hydroperoxides on drying. The relationship between these observations and the antioxidant systems showed that the antioxidant enzymes CAT, ASC POD, AFR reductase, DHA reductase, guaiacol POD and SOD declined markedly during the unscheduled drying, whereas GSH and ASC declined only slightly. On rehydration, most of the enzymes,

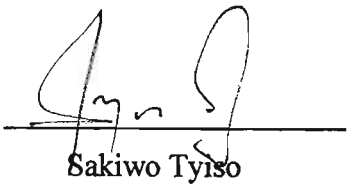
total glutathione, and total ascorbate pool increased, the only exception being the loss of ASC POD activity. DHA reductase, which was seen to decrease as a part of normal germination, declined progressively also in H-D-R treatments. These results suggested that loss of viability was not attributable to a decline of the antioxidant systems but rather to the combined deleterious effects of increased lipid peroxidation, and a generalized and moderately compromised antioxidant system.

These studies have indicated that the occurrence of lipid peroxidation can be seen as a normal part of seed development and germination. The H-D-R studies, on the other hand, supported the concept that the balance between peroxidation reactions and the protective systems was critical to the development of desiccation tolerance.

PREFACE

The research work described in this thesis was carried out in the Biochemical Research Laboratory, School of Life and Environmental Sciences, University of Natal, Durban, from August 1998 to September 2003, under the supervision of Professor M.T. Smith (University of Natal).

The study represents original work by author and has not, otherwise, been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work done by others, it has been duly acknowledged in the text.



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ABBREVIATIONS

ABS	absorbance
AFR	ascorbate free radical
AOS	active oxygen species
ASC POD	ascorbate peroxidase
ASC	ascorbate (ascorbic acid)
$BaCl_2$	barium chloride
BSA	bovine serum albumin
CAT	catalase
DAF	days after flowering
DHA	dehydroascorbic acid
DPG	diphosphatidylglycerol
DTT	dithiothrietol
DW	dry weight
EDTA	ethylenediamine tetra-acetic acid
Fe_2SO_4	ferrous sulphate
$FeCl_2$	ferrous chloride
$FeCl_3$	ferric chloride
FW	fresh weight
g	gram
GC	gas chromatography
GSH	reduced glutathione
GSSG	oxidised glutathione
HCl	hydrochloric acid
H-D-R	hydration-dehydration-rehydration
KCl	potassium chloride
KSCN	potassium thiocyanate
LOOH	lipid hydroperoxides
µM	micromole
M	mole
MC	moisture content
mM	millimole
nmol	nanomole
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)

NaOH	sodium hydroxide
NBT	nitro blue tetrazolium chloride
NEM	N-ethylmaleimide
P	phosphorus
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PL	phospholipid
PM	physiological maturity
POD	peroxidase
PS	phosphatidylserine
PVP	polyvinylpolypyrrolidone
RH	relative humidity
ROS	reactive oxygen species
SE	standard error
SOD	superoxide dismutase
TAG	triacylglycerol
TEMED	N,N,N,N-tetramethyl-ethylendiamine
TLC	thin layer chromatography
TRIS	tris-(hydroxymethyl)methylamine
v/v	volume by volume

Chapter 1

1.1 INTRODUCTION

Three phases of seed development have been identified in crop species. These include rapid cell division, active biosynthesis of reserve material leading to a rapid increase in seed fresh and dry weight, and seed maturation when dry weight accumulation ceases and fresh weight declines markedly (Adams and Rinne, 1980; Ellis *et al.,* 1987; Welbaum and Bradford, 1988). The pattern of dry weight accumulation is considerably influenced by the seed water status during development (Adams and Rinne, 1980). In many orthodox seeds seed maximum dry weight occurs soon after the mid-point of development and is preceded by a cessation of net increase in the amount of water per seed (Ellis *et al.*, 1987). The transition from the second to the third phase is marked by the achievement of maximum dry weight and coincides approximately with the acquisition of the ability to be dried rapidly without severe loss of viability (Kermode and Bewley, 1985; Ellis *et al.*, 1987; Welbaum and Bradford, 1988). This last phase, maturation drying (dessication) is characterised by a general reduction in metabolism as water is lost from the seed, which then passes through a quiescent, and sometimes dormant, state.

It is well-documented that soybean seed composition changes during development. Dry weight, protein, oil and fatty acid content increase during soybean seed development while moisture percentage decreases (Rubel *et al.,* 1972). Investigations into the complete chemical composition of mature soybean seed has revealed the seed to contain approximately 40% protein, 20% oil, 17% cellulose and hemicellulose, 7% sugar, 5% crude fibre, and about 6% ash on a dry weight basis (Bils and Howell, 1963; Rubel *et al.,* 1972). The concentration of protein in the seed is constant during most of the development. However, synthesis of the individual storage proteins varies with stage of development (Yazdi-Samadi *et al.,* 1977; Wilson, 1987). The oil concentration increases during early development and reaches maximum level before physiological maturity (Yazdi-Samadi *et al.,* 1977). Starch reaches maximum levels near podfill before declining to low levels during seed maturation (Yazdi-Samadi *et al.*, 1977; Wilson, 1987). Because protein, oil and starch comprise nearly 70% of the seed dry weight, a clear understanding of when and how these components accumulate is necessary.

Cell division and growth are processes that take place early during seed formation, leading to morphogenetic events in the embryo and endosperm. These and other structures mature as seeds develop while seed reserve formation and deposition proceed simultaneously. Several weeks elapse from the end of the proliferation stage during embryogenesis until seed maturation. Imbibition of water by mature seeds

will trigger the germination process, during which the cells in the different soybean tissue will be reactivated and thus re-establishing metabolism, ultimately resulting in radicle protrusion and the end of the germination process (Bewley and Black, 1986). Respiration and metabolism of reserves are fundamental for germination, implying that organelles, such as mitochondria, are also activated (Bewley and Black, 1994; Bewley, 1997). Synthesis of macromolecules, such as proteins and nucleic acids, can be observed within minutes after imbibition, increasing in rate as germination advances (Bewley, 1997), and leading to renewed cell expansion and cell division as the seedling becomes established.

During tissue-regulated loss of water at seed maturation on the mother plant, several metabolic processes are curtailed, and the seed becomes quiescent. These seeds are able to resume the germination process upon wetting (Bewley, 1997), and are at first undamaged by any subsequent drying (Boubriak *et al.*, 1997). This remains so until a certain critical stage in the germination programme is reached, after which dehydration leads to death of the embryo. At this stage the seed converts from desiccation-tolerance to desiccation-intolerance (Osborne and Boubriak, 1994). Dehydrated, desiccation-sensitive tissues leak almost all of their cytoplasmic solutes when they are rehydrated (Simon, 1974). This points to the inability of the plasma membranes to cope with dehydration. In desiccation-tolerant tissues, membranes remain intact on dehydration and subsequent rehydration (Crowe *et al.*, 1986). Apparently, there is a mechanism in such organisms which protects membrane structure from the dehydration-rehydration process.

Linolenic acid (18:3) accounts for about 7 to 9% of the fatty acids in many cultivated crops of soybeans [*Glycine max* (L.) Merr.] while linoleic acid (18:2) accounts for 55 to 59%. The latter two unsaturated fatty acids, and particularly 18:3, are prone to oxidation (Priestley, 1986) producing highly reactive free radical intermediates and their by-products, and an increase the amounts of free fatty acids (Priestley, 1986; McKersie *et al.*, 1988). While lipids, especially membrane lipids, are particularly susceptible to peroxidative attack, the by-products of these reactions also affect protein and nucleic acid function (Gardner, 1979; Wolff *et al.*, 1986). Alteration of membrane structure could lead to membrane fusion, loss of compartmentalisation, and irreversible membrane disorganisation (Simon, 1974; Priestley, 1986).

Susceptibility to peroxidation may increase with drying (Bewley, 1979; LePrince *et al.*, 1990; McKersie *et al.*, 1988; Dhindsa, 1991; Hendry *et al.*, 1992) and subsequent rehydration (Cakmak *et al.*, 1993; Smith and Berjak, 1995). This implies that mechanisms for preventing loss of membrane and thus cytoplasmic integrity, as well as protectants against free radical oxidation damage of lipids should be an important part of seed development and germination. High levels of non-reducing sugars have been closely linked with maintenance of the bilayer structure of the membranes (Horbowicz and Obendorf, 1994). Reports on changes in activity and importance of free radical scavenging antioxidants and enzymes with seed development and germination, on the other hand, are variable (Puntarulo *et al.*, 1988; LePrince *et al.*, 1990; Arrigoni *et al.*, 1992; Cakmak *et al.*, 1993). However, there appears to be a general trend towards

increasing enzyme-scavenging systems with increasing mitochondrial activity. The capacity of the seed to control peroxidative reactions may be correlated with vigour. An inability to eliminate hydroperoxides (product of peroxidative reactions) and so quench further autocatalytic peroxidation may lead to a steady increase in membrane peroxidation, and cellular compartmentalisation may break down. This may then lead to a massive surge in peroxidation and to viability loss.

Quite naturally, seed formation has receive enormous amount of attention from an anatomical and morphological standpoint. The fascinating sequence of cell positioning that brings about embryo sac formation, fertilisation, and the early divisions of the embryo and the endosperm cells have been well-defined and documented for many species. In many cases the anatomical and morphological descriptions have thoroughly covered the entire process of seed formation for individual species. However, the biochemical and physiological changes associated with the establishment of a viable seed still remain unresolved.

The aim of this study was to examine the biochemical and physiological changes associated with seed development, normal imbibition and also those taking place during hydration-dehydration-rehydration (H-D-R) cycles. It was envisaged that the establishment of such biochemical and physiological data could provide information on factors influencing seed viability and/or vigor, characterise aspects of desiccation-tolerance and desiccation-sensitivity, and contribute towards an understanding the possible mechanisms operating during seed priming.

Chapter 2

LITERATURE REVIEW

2.1 Stages of seed development

Development of the angiosperm seed can be divided into three stages. Initially, during histodifferentiation, the single-celled zygote undergoes extensive mitotic divisions, and the resultant cells differentiate to form the basic body plan of the embryo, namely the axis and cotyledons. The final cell number of the embryo is thought to be reached rather early in its ontogeny, and the subsequent increase in mass is the result of cell expansion and the concomitant deposition of reserves. This marks the second stage of development, or maturation. The cotyledons serve as a gigantic "sump" for the accumulation of proteins, oil and sugars, and ultimately occupies the bulk of the seed mass. Desiccation completes the process of seed development and is characterised by the gradual reduction in metabolism, as water is lost from the seed tissue. At this point, the embryo passes into a metabolically inactive (quiescent), or sometimes dormant, state.

2.2 Compositional changes during seed development

Several reports agree that the increase in fresh and dry weight of developing soybean seeds is largely due a deposition of protein and oil in the cotyledons (Bils and Howell, 1963; Rubel *et al.,* 1972; Dornbos and McDonald, 1986). At the earliest sampling times (lag phase), oil is less than 5% of the dry weight of the seed, and represents less than 1% of the total oil of the mature seed (Bils and Howell, 1963). In contrast, protein constitutes about 30% of the seed dry weight at the same period, and represents about 2% of the total protein in mature seeds. The protein synthesised during the lag phase of soybean seed development is termed "metabolic protein", and not storage protein (Bils and Howell, 1963). At this stage, monosaccharides (glucose, fructose, raffinose) are the predominant carbohydrate component, although sucrose shows a steady accumulation.

The rate of oil and protein accumulation increases during the growth phase of seed development in most soybean cultivars (Egli, 1975, 1994). Similarly, it is during this phase of development that the cell volume increases to about 10-fold, whereas the dry weight of the cotyledons increases to about 25-fold (Bils and Howell, 1963). The growth phase usually begins around 20 to 27 days after flowering (DAF), depending on the cultivar, and ends between 40 and 55 DAF (Fehr *et al.,* 1971; Dornbos and McDonald, 1986). An oil increase of about 30% of the total oil of mature seeds is usually synthesised during the first 20 days of the

growth phase (Bils and Howell, 1963). In three cultivars of soybean, "Acme", "Chippewa" and "Harosoy 63", oil content was found to increase from 3.5 to 20.1% in 16 days of the growth period (Rubel *et al.*, 1972). Similarly, during the same period, a change in the percent fatty acid composition of the oil was observed.

Normally soybean oil consists of 10-12% palmitic acid, 3-5% stearic acid, 25-26% oleic acid, 48-52% linoleic acid, and 5-8% linolenic acid (Rubel *et al.*, 1972). Fehr *et al.* (1971) reported a change in palmitic acid from 19 to 10% and stearic acid decreased from 8 to 3% during the 20 days of the growth phase. Oleic acid and linoleic acid, on the other hand, increased from 7 to 25 % and 35 to 50%, respectively, while linolenic acid decreased from 30% to about 9% during the same period (Fehr *et al.*, 1971). About 30% of the total protein in mature seeds is synthesised during the first 16-20 days of the growth period, while the remaining 70% of the total protein and oil found in mature seeds is synthesised or deposited during the remaining days of the growth phase, depending on the growing season, and the variety (Fehr *et al.*, 1971). The levels of sucrose reach a maximum near the midpod fill, before declining slowly during maturation. At the same time, the monosaccharides decreased abruptly during late growth phase, and only traces of these carbohydrates are detected.

Dehydration and maturation are completed during the final phase of seed growth. A rapid accumulation of raffinose and stachyose (polysaccharides) is observed and reaches a maximum at maturity (Lowell and Kuo, 1989). It is also during this period that the moisture content and respiration rates of the mature seed are quite low (Bils and Howell, 1963). It is postulated that the starch deposited during early development serves as a temporary storage carbohydrate, supporting lipid, protein, and other syntheses in the cotyledons. After the supply of carbohydrates from the leaves is reduced or terminated by leaf yellowing, synthetic activities consume the remaining starch so that, ordinarily, none is present in mature seeds (Bils and Howell, 1963).

2.3 Oil accumulation and composition

Generally, a triphasic pattern of oil deposition has been established from flowering to seed maturity during seed development (Gurr *et al.*, 1972; Norton and Harris, 1975). During the first phase, after flowering, only traces of triacylglycerols (triglycerides; TAGs) are present in the seed or fruit tissue and the structural membrane lipids (phospholipids and glycolipids) are the main lipid constituents. The overall fatty acid composition is typically similar to that of vegetative tissue of the plant. The second phase marks to the onset of oil deposition and the gradual accumulation of TAGs during a stage of more active seed growth. In parallel, the amount, and proportion of the storage oil fatty acids increases dramatically. During this period phospholipids (PLs) and glycolipids are also synthesised, though to a much lesser extent. By the end of the second phase, TAGs are the predominant lipids. Phospholipids and glycolipids account for less than 10% of

the total lipid content and the overall fatty acid composition mostly reflects that of TAGs. Finally, lipid accumulation ceases during the third phase of development leading to seed maturity. The dry weight does not increase significantly, and the moisture content decreases progressively.

At maturity the total lipid composition of soybean seeds consists of three fractions. The neutral lipid group, which makes up 92% of the total lipids at maturity, is made up triglycerides (TAGs), and sterols. The remaining fraction of the total lipids is made up of glycolipids (0.6%) and phospholipids (ca. 7%). The TAG: PL ratio may be as high as 9:1 in oily seeds such as soybeans (Privett *et al.*, 1973). In other legumes such as pea, lentils and broad bean, where total extractable lipids are in the range 1 to 2% (Ponquett *et al.*, 1992), the TAG: PL ratio is heavily biased in favour of membrane lipids. There is little information on whether on not there are differences in the autoxidative potential of TAGs and PLs, although the latter present a greater surface area, and thus greater susceptibility to peroxidation (Wilson and McDonald, 1986).

Seeds store TAGs as food reserves for germination and postgerminative growth of the seedlings. The TAGs are present in small, discrete intracellular organelles called oil bodies (Yatsu and Jacks, 1972; Appelqvist, 1975; Huang, 1992). In addition to TAGs, oil bodies also contain small amounts of phospholipids and proteins called oleosins. It is generally agreed that the oily body has a matrix of TAGs surrounded by a layer of phospholipids embedded with oleosins. The phospholipids form a monolayer such that the acyl moieties of the molecules face inwards to interact with the hydrophobic TAG in the matrix, and the hydrophylic phospholipid head groups are exposed to the cytosol (Miquel and Browse, 1995).

2.4 Phospholipid composition

Lipid composition analysis on higher plant plasma membranes has revealed that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major phospholipid classes (Gronewald *et al.*, 1982; Yoshida and Uemura, 1984). Reports differ, however, as to which of these two phospholipid species constitutes the majority of lipids in the plant membrane. In both meristematic and mature soybean roots, PC comprises approximately 50% of membrane phospholipids (Whitman and Travis, 1985). On the other hand, studies on potato tuber and oat root have reported PE to be the major phospholipid component of the plasma membrane (Mazliak, 1977; Liljenberg and Kates, 1982). This basic difference in membrane composition may reflect genotypic variation or may be the result of endogenous lipases (Scherer and Morre, 1978). High endogenous lipolytic enzymes such as phospholipase D and phosphatidic acid phosphatase (Scherer and Morre, 1978) may significantly alter the composition of recoverable phospholipids. The minor phospholipid constituents include phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidyl-glycerol (PG) and diphosphatidylglycerol (DPG) (Whitman and Travis, 1985).

The functional properties of membranes including fluidity, permeability, protein activity and the structural configuration that the lipid assumes under a given set of conditions is influenced by its chemical composition (Cullis and deKruijff, 1979). The major phospholipid classes in the membrane are thought to confer stability to the bulk of the membranes in the bilayer. Minor lipids, on the other hand, have been speculated to play a role in providing special environments for membrane proteins (Quinn and Williams, 1978). Recent studies indicate that the variety of phospholipids in biological membranes may be present to reduce the permeability of the membranes at the protein-rich boundaries (Van der Steen *et al.*, 1982). This, therefore, implies that the phospholipid composition of the membrane should change during cell differentiation with the protein complement (Booz and Travis, 1980) in order to maintain membrane integrity.

The fatty acids in phospholipids are normally enriched in palmitic, stearic, oleic, linoleic and linolenic acids. Oleic, linoleic and linolenic acids comprise the unsaturated fatty acid component of lipids and account for 60-70% of the membrane fatty acids (Whitman and Travis, 1985). A trend towards increasing desaturation (unsaturation) of plasma membrane fatty acids is normally encountered as seed develops (Fehr *et al.*, 1971; Dornbos and McDonald, 1986). Increasing desaturation of the acyl chains within the membrane may be associated with development-related increases in membrane fluidity (Whitman and Travis, 1985).

2.5 Physiological maturity

Physiological maturity (PM) is normally defined as occurring when the seed reaches its maximum dry weight (Harrington, 1972). Seed scientists generally agree with this definition, but contend that PM should also represent maximum viability and vigour of planting seed (Knittle and Burris, 1976; Delouche, 1974). However, in other species, maximum seed quality is reached sometime after attaining maximum dry weight, as has been reported for *Vicia faba* L. and *Lens culinaris* Medik. (Ellis *et al.*, 1987), *Hordeum vulgare* L. (Pieta *et al.*, 1991; Ellis and Pieta-Filho, 1992), and *Lycopersicon esculentum* Mill. (Demir and Ellis, 1992). The introduction of the term "mass maturity" by Ellis *et al.* (1992) to distinguish the time of maximum dry weight accumulation, from that of physiological maturity might be important for distinguishing harvesting time for seeds to be sown, from those to be consumed. They proposed a model to illustrate that maximum dry weight (mass maturity) precedes maximum viability (physiological maturity). This is in contrast to the hypothesis of Harrington (1972) who postulated that maximum viability in seeds generally coincides with maximum dry weight attainment. Aiazzi *et al.* (1998) have also shown with seeds of *Atriplex cordobensis* that physiological maturity (measured as maximum viability) is attained over a month after maximum dry weight accumulation or mass maturity is completed.

Attempts to describe PM in soybeans have relied primarily on pod and leaf characteristics. TeKrony *et al.* (1979) defined PM as occurring when the pods were yellowing and 50% of the leaves were yellow. A similar definition was given by Fehr *et al.* (1971), who found PM to occur at reproductive (growth) stage R7. Major *et al.* (1975) defined the occurrence of PM when 75% of the leaves had senesesced. In contrast, Fehr *et al.* (1977) found yield reduction to occur when plants were defoliated at R7, thus indicating that PM had not been attained. Harrington (1972) stated that at PM nutrients were no longer flowing into the seed from the mother plant. Precise, rapid determination of physiological maturity (PM) is of interest because it permits an accurate measure of grain filling period or the time factor of yield.

Nkang and Umoh (1996) have noticed significant differences in the germinability of stored soybean seeds harvested from different stages of development. They employed three maturity stages: physiological maturity (pods beginning to yellow); agronomic maturity (pods completely brown and dry); late harvest (two weeks after agronomic maturity). Seeds harvested at agronomic maturity had the highest germination percent while seeds harvested at physiological maturity had the lowest. They concluded that harvesting at agronomic maturity rather than at physiological maturity gave better quality seeds for subsequent storage. Similarly, Pieta *et al.* (1991) reported that further increases in longevity occur after physiological maturity has been reached. These results are, however, incompatible with reports (Harrington, 1972; Chamma *et al.*, 1990) that seeds attain maximum longevity at physiological maturity, and thereafter viability and vigour may decline. At physiological maturity, moisture content in soybean seeds is about 50% while ideally it should be 15% or less to permit storage without immediate drying (Kelly, 1988). At agronomic maturity, seed moisture is further reduced through maturation drying to levels more suitable for seed storage. Delayed harvesting, by as little as two weeks after agronomic maturity, can cause significant reductions in the germination percentages (Nkang and Umoh, 1996).

2.6 Maturation drying

During seed development, seed water content increases in parallel with dry weight, but reaches a maximum level and begins to decline before the seed reaches physiological maturity (Fraser *et al.*, 1982). The water content in the seed is initially high and declines steadily to a relatively constant level at physiological maturity (TeKrony *et al.*, 1979). That drying is important for the acquisition of germinability has been well-documented for cereals (Mitchell *et al.*, 1978). As early as 1852, Ducharte noted the beneficial effects of drying upon subsequent germination of rye, wheat, and barley grains. Harlan and Pope (1922) showed that barley will germinate at 5 days post-anthesis, if dried *in situ* on the straw. Similar observations were made that immature wheat grains will germinate after drying (King, 1976). In fact, air-dried grains of wheat not only germinate at an earlier stage of development than nondried grains, but at later stages they also germinate at a faster rate than their non-dried counterparts (Kermode and Bewley, 1985).

2.7 Development of seed quality

Soybean seed quality is highly variable across location and years, indicating that environmental conditions have a significant effect on seed quality during production. Environmental conditions can influence final seed quality during development, desiccation, or after harvest maturity when the seed is essentially in storage in the field (TeKrony *et al.*, 1987). Thus, high germinability and seedling vigor are important attributes of soybean seed quality (Kiegley and Mullen, 1986) and are affected by environmental conditions during seed development and maturation (TeKrony *et al.*, 1987).

a) Temperature

The effects of temperature on seed development and maturation have been demonstrated by a number of workers. Green *et al.* (1965) reported that seed produced from later dates of planting and which reached maturity after hot, dry weather generally exhibited higher germination and field emergence than seed, which matured during hot, dry weather. Gibson and Mullen (1996) reported similar results. They attributed this to the influence of high temperatures on seed filling. Similarly, TeKrony *et al.* (1979) attributed lower initial germination and vigor at harvest maturity to high temperatures during the period from physiological maturity to harvest maturity.

A consideration of both day and night temperatures is also critical. Gibson and Mullen (1996) evaluated the cultivar Gnome for different day and night temperature combinations, at different reproductive growth stages. They found that the period of seed fill (R5 to R8) to be most sensitive to temperature changes, and increasing either day or night temperature resulted in reduced germination and vigor. The interaction of day and night temperatures significantly impacted on the percentage of abnormal seedlings when the higher temperature treatments were imposed during the R5 to R8 period (Gibson and Mullen, 1996). These authors also noted that high temperatures during seed fill, and the entire reproductive period, decreased soybean seed viability and vigor when measured by accelerated aging, seedling growth rate, and conductivity of leachates. Kiegley and Mullen (1986) found that increasing the temperature from 27/20° C to 32/28° C during seed fill decreased germination percentage of soybean seeds by as much as 34%. However, increasing only day temperatures from 29/20° C to 35/20° C resulted in only a 12% decrease in germination (Dornbos and Mullen, 1991). These clearly indicate that differences in germination are impacted upon by differences in night temperatures.

b) Relative humidity

Under dry conditions or low relative humidity (RH) transpiration rate is high and, conversely, under conditions of high RH transpiration is reduced (Keiser and Mullen, 1993). At high temperature, or low RH,

plants usually experience water stress due to high soil and/or plant evapotranspiration, causing the water vapour pressure deficit (WVPD) to increase. The literature suggests that a combination of temperature and WVPD determine the final effect of environmental factors on seed viability. Potts *et al.* (1978), investigating factors influencing seed deterioration, found that seeds maturing under excessively moist environmental conditions tend to be more susceptible to field weathering. Mechanical damage during harvest was a symptom of seeds maturing under excessively dry conditions.

Dornbos and Mullen (1991) reported that germination percentage and vigor of harvested seeds was reduced by water stress. They found seed weight, germination and seedling growth to be highly, and positively, correlated with plant available water. Drought stress was found to reduce germination by 5%, reduce seedling axis dry weight by 12% and increase single-seed conductivity by 19% (Dornbos *et al.*, 1989). This observation could be explained by the fact that seeds submitted to heat and drought stress are smaller, immature, or even shriveled, and the occurrence of such symptoms relates to the level of stress (Green *et al.*, 1965; Dornbos *et al.*, 1989).

2.8 Seed germination

Imbibition is an essential process initiating seed germination. It is the first key event in the transition of the seed from a dry, quiescent, or dormant state to one of embryo growth. Subsequent to imbibition, an orderly transition of increasing hydration, coupled with increased enzyme activity, storage product breakdown, and resumption of seedling growth occurs. Also, there is reactivation of existing metabolic systems, supplemented by synthesis of new components, which lead to renewed cell expansion and cell division as the seedling becomes established (Bewley, 1997). Mobilisation of reserves is an essential component of postgerminative growth (Bewley and Black, 1994).

Water uptake during seed imbibition is a three-phase process. The initial rapid water uptake phase (phase 1) is associated with rapid increase in oxygen uptake and respiratory activity supporting ATP synthesis (Bewley and Black, 1986; Bewley, 1997). Also of note, during phase 1 is the resumption of protein synthesis from residual mRNAs from previous developmental processes (Lane, 1991), and the possible repair to mitochondria, which become poorly differentiated as a consequence of maturation drying (Bewley, 1997). Phase 2 of water uptake is also referred to as a lag phase since no further increase or change in water content is observed (Bewley and Black, 1994; Bradford, 1995). Apart from some of the activities that overrun from phase 1, mitochondrial synthesis, and protein synthesis from new mRNAs are the main features of phase 2 (Bewley, 1997). A further increase in water uptake is one of the main features associated with phase 3 of imbibition. Also associated with this phase are radicle elongation, cell division and DNA synthesis and reserve mobilisation (Bewley and Black, 1994; Bewley, 1997). The replication of DNA is often delayed until phase 3 (Bray, 1979, 1995) to allow for all repairs that are thought to be

operative during the first few hours of germination to be completed prior to resumption of growth processes (Osborne, 1983).

Seeds are desiccation tolerant during phases 1 and 2, but frequently become intolerant during phase 3. For example, soybean seeds can be imbibed for 6 hours, and dehydrated to 10% moisture without loss of seed viability or vigor. If the same seeds are imbibed for 36 hours, at which time the radicle is beginning to emerge from the seed coat, and then dehydrated to 10% moisture, seed viability is lost (Senaratna and McKersie, 1983). The ability of the plant tissue to tolerate dehydration is thought to reflect the inherent protoplasmic properties of these tissues (Bewley, 1979) and in seeds, the protoplasmic properties which impart tolerance are presumably lost as the seed germinates. The loss of tolerance has been associated with the initiation of cell elongation and hydration of vacuoles (Hegarty, 1978), onset of DNA replication and RNA transcription, and changes in cellular membranes (McKersie and Stinson, 1980).

2.9 Pre-hydration treatments (priming)

Pre-sowing hydration treatments of seeds is a widely used technique to enhance seed performance, with respect to rate and uniformity of germination, thereby enabling better crop establishment (Hegarty, 1978; Taylor *et al.,* 1998). The basis of this technique is that seed water uptake during germination follows a triphasic pattern with an initial rapid imbibition phase (phase 1), followed by a lag period (phase 2) and finally by a second uptake phase associate with start of seedling growth (phase 3) (Come and Thevenot, 1982). Since all preliminary processes for germination are presumed to take place during priming (Heydecker and Coolbear, 1978), the objective of priming is to perform a controlled water uptake by the seed up to the end of phase 2, before the radicle protrusion from the seed coat. Furthermore, since most seeds are desiccation-tolerant up to this developmental stage, drying back can arrest the germination process. The major problem encountered in seed priming is to control seed imbibition to a level permitting pre-germinative processes to proceed, but to block radicle emergence. Otherwise, the consequence of drying back might be the total loss of viability.

The beneficial effect of osmopriming is associated with various biochemical, cellular and molecular events (Karssen *et al.,* 1989; Bray, 1995). Most of the studies have dealt with changes in nucleic acid and protein synthesis (Bray, 1995), and the induction of the cell cycle (Lanteri *et al.,* 1994; Ozbingol *et al.,* 1997) during priming. The respiratory activity of the seeds is also enhanced by priming during the first hours of subsequent rehydration in water (Haplin-Ingham and Sundstrom, 1992; Chojnowski *et al.,* 1997), and the ATP levels are increased (Mazor *et al.,* 1984). Various studies have shown that the respiratory activity of seeds is high during priming (Fu *et al.,* 1988; Smok *et al.,* 1993; Dahal *et al.,* 1996). Oxygen uptake measured before radicle protrusion is highly correlated with subsequent germination rate in pea (Carver and

Matthews, 1975) and pepper (Haplin-Ingham and Sundstrom, 1992), but is not a good indicator of the germination ability in other species (Come and Corbineau, 1989).

2.10 The nature of desiccation stress

One of the unique features of a seed is its ability to withstand drying to very low moisture contents. During the development of orthodox seeds, a phase of slow desiccation is usually followed by one of rapid desiccation (Hong and Ellis, 1990). Although orthodox seeds are tolerant of slow dehydration early in development, tolerance to rapid desiccation is achieved comparatively late in development (Hong and Ellis, 1990).

a) Metabolism-derived stress

The respiratory pathway consists of various reactions, which respond differently to water content (Leprince and Hoekstra, 1998; Leprince *et al.,* 2000). Such differences in response to water stress among, and within, metabolic pathways can lead to imbalances in metabolism. Similarly, continued respiration, when other metabolic processes are shut off, may result in the accumulation of high energy intermediates that leak out of the mitochondria and form reactive oxygen species (ROS) or free radicals (Puntarulo *et al.,* 1991; Hendry, 1993; Foyer *et al.,* 1994; Halliwell and Gutteridge, 1999). Free radicals react with proteins, lipids and nucleic acids, causing permanent damage to enzymes (Wolff *et al.,* 1986; Dean *et al.,* 1993) and membranes (Seneratna and McKersie, 1983; Finch-Savage *et al.*, 1996; Leprince *et al.*, 2000). Peroxidation of lipids decreases the fluidity within membranes (McKersie *et al.*, 1988, 1989), interfering with their selective permeability upon rehydration.

Tissue drying requires that there be a controlled shutdown of metabolism to ameliorate the consequences of unbalanced metabolism (Leprince *et al.*, 2000; Vertucci and Farrant, 1995). This is especially true for cells with more organelles, and greater definition of organelle structure, which appear to be more sensitive to desiccation (Berjak *et al.*, 1990; Farrant *et al.*, 1997). Also, conditions that reduce metabolism such as low temperature (Leprince *et al.,* 1995) or highly complex substrates (Leprince *et al.*, 1990) also tend to reduce sensitivity to desiccation. Desiccation-sensitive cells respire at comparatively greater rates than tolerant cells at the same water content (Leprince *et al.*, 1999; Walters *et al.*, 2001), which may reflect properties of the mitochondria, or of the cellular matrix. It has been suggested that changes in viscosity with dehydration are not as marked in desiccation-sensitive cells, and so metabolism is not as restricted (Leprince and Hoekstra, 1998). It has also been suggested that the packing of macromolecules during dehydration of desiccation-sensitive cells is not as dense (Wolkers *et al.*, 1998), and this might facilitate the diffusion of oxygen through the cell matrix.

b) The drying regime

The rate at which water is removed from the tissue before reaching dryness is an unavoidable aspect of desiccation tolerance experiments (Ellis *et al.*, 1987; Kermode *et al.*, 1989), yet is often overlooked. In some recalcitrant seeds, expression of desiccation tolerance is also dependent on drying rates. Pammenter *et al.* (1991) have remarked that dehydration to low moisture contents by 'flash drying' (40 minutes) could be attained without dramatic loss of viability, while a slower rate of dehydration (2 days) caused lethal damage at the same water content. The approach of mimicking physiological maturation and desiccation, in which desiccation tolerance is progressively attained, is acknowledged. However, it is believed that rapid drying is a more satisfactory way to quantify the degree of desiccation tolerance at a specific developmental stage. The reason for this is that seeds dried slowly over several days will continue to metabolise, or grow before reaching the critical water content (Pammenter *et al.*, 1991). As such, the acquired increase in desiccation tolerance might have taken place during the actual drying process. Hence, the subsequent measurement of desiccation tolerance will not necessarily correspond to the developmental stage before the onset of slow drying.

2.11 Desiccation injury

The number of different stresses that can be associated with removal of water from cells can be attributed to the multiple roles that water plays to support life. Water plays a structural role, *i.e.* at a cellular scale, water fills spaces and provide turgor. At a molecular scale, water provides hydrophilic and hydrophobic associations and controls intermolecular distances that determine the conformation of proteins, polar lipids, and the partitioning of molecules within organelles. With water present, reactive surfaces of metals or molecules are not exposed, and this limits reactivity among molecules. Water also plays a role in controlling metabolism, as it is a reactant and product of many reactions. As a diluent, water affects the chemical potential of other molecules and as a result changes the likelihood of reactions. Changes in water availability affect viscosity of the matrix and the overall mobility of dissolved and suspended molecules. The drier the medium becomes, the more viscous it becomes, until it is essentially a solid matrix trapping molecules (Slade and Levine, 1991; Leopold *et al.*, 1994; Buitink *et al.*, 1998; Wolfe and Bryant, 1999). As one would expect from all the roles of water, there would be a number of strains that the tissue undergoes when water is removed.

The loss of water during drying induces several changes in the cellular environment (Wolfe and Bryant, 1999) such as: (1) reduced hydration of macromolecules and consequent conformational changes; (2) reduced cytoplasmic and intracellular transport; (3) shift in the cytoplasmic pH and ionic concentrations; and (4) accumulation of organic and inorganic ions. All, or any, of these changes might be expected to cause transient dysfunction in specific enzymes and/or electron transport chains (Leprince *et al.*, 1990,

1992). Such dysfunction may lead to the production of free radicals, or promote chemical reactions which would not normally occur in fully hydrated systems. Under normal metabolic conditions, free radicals would be scavenged by enzymatic mechanisms such as superoxide dismutase (SOD) (Halliwell, 1987; Smirnoff, 1993), but because of the reduced hydration, these enzymes may be reduced, or not be able to function. The likelihood that free radicals are produced by the electron transport chain, and possibly by membrane-bound enzymes implies that they may be produced in the membrane, thus finding the ester linkage of phospholipids a convenient reaction site (Wilson and McDonald, 1986; Winston, 1990). The free radical-mediated de-esterification of phospholipids is self-perpetuating and may even be amplified with time, so that the reactions continue, once started, until two radicals conjugate, thereby pairing the electrons, or until the radical reacts with a scavenger (Winston, 1990).

2.12 Oxidative processes and lipid peroxidation

In seed tissues hydrogen peroxide and superoxide are produced in the mitochondria. Concentrations of these reactive oxygen species have been found to increase during germination in association with an increase in respiration rates (Puntarulo *et al.*, 1991; Simontacchi and Puntarulo, 1992), but direct evidence of desiccation-induced superoxide formation in relation to the loss of desiccation tolerance is lacking. In germinating maize seeds, Leprince *et al.* (1992) showed a negative correlation between respiration rates and desiccation tolerance, and a positive correlation between respiration and accumulation of stable free radicals. They suggested that the development of respiration in the mitochondria could contribute to the loss of desiccation tolerance. Desiccation of actively respiring mitochondria may divert the transport of electrons in the respiratory chain to initiate peroxidative damage to membranes. In dried, intolerant radicles, the impairment of the mitochondrial electron transport became evident (Leprince *et al.*, 1992) and the respiratory capability was lost, in marked contrast to desiccation-tolerant tissues in which oxygen uptake was resumed upon rehydration

Free radical-mediated phospholipid degradation is commonly thought to involve peroxidation of unsaturated fatty acids (Wilson and McDonald, 1986). The process can be initiated by the abstraction of hydrogen by a hydroxyl radical from a methylene group, forming a conjugated diene. The superoxide radical is too reactive, and too polar to abstract hydrogen from lipids (Smirnoff, 1993). The conjugated diene reacts readily with oxygen to form a peroxyl radical ($ROO^{\cdot}$), which can abstract hydrogen from another unsaturated fatty acid, thus initiating a chain reaction of lipid peroxidation (Wilson and McDonald, 1986). These reactions result in the formation of lipid peroxides (ROOH), oxygenated fatty acids, and more free radicals. Hydroperoxides can fragment to form ethane and aldehydes (Smirnoff, 1993), including malondialdehyde. Aldehydes, unlike free radicals, are more stable and provide a mechanism for long distance detrimental effects (Esterbauer, 1982). They produce a variety of cytotoxic effects, among which is their reaction with sulfhydryl groups leading to inactivation of proteins (Benedetti *et al.*, 1980). Metal

ions can influence peroxidation by reacting with lipid peroxides to form alkoxy ($RO^{\cdot}$) and peroxy radicals, both of which can further the process of lipid peroxidation (Halliwell and Gutteridge, 1989). Sites of attack would increase via the free radical chain reactions, in the process damaging membranes, and thus leading to membrane leakage (Simon, 1974; Parish and Leopold, 1978).

Direct evidence for the involvement of free radicals in loss of viability of seeds is accumulating, although it is difficult to detect, identify and quantify free radical species. Furthermore, free radical processes differ quantitatively and qualitatively between dead and living tissues. The longer the seed has been dead, the more difficult it becomes to correlate radical processes with loss of viability (Hendry, 1993). Using electron paramagnetic resonance (EPR) techniques, free radicals in various seed tissues including the cotyledon and axes of soybean, as well as the endosperm and embryo of maize (Buchvarov and Gantcheff, 1984; Priestley *et al.,* 1985), were found to increase during natural and accelerated aging, and loss of viability. However, Hepburn *et al.* (1986) were unable to correlate the free radical level of different cultivars of *Brassica,* and legume species, with seed viability and seedling vigour. Recently, Hendry *et al.* (1992) found that EPR-detected free radicals accumulation in the embryonic axis of *Quercus robur*, a seed with recalcitrant storage behaviour, coincided with loss of moisture and viability. In maize, an EPR response similar to that of mosses and acorn seeds was obtained in desiccated, germinating radicles (Leprince *et al.*, 1990). In addition, a significant increase in free radicals also occurred in desiccated, intolerant tissues following 8 hours of rehydration (Leprince *et al.*, 1992). Water is considered to be a major factor in controlling lipid oxidation in dehydrated systems, since it can be expected to influence free radical interactions between molecules such as proteins and lipids (Karel and Young, 1981, cited in Smith and Berjak, 1995).

Peroxidation reactions result in lower levels of fatty acid unsaturation, and higher levels of both lipid hydroperoxides and their by-products, and free fatty acids (Priestley, 1986; McKersie *et al.,* 1988). Saturated fatty acids and free fatty acids tend to increase membrane phase transition temperature, thus promoting the formation of gel phase domains (Senaratna *et al.,* 1985; McKersie *et al.,* 1988, 1989; Crowe *et al.,* 1989, 1992). Because free fatty acids cause membrane fusions at all moisture levels, regardless of the presence of protectants (Crowe *et al.,* 1989; McKersie *et al.,* 1989), they are considered to be quite destabilising to membrane structure (McKersie *et al.,* 1988). The result is a loss in membrane integrity, and an increase in membrane permeability (Simon, 1974) which can readily be demonstrated by measuring leakage of electrolytes and other cytoplasmic components into the external medium (Parish and Leopold, 1978)

In model membranes composed of phospholipid mixtures, the addition of saturated and unsaturated free fatty acids has quite complex, and different effects on the physical properties of the bilayer (Katsaras *et al.,* 1986 cited in McKersie *et al.,* 1988). An addition of saturated free fatty acids in amounts approximating those found in the microsomal fractions containing gel phase domains (Senaratna *et al.,* 1985), results in

increased phase transition temperature and increased microviscosity (Kendall *et al.,* 1986). However, the addition of unsaturated free fatty acid to the same lipid extract does not change the phase transition temperature, but the microviscosity is reduced, indicating slight perturbing effect on the bilayer (Senaratna *et al.*, 1985). The addition of both saturated and unsaturated fatty acids, which is a situation occurring in the membranes naturally, mimicks the response, although slightly modulated, induced by the saturated fatty acids alone. The slight modulation is presumed to be the result of the counteracting perturbing effect of the unsaturated fatty acids.

2.13 Other biological reactions of reactive oxygen species (ROS)

a) Oxidative damage to proteins

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis (Davies *et al.,* 1987; Halliwell and Gutteridge, 1989; Reuzeau *et al.,* 1992). The amino acids in a peptide differ in their susceptibility to attack, and the various forms of ROS differ in their potential reactivity (Davies *et al.,* 1987; Casano and Trippi, 1992). Primary, secondary and tertiary protein structures alter the relative susceptibility of certain amino acids. Sulphur-containing amino acids, and thiol groups specifically, are very susceptible sites. ROS can abstract a hydrogen atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges (Pryor, 1976). Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives. Other forms of free radical attack on proteins are not reversible. For example, the oxidation of iron-sulphur centres by superoxide destroys enzymatic function (Gardner and Fridovich, 1991). Many amino acids undergo specific irreversible modifications when a protein is oxidised. Oxidative modification of specific amino acids is one mechanism of marking a protein for proteolysis (Stadtman, 1986).

b) Oxidative damage to DNA

Activated oxygen and agents that generate ROS induce numerous lesions in DNA that causes deletion, mutations and other lethal genetic effects (Imlay and Linn, 1986). Characterisation of this damage to DNA has indicated that both the sugar and base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to proteins (Imlay and Linn, 1986). Degradation of the base will produce numerous products, including 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine ring-opened and –saturated products. Cross-linking of DNA to protein is a consequence of hydroxyl radical attack on either DNA or its associated proteins (Oleinick *et al.,* 1986). When such cross-linkages exist, separation of protein from DNA by various extraction methods is ineffective. Although DNA-protein cross-links are about an order of magnitude less abundant than single

strand breaks, they are not as readily repaired, and may be lethal if replication or transcription precedes repair (Oleinick *et al.*, 1986).

Upon dehydration, the same destabilising forces that perturb lipid and protein structure may also affect nucleic acid structure (Ran *et al.*, 1984). DNA is a particularly stable molecule (Wayne *et al.*, 1999) that maintains structure in the absence of water and reversibly unfolds at high temperatures (Bonner and Klibanov, 2000). The intermolecular distances of dehydrating DNA strands is comparable to that of condensed DNA in hydrated nuclei (Ran *et al.*, 1984), suggesting that DNA structures are resistant to perturbations resulting from dense packing. When DNA is replicated and decondensed during germination, the cell concomitantly become susceptible to desiccation injury (Deltour and Jacqmard, 1974; Crevecoeur *et al.*, 1988), and rapidly dividing cells during embryogenesis also appear to be sensitive to desiccation (Myers *et al.*, 1992).

2.14 Membranes as the site of desiccation injury

Membrane systems are considered to be particularly susceptible to dehydration and subsequent re-hydration injuries. The loss of membrane function has been attributed to "demixing" (segregation of the various components) of the membrane constituents with different hydration characteristics, and/or to phase transitions of the polar lipid components (Crowe *et al.*, 1987, 1992; Wolfe, 1987; Webb *et al.*, 1993). Two types of phase transitions are commonly reported: lamellar liquid crystalline-to-gel in which the bilayer configuration is maintained, and the lamellar liquid crystalline-to-hexagonal in which a non-bilayer structure is formed (McKersie *et al.*, 1988). The former phase transition has been reported in desiccation-tolerant organisms (McKersie *et al.*, 1988; Crowe *et al.*, 1992). The formation of the hexagonal phase, on the other hand, is associated with irreversible damage, probably because it leads to membrane fusion, loss of compartmentalisation and irreversible membrane disorganisation (Quinn, 1985; Senaratna *et al.*, 1987; Webb and Steponkus, 1993).

Wide-angle X-ray diffraction has been used to examine the phase properties of isolated membranes, and both liquid-crystalline and gel phases can be detected, co-existing in membranes from dehydrated tissues. When phospholipids are in liquid-crystalline phase, as in normal functional biological membranes, the fatty acid side chains exhibit considerable rotational and lateral motions (McKersie *et al.*, 1988). But when phospholipids are in the gel phase, the fatty acids are packed into a more regular hexagonal lattice. The motion is restricted to rotation about the long axis of the chain, and the spacing between the planes of the acyl chains of the phospholipids molecules is more constant. The latter organisation in phospholipids characterises tissues which have lost desiccation tolerance (McKersie *et al.*, 1988; Senaratna *et al.*, 1987; Webb and Steponkus, 1993). The presence of gel phase domains in the cellular membranes would be expected to contribute to the loss of membrane function, and thus loss of cell viability (McKersie *et al.*,

1988; Simon, 1974). The co-existence of liquid-crystalline and gel phase domains is indicative of lateral phase separation of the phospholipids in the bilayer, which would alter the pattern of protein organisation, thus affecting enzyme activity (Sanderman, 1978), and transport processes (Baldassare *et al.,* 1979). The formation of gel phase domains, with consequent elevation of the lipid phase transition temperature, may also result in increased microviscosity (McKersie *et al.*, 1988, 1989; Crowe *et al.,* 1989, 1992). Membranes in the latter condition do not provide adequate barrier properties and leakage occurs, which is thought to result from a mismatch between molecules in the gel and those in the liquid-crystalline state (Crowe *et al.*, 1989, 1997; Clerc and Thompson, 1995).

Soybeans have been shown to retain their viability if they are dehydrated to 6% moisture following 6 hours of imbibition, but lose their viability when dehydrated to a similar extent following 36 hours of imbibition (Senaratna *et al.*, 1984). Studies using wide angle X-ray diffraction indicate the occurrence of regions of gel phase lipid, in addition to regions of liquid-crystalline lipid, in soybean axes imbibed for 36 hours and subsequently dried to 19% moisture content (McKersie and Stinson, 1980). A similar dehydration treatment has been shown to have no effect on membranes of soybeans axes imbibed for 6 hours. The formation of gel phase is an indication of lateral phase separation of phospholipids within the plane of the membrane, and would be expected to contribute to the loss of viability (Senaratna *et al.,* 1984). Similar phase separations have been observed during leaf and cotyledon senescence following treatment of plants with the herbicide paraquat (Chia *et al.*, 1981), and after exposure of isolated membranes to ozone (Pauls and Thompson, 1980).

2.15 Membrane leakage

One of the earliest symptoms of injury following desiccation is the loss of structure or function of either the plasmalemma or organelle membranes (Simon, 1974). During the episodes of drying and rewetting, considerable physical disruption of the plasmalemma may occur, particularly along the outer cell layers which contribute significantly to solute leakage (Powell and Matthews, 1979). The kinetics of solute efflux from a seed as it imbibes water follows a biphasic profile (Simon, 1974). This is characterised by a rapid release of solutes during the first minutes of imbibition, followed by a slower, linear rate of efflux. The rate of the linear phase of solute efflux, which is an indicator of the integrity of the cellular membranes, is increased in seeds with reduced viability and vigour. Several lines of evidence suggest that dehydration stress induces a change in the organisation of the cellular membranes that is reflected in altered semipermeability (Senaratna and McKersie, 1983).

2.16 Development of desiccation tolerance

Seeds do not appear to be tolerant to drying at all stages during their development. Instead, they appear to have an episode of transition from a dessication-intolerant to a dessication-tolerant state at a particular time in the course of their development. Seeds of *Phaseolus vulgaris* L., for example, will not germinate if removed fresh from the pod and placed in water at stages of development prior to 40 days (Dasgupta *et al.*, 1982). Seeds at 26 and 32 days of development can be induced to germinate to increasing extents if first dried over silica gel, whereas those dried at 22-day stage fail to germinate when rehydrated, and eventually deteriorate (Dasgupta *et al.*, 1982). Subsequent studies have confirmed that seeds at the 22-day stage of development do not recover their full metabolic integrity following premature drying treatment (Kermode *et al.*, 1986). At later stages of development, however, seeds acquire tolerance to desiccation and are induced by dehydration treatment to germinate (Dasgupta *et al.*, 1982). A similar situation exists for castor bean seed (Kermode and Bewley, 1985). Here, germinability is not achieved until some 50 to 55 days of development, whereas premature drying will promote the germination of seeds as early as 25 days of development. Drying at an earlier stage of development not only fails to induce germination, but it also results in the death of the seed (Kermode *et al.*, 1986).

It is evident, therefore, that a considerable proportion of the time taken to complete development does not appear to be essential for the production of viable seed, but it is the time required to increase the seed mass, especially through deposition of reserves. For example, castor bean seed at 25 days of development (the earliest desiccation-tolerant stage) has reached only 20% of the maximum dry weight achieved in the mature seed (Kermode and Bewley, 1986). Similarly, even at 30 days of development, the stage at which full germination can occur after drying, the seed had achieved only 40% of maximum dry weight (Kermode and Bewley, 1986). The question, therefore, is to characterise the features that occur within the seed during the brief period of development when it changes from being intolerant of desiccation to being tolerant. Maturation is considered to be a metabolically active phase, involving protein modification, starch utilisation, and the production of soluble sugars (Adams *et al.*, 1983).

2.17 Water status and desiccation tolerance

The final water content of dry seeds appears to be important in determining survival over long periods of storage (Robert and Ellis, 1989). Furthermore, water properties in dried seed tissues appear to be of obvious and primary importance in desiccation tolerance. Several studies on the thermodynamics of hydration in dry seeds have documented the role of water binding as a component of desiccation tolerance. Different concepts have been used to characterise water properties in plant tissues. The term 'bound water' is defined as water associated with the cell matrix. It is sufficiently structured so that its thermodynamic and/or

motional properties differ from 'free and/or bulk water' (Vertucci and Leopold, 1987; Leopold and Vertucci, 1989).

According to sorption isotherms obtained on a range of biological material, at least 5 states of water or hydration levels have been classified in the recalcitrant and orthodox seed tissues (Vertucci, 1989, 1990; Berjak *et al.*, 1990, 1992; Pammenter *et al.*, 1991). In region 1 (0 to 8-10% g H_2O/g DW), the water molecules are very strongly associated with macromolecular surfaces by ionic bonding and behave as a ligand rather than a solvent. The energy of attraction is estimated as -50 kJ mol^{-1}. In region 2 (8-22%) water gains its ability to form glasses (Williams and Leopold, 1989) and the energy of attraction decreases to about -2 kJ mol^{-1}. This suggests a weak interaction with the macromolecular surface (Vertucci, 1989). Above 22%, water gains its solvent properties and some metabolic activities are allowed. Thermal properties of the third hydration level (22-33%) appear to be that of a concentrated solution. Water freezes between -18 ^{0}C and -36^{0}C, has negligible energy of attraction and is believed to initiate bridges over hydrophobic sites. At that hydration level, respiration is measurable (Leopold and Vertucci, 1989). In region 4 (33-55%) and 5 (above 55%), water exhibits thermal properties similar to those of a dilute solution. Above 55% water content, the seed tissues are considered to be fully hydrated and germination processes are possible.

Data obtained from several studies confirm that the structure of the water matrix characterised by the loss, or lack of strong binding sites in region 1 is associate with desiccation sensitivity (Vertucci, 1989; Berjak *et al.*, 1990; Pammenter *et al.*, 1991). However, the amount and role of bound water in desiccation tolerance has not been elucidated. There is evidence suggesting that desiccation tolerance should not be interpreted in terms of the proportion of non-freezable water, but rather, of differing responses to the removal of this type of water. Pammenter *et al.* (1991) and Berjak *et al.* (1992) observed that in recalcitrant *Landolphia kirkii* seeds, the different types of water exhibited similarities to those of desiccation-tolerant seeds. They suggested that drying rates have more marked effect on desiccation sensitivity than the proportions of different water types.

2.18 Mechanisms implicated in desiccation tolerance

2.18.1 Fatty acid composition

During seed development, a trend towards lipid unsaturation is observed in most oily seeds. Increased levels of unsaturated fatty acids can have beneficial effects by increasing tolerance to moderately low water potentials (Uemura and Stoponkus, 1989) and decreasing the likelihood of "demixing" (Quinn, 1985; Crowe *et al.*, 1992). Alternatively, unsaturated fatty acids could have a detrimental effect by increasing the possibility of lethal hexagonal changes (Quinn, 1985; Bryant and Wolfe, 1989, 1992). A consistent trend

towards loss of linolenic acid (18:3) occurs during the later stages of seed maturation (Cherry *et al.*, 1984; Chen and Burris, 1991; Dulta and Appelqvist, 1991). These changes might also be important to the longevity of seeds in the dry state, since unsaturated fatty acids are believed to be more sensitive than saturated fatty acids to peroxidative reactions (Priestley, 1986; McKersie *et al.*, 1988, 1990).

2.18.2 Sugars as stabilising agents

Another important component of desiccation tolerance may be the accumulation of a high level of soluble sugars, a characteristic of mature orthodox seeds (Amuti and Pollard, 1977). They have been implicated by correlation as adaptive agents for desiccation- tolerance during seed development and germination (Chen and Burris, 1990; LePrince *et al.*, 1990). High levels of oligosaccharides, which include raffinose and stachyose, have been shown to accumulate in soybeans, in addition to sucrose. Evidence for the protective role of soluble sugars has also been inferred from model systems (Caffrey *et al.*, 1988; Crowe *et al.*, 1984). It is thought that the hydroxyl constituents of sugars may replace the hydration shell around membranes and thus prevent the structural damage as water is removed (Crowe *et al.*, 1984). The presence of larger oligosaccharides along with sucrose may enhance vitrification rather than crystallisation (Bruni and Leopold, 1991; Chen and Burris, 1990; Koster, 1991).

Several lines of evidence correlate acquisition of desiccation tolerance with increases in soluble sugars during seed maturation of mustard (Fischer *et al.*, 1988), maize (Chen and Burris, 1990), *Brassica campestris* (Leprince *et al.*, 1990), and soybean (Blackman *et al.*, 1992), and loss of desiccation tolerance with decreases in soluble sugars during germination of pea, soybean, maize (Koster and Leopold, 1988), and *Brassica campestris* (Leprince *et al.*, 1992). Desiccation-tolerant tissues are characterised by high amounts of sucrose and oligosaccahrides (stachyose, or raffinose being the most often detected depending on the species) (Kuo *et al.*, 1988), and by the absence, or at least very low amounts, of monosaccharide reducing sugars such as galactose, mannose, glucose and fructose. The opposite trend is found in desiccation-intolerant tissues which are generally endowed with large concentrations of monosaccharides and low concentrations of di- and higher saccharides (Leprince *et al.*, 1990, 1992). These data support the biophysical data from model experiments suggesting that sugars confer desiccation tolerance by replacing water (Vertucci and Farrant, 1995). However, several lines of evidence suggest that the role of sugars may not be ascribed in a unified model of protection, applicable to all kinds of seeds.

Because of its high concentration and ability to induce tolerance *in vitro*, sucrose was believed to represent a major protective agent. However, sucrose is known to have a strong tendency to crystallise in dry conditions and this could induce deleterious effects on tissues under dry conditions (Crowe *et al.*, 1988). Oligosaccharides, on the other hand, appear to inhibit this tendency and afford better protection to dry tissues than sucrose alone (Caffrey *et al.*, 1988). In immature soybean seeds, an *in vitro* slow-drying

treatment, which induced desiccation tolerance, was found to increase stachyose and raffinose concentrations and decrease sucrose concentration (Blackman *et al.*, 1992). In this system, sucrose is not the limiting factor in desiccation tolerance. Therefore, it appears that the presence of oligosaccharides may supplement sucrose in conferring protection against desiccation-induced damage.

a) Sugar mediated-stabilisation of membrane lipids and protein

Several lines of evidence have confirmed that disaccharides interact with lipids by hydrogen bonding of their hydroxyl groups with the polar head groups of the phospholipids (Gaber *et al.*, 1986; Crowe and Crowe, 1986). The recent use of carbohydrate derivatives that are partly embedded inside the lipid bilayer permits a precise evaluation of the association of the carbohydrate with the bilayer (Goodrich *et al.*, 1991). Using computer modeling techniques, Gaber *et al.* (1986) succeeded in displaying accurate models of lipid-sugar interaction which also take into account molecular and conformational dynamic constraints. The model best depicts a bridge consisting of three hydrogen bonds between three hydroxyl groups of one trehalose molecule, and two non-esterified phosphate oxygens of one phospholipid molecule, and one oxygen of a second adjacent phospholipid molecule.

The protective effects of sugars during desiccation also include protein stabilisation (Leopold, 1990; Carpenter *et al.*, 1987). For example, the activation of three enzymes (glucoamylase, cellulase and glucose oxidase) which contained carbohydrate residues stabilising their tertiary structures, was not affected by dehydration (Darbyshire, 1974). When 70% of the carbohydrates were removed by periodate oxidation, subsequent dehydration caused a loss of 80% of enzyme activity that was overcome if the oxidised enzymes were dehydrated in the presence of Dextran T500, suggesting a protection by carbohydrates against dehydration. More recently, a comprehensive study was undertaken in order to characterise the protein-sugar interaction in relation to anhydrous biology (Carpenter *et al.*, 1987, 1990). Using phosphofructokinase (PFK), an extremely labile enzyme when freeze-dried, Carpenter *et al.* (1987) characterised its stabilisation using a variety of sugars, and divalent cations up to 50 mM. The disaccharides, sucrose, maltose and trehalose, were very effective stabilising agents even without Zn^{2+}, whereas the monosaccharides, galactose and glucose, were only effective in the presence of 100-200 mM Zn^{2+}. It is also interesting to note that cryoprotectants such as glycerol, inositol, proline and glycine could not offer protection during freeze-drying, alone or with Zn^{2+} (Carpenter *et al.*, 1987).

b) Cytoplasmic stabilisation

Extreme desiccation of cytoplasm could result in crystallisation of proteins and solutes which undoubtedly would induce severe injury to the cell (Leopold, 1990). In this respect, Burke (1986) has suggested an attractive hypothesis relevant to cytoplasmic protection, *i.e.* vitrification or glass formation within the

cytoplasm. This relates to the creation of a liquid solution with the viscous properties of a solid and is also considered as a supercooled liquid which is metastable from 0 to 363 K (Koster, 1991). Interchange between these two states involves no chemical or physical changes (Williams and Leopold, 1989).

The benefits of vitrification to a cell coping with desiccation are several. Because of its high viscosity, molecular diffusion in a glass is impeded, so chemical reactions are slowed, if not inhibited (Leopold, 1990; Koster, 1991). Consequently, degradative processes are prevented, and quiescence and dormancy are ensured. The glass phase fills space, preventing cellular collapse following desiccation (Burke, 1986). In addition, it may trap chaotropic solutes and prevent them from being too concentrated; avoid alterations in ionic strength or pH; and prevent crystallisation of cytoplasmic solutes (Leopold, 1990). The glass may permit the occurrence of hydrogen bonding at the interface between itself and the strong bonding sites of the cell components (Koster, 1991), and may reversibly "melt" into a liquid phase upon addition of water without cellular injury (Bruni and Leopold, 1991).

Sugars are accumulated up to 20% in dry anhydrobiotic seeds, and are suspected to be the solutes which enhance cytoplasmic vitrification at ambient temperature. Williams and Leopold (1989) reported that the slope of the vitrification phase diagram of maize embryos was identical to thermal data for sucrose alone. Examination of thermograms of sugar solutions in proportions equivalent to those of desiccation-tolerant cells indicated that glass formation was possible at ambient temperature (Koster, 1991), supporting the hypothesis that cytoplasmic glass formation is specific to desiccation-tolerant tissues and that sugars play an important role in its formation.

2.18.3 Late embryogenesis abundant proteins (LEAs)

Among the protective components that have been proposed to be important in the acquisition of desiccation tolerance during seed development are proteins (Blackman *et al.*, 1992). Temporal differences in the relative rates of accumulation of the proteins in maturing soybean have been demonstrated by Dure *et al.* (1989). A particular class of proteins referred to as Late Embryogenesis Abundant (LEA) (Dure *et al.*, 1989) proteins are known to accumulate during the maturation drying phase of seed development. Some of these proteins have been correlated with desiccation tolerance (Bartels *et al.*, 1988; Blackman *et al.*, 1991).

2.18.4 Operation of antioxidative systems

A common stress progressively applied to a varied biota is likely to force parallel, and independent, adaptation among the surviving species. Plants have, thus, evolved complex defense mechanisms to curb damage to cellular membranes and organelles, which under normal circumstances minimise the occurrence of overt oxidative damage. Essentially, antioxidant defenses fall into three categories which include: (1)

enzymatic antioxidants, *e.g.* superoxides dismutases, catalase, and peroxidases; (2) the water-soluble reductants, *e.g.* ascorbate (ascorbic acid), glutathione; (3) the fat-soluble vitamins, *e.g.* alpha-tocopherols.

a) Superoxide dismutase

Superoxide dismutase (SOD) was first isolated by Mann and Keilis (1938) and thought to be a copper storage protein. Subsequently, the enzyme was identified by a number of names, until McCord and Fridovitch (1969) discovered its catalytic function. SOD is known to catalyse the dismutation of superoxide to hydrogen peroxide and oxygen:

$$O_2^- + O_2^- \Rightarrow H_2O_2 + O_2$$

Therefore, the activity of this enzyme determines the relative proportions of the two constituents of the Haber-Weiss reaction that generates hydroxyl radicals. Since SOD is present in all aerobic organisms, and most subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defence against oxidative stress (Beyer *et al.*, 1991; Bowler *et al.*, 1992; Scandalios, 1993). To date it has been shown that SOD activity is increased in cells in response to diverse environmental and xenobiotic stresses including paraquat, high light, waterlogging and drought. Apparently, each of the SOD isozymes (Cu/Zn – SOD, Mn – SOD, Fe – SOD) are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments. Bowler *et al.* (1992) have suggested that this role may be served by unique lipid peroxidation products from each organelle that diffuse from the site of oxidative damage to the nucleus where they would enhance transcription of specific SOD genes.

b) Catalase

Catalase is a heme-containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen. The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (Smirnoff, 1993). Catalase was one of the first enzymes to be isolated in a highly purified state. All forms of the enzyme are tetramers in excess of 220 000 molecular weight. Multiple forms of catalse have been described in many plants. Interestingly, catalase has been found to be very sensitive to light, and has a rapid turnover rate similar to that of the D1 protein of photosystem II (Hertwig *et al.*, 1992). This may be a result of light absorption by the heme group or perhaps hydrogen peroxide inactivation. Stress conditions which reduce the rate of protein turnover, such as salinity, heat shock or cold, cause the depletion of catalase activity (Hertwig *et al.*, 1992; Feirabend *et al.*, 1992). This may have significance in the ability of plants to tolerate the oxidative components of environmental stresses.

c) Ascorbic acid

Ascorbic acid (ascorbate) has been shown to have an essential role in several physiological processes in plants, including growth, differentiation and metabolism (Foyer, 1993). Ascorbate (ASC) also functions as a reductant for many free radicals, thereby minimising the damage caused by oxidative stress (Noctor and Foyer, 1998). Ascorbate can directly scavenge oxygen free radicals with, and without, an enzyme catalyst (Nishikimi, 1975; Foyer, 1993). By reacting with activated oxygen more readily than any other aqueous component does, ascorbate protects critical macromolecules from oxidative damage (Doba *et al.*, 1985). The indirect role of ascorbate as an antioxidant is to regenerate membrane-bound antioxidants, such as alpha tocopherol, that scavenge peroxyl radicals and singlet oxygen, respectively (Fridovich, 1976).

Other investigators have suggested that through its ability to scavenge alkoxyl radicals, ascorbate inhibits propagation reactions (Haliwell and Gutteridge, 1985). The hydroperoxyl radical and superoxide (O_2^-) are both radicals likely to be found under normal physiological conditions, and both have a fairly high rate constant for interaction with ascorbate (Fridovich, 1976). Being hydrophilic, it has been suggested that ascorbate probably cannot afford protection against radical-mediated oxidative damage within membrane lipids (Doba *et al.*, 1985). It has been proposed that ascorbate can protect membranes by scavenging free radicals in the aqueous phase of the cell, thereby preventing an attack within the lipid phase of the membrane (McCay, 1985). It has been suggested that the interaction of vitamin E with ascorbate affords a synergestic antioxidant protective effect, as compared to either of the compounds alone (Doba *et al.*, 1985).

The oxidation of ascorbate results in the formation of two different products, monodehydroascorbate and dehydroascorbate, that represent one and two electron transfers (Noctor and Foyer, 1998), respectively. Monodehydroascorbate can either spontaneously dismutate, or is reduced to ascorbate either via monodehydroascorbate reductase or via dehydroascorbate reductase and glutathione (Nakano and Asada, 1981; Noctor and Foyer, 1998). The terminal electron donor is NADPH (Asada, 1992). This pathway serves two functions. One is the detoxification of hydrogen peroxide that might otherwise participate in Fenton reactions, and the second is the oxidation of NADPH (Foyer and Halliwell, 1976; Asada, 1992). The latter function is an apparently energy-consuming, wasteful process analogous to photorespiration. Dehydroascorbate is unstable at a pH greater than 6, decomposing into tartrate and oxalate. To prevent this, dehydroascorbate is rapidly reduced to ascorbate by dehydroascorbate (DHA) reductase using reducing equivalents from glutathione (GSH) (Foyer and Halliwell, 1976; Asada, 1992).

Although ascorbate metabolism has been studied in more detail in the chloroplast, it is likely that all enzymes for its regeneration also exist in the cytosol of both photosynthetic and non-photosynthetic cells. For example, different isozymes for ascorbate peroxidase have shown to exist in the cytosol and chloroplast

components (Chen and Asada, 1989). The cell wall is also an important site of ascorbate metabolism because it contains millimolar concentrations of ascorbate. Here, the ascorbate may have a role in cell wall biosynthesis (Polle *et al.*, 1990). The cell wall does not contain ascorbate peroxidase, but contains ascorbate oxidase (Chichirocco *et al.*, 1989). Since the enzymes to recycle oxidised forms of ascorbate are not present in the cell wall, it has been proposed that the plasmalemma may have an ascorbate translocator to shuttle oxidised and reduced forms between the cytosol and cell wall (Foyer, 1993).

d) Glutathione

GSH is a tripeptide (Glu-Cys-Gly), whose antioxidant function is facilitated by the sulphydryl group of cysteine (Rennenberg, 1982). On oxidation, the sulphur forms a thiyl radical that reacts with a second oxidised glutathione forming a disulphide bond (GSSG). GSH is found in most tissues, cells and subcellular compartments of higher plants. Its levels decline with tissue age and variations in levels are also seen in plants in different growth environments. For example, GSH levels are higher in the light than in the dark. At subcellular levels, GSH concentration is highest in the chloroplast, averaging between 1 and 4 mM, but significant quantities also accumulate in the cytosol. GSH exists predominantly in the reduced form with estimates varying from 70% in barley chloroplasts (Smith *et al.*, 1985) to 90% in pea chloroplasts (Bielawski and Joy, 1986).

GSH can function as an antioxidant in many ways. It can react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger. GSH may stabilise membrane structure by removing acyl peroxides formed by lipid peroxidation reactions (Price *et al.*, 1990). GSH is a reducing agent that recycles ascorbic acid from its oxidised, to its reduced form, by utilising the enzyme dehydroascorbate reductase (Loewus, 1988). GSH can also reduce dehydroascorbate by a non-enzymatic mechanism above pH 7 and at GSH concentrations greater than 1 mM (Loewus, 1988).

e) Tocopherols

Vitamin E (alpha-tocopherol) is probably the most important antioxidant that is incorporated into the lipid membranes of cells (Winston, 1990). It is estimated that one molecule of this compound can afford antioxidant protection to several thousand fatty acid molecules (Bewley, 1986). Most of the fatty acids of soybean seed lipids are unsaturated (Bils and Howell, 1963), thus, soybeans can be expected to contain large amounts of alpha-tocopherol to protect the membrane against oxidative damage. Not only does it protect against oxygen radicals that might initiate lipid peroxidation of cell membranes, but alpha-tocopherol can also serve as a scavenger of chain-propagating free radicals such as lipid peroxyl radicals (Niki *et al.*, 1984). It does so by donating a hydrogen atom to the lipid peroxyl or lipoxyl radical, forming the corresponding peroxide or alcohol, respectively, thereby breaking the propagating chain reaction

(McCay, 1985). Ascorbate and glutathione, in combination with alpha-tocopherol, can result in synergistic inhibition of oxidative damage to cell membranes (Leung *et al.*, 1981; Doba *et al.*, 1985) presumably through the regeneration of alpha-tocopherol.

The antioxidant properties of tocopherol are the result of its ability to quench both singlet oxygen and peroxides (Fryer, 1992). Tocopherol is a less efficient scavenger of singlet oxygen than β-carotene and therefore in thylakoid membranes it may function to break radical chain reactions by trapping peroxyl radicals. The resulting tocopheroxyl radical is stabilised by the fully substituted benzoquinone ring and therefore does not propagate the radical reaction, thus making tocopherol an effective radical trap. Because the active oxygen of the α-tocopherol is located near the surface of the bilayer and because it can readily diffuse laterally in the plane of the bilayer, tocopherol is able to react with peroxyl radicals formed in the bilayer as they diffuse to the aqueous phase. This also allows the tocopheroxyl radical to be reduced by ascorbate in the aqueous phase to regenerate α-tocopherol. Glutathione reduces tocopheroxyl radicals in alcohol solutions, but there is only limited evidence that this occurs in biological systems (Hess, 1993).

2.19 Rationale and aims of the project

High metabolic activity has been associated with the desiccation intolerant state (Farrant *et al.*, 1986; Leprince *et al.*, 1994, 1999) whereas reduced metabolism is partly responsible for desiccation tolerance (Leprince *et al.*, 1994, 1995; Farrant and Walters, 1998; Walters *et al.*, 2001). Decreasing respiration with decreasing water content can account for greater viability in developing seeds and greater tolerance to low water contents in rapidly dried axes. However, metabolism at immediate moisture levels is hypothetically damaging to cells because ROS are produced as by-products of respiration (Leprince *et al.*, 1999, 2000). Oxidative activity increases with water content in orthodox seeds (Ibrahim *et al.*, 1983; Dahal *et al.*, 1996) and decreases with decreasing water content in recalcitrant seeds (Salmen-Espindola *et al.*, 1994; Leprince *et al.*, 1999).

A free radical damage hypothesis has been advanced to explain the loss of desiccation resistance of soybean embryos (Senaratna *et al.*, 1984, 1985; Senaratna *et al.*, 1987) leading to the rapid loss of solutes, which is characteristic response of all damaged non-tolerant tissues (Senaratna *et al.*, 1984). However, plants are equipped with defence antioxidants in the form of enzymes and scavengers that quench free radicals. There is considerable support for the hypothesis that the activity levels of one or more of these antioxidants are generally increased in plants exposed to stress (Allen, 1995; Scandalios, 1993; McKersie and Leshem, 1994) and that this increased activity correlates with enhanced tolerance. Therefore, the activity levels of the defence antioxidants or scavengers determine the abundance of the free radicals, and a critical balance between the two is required to achieve and maintain viability. Disorder or damage to cells occurs when either compromised defence or repair mechanisms fail to provide adequate protection, or an

increase in degradative reactions exceed the capacity of defence and repair mechanisms to provide adequate protection (Winston, 1990), or a combination of these processes. Loss of the ability to scavenge free radicals effectively is a generally acknowledged feature of loss of viability and/or vigor in plant tissues (Dhindsa *et al.*, 1981).

The project examined the biochemical and physiological changes at different harvest intervals from early seed development of soybean (*Glycine max* (L.) Merr.) to the stage when the seed was fully matured and ready for subsequent planting; changes during normal imbibition; and changes in seeds and axes subjected to a hydration-dehydration-rehydration cycle. The aim was to characterise desiccation tolerance and sensitivity by examining the changes in lipids, both membranal and storage; levels of lipid peroxidation in conjuction with the changes in the antioxidant defense systems; and leakage of substances such as proteins and phosphorus from seeds as a measure of membrane damage.

2.20 Thesis Organisation

The thesis began with a review of the literature on the compositional changes associated with seed development, processes associated with seed germination, possible mechanisms of damage to, and malfunction of structures and organelles as a results of desiccation stress, and the mechanisms which seeds have evolved and/or adopted to withstand desiccation (Chapter 2).

Investigation of the biochemical and physiological changes during seed development are the focus of Chapter 3. Soybean plants were established in the greenhouse. At regular intervals, seeds were harvested and analysed for changes in water content, fresh weight, dry weight, lipids and lipid peroxidation, sugars, and the antioxidant defense systems. Changes in these parameters, in relation to the acquisition of desiccation tolerance, are discussed.

Chapter 4 examines the biochemical and physiological events taking place during seed imbibition and germination. Events that accompany the seeds and axes as they shift from a desiccation-tolerant state to a desiccation-sensitive state, with the progress of germination, are highlighted.

In Chapter 5, the effects of dehydration, and subsequent rehydration of imbibed embryonic axes are investigated. Changes in phospholipid levels, lipid peroxidation and membrane integrity, and the efficiency of mechanisms operating to combat free-radical processes are explored. Mechanisms of desiccation tolerance and desiccation sensitivity are further explored.

The main conclusions drawn from Chapters 3, 4, and 5 are discussed and presented in Chapter 6. This concluding chapter of the thesis also provides recommendations for future studies.

Chapter 3

SEED DEVELOPMENT

3.1 Introduction

The significance of lipid peroxidation in relation to the ability of the seed to attain desiccation tolerance during seed development has received very little attention. Seeds have been reported to be sensitive to desiccation in their early stages of development. On the same note, the most documented degradative reaction linked with desiccation-sensitivity in seeds is the accumulation of peroxidative damage following drying. Thus, the removal of water during seed development could induce peroxidative reactions that could result in cellular damage.

Seed development and maturation are accompanied by an overall loss of moisture (Adams and Rinnie, 1980). In most documented cases, young seeds have the highest moisture content but this then declines as the seed matures. When moisture has declined to about 50-60%, many seeds cease or slow down development and enter a final phase of maturation (TeKrony *et al.*, 1979; Crookston and Hill, 1978). This observation has led to suggestions that desiccation is necessary for most seeds to transform from a developmental, to a germination, phase of development (Adams and Rinnie, 1981; Kermode and Bewley, 1985; Dasgupta *et al.*, 1982). The occurrence and extent of this drying varies among species, but in excess of 90% of the original water may be lost (Adams and Rinne, 1980). As a consequence, maturing, desiccation-tolerant seeds have adopted several strategies for coping with the injurious effects that water loss may impose.

As water leaves a cell that does not tolerate desiccation, membranes become disrupted leading to solute leakage and loss of compartmentation (Simon, 1974). Additionally, water removal leads to crystallisation of solutes and denaturation of proteins. The most documented degradative reaction linked with desiccation sensitivity in seeds is the accumulation of peroxidative damage following drying (Hendry *et al.*, 1992; Leprince *et al.*, 1994). Such oxidative stress is likely to originate from reactive oxygen species (ROS) that are generated as a result of uncontrolled respiration and impaired electron flow in the mitochondria (Leprince *et al.*, 2000). Previous studies on desiccation-intolerant embryos (LePrince *et al.*, 1994, 1995) showed that respiration is involved in free radical processes leading to lipid peroxidation and membrane damage, suggesting that the normal tight control of ROS production is lost during drying. This can be taken to imply that in both orthodox and recalcitrant seeds, a controlled down-regulation of metabolism must

occur during drying to avoid overproduction of ROS and free radical damage (Leprince *et al.*, 1994, 1995; Vertucci and Farrant, 1995)

The ability to tolerate desiccation in plant tissues is a multifactorial trait in which the synthesis of protective substances and the repression of degradative processes that are induced during dehydration are equally critical (Vertucci and Farrant, 1995). Among the protective components that have been proposed to be important in the acquisition of desiccation tolerance during seed development is the presence of high amounts of non-reducing sugars and the activity of the free radical scavenging systems (Leprince *et al.*, 1992). Non-reducing sugars may substitute for water by forming hydrogen bonds, thus maintaining hydrophilic structures in their hydrated orientation (Crowe *et al.*, 1988). This may also contribute to the stabilisation of proteins and membranes in dry conditions. Moreover, non-reducing sugars are reported to promote the formation of a "glass phase" in the cytoplasm, a process known as vitrification, and this leads to numerous benefits for the quiescent tissue coping with desiccation (Burke, 1986).

Since free radicals are naturally produced during plant metabolism (Halliwell, 1987), the importance of a well-balanced antioxidative and/or enzymic protection of cells against toxic forms of activated oxygen has been documented in numerous publications (Smirnoff, 1993; Elstner and Osswald, 1994). Accumulation of free radicals has been demonstrated in desiccation-tolerant (Seel *et al.*, 1991) and desiccation-sensitive species (Seel *et al.*, 1991; Hendry *et al.*, 1992). Cellular protection from drought stress-induced free radicals in desiccation-tolerant species is mediated by antioxidants such as glutathione, ascorbic acid and free-radical processing enzymes such as superoxide dismutase, peroxidases, and catalase (Hendry *et al.*, 1992; Seel *et al.*, 1992). Changes in activity and importance of some of these protective compounds are variable (Arrigoni *et al.*, 1992; Cakmak *et al.*, 1993) but there appears to be a trend towards increasing ROS scavenging systems with increasing mitochondrial activity. The capacity of the developing seed to control peroxidative reactions may be correlated with seed vigour. An inability to eliminate hydroperoxides and so quench further autocatalytic peroxidation may lead to a steady increase in membrane peroxidation, and cellular compartmentalisation may break down (Simon, 1974). This may then lead to a massive surge in peroxidation and to viability loss on germination.

This chapter examines the relationship between lipid peroxidation, the anti-oxidative defence systems, and the levels of reducing sugars in developing soybean seeds that retain cellular integrity at maturity. It was envisaged that a better understanding of the events taking place during seed development, and their relationship could shed more light on the phenomena of desiccation-tolerance and desiccation-sensitivity.

3.2 Materials and methods

3.2.1 Greenhouse studies

Soybean plants of cultivar Braithwaite were established in the greenhouse at the University of Natal and allowed to set seed. To obtain the plants, commercial seeds were obtained from Pannar Seeds and sown in sand-filled pots (30 cm diameter and 28 cm length) in August 1999, February 2000 and August 2000, respectively. The soybean seeds were first inoculated with a commercial *Rhizobium* inoculant prior to sowing. Five seed were sown in each of the twenty plastic pots. The population was then thinned to two plants per pot when the first two trifoliate leaves had unfolded. The plants were grown under a photoperiod of 12 hours of natural light. The temperatures were 27° C for 12 hours during the photoperiod (day) and 20° C for the remaining 12 hours. Pots were watered twice daily and fertilised three times a week utilising a modified Hoagland's Solution (See Table 1). Flowers were tagged upon opening (anthesis), and tagging was done daily. Seed age was measured as days after flowering (DAF). At approximately 5 day-intervals, pods were harvested randomly by hand at different stages of development. Harvesting took place between 20 and 80 DAF. Seeds were carefully shelled from the harvested pods by hand for further processing and studies.

Seed growth analysis was achieved through direct observation of the seed size to determine the initiation of seed growth. This was intended to improve the precision in measurements of initiation of seed fill. The pod wall of a soybean pod is translucent, and the developing ovule is opaque. The early ovule can be observed nondestructively since an ovule of greater than 5 mg fresh weight appears as a well-defined dark spot within the pod.

3.2.2 Moisture content and dry weight of seeds

Seed moisture content (MC) of whole seeds was determined immediately after harvesting using the oven drying method. Sub-samples of freshly harvested seeds were selected at random and fresh weight recorded from three samples of each of the developmental stages. The seeds were then dried at 90° C for 48 hours, and re-weighed after cooling in a desiccator with silica gel. Moisture content was calculated on a fresh weight basis.

Table 1: A revised composition of Hoagland's nutrient solution (Hoagland and Arnon, 1950.)

Macronutrients	**Amount (grams per 100 L)**
Potassium sulphate (K_2SO_4)	7.65
Ferric citrate ($FeC_6H_5O_7$)	1.0
Potassium dihydrogen orthophosphate (KH_2PO_4)	15.38
Calcium nitrate [$Ca(NO_3)_2$]	27.0
Magnesium sulphate ($MgSO_4$)	29.84
Magnesium chloride ($MgCl_2$)	13.05
Micronutrients	
Copper sulphate ($CuSO_4$)	1.9
Zinc sulphate ($ZnSO_4$)	4.4
Manganese sulphate ($MnSO_4$)	17.0
Boric acid (H_3BO_3)	29.0
Ammonium molybdate [$(NH_4)_6MO_7O_{24}$]	0.9

3.2.3 Lipid extraction

Lipids were extracted from whole seeds using the solvent system of Khor and Chan (1985). Seeds were placed in methylation tubes and heated at 70° C in isopropanol (3 ml per 0.5 g tissue) for 30 minutes. After cooling, the tissue was homogenised in chloroform/methanol (2:1 v/v) using a pestle and mortar. The homogenate was then filtered through a Whatman No. 1 filter paper. To the filtrate, 2 ml of water and 2 ml of Garbus solution (2 M potassium chloride in 0.5 M potassium phosphate buffer, pH 7.4) (Garbus *et al.*, 1963) were added, and mixed thoroughly using a vortex mixer. The resulting emulsion was centrifuged in a bench centrifuge which resulted in the formation of two phases. Using a glass Pasteur pipet, the upper phase was carefully removed leaving the lower phase that contained lipids. The lower chloroform phase was then transferred to a pre-weighed Pyrex tube and evaporated to dryness under a stream of nitrogen at 30° C. The tube was then re-weighed to determine the dry weight of total lipids before re-dissolving the dry lipid extract in 1 ml of chloroform. Air was flushed out of the tube with nitrogen before sealing and storing the lipid extract in a freezer until further analysis.

3.2.4 Thin layer chromatography of lipids

Two-dimensional thin layer chromatography (TLC) on 10x10 cm plates precoated with silica gel G as adsorbent was used for analytical separation of individual lipid classes.

a) Polar lipids

The TLC plates were pre-washed in chloroform and then dried at 110° C. After cooling, 20 µg of lipid was applied at the lower right-hand corner of the plate and the solvent was allowed to evaporate. The plate was then developed in a TLC tank containing solution **I** [Chloroform - methanol - benzene - 28% ammonium hydroxide (65:30:10:6, v/v)]. When the solvent front was about 3 mm from the top of the plate, it was removed and dried with a stream of unheated air from a hair dryer. The plate was then turned through a 90° degrees clockwise from the first development direction and placed in a TLC tank containing solution **II** [Chloroform - methanol - benzene - acetone - acetic acid (70:30:10:5:4:1)] until the solvent front was about 1.5 cm from the top edge. After drying, the plate was placed in a tank of iodine vapour for visualisation of the individual lipids

b) Neutral lipids (TAG)

A one-dimensional TLC on 10x10-cm plates, precoated with silica gel G, using two solvent mixtures, was used for the separation of neutral lipids. Lipid was applied as a streak at about 1.0 cm from the bottom edge of the plates. The plate was first developed in a tank containing solvent mixture **I** [Toluene - hexane - formic acid (140:60:1, v/v)]. A second development, in the same direction, was achieved in a tank containing solvent mixture **II** [Hexane - diethyl ether - formic acid (60:40:1, v/v)].

3.2.5 Determination of phospholipids (PL)

The phosphorus assay (Vaskovsky *et al.*, 1975) was used determine and quantify total phospholipids (PL) and PL classes from the crude whole seed lipid.

Solution A: 0.272 M Hydrazine dihydrochloride in 4M hydrochloric acid
Solution B: 0.69 M Sodium molybdate in 4M hydrochloric acid
Stock Reagent: Solution A was mixed with solution B and heated in a boiling waterbath for 20 minutes. After allowing to cool, 14 ml concentrated sulphuric acid was added before adjusting the volume to 100 ml with distilled water.
Working reagent: 26 ml 1N sulphuric acid was added to 5.5 ml of the stock solution.The volume was then adjusted to 100 ml with distilled water.

To a test tube containing lipid samples, 0.05 ml of perchloric acid (72%) was added. Samples were digested by heating in an electrically heated metal block at 180-200° C for 20 minutes. After cooling, 0.45 ml of working reagent was added into the tube before heating in boiling water-bath for 15 minutes. The absorbance of the resulting solution was measured at 815-830nm. A standard curve was prepared using 0.01-1.1 μg of phosphorus.

3.2.6 Determination of triacylglycerols (TAGs)

Ester bonds in triacylglycerols were determined by a modification of the method of Snyder and Stephens (1959), and Higgins (1987).

Stock ferric perchlorate solution: 5 g of $Fe(ClO_4)_3.6H_2O$ was dissolved in 10 ml 70% perchloric acid and 10 ml distilled water. The volume was adjusted to 100 ml with cold absolute ethanol.
Working reagent: 4 ml of the stock solution was mixed with 3 ml of 70% perchloric acid. The volume was adjusted to 100 ml with cold absolute ethanol.
Solution A: 2 g of hydroxylamine hydrochloride was dissolved in 2.5 ml of water before adjusting the volume to 50 ml with absolute ethanol (0.576 M solution).
Solution B: 4 g of NaOH was dissolved in 2.5 ml of water before adjusting the volume to 50 ml with absolute ethanol (2 M solution).
Solution C: equal volumes of solutions A and B were mixed and filtered.

To a tube containing 3 μg lipid from whole seeds, 1.0 ml of solution C was added and the mixture was heated at 65° C in a water bath for 2 minutes. After allowing to cool, 3.0 ml of working reagent was added and the solution was allowed to stand for 30 minutes. Absorbance was measured at 530mn. A standard curve was prepared using triacylglycerol at concentrations between 0.1 and 10 μM.

3.2.7 Hydroperoxide determination

Reagents
Solution A: Benzene/Methanol (7:3 v/v) analytical grade
Solution B: Ferric chloride solution: 0.04 M $BaCl_2$ was mixed with 0.07 M Fe_2SO_4 in water. Exactly 2 ml of 10M HCl was then added and the resulting precipitate was cleared by centrifugation.
Solution C: 10M HCl: 95.24 ml HCl was brought to 100 ml with distilled water
Solution D: 30% KSCN solution: 15 g of KSCN was dissolved in 50 ml of water

Hydroperoxide levels of whole seeds were determined using a modification of the test of Stine *et al.* (1953). Approximately 4 µl of $FeCl_2$ solution was added to 1 ml of benzene/methanol (7:3 v/v) and shaken. 10 µg of lipid was then added followed by the addition of 4 µl 30% KSCN. The mixture was vortexed before reading the absorbance at 505nm against a blank of the reagents.

3.2.8 Lipid esterification and fatty acid determination

Methyl esters of fatty acids were obtained using the organic base-catalysed technique of Metcalfe and Wang (1981). An aliquot of lipid was dissolved in diethyl ether, to which 1 M tetramethylammonium hydroxide (TMAH) in methanol was added, and the mixture shaken at room temperature. Following the addition of 1 ml of water, 1 µl of the upper ether phase was analysed using a Varian 3700 gas chromatography fitted with a flame ionisation detector and a 1.8 m X 2 mm internal diameter glass column packed with 10% Silar 5CP on 100/120 mesh Supelcoport. During analysis, injection port and detector temperatures were 270° and 300° C, respectively, while the column was maintained isothermally at 185° C. Nitrogen was used as a carrier at 25 ml/min and peaks were integrated using a HP 3380A integrator and expressed as area percentages. Comparison of retention time of the fatty acids was made against those of an authentic fatty acid methyl esters mix (Sigma) which included the esters of palmitic, stearic, oleic, linoleic and linolenic acids

3.2.9 Extraction of ascorbate (ASC) and glutathione (GSH)

Whole seeds were homogenised with four volumes of cold 5% metaphosphoric acid using a pestle and mortar. The homogenate was centrifuged at 20 000 g for 15 minutes at 4° C, and the supernatant was collected and analysed for total ascorbate and glutathione pools.

3.2.10 ASC and GSH Analysis

ASC and DHA were measured according to Kampfenkel *et al.* (1995) with minor modifications. Total ascorbate was determined after reduction of DHA to ASC with DTT, and the concentration of DHA was estimated from the difference between total ascorbate pool (ASC and DHA) and ASC. The reaction mixture for total ascorbate pool contained 0.1 ml aliqout of the supernatant, 0.25 ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, and 0.05 ml of 10 mM DTT. After incubation for 10 minutes at ambient room temperature, 0.05 ml of 0.5% N-ethylmaleimide (NEM) was added to remove excess DTT. Ascorbate was determined using a similar reaction mixture except that 0.1 ml water was added instead of DTT and NEM. Color was developed in both reaction mixtures after the addition of the following reagents: 0.2 ml 10% trichloroacetic acid, 0.2 ml of 44% ortho-phosphoric acid, 0.2 ml of 4% α,α'-dipyridyl in 70% ethanol and 0.3% (w/v) $FeCl_3$. After vortexing, the mixture was incubated at 40 °C for 40 min and the

absorbance was read at 525nm. A standard curve was developed using ascorbic acid in the concentration range of 0-50 μg/ml.

The glutathione pool was assayed according to Zhang and Kirkhman (1996) utilising 0.4 ml aliqouts of supernatant neutralised with 0.6 ml of 0.5 M phosphate buffer (pH 7.5). For GSSG assay, the GSH was masked by adding 20 μl of 2-vinylpyridine to the neutralised supernatant, whereas 20 μl of water was added in the aliqouts utilised for total glutathione pool (GSH plus GSSG) assay. Tubes were mixed until an emulsion was formed. Glutathione content was measured in 1 ml reaction mixture containing 0.2 mM NADPH, 100 mM phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM 5,5'-dithiobis (2-nitrobenzoic acid), and 0.1 ml of sample obtained as described above. The reaction was started by adding 3 units of glutathione reductase and was monitored by measuring the change in absorbance at 412nm for 1 min. GSH was estimated as the difference between the amount of total glutathione and that of GSSG. A standard curve for GSH in the concentration range of 0-30 μM/ml was prepared.

3.2.11 Preparation of the enzyme extract

Whole seeds (10 at a time) were ground in 0.2 M Tris-HCl buffer (pH 7.8), containing 1 mM DTT, 0.2 mM ethylenediamine tetra-acetic acid (EDTA) and 2% polyvinylpolypyrrolidone (PVP) at a ratio of seed material: buffer of 1:3, and subsequently homogenised using a Virtis homogeniser. The homogenates was then centrifuged at 20 000xg using an Eppendorf centrifuge 5403 for 20 minutes. The entire procedure was performed at 4°C to minimise the effect of temperature on enzyme denaturation. The supernatants containing soluble proteins were stored in Eppendorf tubes at -70° C until further use.

3.2.12 Estimation of protein content

Protein estimation was carried out using the Bradford method (Bradford, 1976). Enzyme extract (3 μl) was mixed with 1 ml of Coomassie blue dye reagent. The mixture was allowed to stand for 15 minutes before measuring the absorbence at 595nm. A standard curve was prepared using bovine serum albumin (BSA).

3.2.13 Separation of isoenzymes of SOD, POD and CAT by gel electrophoresis and their specific detection

Electrophoretical separation of isoenzymes SOD, DHA reductase, ascorbate and guaiacol peroxidases, and CAT was achieved at 4°C on 7.5% polyacrylamide gels (pH 8.9), according to the procedure devised by Davis (1964).

3.2.13.1 Stock solutions for gels

Solution A – 3 M Tris adjusted to pH 8.9 with HCl.

Solution B - Solution A (13.66 ml in 100 ml) + TEMED (0.46 ml/100 ml). pH 6.9.

Solution C - Acrylamide (29.2 g/100 ml) + bisacrylamide (0.8 g/100 ml)

Solution D - Acrylamide (10 g/100 ml) + bisacrylamide (2.5 g/100 ml)

Solution E - 26.5 µM Riboflavin

All the above solutions were filtrated and then stored at 4° C

Solution F - 6 mM Ammonium persulfate

3.2.13.2 Running gel (7.5% acrylamide)

This was prepared by adding 6 ml of solution A to 12 ml of solution C, after which 4 ml of water and 16 ml of solution F were added. The resulting solution was first degassed for 5 minutes at 800 mbar before adding 10 µl of TEMED. The gels were allowed to polymerise at ambient room temperature for approximately 45 minutes.

3.2.13.3 Stacking gel (4.3% acrylamide)

This was prepared by adding 2.25 ml of solution B to 3.75 ml of solution E, after which 4.5 ml of solution D and 2.5 ml of water were added. The resulting solution was first degassed for 4 minutes before pouring onto the running gel. Polymerisation was allowed to take place under fluorescent light at ambient temperatures for approximately one hour.

3.2.13.4 Loading the samples

Polymerised, unloaded gels were subjected to pre-electrophoresis at 100 volts, provided the current was below 20 amperes, for an hour to remove catalysts (*e.g.* ammonium persulfate). Different volumes of enzyme extracts from the different treatments were loaded into channels onto the stacking gel with a micropippete. Enzyme samples, loaded in the sample wells were equalised on a protein basis.

3.2.13.5 Running the gels

The gels were run vertically using Tris-glycine pH 8.3 as a buffer. Loaded gels were first run at 100 V (constant voltage) for 15 minutes. This facilitated the concentration of the extract on to the upper layer of the gel surface and minimised heating, as molecules entered the gel. Further electrophoresis was run at 220 V at 4^0C for 2-3 hours until the tracking dye (0.1% bromophenol blue in extraction buffer) had reached the

bottom of the gel. The gel plates were then removed from the tank and the gel removed from the glass plates for staining. Control gels, without samples, were run parallel for detection of possible artificial activity caused by reagents.

3.2.13.6 Gel staining procedure

a) Superoxide dismutase (SOD)

SOD isozymes were stained according to Beauchamp and Fridovich (1971). Gels were first incubated in 0.2 mM nitro blue tetrazolium chloride (NBT) for 20 minutes and thereafter in a solution 0.25 M TEMED and 0.28 mM riboflavin in 0.036 M phosphate buffer (pH 7.8) for 15 minutes. All staining was conducted in the dark. The gels were then transferred into 0.036 M phosphate buffer (pH 7.8) and illuminated with flourescent light until white bands appeared against a blue background.

b) Ascorbate oxidase and peroxidase, and dehydroascorbate (DHA) reductase

The incubation medium for ascorbate oxidase was 0.1 M sodium phosphate (pH 6.4) containing 2 mM ascorbic acid. The incubation medium for ascorbate peroxidase was 0.1 M sodium phosphate (pH 6.4) containing 4 mM ascorbic acid and 4 mM hydrogen peroxide. For DHA reductase, the incubation medium consisted of 0.1 M sodium phosphate (pH 6.4) containing 2 mM DHA and 4 mM reduced glutathione (GSH). Gels for ascorbate oxidase, ascorbate POD and DHA reductase were incubated in their respective incubation media for 15 minutes at ambient temperatures. The gels were then rinsed three times in distilled water. All gels were then stained in a solution containing 0.125M hydrochloric acid, 0.1% ferric chloride and 0.1% potassium ferricyanide at ambient temperatures for apporoximately 10 minutes.

c) Guaiacol peroxidase

The isoenzymes of POD were detected using the staining procedure devised by Buttery and Buzzell (1968). Gels were incubated in a solution of 0.05% benzidine and 0.135% guaiacol in 0.25 M acetate buffer (pH 5) for five minutes, after which a total of 50 µl of 30% H_2O_2 was added until bands appeared as dark brown streaks on a colourless background. The developed gels were then washed three times in distilled water to remove excess substrate.

d) Catalase

Visualisation of CAT activity on gels was achieved by using the revised staining procedure devised by Stuber *et al.* (1988) and Acquaah (1992). Gels were incubated in 0.05% hydrogen peroxide for up to 5

minutes, followed by rinsing in distilled water before the gels were transferred to a staining solution containing 1% ferric chloride and 1% potassium ferricyanide. Catalase bands appeared as white bands against a blue background within 2-3 minutes after which the gels were rinsed and stored in distilled water. Photographs of gels were taken immediately after staining. Thereafter, the gels containing POD and CAT were stored in 50% glycerine in the cold room (5°C) whereas gels containing SOD isozymes were stored in diluted phosphate buffer (0.036 M) at 5°C.

3.2.14 Spectrophotometric quantification (*in vitro* assay) of enzymes

a) AFR reductase

AFR reductase was assayed spectrophotometrically by following a decrease in absorbance at 340nm due to NADH oxidation using an absorbance coefficient of 6.2 $mM^{-1}cm^{-1}$. The reaction mixture (1 ml) was composed of 0.2 mM NADH, 1 mM ascorbic acid, and 0.1 M Tris-HCl, pH 7.2. The reaction was started by adding 0.2 units ascorbate oxidase to generate saturating concentrations of AFR.

b) DHA reductase

The enzyme was assayed according to Asada (1984), following an increase in absorbance at 265nm due to the GSH-dependent production of ascorbate for 1 minute. The extinction coefficient used was 14 $mM^{-1}cm^{-1}$. The assay mixture (1 ml) consisted of 1 mM DHA, 1 mM GSH, and 100 mM potassium phosphate buffer, pH 6.4. The reaction was corrected for the non-enzymatic reduction of dehydroascorbate by GSH.

c) ASC POD

The enzyme was determined by following the decrease in absorbance at 265nm for 1 minute. The decrease in absorbance was due to hydrogen peroxide-dependent oxidation of ascorbate. The reaction mixture was composed of 50 µl of 5 mM ascorbic acid, 90 µl of 17 mM hydrogen peroxide, and 100 mM potassium phosphate buffer, pH 6.5.

d) Superoxide dismutase

The activity of SOD was assayed in terms of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The 1 ml reaction mixture contained 63 µM NBT, 1.3 µM riboflavin, 13 mM methionine, 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), and an enzyme extract. Riboflavin was added last. The tubes containing the mixtures were placed under two flourescent lamps for 8 minutes. Switching off the light stopped the reaction and the change in

absorbance was read at 560nm. One unit of SOD was defined as the volume of enzyme extract that would cause a 50% inhibition of photochemical reduction of NBT.

e) Catalase

Activity of catalase was measured by the method of Chance and Maehly (1955). The reaction involves the decomposition of H_2O_2 and monitoring the decline in absorbance at 240nm for 1 minute to follow this. The 1 ml reaction mixture contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and an enzyme extract. The reaction was initiated with addition of H_2O_2.

f) Guaiacol POD

For guaiacol POD, the oxidation of guaiacol was measured by an increase in absorbance at 470nm for 1 minute using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ (Chance and Maehly, 1955). The 1 ml assay mixture contained 16 µl of 20 mM guaiacol, 10 mM phosphate buffer (pH 7.0) and an enzyme extract. The reaction was started with the addition of 6 µl of 40 mM H_2O_2.

3.2.15 Sugar extraction

Whole seeds harvested at different stages of development were plunged in liquid nitrogen for few minutes before they were placed in a freeze-drier for approximately 5 days. The seeds were then stored in a vacuum-sealed container at -65° C until further processing. Three samples of 50 mg from each developmental stage were weighed and then homogenised using a pestle and mortar in 3 ml of boiling methanol. The extract was shaken for 15 minutes and thereafter centrifuged in a benchtop centrifuge for 10 minutes. The supernatant was removed and dried at 45° C under a stream of nitrogen.

3.2.16 Sugar derivatisation

Sugars were analysed by gas chromatography (GC) after conversion to volatile derivatives. This involved the production of oximes (Tanowitz and Smith, 1984) followed by trimethylsilyl derivatisation of the oximes (Sweeley *et al.*, 1963). The dried sugar extract was resuspended in 500 µl STOX, a pyridine solution containing 25 mg/ml hydroxylamine hydrochloride and 6 mg/ml phenyl-β-D-glucopyranoside as an internal standard (Pierce Chemicals). The resulting solution was heated at 40° C for 20 minutes in stoppered vials. Thereafter an aliqout of 100 µl of the solution was taken to dryness under nitrogen, after which 100 µl of silylating reagent, Sylon BTZ (SUPELCO), was added. The resulting solution was then allowed to stand for 15 minutes at ambient room temperature. 1 µl of the derivatised sugars was analysed.

Sugars were identified by comparison of retention times with known authentic standards (MERCK) of fructose, glucose, galactose, sucrose, raffinose and stachyose.

3.2.17 Sugar determination

The separation of sugars was achieved by using a Varian 3700 gas chromatograph fitted with a flame ionisation detector and a 1.8 m X 6 mm I.D. glass packed with 5% OV17 on chromosorb WHP 100/120 mesh. Initially, the column was held at 150° C for 4 minutes. Thereafter, the temperature was increased to 300° C at a rate of 5° C per minute. The final temperature was maintained for 10 minutes. Sugars were quantified as the percentage of the ratio of the peaks of the sugar to the internal standard.

3.3 RESULTS

3.3.1 Changes in fresh weight, dry weight and moisture content

The development of soybean seeds was accompanied by increases in both the fresh weight and the dry weight of whole seed during the first 50 DAF, at which time the seeds had accumulated approximately 17 times the initial weight of seeds from first harvest (20 DAF) (Figure 3.1). Fresh weight increased from 30 mg per seed at 20 DAF up to 540 mg per seed at 50 DAF, after which fresh weight decreased steadily to a final value of 230 mg per seed at 80 DAF. At 20 DAF, the dry weight was only 5 mg per seed but increased rapidly thereafter and reached approximately 200 mg per seed at 50 DAF. Seed dry weight increased only slightly from 50 DAF with seeds harvested at 80 DAF recording only a further 20 mg increase.

Seeds showed a high moisture content (MC) at 20 DAF but the levels declined steadily thereafter up until 60 DAF (Figure 3.2). Approximately 40% moisture was lost during this period, of which 27% was lost after the seed had attained maximum fresh weight. Water loss was marked between 60 and 80 DAF, and this period accounted for approximately 55% of the total moisture lost by the seed. Interestingly, the stage 60 DAF coincided with the first visible signs of seed coat browning. The final seed moisture content at 80 DAF was 0.05 g H_2O per gram fresh weight.

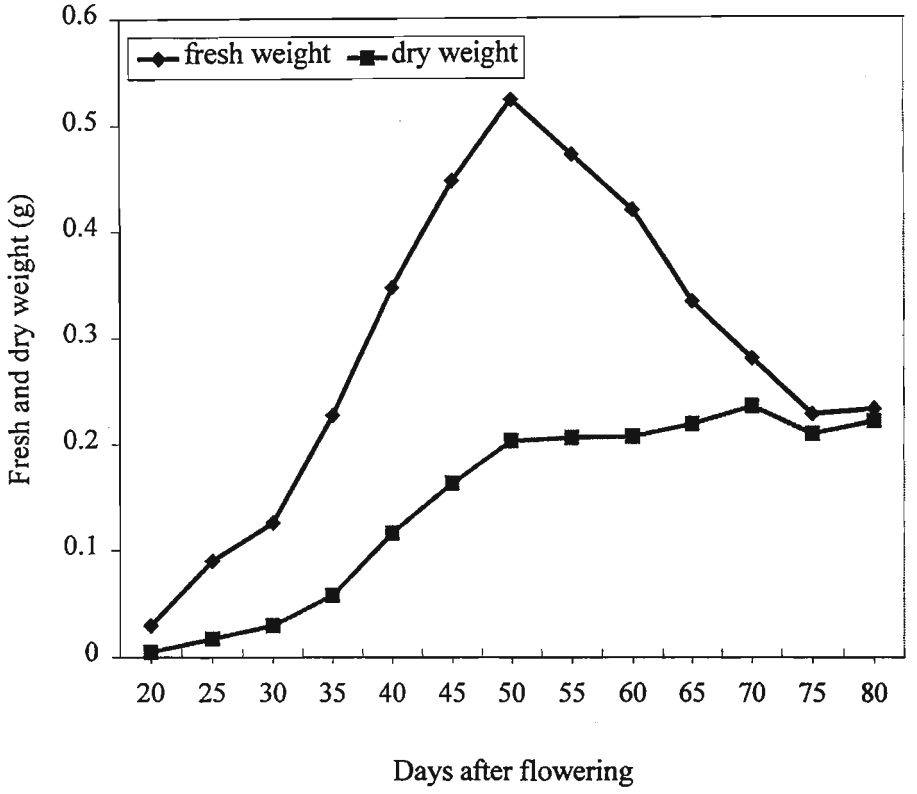

Figure 3.1. Changes in the fresh weight and dry weight (per seed) during the development of soybean seeds. Values are the means of three experiments.

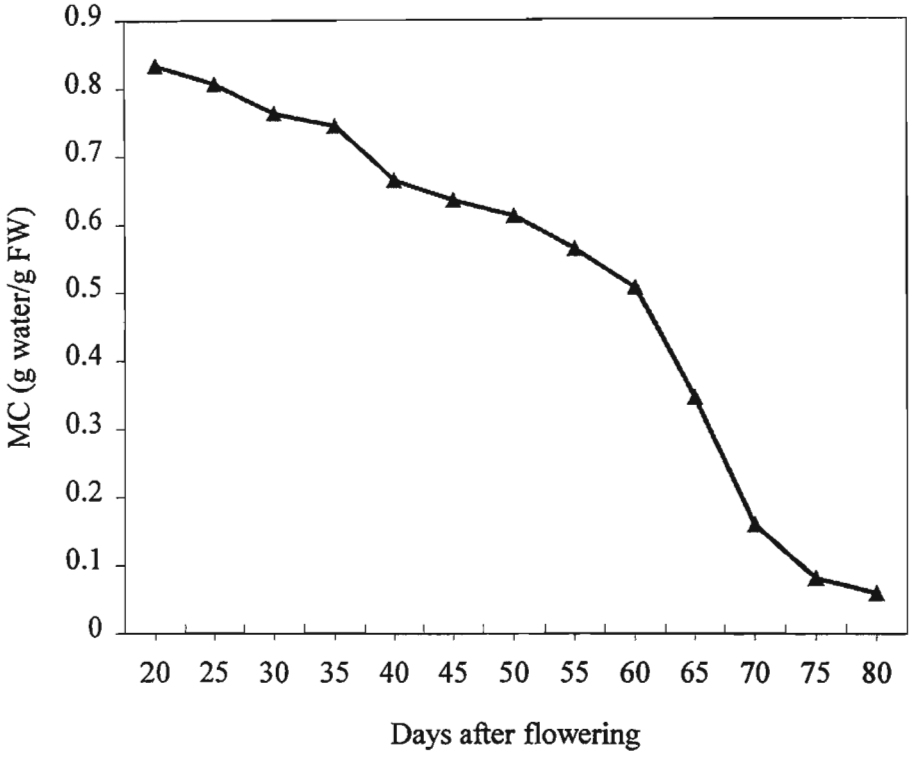

Figure 3.2. The changes in the moisture content of soybean seed during development. Values are the means of three experiments.

3.3.2 The accumulation of lipids during seed development

Figures 3.3 and 3.4 give a summary of the results obtained regarding the changes in the triglycerides, and phospholipids, respectively, during the development of soybean seeds. Generally, both lipids increased progressively as the seeds approached maturity.

Approximately 43.6 μM of triacylglycerol (TAG) per gram dry weight was detected as early as first harvest (Fig. 3.3). The rate of TAG accumulation increased continuously from 20 DAF up until 50 DAF, at which time TAG content reached maximum. Thereafter, TAG content began to decline between 50 and 60 DAF. At 80 DAF, TAG content was 32.6-fold higher than the content recorded at 20 DAF.

The amount of phosphorus released following acid digestion of lipid was used to quantify the changes in phospholipids during seed development. Figure 3.4 shows that phospholipids levels increased quite dramatically between 20 and 25 DAF, followed by a sharp decline between 25 and 40 DAF. Another increase in phospholipid levels was again noted between 40 and 50 DAF, and between 65 and 80 DAF.

Four phospholipid classes: phosphatidylserine/inositol (PS/I), phosphatidic acid (PA), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were identified after TLC separation (Figure 3.5). All four phospholipid classes were present at 20 DAF, where PC and PE were the dominant classes. The polar lipids increased dramatically at 25 DAF but decreased thereafter. In contrast, PS/I increased continuously up to 30 DAF before declining. PC and PE increased again between 40 and 50 DAF, and between 65 and 80 DAF. Phosphatidic acid, on the other hand, declined continuously from 30 DAF up to 80 DAF. Also of note was the gradual decline in the PC to PE ratio between 25 and 45 DAF. However, the ratio increased progressively thereafter as the seeds approached maturity at 80 DAF. At final harvest (80 DAF), PC was the most dominant phospholipid class, followed by PE and then PS/I. Phosphatidic acid, on the other hand, was only present in trace amounts.

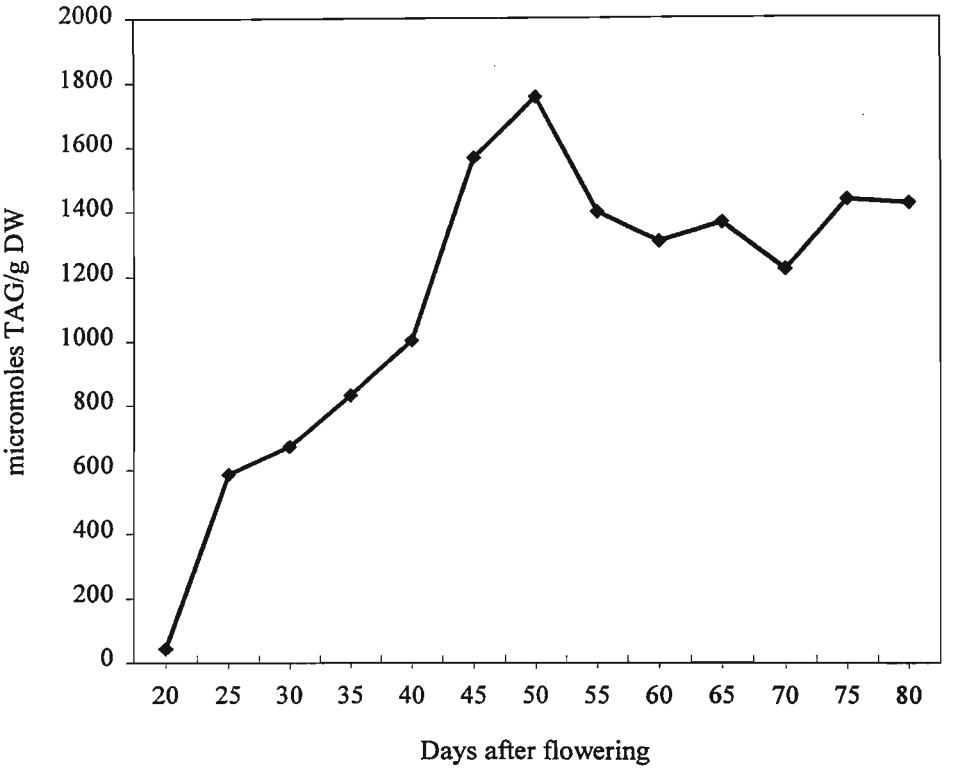

Figure 3.3. Levels of TAG at different stages of soybean seed development. Values are the means of three experiments.

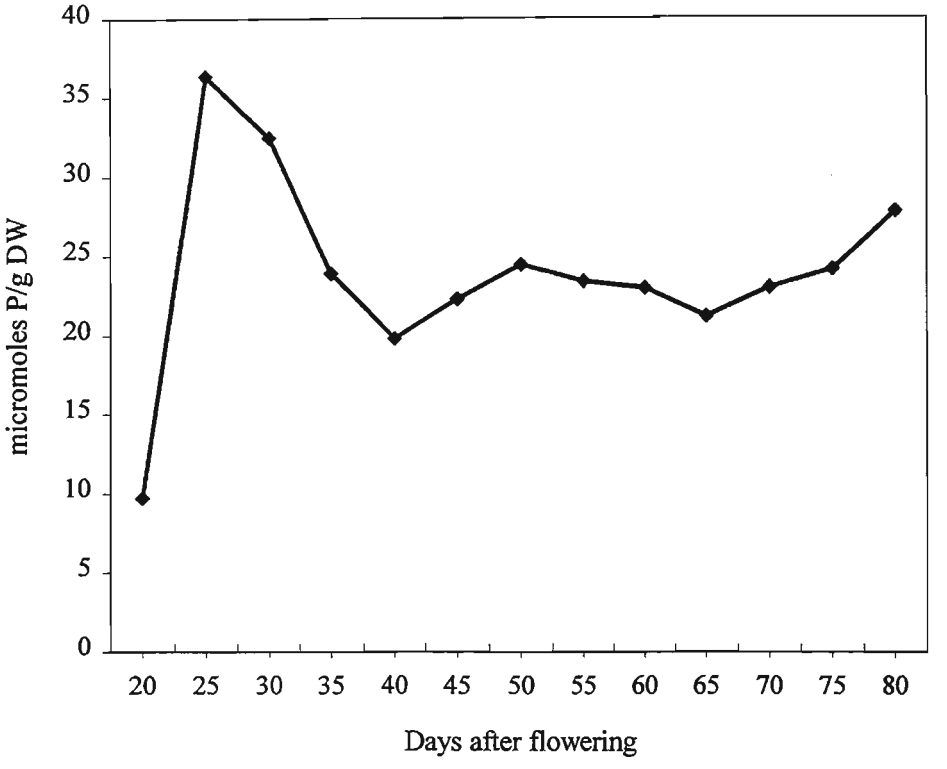

Figure 3.4. Accumulation of phospholipids during soybean seed development. Values are the means of three experiments.

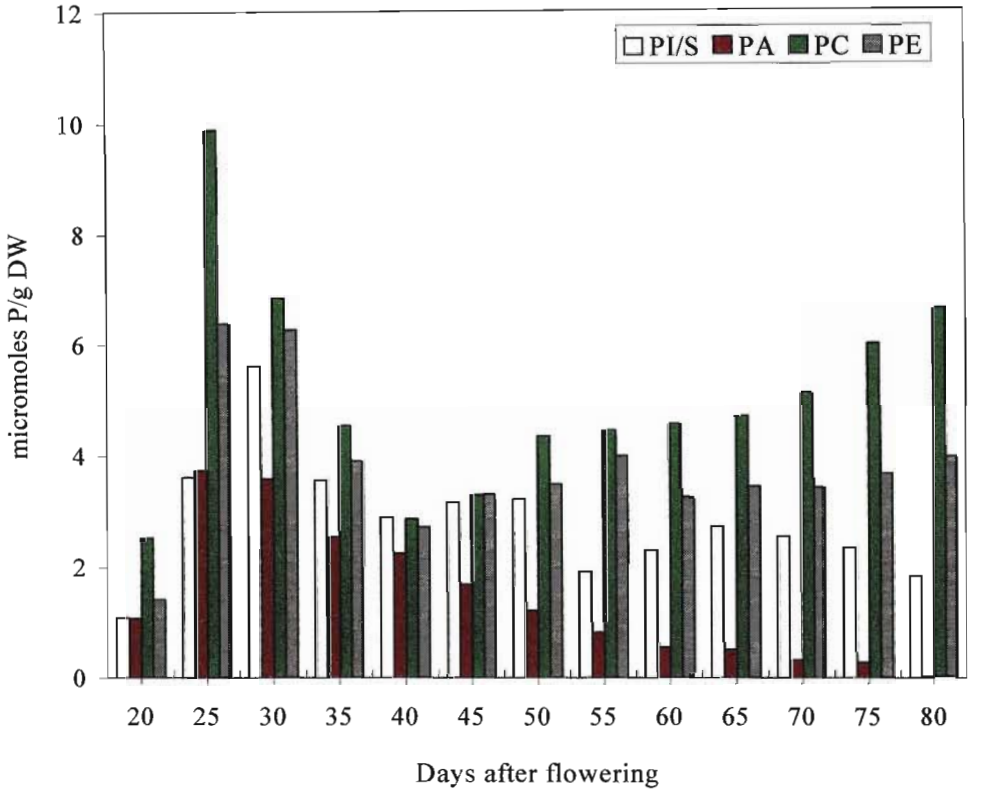

Figure 3.5. Changes in different phospholipid classes in soybean seeds harvested at different stages of seed development. Values are the means of three experiments.

3.3.3 Developmental changes in fatty acids

Five fatty acids were identified and their pattern of change followed during the development of seeds (Figure 3.6). The levels of linolenic acid (18:3) were high at 20 DAF but then declined steadily afterwards as the seed matured. Levels of linolenic acid remained fairly constant from 45 to 80 DAF, and were half the initial recorded levels. Oleic acid (18:2) was the predominant fatty acid throughout the development of seeds, representing almost 53% of the total fatty acids, followed by stearic acid (18:1) at 25%. Oleic acid increased only slight during seed development, with only 10% added to the initial recorded value at 75 DAF. Stearic acid, on the other hand, increased gradually and reached maximum at 45 DAF but then decreased, reaching a value that was only 5% more than the initial recorded value at 25 DAF. The 16:0 fatty acid decreased, but only slightly before remaining constant for the remainder of maturation. No pattern of changes could be established for the 18:0 fatty acid.

3.3.4 Changes in lipid hydroperoxides

The changes in the magnitude of lipid peroxidation during seed development were measured indirectly as changes in the levels of lipid hydroperoxides (LOOH). Low levels of LOOH were recorded at 20 DAF but thereafter increased gradually and reached maximum at 45 DAF (Figure 3.7), this corresponding with the pattern of fresh weight increase (Figure 3.1). LOOH increased by approximately 3.6-fold over the period 20-40 DAF, the greatest increase occurring between 40 and 45 DAF just before the seeds attained maximum dry weight. Thereafter, the levels of LOOH began to decline, coinciding with the decline in fresh weight (Figure 3.1). The decrease was gradual between 45 and 55 DAF but changed abruptly between 55 and 60 DAF where there was an approximate 80% decrease in LOOH levels. Lipid peroxidation changed slightly after 60 DAF, and LOOH reached their lowest value at final harvest (80 DAF).

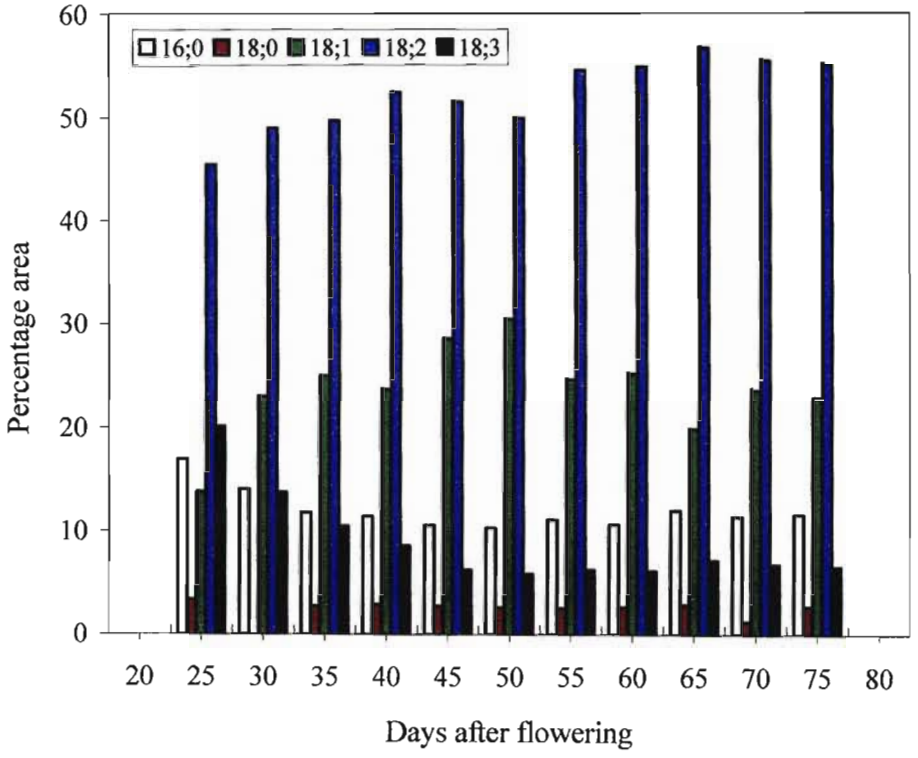

Figure 3.6. The fatty acid composition of the total lipids in soybean seeds harvested at different stages of seed development. Values are the mean of three experiments.

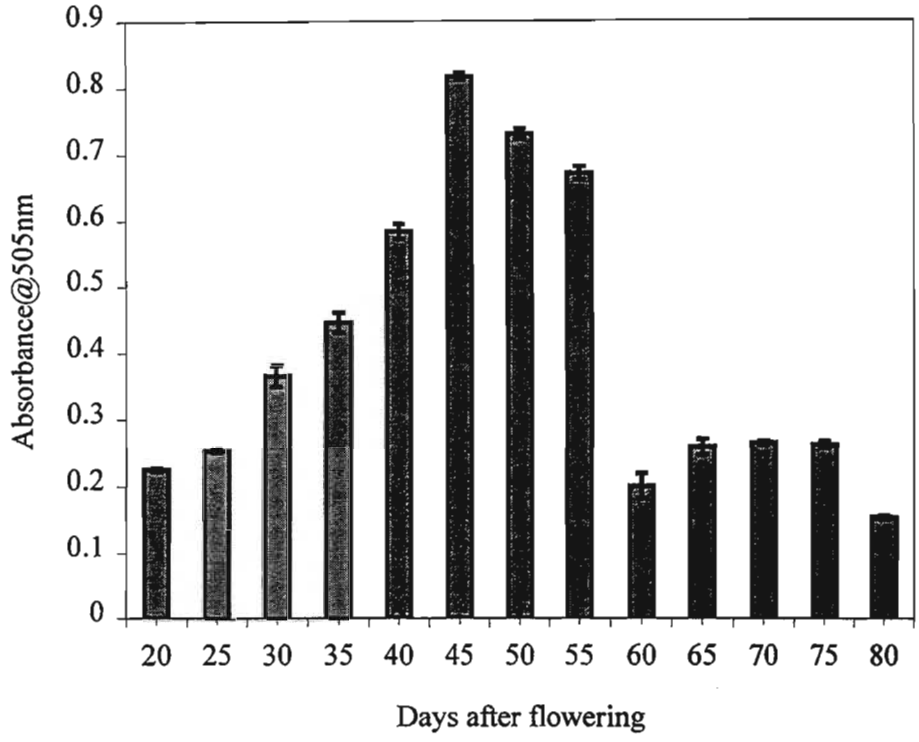

Figure 3.7. The changes in the levels of lipid hydroperoxides during the development of soybean seeds. Values are the mean ± SE of three experiments.

3.3.5 Changes in the antioxidant systems

Changes in total ascorbate pool (ascorbic acid and dehydroascorbate) per seed were analysed during seed development and are summarised in Figure 3.8. The total ascorbate content increased gradually during the first 40 DAF. The increase, which was due to increases in both ascorbic acid (ASC) and dehydroascorbate (DHA), was gradual between 20 and 35 DAF, but very pronounced between 30 and 40 DAF. Maximum total ascorbate content was recorded at 40 DAF, of which 72% was ASC. Also of note was that during the period between 20 and 40 DAF, there was a 10-fold increase in ASC whereas DHA increased by 36-fold. The total ascorbate pool began to decrease after 40 DAF. The decline was more dramatic between 40 and 45 DAF, where approximately 77% and 27% of DHA and ASC were lost, respectively. Thereafter, the decline in total ascorbate was gradual and reached fairly constant levels between 65 and 80 DAF. The decrease in ascorbate pool from 45 DAF was attributed to a decrease in ASC whereas the increase in DHA contributed in maintaining the total ascorbate levels fairly constant from 65 DAF.

The changes in total glutathione pool, which includes both reduced (GSH) and oxidised (GSSG) forms of glutathione, during soybean seed development are presented in Figure 3.9. The total glutathione pool increased gradually from 20 to 80 DAF and reached a maximum at 80 DAF, and at which time it was 21-fold higher than the initial recorded levels at 20 DAF. The increase in GSH was most prominent in the increase in total glutathione pool as it constituted the bulk of the total glutathione. Also of note, was the observation that only GSH contributed to the total glutathione pool after 65 DAF, and no GSSG was detected. The oxidised form of glutathione, GSSG, was also present as early as 20 DAF but thereafter increased 6–fold between 20 and 40 DAF. The levels of GSSG declined dramatically from 60 DAF until no GSSG could be detected after 65 DAF

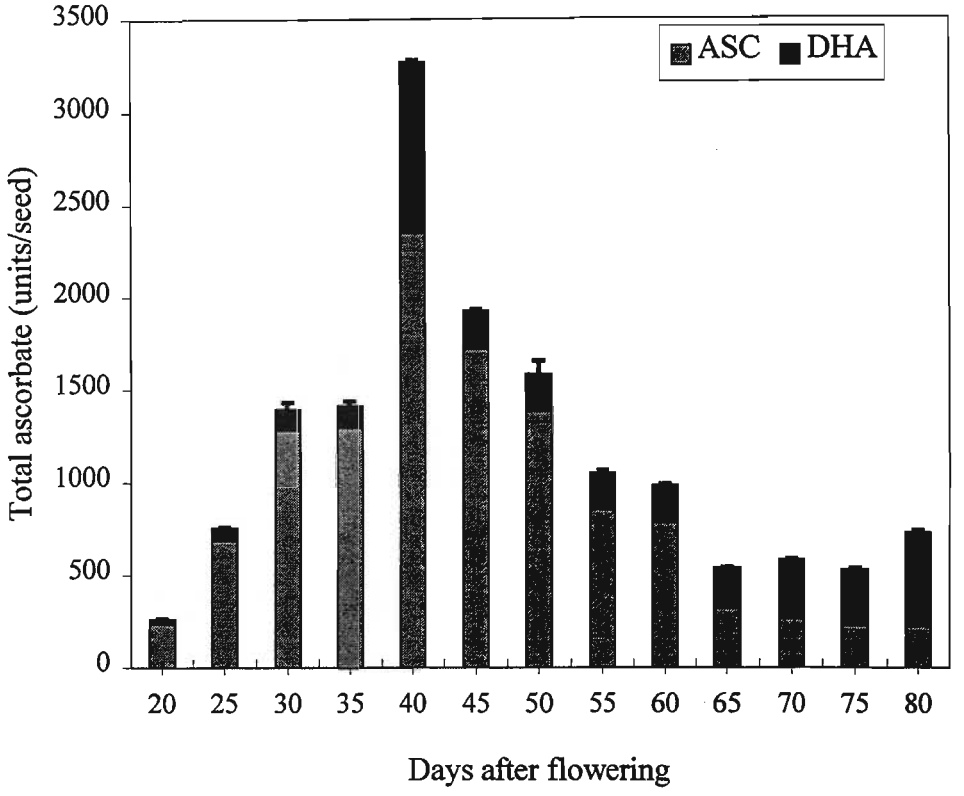

Figure 3.8. Ascorbic acid (ASC) and DHA contents of seed at different stages of soybean seed development. Units equivalent to nmoles per gram fresh weight. Values are the mean ± SE of three experiments.

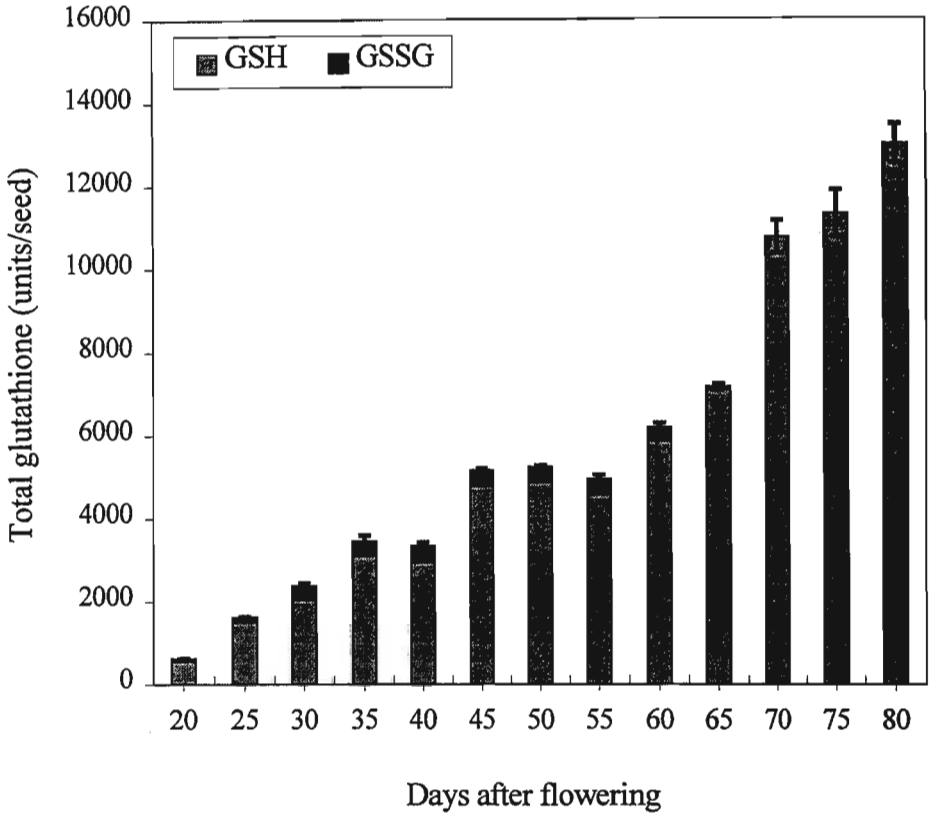

Figure 3.9. Changes in total glutathione (GSH + GSSG) content per seed during soybean seed development. Units equivalent to nmoles per gram fresh weight. Values are the mean ± SE of three experiments.

3.3.6 Changes in DHA reductase

Spectrophotometric quantification of DHA reductase activity, an enzyme catalysing the reduction of DHA to ASC, revealed that the activity levels of the enzyme per seed were quite low at 20 DAF (Fig. 3.10). Thereafter, levels of the enzyme increased gradually between 20 and 30 DAF, followed by a sharp increase between 30 and 40 DAF. The enzyme increased more 20-fold in the period between 20 and 45 DAF. DHA reductase began to decrease after 45 DAF, losing almost 40% of its activity after which the enzyme remained fairly constant up until 80 DAF. DHA reductase activity closely paralleled changes in seed fresh weight (Figure 3.1), but did not decline completely in the final stages of seed maturation.

The electrophoretic pattern showed that 8 protein bands with DHA reducing properties were present in soybean seeds (Figure 3.11). Band 4 was prominent in all the stages of soybean seed development and appeared to represent most of the DHA reductase activity present in the seeds. Bands 1 and 2 were the least mobile of the 8 bands and their intensity developed as the seeds matured. A similar increase in intensity was also noted in band 3. The remaining bands (5, 6, 7, and 8) were detected at 20 DAF, but disappeared at mid-maturation only to re-appear again late during seed maturation. Data obtained from electrophoresis regarding the pattern of DHA reductase change did not concur with data obtained from spectrophotometric assays. It is noteworthy, though, that gels were loaded on a protein basis, whereas in spectrophotometric assays the enzyme levels were expressed per seed.

3.3.7 Changes in AFR reductase

This enzyme co-operates with DHA reductase in ASC recycling from AFR (the first product of ASC oxidation) and DHA, respectively. AFR reductase activity was low during the early stages of seed development, but increased gradually as the seeds matured (Figure 3.12). Quantitatively, AFR reductase increased gradually from 47 units at 20 DAF to 240 units at 35 DAF, an increase of approximately 4-fold in the enzyme activity. A further increase in AFR reducatse activity was recorded from 40 DAF to 50 DAF; this was followed by a decrease in the activity of the enzyme in the period between 50 and 65 DAF. The final AFR reductase value at 80 DAF was approximately 200 nmol and was approximately 4-fold higher than the initial recorded value at 20 DAF. Of note was the similar overall pattern of change in both AFR and DHA reductases, although AFR reductase appeared to peak 5 days after DHA reductase had reached its maximum.

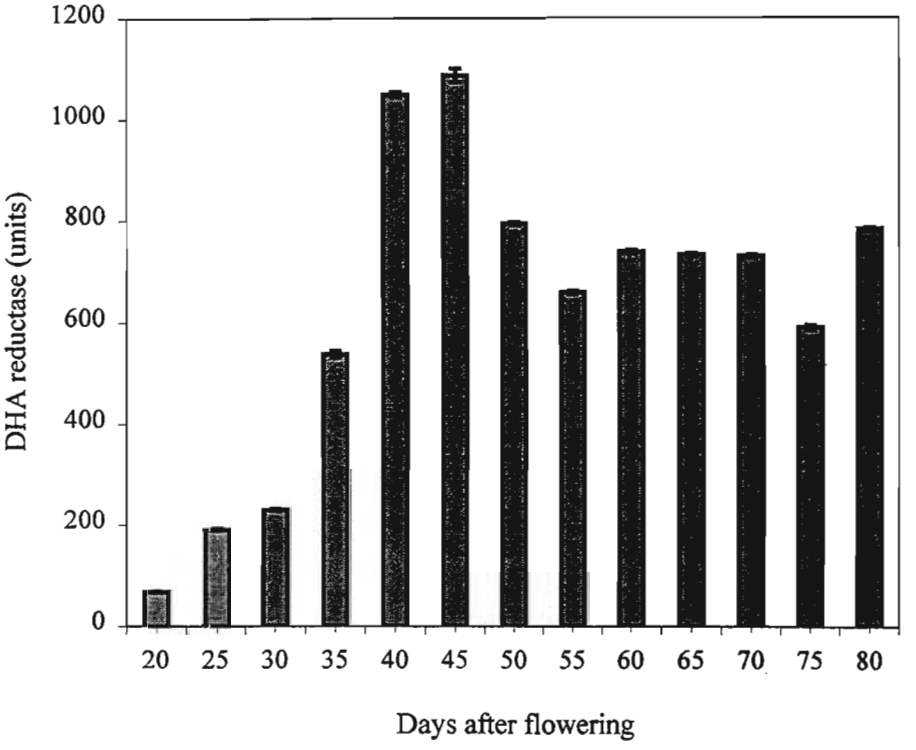

Figure 3.10. Changes in the activity levels of DHA reductase during the development of soybean seeds. Values are the mean ± SE of three experiments. One unit of DHA reductase equates to 1 nmol DHA reduced per milligrams protein per minute per seed.

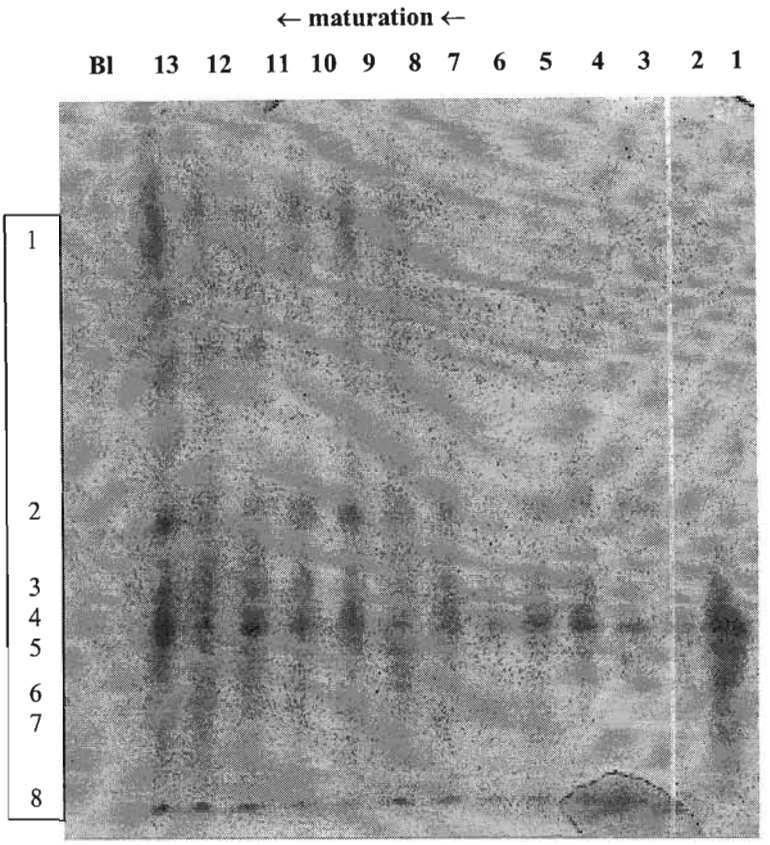

Figure 3.11. Changes in the electrophoretic pattern of DHA-reducing proteins for different stages of soybean seed development. **Bl** denotes a blank of tracking dye bromophenol blue, without any enzyme extract, whereas numbers 1-13 represent successive 5-day sampling intervals between 20-80 DAF, respectively. The enzymes appeared as light blue bands on a clear background after 50 μg protein was loaded per lane for each developmental stage.

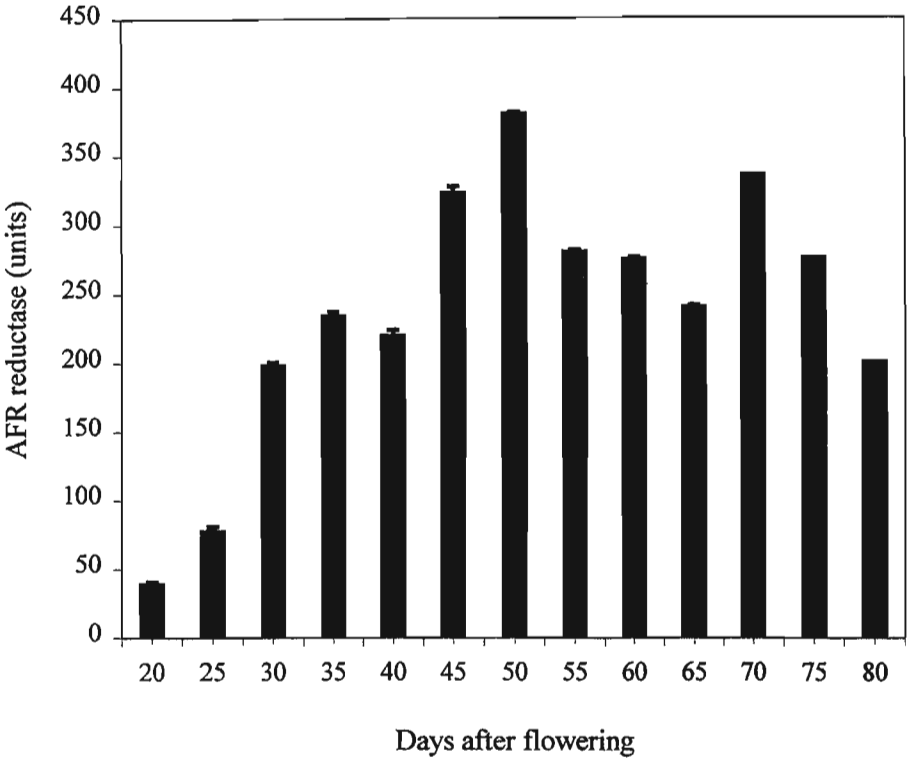

Figure 3.12. AFR reductase activity at different stages of soybean seed development. Values are the mean ± SE of three experiments. One unit AFR reductase equates to 1 nmol NADH oxidised per milligram protein per minute per seed.

3.3.8 Changes in superoxide dismutase

Both electrophoresis and spectrophotometric enzyme assays established that extractable SOD was present as early as first harvest during the development of soybean seeds. Nine distinct bands characteristic of SOD were detected on gels following PAGE (Figure 3.13). These bands are numbered in order of increasing relative mobility. Six of the nine bands were detected as early as 20 DAF when first harvest took place whereas bands 2, 3, and 7 only became clearly evident at 35 DAF.

With 50 μg protein loaded per lane, qualitative differences were noted between the SOD bands from different developmental stages as denoted by the differences in band intensity and number. An increase in SOD band intensity was observed as the seeds matured. The increase in band intensity was gradual, with low-intensity and clearly defined bands evident at first harvest. By the time seeds reached maturity, the intensity was so great that all the bands appeared to merge. Bands 4, 5, and 6 with intermediate mobility exhibited the highest intensity and thus appeared to represent most of the SOD activity recorded in all the developmental stages examined.

That the levels of SOD were low during the early stages of seed development was further confirmed by the spectrophotometric quantification of the enzyme. However, the decrease in SOD during late maturation as indicated in the spectrophotometric assay is not reflected on the gels. Figure 3.14 illustrates that SOD was low at 20 DAF and showed an initial increase between 20 and 30 DAF. The activity levels of the enzyme increased a further 2-fold between 40 and 45 DAF, and by 50 DAF the enzyme had reached its maximum activity, which was 8-fold higher than the initial value recorded at 20 DAF. The levels of the enzyme decreased abruptly after 55 DAF and remained at a constant level between 70 and 80 DAF. As noted earlier, the overall levels of the enzyme activity closely paralled the changes in seed fresh weight (Figure 3.1).

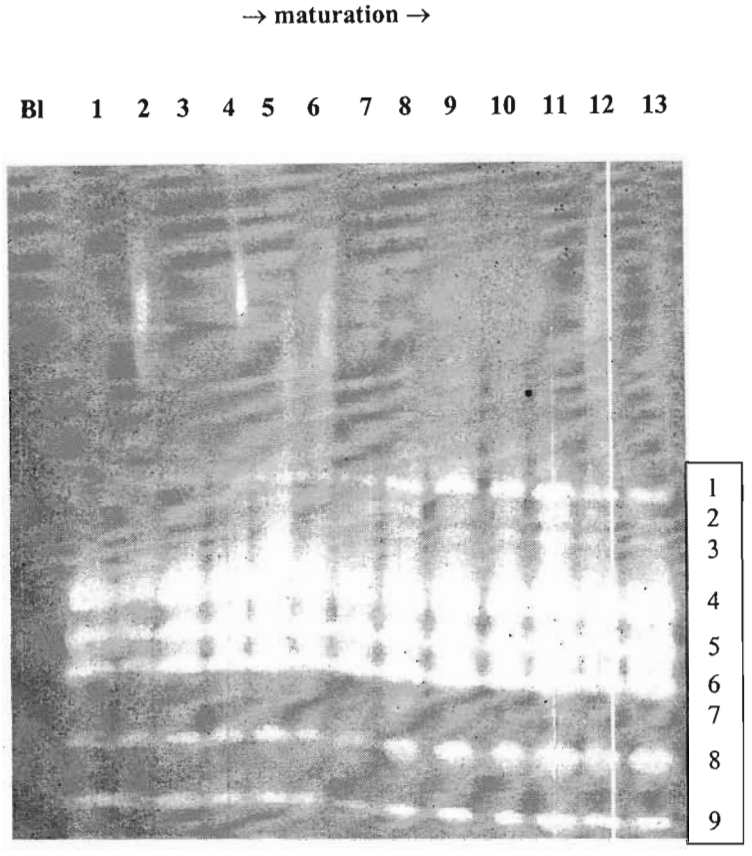

Figure 3.13. Electrophoretic separation of SOD isozymes during seed development. Numbers from 1 to 13 denote the different stages of development. **Bl** denotes a blank of tracking dye bromophenol blue, without any enzyme extract, whereas lanes 1-13 represent successive 5-day sampling intervals between 20-80 DAF, respectively. The enzymes appeared as achromatic bands on a blue-stained background after 50 μg protein was loaded per lane for each developmental stage.

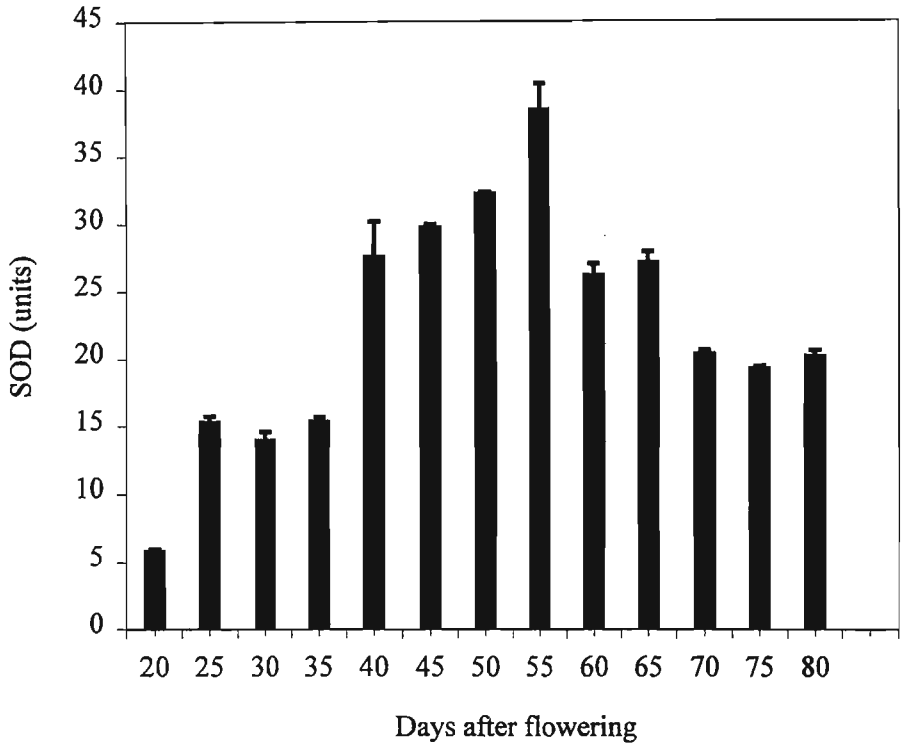

Figure 3.14. Developmental changes in the activity levels of SOD during soybean seed maturation. Values are the mean ± SE of three experiments. One unit of SOD was defined as the amount that inhibits the NBT photoreduction by 50 %.

3.3.9 Changes in catalase

Visual observation of the PAGE gels after staining revealed two CAT isoenzymes as two close bands with low mobility (Figure 3.15). In some cases though (*i.e.* lanes 1-3) the bands appeared as a single diffuse band. Changes in CAT activity were detected by the differences in band intensities. High band intensities were recorded during the early stages of development (i.e. between 20 and 30 DAF; lanes 1-3), thereafter the intensity of the bands decreased (lanes 5-8; Figure 3.15) before increasing again between 60 and 80 DAF (lanes 9-13).

Spectrophotometric quantification of CAT revealed a lower CAT activity per seed during the early stages of seed development (Figure 3.16). The activity of the enzyme increased gradually thereafter and reached a peak at 40 DAF. Approximately 50% of the enzyme activity recorded at 40 DAF was accumulated in the developmental stage between 35 and 40 DAF. Catalase levels declined after 40 DAF and 45% of its activity was lost in the period between 40 and 60 DAF. Another peak in CAT activity was recorded at 65 DAF, after which the enzyme decreased and reached a final value that was approximately 5.5-fold higher than the recorded initial value. This bimodial activity was at variance with the electrophoretic results.

→ maturation →

Bl 1 2 3 4 5 6 7 8 9 10 11 12 13

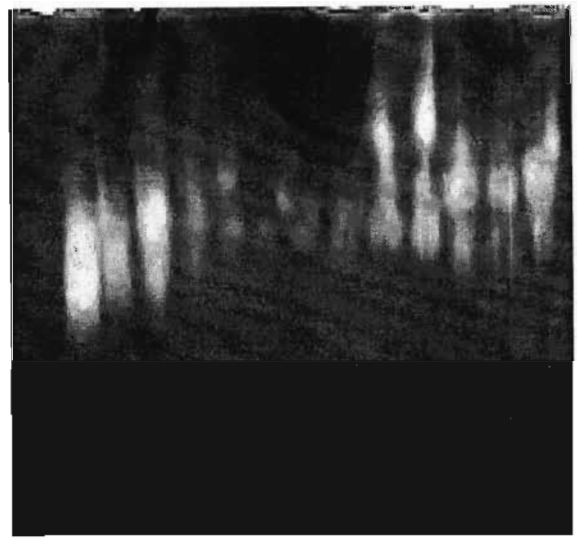

Figure 3.15. The separation of CAT isoenzymes following PAGE of crude ezyme extracts from different stages of soybean seed development. Numbers from 1 to 13 represent successive 5-day sampling intervals between 20-80 DAF, respectively. **Bl** denotes a blank of tracking dye bromophenol blue, without any enzyme extract. The enzymes appeared as achromatic bands on a blue-stained background after 50 μg protein was loaded per lane for each developmental stage.

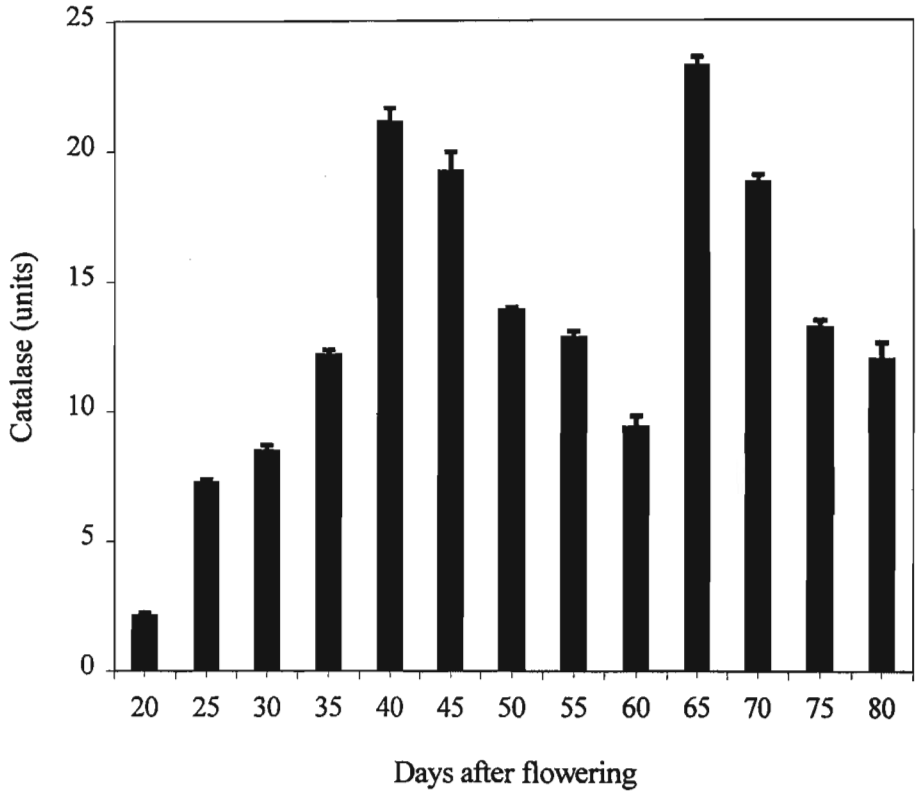

Figure 3.16. Catalase activity during the development of soybean seeds. Levels are expressed as mean ± SE of three experiments. One unit of catalase is equivalent to 1 nmol of H_2O_2 decomposed per milligram protein per minute per seed.

3.3.10 Changes in peroxidases

a) Guaiacol POD

This enzyme was present in low quantities during the early stages of development, before increasing gradually as the seeds approached maturity. Both PAGE and spectrophotometric assays of the enzyme confirmed this pattern of change. Unfortunately, gels stained for guaiacol POD activity could not be captured or photographed as the bands faded rapidly after removal from the staining solution.

Two early stages of enzyme increase (i.e. between 20 and 25 DAF, and between 40 and 50 DAF) were identified during the first 50 DAF, by which time the activity levels of the enzyme were approximately 3-fold higher than the recorded initial activity at 20 DAF (Figure 3.17). Thereafter, the enzyme levels fluctuated between 36 and 68 nmol mg^{-1}protein min^{-1} during the final 30 days of the 80-day harvest period. The final recorded guaiacol POD levels were approximately 55 mol.mg^{-1}protein min^{-1}, and these levels were approximately 5-fold higher than the initial recorded levels at 20 DAF.

b) Ascorbate peroxidase

When assayed spectrophotometrically, ascorbate POD levels were low at the start of harvesting (Fig 3.18) and thereafter increased sharply. Peak activity of the enzyme was recorded between 40 and 45 DAF, and during this period the enzyme levels were 4.5-fold higher than the initial recorded levels. Ascorbate POD decreased almost linearly from 45 DAF and no traces of the enzyme were detected after 70 DAF.

Electrophoresis revealed two characteristic bands of ascorbate peroxidase activity during soybean seed development (Fig. 3.19). The intensity staining of the two bands was high during the early stages of seed development (lanes 1-6), but decreased gradually as the seed matured. By mid seed development (i.e. after 45 DAF; lane 7), both bands had disappeared.

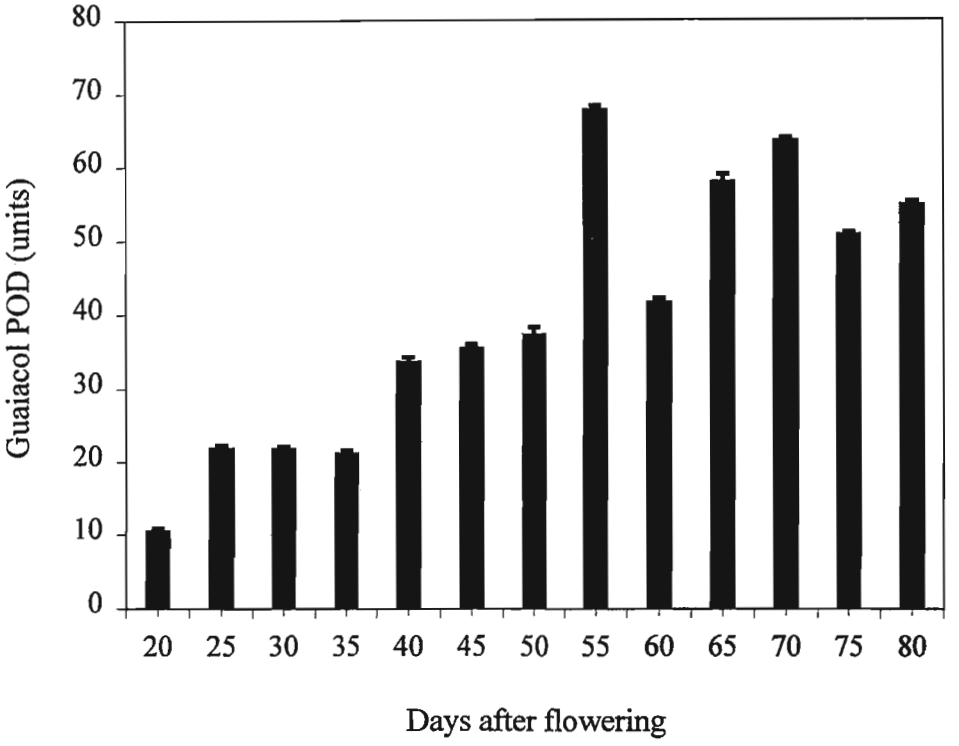

Figure 3.17. The activity levels of guaiacol peroxidase at different stages of soybean seed development. A unit of guaiacol POD is equivalent to nmol guaiacol oxidised per milligrams protein per minute per seed. Levels are expressed as mean ± SE of three experiments.

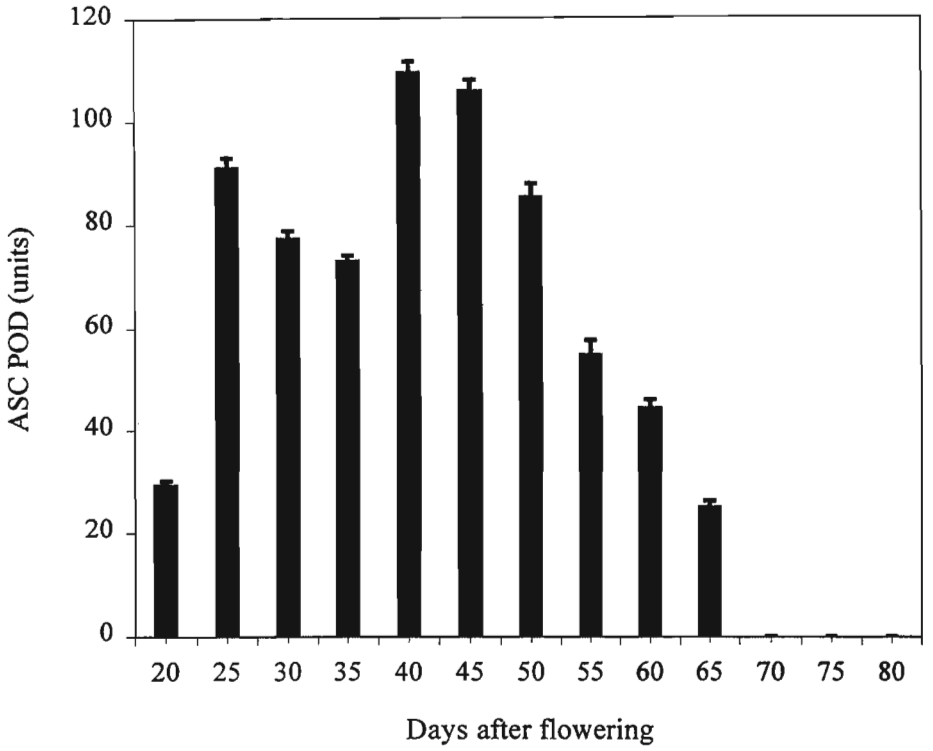

Figure 3.18. Levels of ascorbate peroxidase at different stages of soybean seed development. Values are the mean ± SE of three experiments. One unit of ascorbate peroxidase is equivalent to 1 nmol ascorbate oxidised per milligram protein per minute per seed.

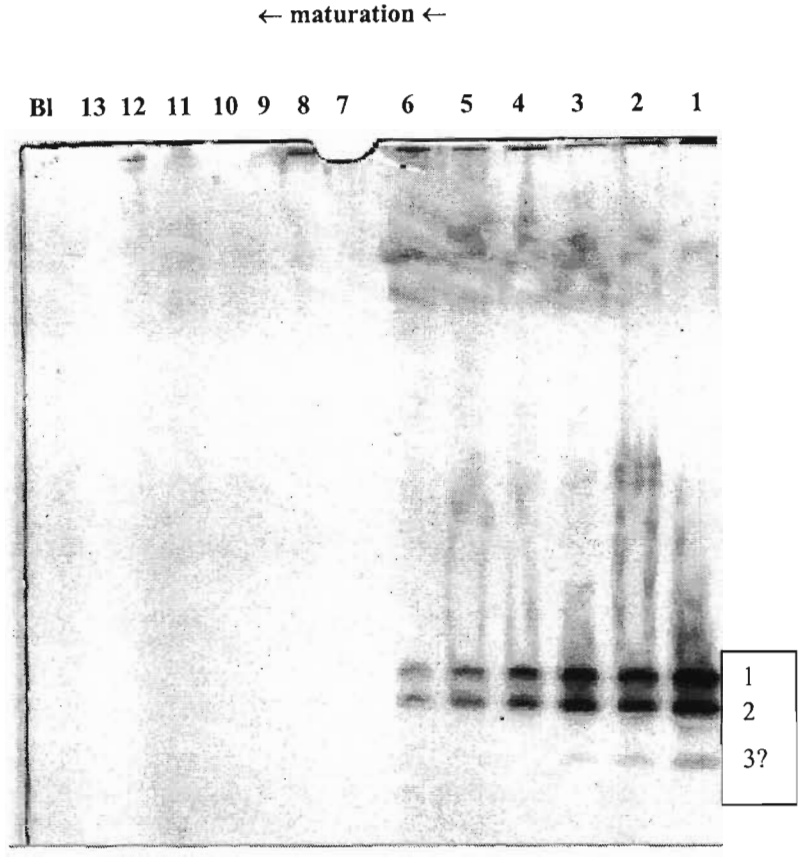

Figure 3.19. The migration of ASC POD isoenzyme following PAGE of crude extracts from different stages of seed development. **Bl** denotes a blank of tracking dye bromophenol blue, without any enzyme extract, and lanes 1-13 represent successive 5-day sampling intervals between 20-80 DAF, respectively. The enzyme appeared as dark blue bands on an achromatic background after 50 μg protein was loaded per lane for each developmental stage.

3.3.11 Changes in the levels of sugars

The changes in the concentration of three soluble sugars were followed during soybean seed development (Figure 3.20), and these included reducing sugars (glucose and fructose) and a non-reducing sugar, sucrose. All three sugars were present at low concentrations at 20 DAF, decreased slightly at 25 DAF before increasing gradually from 30 DAF. Glucose began to decline continuously from 40 DAF until no traces of this sugar were detected at 60 DAF. Low levels of glucose were seen at 65 and 70 DAF but thereafter became undetectable. Fructose concentration, on the other hand, did not exhibit any distinct pattern after the initial gradual increase between 30 and 45 DAF, but the sugar was still detected at final harvest (80 DAF). In contrast to the changes observed for reducing sugars, sucrose, after the initial lag phase, increased almost exponentially from 30 DAF and reached peak concentration at 50 DAF. Thereafter, the concentration of sucrose declined to lower levels during the period between 50 and 80 DAF but remained by far the most abundant of the three sugars.

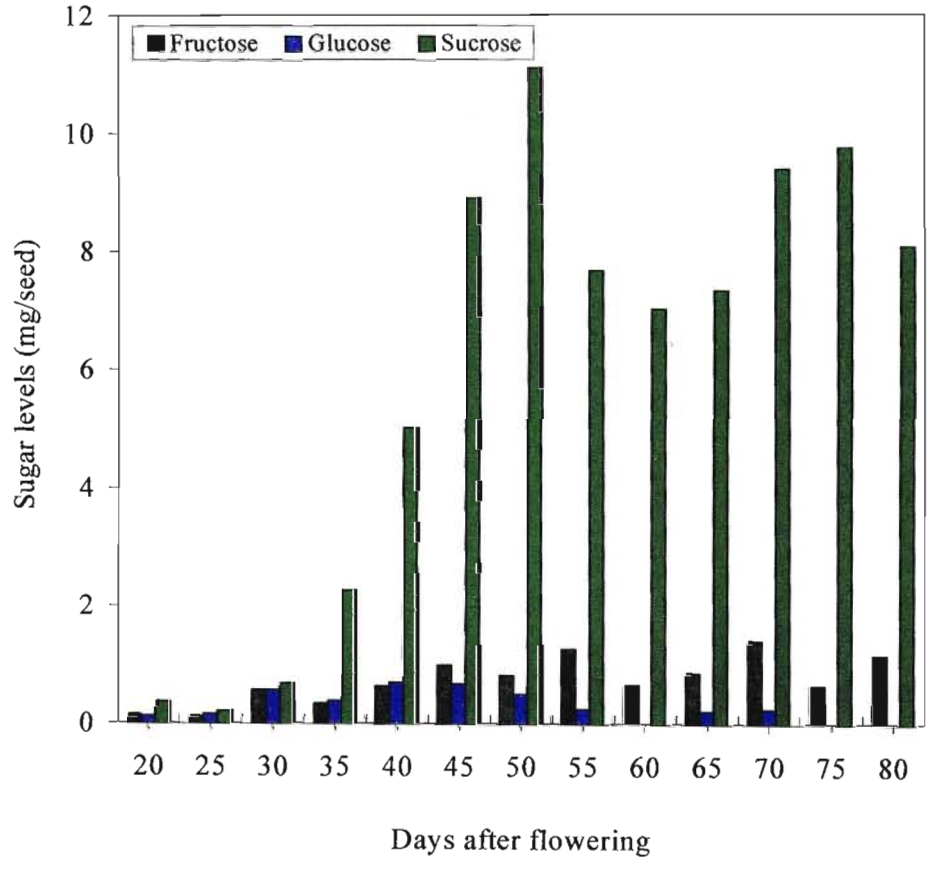

Figure 3.20. Relative levels of soluble carbohydrates at 13 stages of soybean seed development. Carbohydrates were separated by GC. Sugar levels at each stage of development represents a mean of three experiments.

3.4 DISCUSSION

The water relations exhibited by soybean seed development appeared to be typical of seed development in general. Throughout the course of development, soybean seeds characteristically underwent changes in fresh weight, dry weight and moisture content (Figures 3.1 and 3.2). Both fresh and dry weights increased linearly during the first 50 DAF due to cell expansion and the accumulation of reserve materials by the cotyledons. The continued accumulation of dry weight in soybean seed in the absence of net water uptake resulted in the seed moisture content declining to approximately 61% at 50 DAF. At this moisture content, further increases in both fresh and dry weights ceased and the seed is said to be "physiologically matured" (Crookston and Hills, 1978; TeKrony *et al.*, 1979), by which stage they are capable of germination following rehydration (Adams and Rinnie, 1981; Crookston and Hills, 1978). Similarly, growth slows down dramatically in many non-oil grain legumes at moisture contents of 55- 60% (Ellis *et al.*, 1987; Egli, 1990), and in *Phaseolus vulgaris* embryonic axes (Walbot, 1978).

The continual decline in moisture content from 20 to 50 DAF was due primarily to water being displaced by protein, sugars and lipid deposition in the cotyledons (Greenwood and Bewley, 1982). The accumulation of TAG (Figure 3.3) and sugars (Figure 3.20), probably as a result of net increase in carbon flux from the mother plant, paralleled the increase in fresh weight and dry weight (Figure 3.1). It is, however, during this period of rapid dry matter accumulation that the loss of moisture by the seed was rather slow. This relatively high moisture content, in spite of reduced vacuolar volume, has led to suggestion that a non-quantitative relationship exists between vacuole size and desiccation tolerance (Kermode, 1990; Vertucci and Farrant, 1995).

Maturation drying of seeds on the mother plant commenced after about 60 DAF and continued until the completion of maturation at 80 DAF (Figure 3.1). This final stage in the development of soybean seeds was characterised by a dramatic loss in fresh weight, slow-down and/or cessation in dry weight accumulation, and a more abrupt decline in seed moisture content (Figures 3.1 and 3.2). It is during the maturation stage of seed development that changes occur in seed metabolic processes that coincide with the deceleration of seed developmental metabolism (such as the cessation of storage protein synthesis) and the acceleration of metabolism associated with equipping the seed for germination and growth upon rehydration (Adams and Rinne, 1980, 1981; Adams *et al.*, 1983; Kermode and Bewley, 1985). Furthermore, the drying period is believed to be important for legume seed development for attainment of good quality seeds (Rosenberg and Rinnie, 1986).

As with most seeds, soybean seeds became increasingly tolerant of drying with development and were able to withstand drying to very low moisture contents, as is a characteristic feature of orthodox seeds. When desiccation tolerance was evaluated using various biochemical parameters, the acquisition of desiccation

tolerance appeared to be a continuous, rather than all-or-none, process. Orthodox seeds are known to possess desiccation tolerance only in the later stages of seed development. For example, tolerance of desiccation is developed in the seeds of *Phaseolus vulgaris* between 22 and 26 days post-anthesis (Dasgupta *et al.*, 1982), after which the moisture content of the seed declines and a quiescent state is imposed. The final water content of dry seeds (together with temperature) appears to be important in determining survival over long periods of storage (Roberts and Ellis, 1989). Furthermore, water properties in dried seed tissues appear to be of obvious and primary importance in desiccation tolerance.

Phospholipid composition analysis of higher plants has shown that in plasma membranes PC and PE are the major phospholipids (Bartholomew and Mage, 1972; Gronewald *et al.*, 1982). However, reports differ as to which of the two phospholipids species constitute the majority of lipids in the membranes. In the present study, PC was the dominant species, followed by PE in mature soybean seeds. In contrast, in potato tuber (Mazliak, 1977) and oat root (Liljenberg and Kates, 1982) PE has been reported to be the major phospholipid component of the plasma membrane. The differences in membrane composition may reflect genotypic variation, or be a reflection of the physiological state of the plant or may be the result of endogenous phospholipases. High activities of endogenous lipolytic enzymes such as phospholipase D and phosphatidic acid phosphatase (Scherer and Morre, 1978) may significantly alter the composition of recoverable phospholipids. Phosphatidylinositol (PI) and PS were minor phospholipid constituents of soybean seeds.

Although changes have been inferred (LePage-Degivry and Garello, 1991), there have been few studies that actually document changes in membrane components during seed development, and how these changes confer adaptation to the desiccated state. The decline in the ratio of PC to PE in soybean seeds during the first 45 DAF of development (Figure 3.5) may be responsible for their desiccation sensitivity. Hoekstra *et al.* (1989) made a similar suggestion for pollen grains with relatively high levels of PE. From 50 DAF (Figure 3.2), soybean seeds entered maturation drying and this was accompanied by a rise in the levels of PC, and an increase in the PC to PE ratio. A high ratio of PC to PE might be expected during this stage of rapid water loss, as it may help preserve the membrane bilayer structure at low moisture contents. A doubling of PC to PE ratio was observed during pre-conditioning of maize seeds (Chen and Burris, 1991) and acclimation of oat roots to water stress (Liljenberg and Kates, 1985). However, even though the PC to PE ratio was high, PE was still, suprisingly, relatively high for a desiccation tolerant tissue. Phosphatidylethanolamines (PE) can undergo lethal hexagonal phase changes with drying (Vertucci and Farrant, 1995). This suggests that other forms of protection against the formation of non-bilayer phases may be required. In mature soybean and corn embryos, and *Typha* pollen, membranes with 20% PE content were found to undergo hexagonal phase changes at moisture contents at which intermediate and orthodox seeds experience damage.

Numerous studies have demonstrated that membranes are particularly susceptible to structural changes during desiccation and that alteration of membrane structure can be particularly damaging. Based on these findings, one would predict that there should be changes in the lipid composition of orthodox seeds to accommodate the mechanical and biochemical stresses. The major phospholipid in the membrane, PC, probably stabilises the bulk of the membrane in a bilayer (Cullis and DeKruiff, 1979). The role of minor phospholipid classes is less well understood. Some of these lipids/membranes may provide special environments for membrane proteins, but this does not appear to be a general phenomenon (Quinn and Chapman, 1980). Other studies have indicated that the variety of phospholipids in biological membranes may be present to reduce the permeability of the membranes at the protein-lipid boundaries (Van der Steen *et al.*, 1982). In this case, the phospholipid composition of the membrane would change during cell differentiation with the protein complement (Booz and Travis, 1980) in order to maintain membrane integrity. Minor phospholipid components may also be important during non-bilayer membrane processes such as fusion events (Cullis and DeKruiff, 1979).

Saturated fatty acids (16:0 and 18:0) deposition in developing soybean seeds remained fairly low (Figure 3.6). The 18 carbon unsaturated fatty acids, on the other hand, underwent changes in content during seed development. In the early stages of seed development, the relative amount of 18:3 fatty acid declined while those of 18:1 and 18:2 increased. The high amounts of 18:3 acid detected at the early stages of development are supported by the data by Stymne and Appleqvist (1980) for soybean and those of Ichihara and Noda (1980) for safflower. From these results it can be concluded that the rate of synthesis of 18:3 declined while that of 18:1 and 18:2 acids remained relatively high during dry matter accumulation. If 18:2 is a precursor of 18:3 synthesis, it appears that the amount of desaturation of 18:2 would limit 18:3 production during development. Therefore it is suggested that soybean seeds with low levels of 18:3 may be limited by the enzyme system which desaturates 18:2 to 18:3. Limitation to 18:3 production would be advantageous to the seed tissue, as this fatty acid is known to be prone to peroxidative attack (Wilson and McDonald, 1986; Winston, 1990; Smirnoff, 1993).

As mentioned earlier, seed development was accompanied by a general reduction in metabolism, as water was lost from the seed. An important requirement for metabolic arrest is that rates of all cellular processes be reduced in concert so that overall metabolism remains balanced at much lower ATP costs (Hand and Hardewig, 1996). If the down-regulation leading to total metabolic arrest is not well co-ordinated, imbalances in the metabolic rates may result in the accumulation of metabolic by-products to toxic levels, or cause excess free radical build-up.

Lipid peroxidation, estimated as LOOH levels, is an indicator of free radical reactions in tissues (Roskruge and Smith, 1997). The levels of LOOH gradually increased during the first 45 DAF, but then decreased at a later stage of seed development just prior to maturation drying. A high level of lipid peroxidation during

early development suggests that oxidative stress occurred, probably as result of intense metabolic activity. That free radicals are naturally produced during plant metabolism is well documented (Halliwell, 1987; Puntarulo *et al.*, 1991). In developing seeds, hydrogen peroxide and superoxide can be assumed to be produced in the electron transport chains. Concentrations of such radicals can be expected to be high during the early stages of seed development in association with high respiration rates and metabolism. Lambers *et al.* (1991) have concluded that the activity of respiratory pathways is controlled by respirable substrates such as sugars. In germinating maize seeds, Leprince *et al.* (1992) showed a positive correlation between sugar concentration, respiration rates and the accumulation of stable free radicals. Interestingly, the increase in sucrose levels between 25 and 45 DAF was somewhat similar to the pattern seen for LOOH (Figures 3.7 and 3.20). As the seeds matured, they gradually lost moisture. Although the loss of moisture was not marked during the first 50 DAF, it could, in actively respiring mitochondria, be expected to enhance the loss of electrons from the electron transport chain to oxygen, resulting in the formation of active oxygen species and thus the initiation of peroxidative damage.

A role of metabolism in the expression of desiccation damage has been suggested in other studies based on ultrastructural observations (Berjak *et al.*, 1993). It has been shown that the source of the stable free radical lies, at least in part, in the mitochondria, and its generation during drying probably arises from the impairment of the electron transport chain (Leprince *et al.*, 1994). Also, it has been demonstrated that the rate of respiration is associated with increased peroxidative injury and loss of desiccation tolerance (Leprince *et al.*, 1992, 1994). Given that metabolic rates are dependent on the hydration levels, the observed correlation between the levels of LOOH and that of moisture content during development further supports the possible role of metabolism in free-radical damage. Therefore, it can be concluded that a high metabolic activity whilst water is lost influences the susceptibility to desiccation damage as previously suggested (Hendry *et al.*, 1994; Leprince *et al.*, 1992, 1994).

As the seed entered maturational drying, a decrease in the levels of LOOH occurred. The decrease in LOOH levels was first noted after 50 DAF, the period at which the seed had ceased any further increases in fresh and dry weights (*i.e.* physiologically mature state). A reduction in respiratory activity occurs in orthodox seeds just after dry weight accumulation is completed but prior to a severe reduction in moisture content (Fischer *et al.*, 1988; Farrant *et al.*, 1997). Similarly, Rogerson and Matthews (1977) showed that in developing garden pea seeds the acquisition of desiccation tolerance was accompanied by a fall in respiration rates coincident with maturation drying. Thus, the decrease in LOOH levels during maturation drying could, in part, be attributed to the fall in respiration.

The role of active oxygen species in degradative and destructive processes has been discussed in many reviews (Halliwell, 1987; Smirnoff, 1993). Data presented here suggest that free radical attack on the phospholipids may have been partly responsible for the progressive increase in the levels of LOOH during

the first 45 days after flowering (Figures 3.4 & 3.7). This is inferred from the observed increase in lipid peroxidation between 25 and 45 DAF, which occurred simultaneously with the decline in the levels of phospholipids. Also, it is evident from Figure 3.6 that free radical attack might have been directed on linolenic acid (18:3), since this fatty acid declined continuously between 20 and 45 DAF. Unsaturated fatty acids, especially 18:3, have been reported to be more prone to peroxidative attack (Wilson and McDonald, 1986; Winston, 1990; Smirnoff, 1993). The selective attack on phospholipids, on the other hand, may be ascribed to the possibility that this lipid class presents more surface area for free radical attack than other lipid classes (Smirnoff, 1993; Winston, 1990).

The association of lipid peroxidation with the formation of gel phase lipid has been documented from a number of desiccation-intolerant tissues and has been attributed to free radical-mediated lipid peroxidation. For example, treatment of isolated membranes with ozone under conditions in which free radicals are produced induces lipid peroxidation and the formation of a gel phase (Pauls and Thompson, 1980, 1981). Similarly, treatment of photosynthetic tissue with the herbicide paraquat, which "short-circuits" photosynthetic electron tranport and facilitates the catalytic production of superoxide radical, induces lipid peroxidation and the parallel appearance of gel phase lipid in chloroplast and microsomal membranes (Chia *et al.*, 1981). The accumulation or buildup of stable free radicals during seed development can be considered to be an end product of degradative free-radical processes induced by loss of moisture (Artherton *et al.*, 1993; Hendry *et al.*, 1994). Furthermore, its correlation with the desiccation-sensitive stage of seed development has been demonstrated in various plant systems by using different conditions of drying (Hendry *et al.*, 1992; Leprince *et al.*, 1990, 1994).

Active oxygen species are removed by the antioxidant reserves, which react both enzymatically and chemically with toxic oxygen species, and their products (Zhang and Kirkham, 1996). Soybean seeds clearly showed evidence of an activation of their anti-oxidative systems during development. Monitoring of the protective mechanisms operating against activated forms of oxygen indicated both enzymatic protection, with gradual increases in activities of superoxide dismutase, catalase, peroxidases, DHA and AFR reductases, and antioxidants in the form of ascorbic acid and glutathione during development. Such increases, most of which increased in parallel with increases in levels of lipid peroxidation, can be seen as a strengthening of the scavenger mechanisms of the seeds, thereby decreasing or limiting damage caused by free radical production. Similar increases in SOD, CAT, and PODs have been reported in plant tissues exposed to oxidative stress (Dhindsa and Matowe, 1981; Sgherri *et al.*, 1994), indicating the fundamental nature of the protective mechanism of cells and tissues.

Ascorbate is implicated in playing a significant role in vital cell functions such as the regulation of cell cycle, cell elongation, and cell wall expansion (Arrigoni, 1994; Cordoba-Pedregosa *et al.*, 1996). The increasing levels of ascorbate during the early stages of seed development further confirm this, since

histodifferentiation and reserve accumulation are the main events taking place during the first 50 DAF. Meristematic cells of *Vicia faba* have been reported to utilise about 4 μmol ASC $h^{-1}g^{-1}$ fresh weight (Liso *et al.*, 1984). Similarly, when the ASC content of actively proliferating cells is experimentally lowered with lycorine, a specific inhibitor of ASC biosynthesis (Arrigoni *et al.*, 1975), the cell cycle is arrested in the G1 phase (Liso *et al.*, 1984). That cell expansion also requires ascorbate was demonstrated by Cordoba-Pedregotal *et al.* (1996) who reported that the enzyme prolyl hydroxylase specifically utilises ascorbate *in vivo* for the hydroxylation of the proline present in polypeptide chain.

During the first 30 DAF of soybean seed development, the ASC/DHA ratio was high and this can be ascribed to higher ASC utilisation during cell division and elongation growth. The higher ASC/DHA ratio seen at 30 and 35 DAF could be the result of a higher demand on ASC during active growth rather than during cell division. Also, the observed increase in ASC peroxidase activity further explains why more ascorbate was needed as seeds accumulated biomass. The buildup of DHA during seed development can also be taken as an indication of stress in the plant tissues. As observed in Figure 3.8, the period of rapid growth was associated with higher levels of lipid peroxidation, and that ASC was probably utilised by ascorbate peroxidase.

Total ascorbate content decreased quite dramatically during the maturation drying stage of seed development (Figure 3.9) and this decline coincided with a decline in LOOH. This response can be expected if ASC was functioning directly as a scavenger of oxygen radicals. There was no evidence of asorbate recycling after 40 DAF as can be seen from the continued accumulation of DHA, and this must have contributed to the depletion of the ASC pool. There is also the possibility that a high concentration of ASC in drying seed could act to generate potent forms of activated oxygen through the Fenton-type reactions via ascorbate-reduced iron or copper (Rowley and Halliwell, 1983). Thus, its reduction during seed maturation was possibly an adaptation to reduce this mechanism of active oxygen production.

The continual increased in ASC levels from 20 to 40 DAF can also be correlated with the level of AFR reductase activity in the cells. To understand how AFR reductase activity affects the amount of ASC in the cell, it is appropriate to consider the mechanism of ASC oxidation. It is well-known that cell metabolism utilises ASC as an electron donor and that ASC oxidation by ASC oxidase or peroxidase is a two-step reaction in which each step removes one electron (Yamazaki and Piette, 1961). The first ASC oxidation product is a semiquinone-like free radical, i.e. AFR. AFR can be reconverted to ASC by AFR reductase [2AFR + NAD(P)H $\rightarrow$2ASC + NAD(P)+] (Bielski *et al.*, 1981) or spontaneously undergoes disproportionation [2AFR $\rightarrow$ ASC + DHA], thus generating DHA (Yamazaki and Piette, 1961; Bielski *et al.*, 1981). From this it can be argued that when AFR reductase activity is high, a large quantity of AFR is reduced to ASC and a small amount of AFR remains available for disproportionation. Conversely, when AFR reductase is low, a smaller amount of is reconverted to ASC and most of the AFR undergoes

disproportionation, generating DHA in large quantities. Based on this argument, it is possible to explain why DHA accumulation was not pronounced even during periods of active cell division and growth.

It is noteworthy that most seeds are not capable of synthesising ASC until they reach a period of intense growth (Arrigoni *et al.*, 1992). Because the seeds in the present study contained appreciable amounts of ASC during the first 40 DAF, which are characterised by rapid cell growth and reserve accumulation, it is possible that the ASC present in the young seed is furnished entirely by the parent plant. The seed acquires the capability to synthesise ascorbate a few days before the onset of desiccation (Arrigoni *et al.*, 1992), this indicating the necessity for the seed to be endowed with the ASC biosynthetic system before entering the resting state. In this manner, the seed can promptly start the biosynthesis of ASC when imbibition and germination commences.

Glutathione is considered to function in cellular protection against oxidative damage, regulation of enzyme activities and the synthesis of proteins and DNA (Meister, 1995). GSSG accumulation during the early stages of seed development is considered to be an indicator of stress, as it formed during the detoxification of cytotoxic oxygen species. Later during development, stress is alleviated by the cumulative action of other defense mechanisms, resulting in GSSG being reduced back to GSH. This is necessary because GSSG is a potent inhibitor of protein synthesis (Dhindsa, 1987), and its absence in dry soybean seeds underlies the ability of the seed to prepare itself to resume protein synthesis rapidly upon imbibition.

Levitts SH-SS-hypothesis (Levitt, 1980) postulates that plants are protected against stress (including drought) by thiol-disulfide conversions which may include the reactions of glutathione. Protoplasmic stress tolerance would be based on mechanisms which can prevent damage of proteins caused by formation of irreversible disulfide bridges or by reduction of disulfides as soon as they are formed. In addition, there are 'adapted proteins' whose conformation is stabilised by formation of disulfide bridges (Levitt, 1980). This type of adaptation to drought stress could also occur during maturation of seeds. The oxidation of GSH to GSSG at the beginning of stress may have been a trigger for increased glutathione synthesis (Figure 3.8), as is suggested by Smith *et al.* (1985). As previously seen (Sgherri *et al.*, 1994), GSH plays a key role in drought tolerance and limited cellular damage and while the induction of defense mechanisms against activated species of O_2 seem to be consequence of the rate of water depletion.

GSH may also function as a protectant against oxidative stress in the ascorbate-dependent H_2O_2-scavenging pathway (Smith *et al.*, 1990). The DHA produced during ascorbate oxidation is reduced to ascorbate by the action of DHA reductase, using GSH as a reducing compound. Both DHA reductase (Figure 3.10) and the GSH (Figure 3.9) recorded increases during seed development. The reduction of DHA is accompanied by the generation of GSSG, which also recorded increases during seed development, this further confirming the dependence of ASC regeneration on GSH.

Sucrose, the primary carbon source translocated from the mother plant to the developing seeds, accumulated in higher concentrations relative to other soluble carbohydrates (Figure 3.20). Although sucrose uptake by developing seeds is known to decline with maturation (VerNooy *et al.*, 1986), its accumulation in the present study was ongoing until maturity (Figure 3.20) presumable because soybean seeds possess the ability to convert glucose to sucrose (Hageman and Rinne, 1976). The decline in glucose concentration accompanied by sucrose accumulation further supports the latter argument. Upon its initial uptake by the seed, sucrose is presumably catabolised to provide carbon for respiration and other structural skeletons (Sung *et al.*, 1989). This catabolism is usually catalysed by alkaline invertase, although other sucrose catalysing enzymes can be involved, depending on the tissue type (Sung *et al.*, 1989). Alkaline invertase also hydrolyses sucrose to provide hexoses for starch synthesis in soybean suspension cells (McDonald and apRees, 1983). Kuo *et al.* (1997) also observed a correlation between sucrose synthase and sucrose content in developing soybean axis and cotyledons.

In the present study, sucrose accumulation was found to increase in parallel with the accumulation of LOOH. Associated with this increase in sucrose was a marked decrease in reducing sugars, glucose and fructose. Low levels of reducing sugars have been correlated with desiccation tolerance (Tetteroo *et al.*, 1994; Leprince *et al.*, 1994) as the tissue is starved of respiratory substrate required for cell metabolism. Several lines of evidence correlate acquisition of desiccation tolerance and soluble sugars during maturation, for example, in mustard (Fischer *et al.*, 1988), maize (Chen and Burris, 1990) and soybeans (Blackman *et al.*, 1992). In the light of these data, it can be concluded that desiccation-tolerant tissues are characterised by high amounts of sucrose and by the presence of very low amounts (or absence) of reducing sugars such as glucose and fructose.

A possible role of sucrose in seed tissues could be that of 'mopping up' cellular water during the first 50 DAF (Figures 3.2 & 3.20). During the period of rapid water loss (i.e. after 60 DAF), structural damage to the cells is probably limited or reduced as sucrose fills up the spaces left by water and possibly cushions the internal organelles and membranes from collapsing. Sucrose has been suggested as an important protectant of macromolecular structure when water is removed (Crowe *et al.*, 1992; Leopold *et al.*, 1994). Sugars are accumulated up to 20% in dry, anhydrobiotic organisms including seeds, and are suspected to be the solutes which enhance cytoplasmic vitrification at ambient temperatures (Williams and Leopold, 1989; Koster, 1991). Survival of cysts was found to be associated with synthesis of trehalose during desiccation and its degradation during rehydration (Madin and Crowe, 1975). The function of sugars has been studied in detail in model membrane systems. Their interaction with the phosphate head groups during drying is thought to be responsible for the prevention of phospholipids from entering into the gel phase (Crowe *et al.*, 1987). The temperature of phase transition starts to rise below a water content of 12 mol H_2O per mol phospholipid (Chapman *et al.*, 1967; Crowe *et al.*, 1984), which in living cells represents about 20 to 30%

moisture content on a the dry weight basis (Crowe *et al.*, 1984; Hoekstra and Van der Wal, 1988). In liposomes and isolated membranes, trehalose and sucrose have been shown to counteract the increase in the temperature of phase transition during drying, which means the formation of gel phase lipids is postponed and that phase separation, fusion and leakage do not occur (Crowe *et al.*, 1986, 1987).

As the formation of active oxygen species is favoured during the early stage of seed development, one can expect some adaptive changes in the active oxygen scavenging enzymes to ameliorate the situation. Among the enzymes responsible for scavenging the active oxygen species, SOD was clearly increased in parallel with the accumulation of LOOH during the first 45 DAF, this indicating the seed's enhanced the capacity to scavenge superoxide radical during development. The increased activity of SOD may have maximised the generation of H_2O_2 from $O_2^{\cdot}$. Elevated level of both $O_2^{\cdot}$ and H_2O_2 may lead to production of highly reactive hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) radicals through the Haber-Weiss reaction (Smirnoff, 1993). These cytotoxic radicals can eventually bring about lipid peroxidation with consequent membrane damage (Dhindsa *et al.*, 1982; Smirnoff, 1993). This could explain the observed increase in the rate of LOOH accumulation during the active reserve accumulation phase of soybean seed development.

Two enzymes, ASC POD and CAT, are responsible for breaking down H_2O_2 in cells to water and oxygen, and in so doing bring down the levels of LOOH to controllable levels. Ascorbate POD, which is the first enzyme of the ascorbate-glutathione pathway (Asada and Takahashi, 1987; Salin, 1988), detoxifies H_2O_2 using ascorbate as the reductant (Nakano and Asada, 1981). This enzyme increased in parallel with the increase in LOOH during the first 45 DAF, but declined thereafter, by which time the levels of LOOH were probably reduced to controllable levels. It has also been argued that the expression of ASC POD increases under H_2O_2-overproducing conditions, such as those induced by biotic and abiotic stress (Cakmak and Marschner 1992; Foyer *et al.*, 1994; Kubo *et al.*, 1995). The latter observation could indicate that variations in H_2O_2 production in some way also regulate ASC POD. Similarly, with an increase in ASC content noted during the first 40 DAF, it can be argued that the higher levels of ASC POD under H_2O_2-overproducing conditions is also triggered by an increase in ASC.

The increase in catalase also paralleled that of LOOH levels during the first 40 DAF and thereafter, CAT decreased linearly as the levels of LOOH were declining. Also of note was the marked increase in CAT at the start of rapid water loss stage by the seeds (i.e. after 60 DAF), at which time ASC POD was very low. Catalase is known to have an extremely high maximum catalytic rate (Willekens *et al.*, 1995 cited in Noctor and Foyer, 1998) and can be assumed to supplement and/or complement ASC POD activity in breaking down H_2O_2. However, the low substrate affinity of CAT (Smirnoff, 1993) positions ASC POD as the most effective and efficient enzyme for H_2O_2 elimination during periods of high metabolic activity. Later during development, when processes associated with H_2O_2 production have slowed down, CAT appears to become the major protectant in the absence of ASC POD. The recorded increase in guaiacol

POD during seed development (Figure 3.18), on the other hand, was a reflection of enhanced seed growth and development (Cakmak *et al.*, 1993). There are several lines of evidence indicating that guaiacol POD is involved in cell wall metabolism and development (Gasper *et al.*, 1985) rather than in free radical processes.

In conclusion, the results show that peroxidative reactions are an integral part of soybean seed development, and are enhanced during active, seed-filling stages. Also, there appears to be a good correlation between the ability of the seed to elevate the activities and functioning of the antioxidant defense systems, and its ability to limit lipid peroxidation. Complementary to this is the ability of the seed to accumulate sugars which limit chemical reactions, and also act as a replacement for water during maturation drying. It is concluded that a capacity to limit cellular or membrane damage to reparable levels by controlling peroxidative chain reactions may be an important facet of maturational development and desiccation tolerance.

Chapter 4

SEED GERMINATION

4.1 INTRODUCTION

Chapter 3 has given insights into the possible biochemical and physiological changes that accompany the orthodox seed to safe maturational drying on the mother plant. If maturation on the mother plant, however, is to be considered safe, it must be accompanied by the development of high quality seeds, which upon hydration should produce highly viable and vigorous plants. Germination is one way of measuring seed quality and has been used to test seed viability in the present study. But, since germination is variably defined, here we have defined the process of germination as those events that commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryonic axis (Bewley and Black, 1994). The visible sign that germination is complete is usually the penetration of the structures surrounding the embryo by the radicle and subsequent events, including the mobilisation of the major storage reserves, that are associated with growth of the seedling (Bewley, 1997).

The physiological and biochemical events such as membrane organisation and metabolic reactivation, taking place during imbibition can have a profound influence on seed germination and on the future growth of seedlings. The influx of water into the cells of dry seeds during the first few hours of imbibition results in temporary structural perturbations, particularly to membranes, which lead to an immediate and rapid leakage of solutes and low molecular weight metabolites into the surrounding imbibition solution (Simon, 1974). This has been interpreted as symptomatic of a transition of the membrane phospholipid components from the gel phase achieved during maturation drying to the normal, hydrated liquid-crystalline state (Crowe and Crowe, 1992). Within a short time of hydration, the membranes return to their more stable configuration, at which time solute leakage is curtailed. It is still uncertain how repair to maturation drying- and imbibition-induced damage to membranes and organelles is achieved. However, in imbibing cottonseeds, the amount of N-acetylphosphatidylethanolamine (PE), a phospholipid with membrane stabilising properties, has been found to increase, together with that of the corresponding synthase (Sandaval *et al.*, 1995). These molecules may be involved in maintaining or enhancing membrane integrity (Sandaval *et al.*, 1995).

Imbibition of water also initiates a series of metabolic steps that lead to germination. The structures and enzymes necessary for this initial resumption of metabolic activity are generally assumed to be present within the dry seed, having survived, at least partially intact, the desiccation phase that terminates seed maturation (Bewley and Black, 1994). Re-introduction of water during imbibition is sufficient for metabolic activities to resume and, one of the changes is the resumption of respiratory activity, which can detected within minutes (Bewley, 1997). After a steep initial increase in oxygen consumption, the rate declines until the radicle penetrates the surrounding structures. At this time another burst of respiratory activity occurs (Bewley and Black, 1994).

In plant mitochondria, besides the well-known tetravalent reduction to water, molecular oxygen is also reduced partially to toxic oxygen species such as superoxide anion radical (O_2^-) and H_2O_2, both in a cyanide-sensitive and in cyanide-insensitive electron transport (Boveris *et al.*, 1978; Huq and Palmer, 1978). The production of O_2^- and H_2O_2 in the mitochondria of germinating seed has been shown by Puntarulo *et al.* (1988) using soybean embryonic axes. Mitochondrial H_2O_2 is suggested to be the most important cytosolic source of H_2O_2 in germinating seeds (Puntarulo *et al.*, 1991) and its reaction with O_2^- can generate a potent oxidant, the hydroxyl radical ($OH^\cdot$). All these toxic forms of oxygen have the potential to inactivate enzymes and damage important cellular components (Smirnoff, 1993; Foyer *et al.*, 1994).

The symptoms of oxidative damage are not easily or reliably measured. They include lipid peroxidation, denaturation of proteins and damage to nucleic acids. Lipid peroxidation is the symptom most easily ascribed to oxidative damage and malondialdehyde (MDA) and lipid hydroperoxides (LOOH), the breakdown product and end product of lipid peroxidation (Smirnoff, 1993; Roskruge and Smith, 1997), respectively, have been widely used as indicators of oxidative damage. Peroxidation of polyunsaturated lipids in the cell membrane can lead to loss of compartmentalisation resulting in the release of degradative enzymes that could adversely affect the integrity and thus the activity of protective enzymes (Mudgett and Clarke, 1993).

To minimise the damaging effects of activated oxygen, plants have evolved various enzymatic and non-enzymatic mechanisms that reduce oxidative stress by detoxifying harmful oxygen species. SOD catalyses the dismutation of O_2^- to H_2O_2 (Bowler *et al.*, 1992; Scandalios, 1993), which maintains the low steady state concentration of superoxide and, therefore, minimises hydroxyl radical formation by the metal catalysed Haber-Weiss reaction (Smirnoff, 1993). CAT and POD (Asada, 1992; Scandalios, 1993) break down hydrogen peroxide to water. However the protective action of CAT is limited, because it has a relatively poor affinity for its substrate (Foyer *et al.*, 1994). In plants cells, an alternative and more effective detoxification mechanism against H_2O_2 is the ascorbate-glutathione cycle (Foyer, 1993; Creissen *et al.*, 1994). In this cycle, ASC POD that is fuelled by ASC reduces H_2O_2 to water. The regeneration of reduced

ASC from AFR or DHA, which are reaction products of ASC oxidation, can be catalysed by either NAD(P)H-dependent AFR reductase or GSH-dependent DHA reductase (Noctor and Foyer, 1998).

It is likely that oxidative stress ensues when the defensive capacity of plants is overwhelmed by the formation of free radicals. In view of the importance of changes in membrane integrity and solute retention in seed viability, the present study seeks to understand the extent of lipid peroxidation during the first 48-hour period of seed imbibition. Also, changes in the enzymatic activities of SOD, CAT, ASC POD, guaiacol POD, DHA reductase and AFR reductase were quantified during the course of imbibition to determine the role of these anti-oxidants towards the establishment of vigorous seedlings. In addition, the contents of glutathione (GSH and GSSG) and ascorbate (ASC and DHA) were determined since they are an important component of the ascorbate-glutathione cycle.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

Soybean plants were established in the greenhouse at the University of Natal and grown until they were approximately matured. To obtain the plants, commercial seeds were obtained from Pannar Seeds (Braithwaite cultivar) and sown in sand-filled pots in August 1999, February 2000 and August 2000, respectively, as described in Chapter 3. Only fully matured soybean seed were used for imbibition trials and the average germination percentage of all seed stock was 98%.

4.2.2 Imbibition responses of seeds

Groups of 20 seeds were massed and then placed on Whatman No. 1 filter paper on Petri dishes. To each Petri dish, 10 ml of water was added and seeds were incubated at ambient temperature. At regular intervals thereafter, whole seeds and axes (excised) were harvested for further analysis.

4.2.3 Lipid extraction

Lipids were extracted from both whole seeds and axes using the solvent system of Khor and Chan (1985) following the procedure outlined in Chapter 3.

4.2.4 Thin layer chromatography of lipids

Two-dimensional thin layer chromatography (TLC) on 10x10 cm plates precoated with silica gel G as adsorbent was used for analytical separation of individual lipid classes.

4.2.4.1. Polar lipids

The TLC plates were pre-washed in chloroform and then dried at 110° C. After allowing to cool, about 20 µg lipid extracted from the axes was applied at the lower right-hand corner of the plate and the plate was developed as outlined in Chapter 3.

4.2.5 Hydroperoxide determination

Hydroperoxide levels were determined for both whole seeds and axes using a modification of the test of Stine *et al.* (1953) as described in Chapter 3.

4.2.6 Extraction of ASC and GSH

Excised axes were homogenised with four volumes of cold 5% metaphosphoric acid using a pestle and mortar. The homogenate was centrifuged at 20 000 g for 15 minutes at 4° C, and the supernatant was collected and analysed for ascorbate and glutathione as described in Chapter 3.

4.2.7 Preparation of the enzyme extract

Enzymes were extracted from both whole seeds and excised axes in 0.2 M Tris-HCl buffer (pH 7.8), containing 1 mM DDT, 0.2 mM EDTA and 2% PVP as outlined in Chapter 3.

4.2.8 Estimation of protein content

The analysis for protein estimation was carried out using the Bradford method (Bradford, 1976) as described in Chapter 3.

4.2.9 Separation of isoenzymes by PAGE and their specific detection

Electrophoretical separation and staining of isoenzymes SOD, DHA reductase, ASC peroxidase, and CAT from excised axes was achieved as outlined earlier in Chapter 3.

4.2.10 Spectrophotometric quantification of enzymes

AFR reductase, DHA reductase, ascorbate peroxidase, superoxide dismutase, catalase, and guaiacol POD were assayed for both whole seeds and excised axes using techniques and methods described earlier in Chapter 3.

4.3 RESULTS

4.3.1 Changes in phospholipids

The changes in the different phospholipid classes, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol/serine (PI/S) and phosphatic acid (PA) were recorded in both whole seeds and excised axes during the course of seed hydration. The difference between the seeds and the axes was not great, even though the seeds recorded the highest levels of phospholipids. Figures 4.1 and 4.2 show that the levels of PC, PE, and PI/S showed a very small change during first 36 hours of hydration in both the axes and seeds. The PC to PE ratio did not vary significantly throughout seed hydration. Phosphatic acid (PA), on the other hand, was present in small quantities at the beginning of hydration before increasing 2-fold between 6 and 12 hours of hydration. Phosphatic acid increased by another 2.5-fold from 12 hours of hydration and reached maximum at 36 hours. By the end of the 36 hour-hydration period, PA was the second most abundant phospholipid in the axis

4.3.2 Changes in lipid hydroperoxides

Changes in lipid hydroperoxides (LOOH), which give an indirect measure of lipid peroxidation, were followed in hydrating soybean seeds. Figure 4.3 illustrates that LOOH were present in low levels in dry soybean axis. The levels of LOOH increased 2.5-fold after 6 hours of hydration relative to the control and thereafter increased gradually over the next 18 hours. A further marked increase in the levels of LOOH was noted between 24 hours and 48 hours of hydration. Overall, LOOH levels increased 8-fold over 48 hours in axes.

The levels of LOOH in whole seeds were much higher (on a tissue fresh weight basis) than those recorded in the axes during the entire 48-hour hydration period (Figure 4.3). The difference between whole seeds and axes was more pronounced after 48-hour hydration. Overall, however, LOOH increased 13-fold in whole seeds over the hydration period. Apart from differences in quantities, LOOH pattern of change, for both whole seeds and axes, was very similar but the slope of the curve for the whole seeds was much steeper.

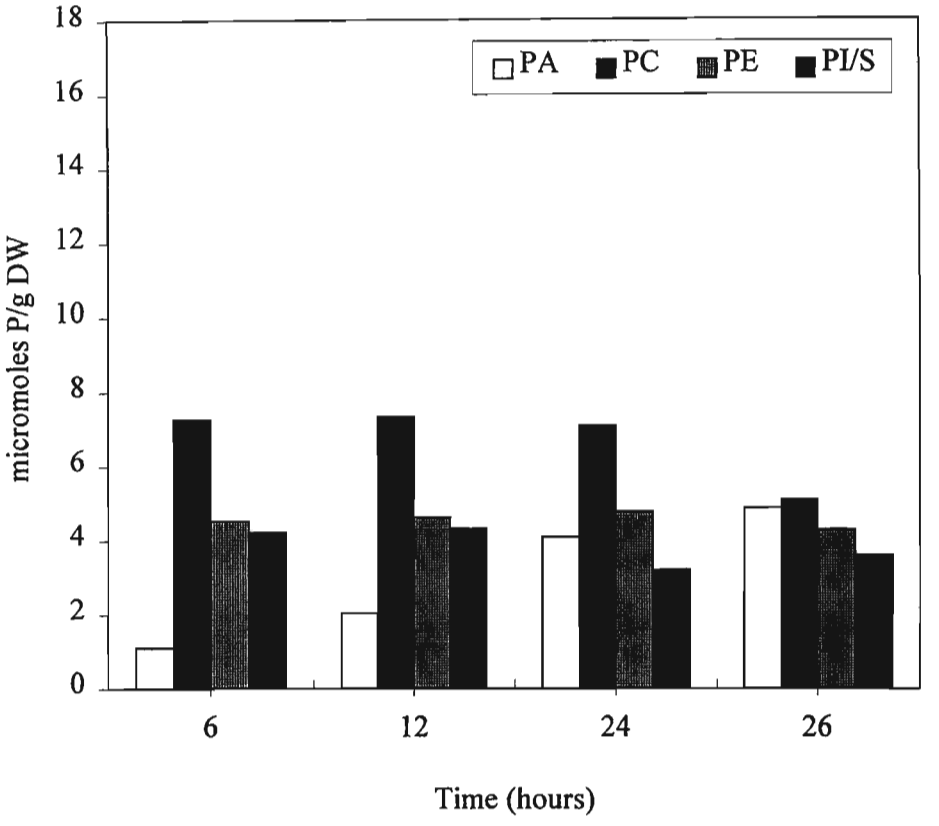

Figure 4.1: Phospholipid classes in axes during seed hydration: phosphatic acid (PA), phosphatidylethanol (PE), phosphatidylcholine (PC), and phosphatidylserine/inositol (PS/I) were determined for soybean axes. Values are the mean of three experiments.

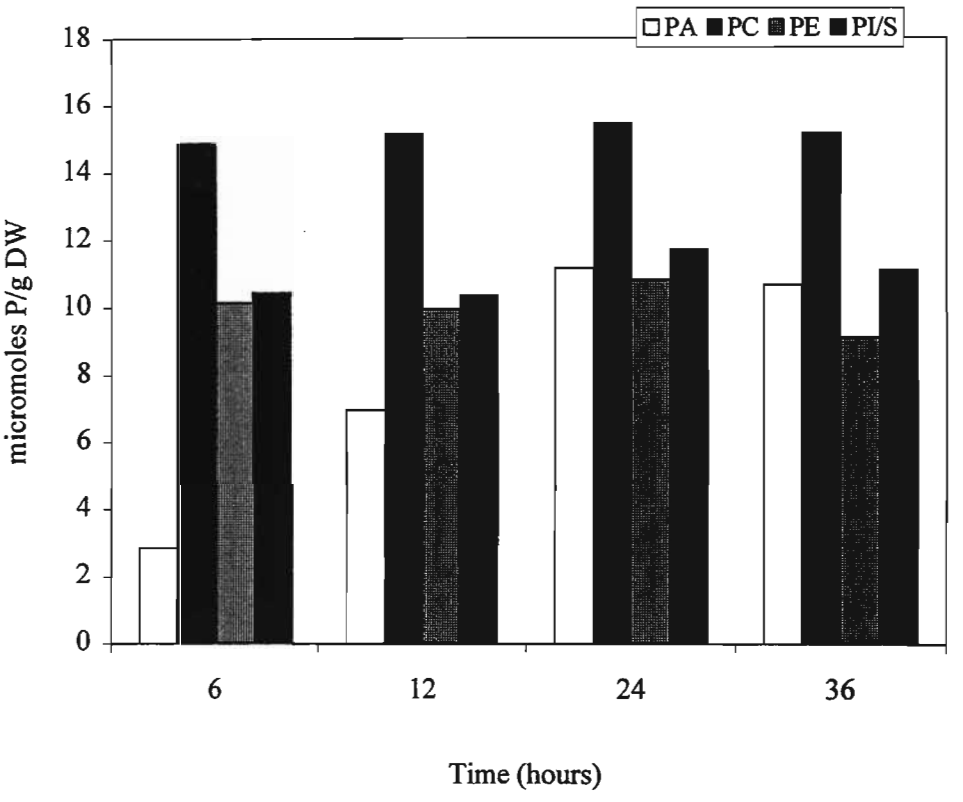

Figure 4.2: Changes in phospholipid classes during seed hydration: phosphatidic acid (PA), phosphatidylethanol (PE), phosphatidylcholine (PC), and phosphatidylserine /inositol (PS/I) in hydrating soybean seeds. Values are the mean of three experiments.

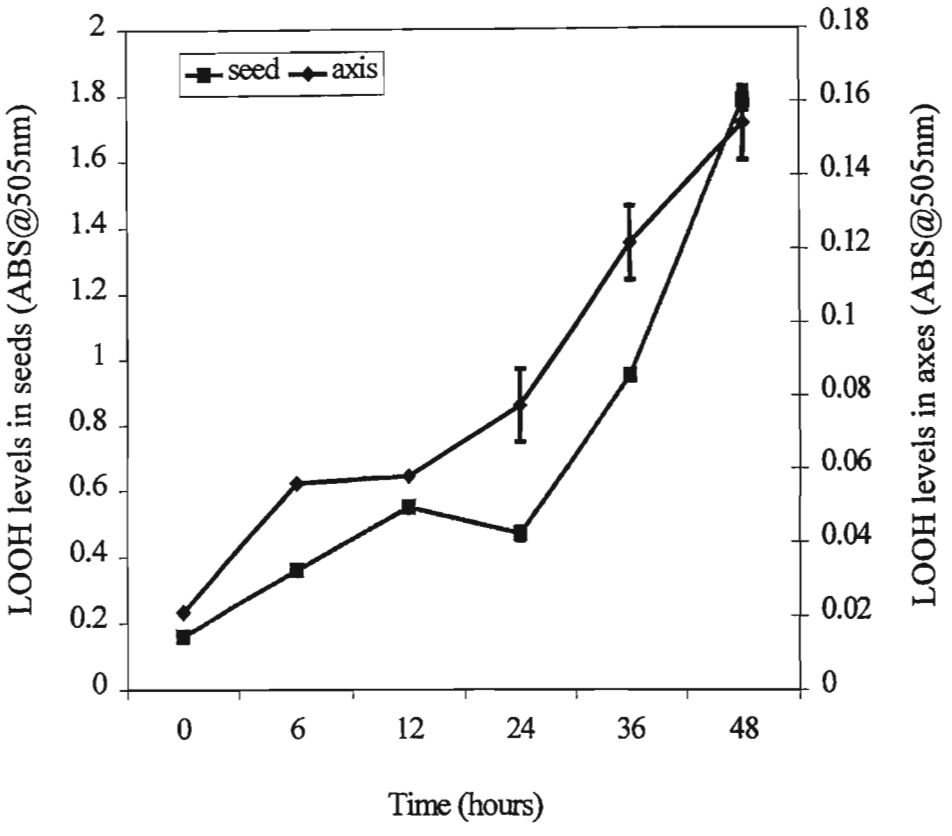

Figure 4.3. Changes in lipid hydroperoxides (LOOH) in the axes and seeds of soybean during seed hydration. Absorbance readings are for 50 mg FW tissue. By 48 hours, 98% germination had been achieved. Values are the mean ± SE of three experiments.

4.3.3 Changes in total glutathione

The germination of soybean seeds was also associated with a gradual increase in the amount of total glutathione (Figure 4.4) which mirrored increases in LOOH. GSH was 14-fold higher than GSSG in the dry control axis, thereafter GSH increased by 94.5% during the first 6 hours of seed imbibition and remained fairly unchanged in the following 18 hours of germination. The increase in GSH/GSSG ratio from 14:1 to 40:1 after 6 hours of germination was due largely to an increase in GSH and a decrease in GSSG relative to the control. Total glutathione increased further after 24 hours of imbibition reaching maximum at 48 hours. During this time, the GSH/GSSG ratio decreased to 13:1 as a result of a dramatic increase in the GSSG content.

4.3.4 Changes in total ascorbate

Figure 4.5 summarises the results obtained for the changes in ASC and DHA during the first 48 hours of soybean seed imbibition and germination. Total ascorbate content (ASC + DHA) varied, but only slightly, during the first 24 hours of imbibition, afterwhich total ascorbate increase mirrored LOOH increase. ASC was 3.7-fold higher than DHA in dry embryonic axis. The first 6 hours of imbibition was associated with a decrease in the ASC/DHA ratio to 1.04 as a result of a slight decrease in ASC and a 3-fold increase in DHA. Thereafter, ASC increased gradually and reached levels that were 12.3-fold higher than the control levels by 48 hours of imbibition. The ASC/DHA ratio increases at 12 hours and 24 hours were partly due to a decrease in DHA as well as an increase in ASC. However, DHA showed marked increases at 36 hours and 48 hours, being some 4.5- and 5.6 –fold, respectively, than dry, control axes.

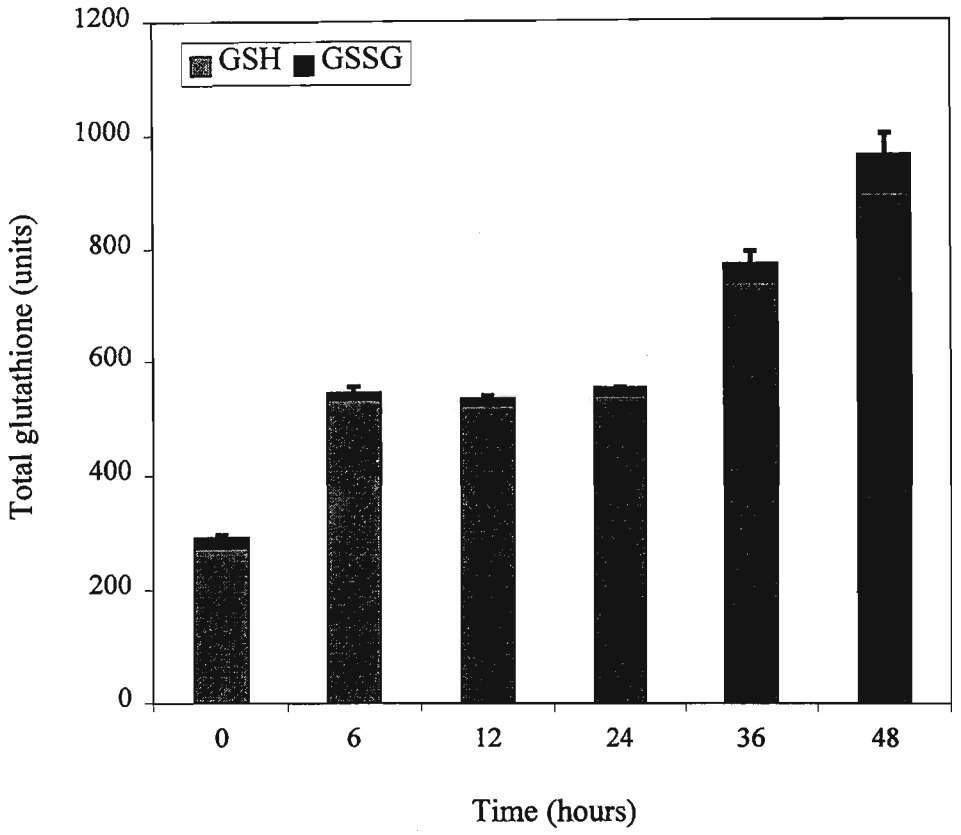

Figure 4.4. Reduced glutathione(GSH) and oxidised glutathione (GSSG) contents in soybean axes during imbibition and germination. Values are the mean ± SE of three experiments. Units expressed as nmol per grams FW per axis.

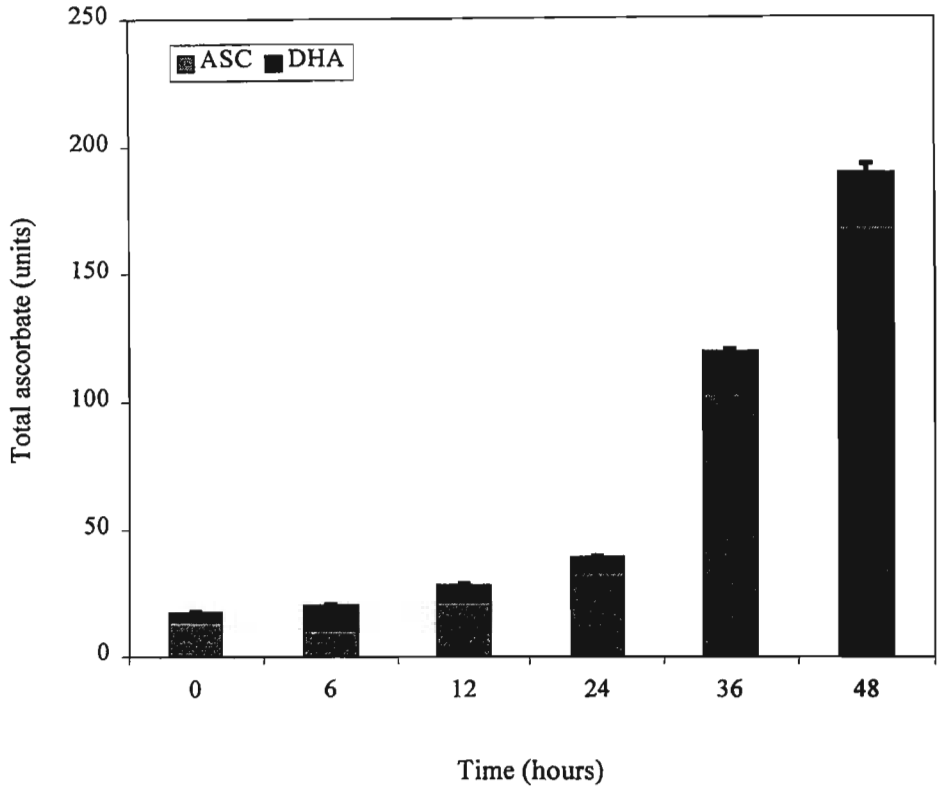

Figure 4.5. Ascorbate (ASC) and dehydroascorbate (DHA) contents of soybean axes at different stages of imbibition and germination. Values are the mean ± SE of three experiments. Units expressed as nmol per grams FW per axis.

4.3.5 Changes in DHA reductase

The changes in DHA reductase, an enzyme responsible for ASC recycling from DHA, were studied in hydrating soybean seeds in an attempt to understand changes that were occurring in ASC. The data from Figure 4.6 indicates that DHA reductase was present in dry soybean axes. Levels increased 2-fold and reached maximum during the first 6 hours of hydration. The level of the enzyme began to decline during the following 6 hours of hydration and decreased progressively thereafter. A value of 5.54 units was recorded at 48 hours of hydration, which was almost half the value recorded in the dry control axis. Approximately 76% of DHA reductase was lost between 6 hours and 48 hours of hydration.

On the other hand, DHA reductase levels in whole seeds were remarkably different from those recorded in axes. Dry control levels of DHA were higher than the levels recorded in the axes. Contrary to changes in DHA levels in the axes, the enzyme decreased to levels below the control after 12 hours of hydration. Thereafter, DHA activity increased sharply at 24 hours of hydration but decreased progressively again afterwards to levels below control levels at 48 hours of hydration.

4.3.6 Changes in AFR reductase

The pattern of change in the activity levels of AFR reductase in the axes of germinating soybean seeds is illustrated in Figure 4.7. AFR reductase was recorded in the dry, control axis and showed a transient increase at 6 hours of hydration, but decreased slightly in the following 6 hours. Thereafter, a progressive and steady increase in AFR reductase was seen, the highest value of 107 nanomoles per axis being recorded at 48 hours of hydration. This value was 7.2-fold higher than the control, dry axes.

Higher levels of AFR reductase were recorded in whole seeds relative to isolated axes. The first 12 hours of hydration were associated with a dramatic decrease in AFR reductase in seeds, after which the enzyme increased by approximately 3-fold between 12 hours and 24 hours of hydration. Thereafter, the enzyme levels decreased steadily and ultimately reached levels lower than those recorded in the control at 48 hours.

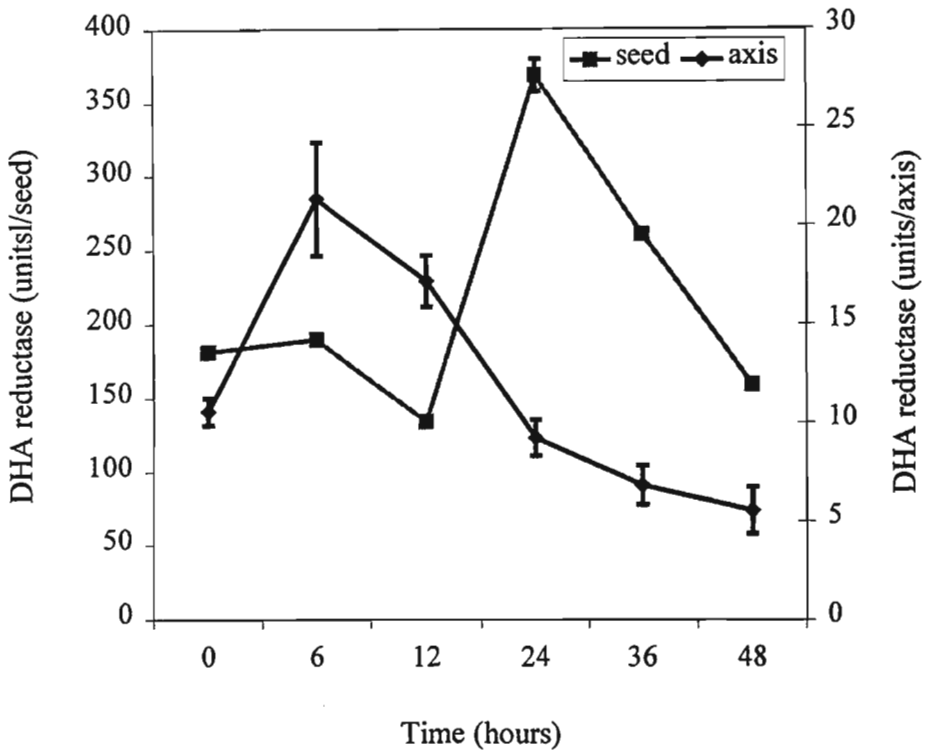

Figure 4.6. Changes in DHA reductase in whole seeds and excised axes during the first 48 hours of seed hydration. One unit of DHA reductase activity was defined as one nmol DHA reduced mg^{-1} protein min^{-1}. Values are the mean ± SE of three experiments.

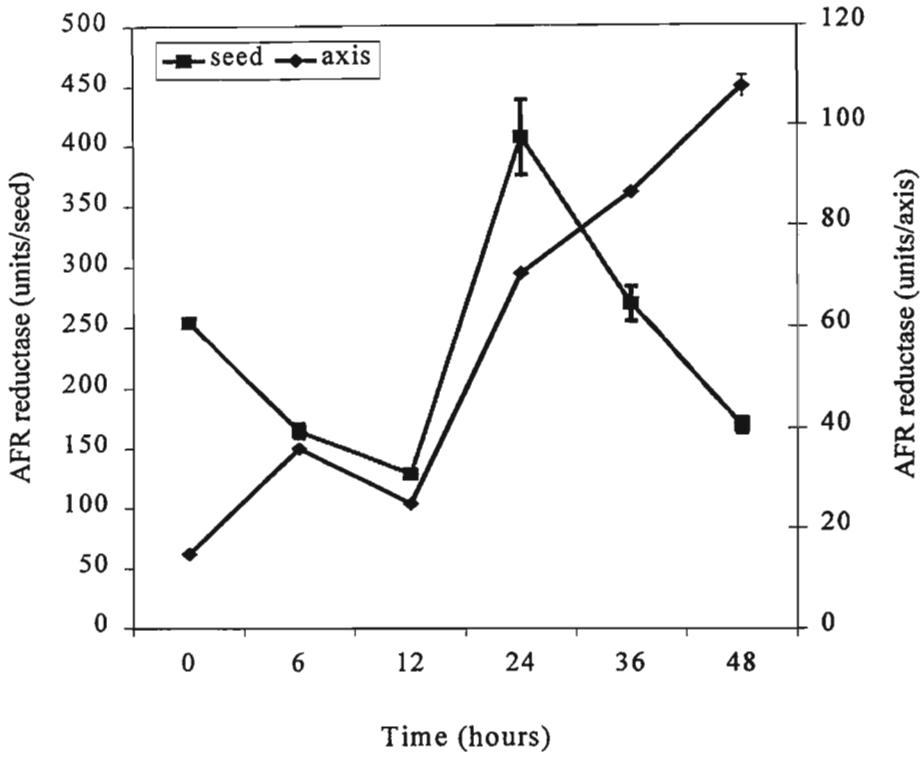

Figure 4.7. Changes in the levels of AFR reductase in whole seeds and isolated axes of soybean during the first 48 hours of seed hydration. One unit of AFR reductase activity was defined as one nmol NADH oxidised mg^{-1} protein min^{-1}. Values are the mean ± SE of three experiments.

4.3.7 Changes in SOD

The germination of soybean seeds was associated with a gradual increase in the activity levels of SOD (Figure 4.8). The low levels of SOD seen in dry axes were followed by a sharp increase during the first 6 hours of hydration. The enzyme level changed only slightly in the following 6 hours, but between 12 hours and 48 hours, the levels increased a further 4-fold. SOD levels reached maximum at 48 hours which was 13.8-fold higher that the dry, control axis.

Consistently higher levels of SOD were recorded for whole seeds compared to the axes during the entire 48-hour hydration period. During the first 12 hours of hydration, SOD levels declined only marginally, but increased quite significantly thereafter. Another decline in SOD levels was noted after 24 hours, before the enzyme levels increased again and reached maximum at 48 hours (germination).

The polyacrylamide gel electrophoresis (PAGE) pattern of SOD isoenzymes is illustrated in figure 4.9. Only 7 of the 9 bands characteristic of seed development (Figure 3.14) were seen in the axis after the gels were stained. Bands 2, 3, and 4 appeared to represent most of the SOD activity and showed increasing staining intensity during seed hydration.

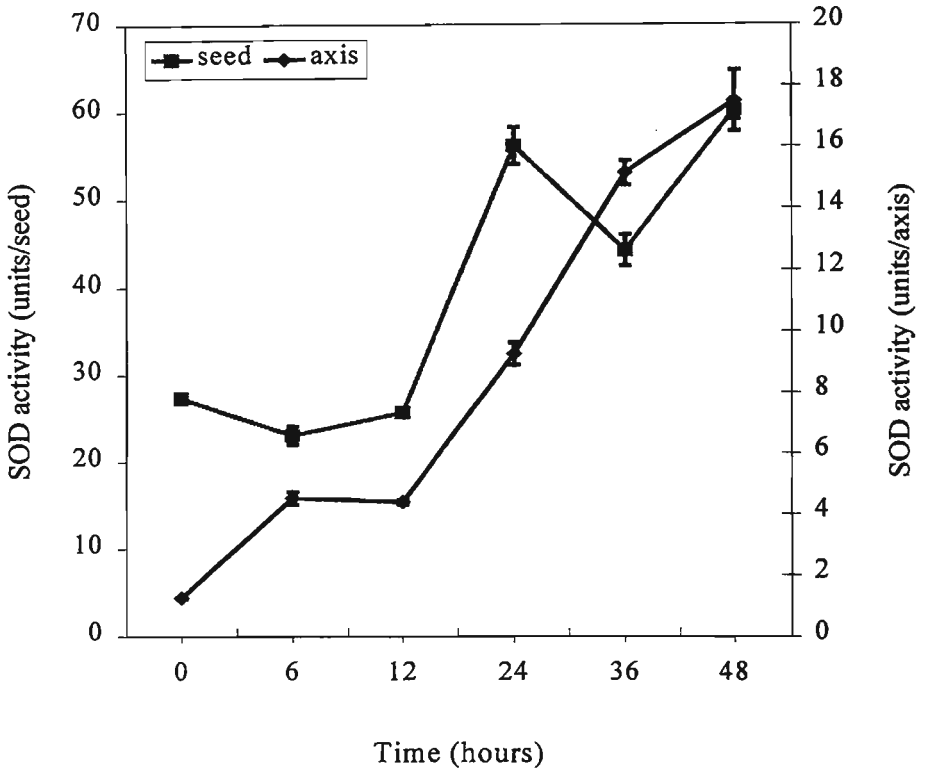

Figure 4.8. The activity levels of superoxide dismutase (SOD) in whole seeds and excised axes during the first 48 hours of seed hydration. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction at 560nm. Values are the mean ± SE of three experiments.

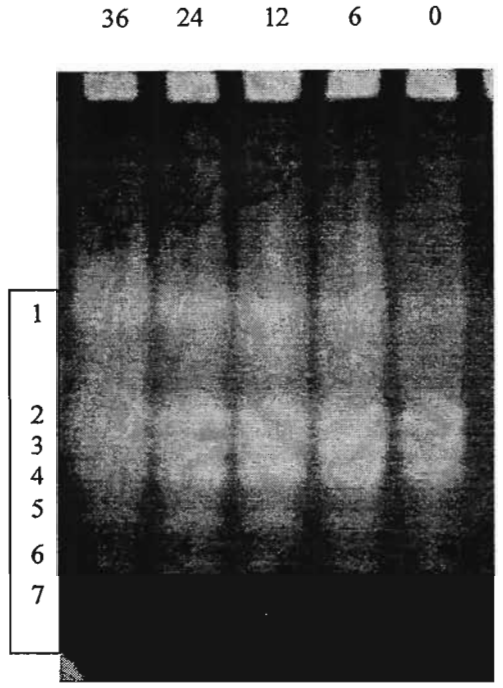

Figure 4.9. Electrophoretic separation of SOD isozymes from hydrating soybean axes. Numbers from 0 to 36 denote the time period (hours) the seeds were allowed to hydrate in water at ambient temperature. The enzymes appeared as achromatic bands on a blue-stained background. 50 μg protein was loaded per lane.

4.3.8 Changes in CAT

Catalase increased gradually during the first 48 hours of soybean seed hydration (Figure 4.10). Low levels of CAT were recorded in dry, control axes. The levels increased 4.4-fold during the first 6 hours of hydration, after which they changed only slightly in the following 6 hours of hydration. Catalase activity increased further from 12 hours of hydration, and approximately a 7-fold increase was recorded between 12 hours and 48 hours. The maximum activity recorded at 48 hours of hydration was 37-fold higher than that of the dry axes.

Catalase activity levels in whole seeds were consistently higher than those recorded in the axes during the entire 48-hour hydration period, although the overall pattern of change in enzyme activity was similar for both. The enzyme remained close to levels recorded for the dry seed during the first 12 hours of hydration, after which an increase of 3-fold was recorded between 12 and 24 hours of hydration. Catalase activity was maximum at 48 hours of hydration following a further sharp increase between 36 and 48 hours.

Visual observation of the gels showed that catalase appeared as a single broad band at the top of the gel in all the treatments. As illustrated in Figure 4.11, the activity levels of CAT were low in dry seeds, but further trends were not observed as a result of the poor overall resolution.

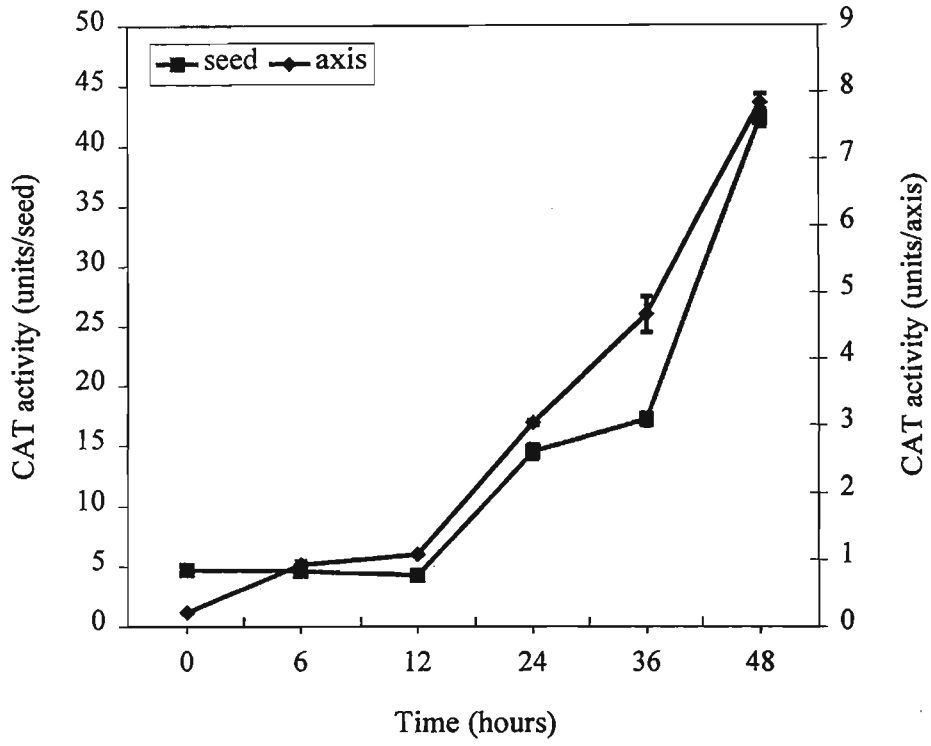

Figure 4.10. Changes in the activity levels of catalase (CAT) in whole seeds and excised axes during the first 48 hours of seed hydration. One unit of CAT activity was defined as one μmol H_2O_2 decomposed mg^{-1} protein min^{-1}. Values are the mean ± SE of three experiments.

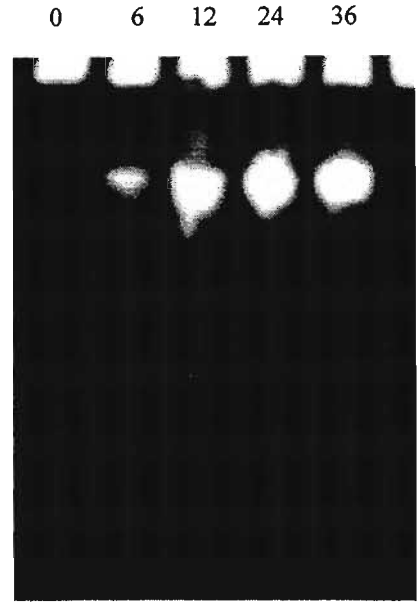

Figure 4.11. The separation of CAT isoenzymes from hydrating soybean axes. Numbers from 0 to 36 denote the time period (hours) the seeds were allowed to imbibe water at ambient temperature. The enzymes appeared as achromatic bands on a blue-stained background. 50 μg protein was loaded per lane

4.3.9 Changes in guaiacol POD

Both dry whole seeds and axes recorded low levels of guaiacol POD (Figure 4.12). The enzyme levels increased only marginally during first 12 hours of hydration for axes, whereas negligible levels of guaiacol POD were detected in seeds over the same period. The axes recorded a near-linear increase in guaiacol POD between 12 hours and 24 hours of hydration, whereas this increase was only recorded after 24 hours in seeds. Maximum levels of the enzyme were noted after 48 hours of hydration with the axes recording higher levels than whole seeds.

4.3.10 Changes in ASC POD

Dry embryonic axes and seeds were devoid of measurable ASC POD (Figure 4.13) and this was maintained for 6 hours and 12 hours hydration, respectively. A small increase in the enzyme levels between 6 and 12 hours of hydration was followed by sharp increase between 12hours and 24 hours, amounting to a 17-fold increase in the axes. ASC POD increased further between 24 hours and 48 hours of hydration. In contrast, ASC POD could be detected only after 24 hours of hydration in whole seeds, with maximum levels being reached at 48 hours.

Ascorbate peroxidase activity was represented by one band after electrophoresis of extracts from the axis. (Figure 4.14). The band could not be detected during the early stages of seed imbibition, and was only evident after 12 hours and 24 hours of hydration in the axes.

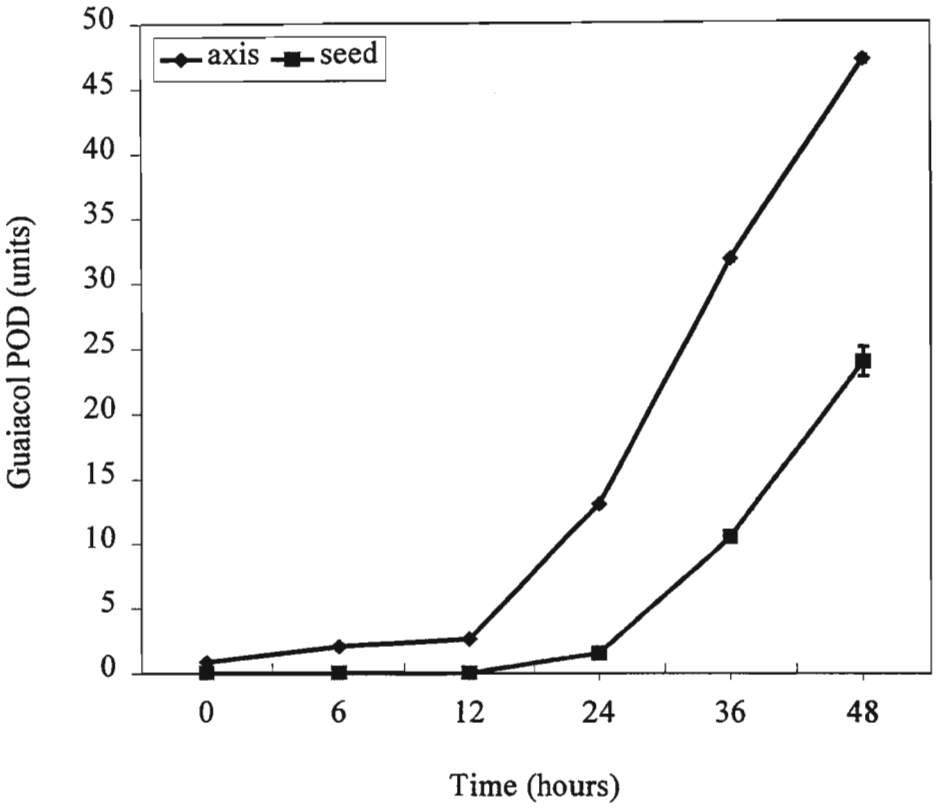

Figure 4.12. The levels of guaiacol POD in whole seeds and excised axes during the first 48 hours of seed hydration. One unit of guaiacol POD activity was defined as the amount equivalent to one nmol guaiacol oxidised per milligrams protein per minute. Values are the mean ± SE of three experiments.

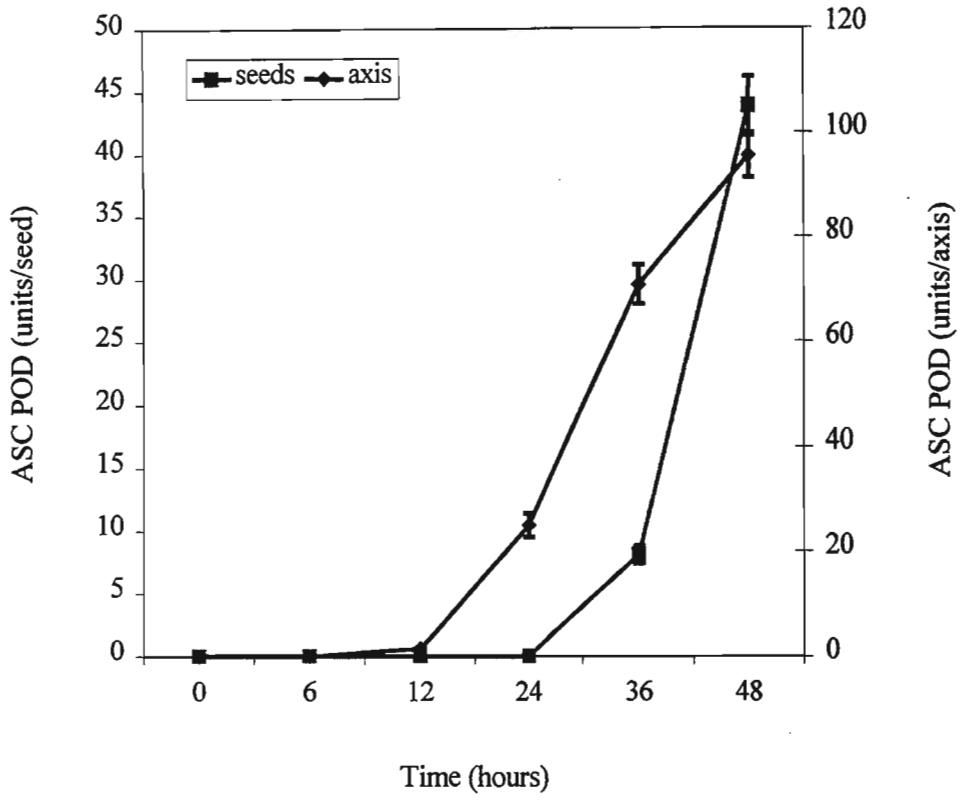

Figure 4.13. Changes in the ascorbate POD levels in whole seeds and excised axes during the first 48 hours of seed hydration. One unit of ascorbate POD activity was defined as one nmol ASC oxidised mg^{-1} protein min^{-1}. Values are the mean ± SE of three experiments.

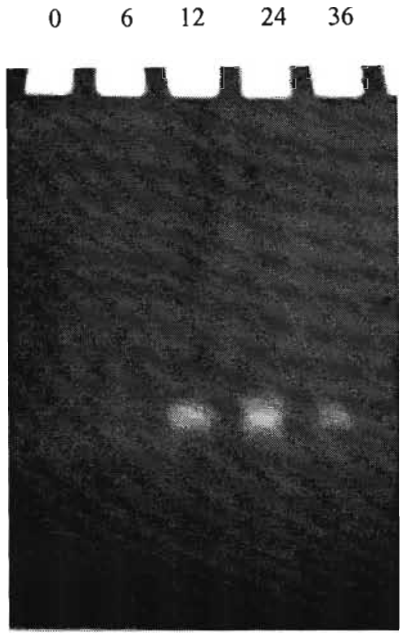

Figure 4.14. PAGE separation of ASC POD isozymes from hydrating soybean axes. Numbers from 0 to 36 denote the time period (hours) the seeds were allowed to imbibe water at ambient temperature. The enzymes appeared as achromatic bands on a blue-stained background. 50 μg protein was loaded per lane.

4.4 DISCUSSION

Imbibition or hydration of dry seeds is a critical event in the germination process, and many authors have related it to reorganisation of cellular membranes (Simon and RajaHarum, 1978; Bewley and Black, 1994; Bewley, 1997) following maturation drying. A transition in the membrane phospholipid components from the gel phase achieved during maturation drying to the normal, hydrated-liquid crystalline state marks the return of membranes to their more stable configuration (Crowe and Crowe, 1992). This transition is critical to the re-establishment of membrane fluidity which is essential if the membrane is to act as a selective barrier to prevent the outflow of cellular constituents upon rehydration (Simon and RajaHarum, 1978).

Increased membrane fluidity has been attributed to increased levels of phosphatidylcholine (PC) in membranes of wheat (Vigh *et al.,* 1979). PC is supposed to stabilise membrane bilayers and its degradation could result in changes in the properties of the membranes (Cullis and DeKruijff, 1979) and consequently the ability of seeds to germinate. From the data in Figure 4.1, it seems likely that the high levels of PC in membranes of hydrating seeds could be part of a common mechanism that results in increased membrane fluidity and prevents the formation of the non-bilayer phase. PC is favoured because it forms less tightly packed structures than PE (Michaelson *et al.,* 1974), and thus extends the membrane surface. Therefore, a high PC to PE ratio should hasten the return of membranes to their normal fluid liquid crystalline state upon imbibition.

No major changes in the ratio in the levels the major phospholipid components, PC and PE, were observed during the 36-hour hydration period. PC and PE were always the predominant constituents, even though they appeared to decline only marginally at 36 hours. The gradual increase in phosphatidic acid (PA) (Figures 4.1 and 4.2), a compound regarded as an intermediary in membrane turnover, could be due to enhanced combined hydrolytic action of various lipid hydrolysing enzymes. Findings by DiNola and Mayer (1986) suggest a possible involvement of phospholipase C and D activity in the degradation of phospholipids. However, the decrease in PI/S recorded after 12 hours could not account for the high-accumulated levels of PA. However, since *de novo* synthesis of membrane phospholipids for the use in building cellular and organellar membranes (Ching, 1972) is taking place at the same time that phospholipids are being degraded, it becomes difficult to determine the source of PA with complete certainity during hydration.

Metabolic activities are resumed immediately upon hydration of the quiescent dry seed (Bewley and Black, 1994). One of the changes upon imbibition is the resumption of respiratory activity, which can be detected within minutes (Bewley, 1997). Lipid peroxidation requires active oxygen uptake (Robinson, 1965 cited in Dhindsa *et al.*, 1981) and involves the production of reactive oxygen species such as superoxide radical,

hydrogen peroxide and hydoxyl free radical (Fridovich, 1976). The results of the present study indicate that free radical-producing and free radical-consuming processes are active early in the hydration process of soybean embryonic axes. Increases in LOOH generation observed during hydration can be expected in view of the high O_2 consumption and respiratory activity following seed imbibition. Accelerated mitochondrial electron transport is associated with an increasing potential for higher leakage of electrons to O_2 with concomitant production of O_2^- and H_2O_2 (Elstner, 1982).

A transient change in LOOH has been suggested to accompany seed hydration under normal conditions (Smith and Berjak, 1995). From the data obtained in Figure 4.3, the first 6 hours of hydration were accompanied by slight increase in the levels of LOOH, before the levels increased quite significantly after 24 hours of hydration. This pattern of LOOH change further supports the view of the dependence of lipid peroxidation on oxygen uptake (Robinson, 1965 cited in Dhindsa *et al.*, 1981). Similarly, the pattern of LOOH changes is in line with the pattern of oxygen uptake as described by Bewley (1997) where he described two regions of rapid oxygen uptake, *i.e.* at the start of seed hydration and in the middle of the germination process.

The increase in lipid hydroperoxides may be a reflection of greater susceptibility to peroxidation in the seed or that a rapid and uncontrolled uptake of water can initiate significant peroxidation. If reactive oxygen species are produced during unrestricted imbibition, then the damaging effects are limited, in as much as they do not impair germination. It is suggested that the LOOH present in seeds has been formed as a result of limited damage from oxygen radicals, generated as a consequence of impaired electron transport in respiring tissues undergoing hydration. With increased oxygen uptake, there is more opportunity for electron 'leakage' to oxygen as visualised by Halliwell (1987).

That peroxidative reactions are increased during the course of seed hydration is also supported by the increased accumulation of GSH in axes, which would be expected to limit peroxidative damage to biomolecules. At low levels of peroxidation (Figure 4.3), the amount of GSH was low (Figure 4.4), but increased significantly later during hydration, probably as a result of higher LOOH levels. It is possible that the relatively low levels at 6 hours, 12 hours, and 24 hours of hydration could be attributable to the higher levels of GSH in conjunction with other defence systems. This observation confirms the importance of GSH as an antioxidant, and that tissues lacking GSH, or with altered glutathione redox status become sensitive to oxidative stress induced by reactive oxygen species (ROS), as well as toxic products of lipid peroxidation. However, the hydration-induced increase in GSH was more than could be explained by the reduction of GSSG only. This additional increase in GSH possibly resulted from reduction of mixed disulfides (Butt and Ohlrogge, 1991) and/or *de novo* synthesis of glutathione (Klapheck *et al.*, 1990; Rouser *et al.*, 1991).

Dry axes recorded higher levels of GSSG, that was reduced to lower levels as early as the first 6 hours of seed hydration. A similar rapid reduction in GSSG has been reported for isolated wheat embryos (Fahey *et al.*, 1980) and pea seeds (Kranner and Grill, 1993) during imbibition. In view of the ability of GSSG to inhibit protein synthesis, it has been suggested (Fahey *et al.*, 1980) that a rapid reduction of GSSG upon imbibition is necessary for optimum protein synthesis. The increase in GSSG after 24 hours of hydration is consistent with the role of GSH as both a free radical scavenger and a co-factor for various antioxidant enzymes, including glutathione peroxidases (Turton *et al.*, 1997; Grant, 2001).

The ASC content of axes decreased during the first 6 hours of hydration, probably as a response to quench the escalating rise in the levels of LOOH at this period. Similarly, ascorbate may also directly reduce $O_2^{\cdot}$, quench $^1O_2$ and regenerate reduced α-tocopherol (Foyer, 1993). Any of the routes of ascorbate oxidation listed above, as well as slow synthesis rate of ASC, or decreased reduction rate of both oxidation products (*i.e.* DHA and AFR) could lead to the decrease in ASC. The increase in ASC content in axes from 6 to 12 hours was probably as a result of DHA reduction. The DHA-reduction capability of the seeds is believed to be temporary until *de novo* synthesis of ASC from glucose is restored and reaches sufficiently high levels to support the intense ASC utilisation (De Gara *et al.*, 1997). The delay in ASC biosynthesis can be attributed to the lack of L-galactono-γ-lactone (GL), synthesis, the last precursor of ASC biosynthesis, from storage carbohydrates (Mutsuda *et al.*, 1995; De Gara *et al.*, 1997). GL is believed to be the limiting factor in the pathway.

To function as an antioxidant, ASC must be maintained in a reduced state. Monodehydroascorbate (MDHA or AFR) is directly reduced to ASC by AFR reductase in the presence of NAD(P)H (Hossain *et al.*, 1984). Ascorbate is also regenerated from dehydroascorbate in a glutathione-dependent reaction catalysed by DHA reductase, which involves the GSH reductase catalysed reduction of GSSG to GSH. In the present study, both DHA- and AFR-reductases increased during the first 6 hours of hydration (Figures 4.6 and 4.7), which strongly suggested that the regeneration of ASC was dependent on their activities. Interestingly, the activity of DHA reductase began to decline after 6 hours of hydration whilst that of AFR continued to increase. The cause of this decline in DHA reductase is not clear, but these results may imply that in hydrating seeds the regeneration of ASC for H_2O_2 scavenging is mostly catalysed by AFR reductase.

That AFR reductase activity affects the amount of DHA in the cell (De Gara *et al.*, 1997) could also explain the decrease in DHA reductase activity observed after 6 hours of hydration. It is well known that cell metabolism utilises ASC as an electron donor and that ASC oxidation by ASC oxidase or peroxidase is a two-step reaction in which each step removes one electron (Yamazaki and Piette, 1961). AFR, a semiquinone-like free radical is the first ASC oxidation product which can be reconverted to ASC by AFR reductase (Bielski *et al.*, 1981) or spontaneously undergo disproportionation to generate ASC and DHA (Yamazaki and Piette, 1961; Bielski *et al.*, 1981). It appears, therefore, that when AFR reductase activity is

high, a large quantity of AFR is reduced to ASC and a small amount of AFR remains available for disproportionation.

The superoxide radical-scavenging enzyme, SOD, exhibited a gradual increase in activity during the course of hydration in both seeds and isolated axes (Figure 4.8). This is to be expected of high vigour seeds which possess appreciable levels of this enzyme in the dry state, and can therefore, on hydration, protect cells from activated oxygen attack. Such protection would be expected to be associated with better germination ability, and this may explain why low-vigour seeds exhibit reduced levels of SOD activity (Nandi *et al.*, 1997). The increase of SOD activity might reflect enhanced production of $O_2^{\cdot}$, probably as result of augmented leakage of electrons from the electron transport to molecular oxygen. The dismutation product of two $O_2^{\cdot}$ by SOD is hydrogen peroxide and oxygen. Therefore, the activity of this enzyme determines the relative proportions of $O_2^{\cdot}$ and H_2O_2.

Singlet oxygen ($^1O_2$) and hydroxyl free radical ($OH^{\cdot}$) are two highly reactive oxygen species that may be produced by the interaction between $O_2^{\cdot}$ and H_2O_2 (Fridovich, 1976). Enzymes that scavenge hydrogen peroxide include catalase and ascorbate-fuelled peroxidase (ASC POD), and their activities mirrored the levels of lipid peroxidation seen during the course of soybean seed hydration (Figures 4.10 & 4.13). These increases provide evidence for enhanced H_2O_2 production during hydration as a result of the high O_2 consumption and respiratory activity following seed hydration. The presence of these enzymes would allow for H_2O_2 to be quenched before it reacts with $O_2^{\cdot}$, and thus control the level of peroxidation. Puntarulo *et al.* (1991) have shown a close relationship between O_2 uptake and mitochondrial $O_2^{\cdot}$ and H_2O_2 production during germination of soybean embryonic axes. Accordingly, these results strongly suggest that conditions associated with H_2O_2 generation potential were linked with higher activities in H_2O_2-scavenging enzymes during hydration-germination in soybean seeds.

Regarding the appearance of the enzyme ASC POD during seed hydration, the data indicated that the expression of this H_2O_2-scavenging enzyme is regulated by ASC. Increases in ASC POD activity during germination occurred in parallel with the rise in ASC content. However, the fact that ASC was present in dry seeds, and after 6 hours of hydration, and yet no ASC POD was detected would appear to contradict this claim. A possible explanation for this would be that, ASC POD affinity for ASC appeared to be dependent on the amount of ASC available in the seeds at the moment in which the enzyme became functional. Thus, soybean seeds were able to accumulate enough ASC after 12 hours of hydration for ASC POD to be functional.

POD activities using guaiacol as an artificial substrate also increased during the course of soybean seed hydration. Much of the recorded guaiacol POD activity was localised in the embryonic axes and thus can be considered to be a reflection of the enhancement of growth and development of the embryo and seedling

(Cakmak *et al.,* 1993). It appears, therefore, that the primary function of guaiacol POD is not H_2O_2 scavenging and, consequently, the measurement of peroxidase activity with guaiacol may provide little information about the H_2O_2-scavenging capacity. There are several lines of evidence indicating that guaiacol POD is involved in cell metabolism and development (Gasper *et al.,* 1985).

From the data obtained it appeared that the efficiency of scavenging enzymes or the balance between the scavenging enzyme activity and free radical attack, both of which are enhanced immediately upon imbibition, may determine the integrity of overall cell structure and function, and ultimately the germination ability of seeds. Even if a low level of cell damage may occur due to free radicals or oxidative chain reactions accumulating within the living cell, the determining factor would be the balance between the rapid build-up of free radicals and the enhancement in scavenging enzyme activity immediately on hydration. Provided that the cell has a free-radical scavenging ability and that the scavenging activity is operative immediately upon imbibition, the cell may survive and grow. It is concluded that it is not the extent of lipid peroxidation (measured as LOOH levels) that is responsible for the loss of seed viability. Rather, it is a balance between the oxidative end product (LOOH levels) or the mitochondria in limiting electron "leakage" and the efficiency or integrity of active oxygen-scavenging enzymes present in dry seeds that determine the fate of membranes and other macromolecules when water is made available to the seed during hydration.

Chapter 5

HYDRATION-DEHYDRATION-REHYDRATION TREATMENTS

5.1 INTRODUCTION

Seeds of all orthodox species are tolerant of dehydration to low moisture contents at maturity. Imbibition of water initiates a series of metabolic steps that lead to germination, and during this sequence of events, desiccation tolerance is lost. During the early stages of germination, and prior to radical emergence in most species, the seed can be dried back to its original moisture content without causing injury (Hegarty, 1978). However, the same degree of drying imposed at progressively later stages of germination dramatically reduces seed vigor and, if imposed after radical elongation has commenced, usually results in seedling death.

Cellular membrane systems appear to be one of the primary sites of desiccation injury. However, it remains unclear what differences occur that allow seeds to tolerate injury to membranes at one stage of germination and yet be sensitive at another. Investigations carried out using soybean seed axes have indicated that physical and compositional properties of cellular membranes are altered after desiccation stress (Senaratna *et al.*, 1987). These changes occur only in the axes from soybean seeds that are in a desiccation-sensitive state (*i.e.* 36 hours of imbibition). In contrast, axes from seeds imbibed for 6 hours are still tolerant of desiccation and membrane properties are not altered by the same degree of desiccation stress. The altered physical properties of membranes may include the presence of gel phase lipid domains, which are an indication of lateral phase separations within the plane of the membranes (Senaratna *et al.*, 1984; McKersie *et al.*, 1989). Such changes in phase properties of membranes are associated with loss of phospholipids and accumulation of free fatty acids (McKersie *et al.*, 1989).

One of the most documented degradative reactions linked with desiccation sensitivity in seeds is the accumulation of peroxidative damage following drying. Desiccation-induced changes in the cellular environment can be expected to cause transient dysfunction in specific enzymes and/or electron transport chains (Leprince *et al.*, 1992, 2000). Such dysfunction may lead to the production of free radicals, or

promote chemical reactions that would not normally occur in a fully hydrated system (Leprince *et al.*, 1994). Under normal metabolic conditions, enzymatic and non-enzymatic defence mechanisms would scavenge free radicals, but because of the reduced hydration, these mechanisms may not function. Previous studies on desiccation-intolerant embryos (Leprince *et al.*, 1994, 1995) have shown that respiration is involved in free radical processes leading to lipid peroxidation and membrane damage, suggesting that the normal tight control of reactive oxygen species (ROS) by cellular defences is lost during drying.

A typical symptom of dehydration injury in seeds is an increase in the quantity, and changes in the kinetics, of solute efflux and other cytoplasmic solutes from the tissue during rehydration (Senaratna and McKersie, 1983; Simon, 1974). During episodes of drying and wetting, considerable physical tearing of the plasmalemma may occur which contributes to solute leakage (Powell and Matthews, 1979). Several lines of evidence suggest that desiccation stress induces changes in the organisation of cellular membranes that is reflected in altered semipermeability (Senaratna and McKersie, 1983).

If irreversible injury to membrane systems is initiated during desiccation via a free radical-mediated mechanism, as suggested, then it might be expected that the tolerance of seed tissues in the early stages of germination would be provided by a free radical tolerance mechanism. To prevent oxidative damage, plants possess antioxidative systems composed of low molecular weight antioxidants such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (PODs). Other components of the defense system, monohydroascorbate radical (AFR) reductase, dehydroascorbate (DHA) reductase, and glutathione (GSH) reductase serve to maintain the antioxidants in their reduced state (Noctor and Foyer, 1998). These compounds are known to prevent the build-up of reactive oxygen species. Because desiccation-tolerant tissues do not exhibit symptoms of ROS-induced injury, they are also expected to be endowed with mechanisms controlling such injury during the loss of water. The absence of an effective antioxidant system has been identified as one of the factors contributing to a lethal accumulation of ROS in plant tissues (Nandi *et al.*, 1997).

It is evident from Chapters 3 and 4 that there are several similarities between imbibing or hydrating seeds and those undergoing maturation drying. Since hydrating seeds shift from a desiccation-tolerant state to a desiccation-sensitive state with the progress of imbibition, they present an ideal system to elucidate further the phenomena of desiccation-tolerance and desiccation sensitivity. In this Chapter, studies were made on the effects of unscheduled dehydration applied to seeds that were hydrated for 6, 12, 24 and 36 hours, and when they were subsequently rehydrated, on lipid levels, lipid peroxidation, solute leakage, and free radical defenses in an attempt to probe further the adaptive nature of antioxidant defences and their relationship to desiccation tolerance.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

In order to limit the seed-to-seed variability, which might arise from commercially, purchased seeds, all seed material used in this study was hand-harvested from experimental material grown in the greenhouse at the University of Natal. To obtain the plants, commercial seeds of cultivar Braithwaite were obtained from Pannar Seeds and sown in sand-filled pots in August 1999, February 2000 and August 2000, respectively, as described in Chapter 3. Only fully matured soybean seeds were used for imbibition trials and the average germination percentage of all seed stock was 98%.

5.2.2 Water uptake studies

Groups of 20 seeds were massed and then placed on Whatman No. 1 filter paper in Petri dishes. To each Petri dish, 10 ml of water was added and seeds were held at ambient laboratory temperature. At regular intervals thereafter, seeds were harvested for further analysis. Water uptake patterns for excised axes were characterised at regular intervals by removing excess moisture from the seeds or axes with absorbent paper towelling and their masses recorded. The increase in fresh weight was determined for each group of axes.

5.2.3 Hydration-dehydration-rehydration (H-D-R) treatments

Seeds hydrated for 6, 12, 24 and 36 hours, respectively, were dehydrated for 36 hours at ambient laboratory temperature by placing the opened Petri dishes in front of a household fan for 36 hours. After this period of fan drying, the seeds were found to have reached their original fresh weight and their axes were harvested for the studies and assays undertaken below. Rehydration of seeds was achieved by placing groups of 20 seeds on a Whatman No. 1 filter paper in Petri dishes for each hydration period. To each Petri dish, 10 ml of water was added and the seeds were held at ambient laboratory temperature. Axes were harvested at 6, 12 and 24 hours of rehydration, respectively.

5.2.4. Leakage studies

Three repetitions of 10 axes excised from dehydrated seeds were placed in glass vials with 10 ml of water. The supernatant was then analysed after 1, 2, 4, 8 and 12 hours for protein using the Bradford method, and phosphorus using the assays previously described in Chapter 3.

5.2.5 Lipid studies

Lipids were extracted from axes excised from hydrated, dehydrated and rehydrated seeds, respectively, using the solvent system of Khor and Chan (1985) and following the procedure outlined in Chapter 3.

4.2.5.1 Thin layer chromatography of lipids

Two-dimensional thin layer chromatography (TLC) on 10x10 cm plates precoated with silica gel G as adsorbent was used for analytical separation of individual lipid classes from the lipid extract.

4.2.5.2 Polar lipids

The TLC plates were pre-washed in chloroform and then dried at 110° C. After allowing to cool, about 20 µg of lipid was applied at the lower right-hand corner of the plate and the plate was developed as outlined in Chapter 3.

4.2.5.3 Hydroperoxide determination

Hydroperoxide levels in axes excised from hydrated, dehydrated and rehydrated seeds, respectively, were determined using a modification of the test of Stine *et al.* (1953) as described in Chapter 3.

5.2.6 Extraction and Analysis of ASC and GSH

Excised axes from hydrated, dehydrated and rehydrated seeds, respectively, were homogenised with four volumes of cold 5% metaphosphoric acid using a pestle and mortar. The homogenate was centrifuged at 20 000 g for 15 minutes at 4° C, and the supernatant was collected and analysed for ascorbate and glutathione as described in Chapter 3.

5.2.7 Preparation of the enzyme extract

Enzymes were extracted from the axes excised from hydrated, dehydrated and rehydrated seeds, respectively, in 0.2 M Tris-HCl buffer (pH 7.8), containing 1 mM DDT, 0.2 mM EDTA and 2% PVP as outlined in Chapter 3.

5.2.8 Estimation of protein content

The analysis for protein estimation was carried out using the Bradford method (Bradford, 1976) as described in Chapter 3.

5.2.9 Separation of isoenzymes by PAGE and their specific detection

Electrophoretical separation and staining of isoenzymes of SOD, DHA reductase, ascorbate peroxidase, and CAT was achieved as outlined earlier in Chapter 3.

5.2.9.2 Spectrophotometric quantification of enzymes

AFR reductase, DHA reductase, ascorbate peroxidase, superoxide dismutase, catalase, and guaiacol POD were assayed in the crude enzyme extract of the axes spectrophotometrically using the techniques described in Chapter 3.

5.3 RESULTS

5.3.1 Fresh weight changes

The dehydration response of axes was influenced by the length of the hydration period after which the seeds were dried. Soybean axes hydrated for up to 12 hours showed the smallest reduction in fresh weight after they were dehydrated to their original fresh weight (Figure 5.1). Severity of dehydration, as measured by water loss, was more pronounced in seeds hydrated for 24 and 36 hours (Figure 5.1). Axes from seeds hydrated for 6 and 12 hours showed an identical dehydration pattern. Of note was that, in seeds hydrated for 36 hours, the radicle had already emerged from the seed coat. As such, axes dehydrated at this stage had retained a slightly higher fresh weight relative to the other treatments after the 36 hours of dehydration. This response was probably a reflection of some reserve mobilisation.

With the exception of axes from seeds dehydrated after 36 hours of hydration, the patterns of fresh weight increase followed a similar pattern upon rehydration (Figure 5.1). The axes from seeds, which had previously been hydrated for 36 hours, showed a sharp rise in fresh weight in the first 6 hours of rehydration, attaining nearly 70% of the value reached after 36 hours hydration. However, this sharp increase levelled off over the following 18 hours of rehydration. Visual signs of possible injury, such as curled hypocotyls and roots, were evident in axes dehydrated at 24 and 36 hours of hydration, and complete seedling establishment was not achieved following a hydration-dehydration-rehydration (H-D-R) treatment. The damage appeared to be mainly confined to the meristematic region of the radicle and was sufficient to affect the increase in fresh weight in axes dried at 36 hours hydration upon subsequent rehydration.

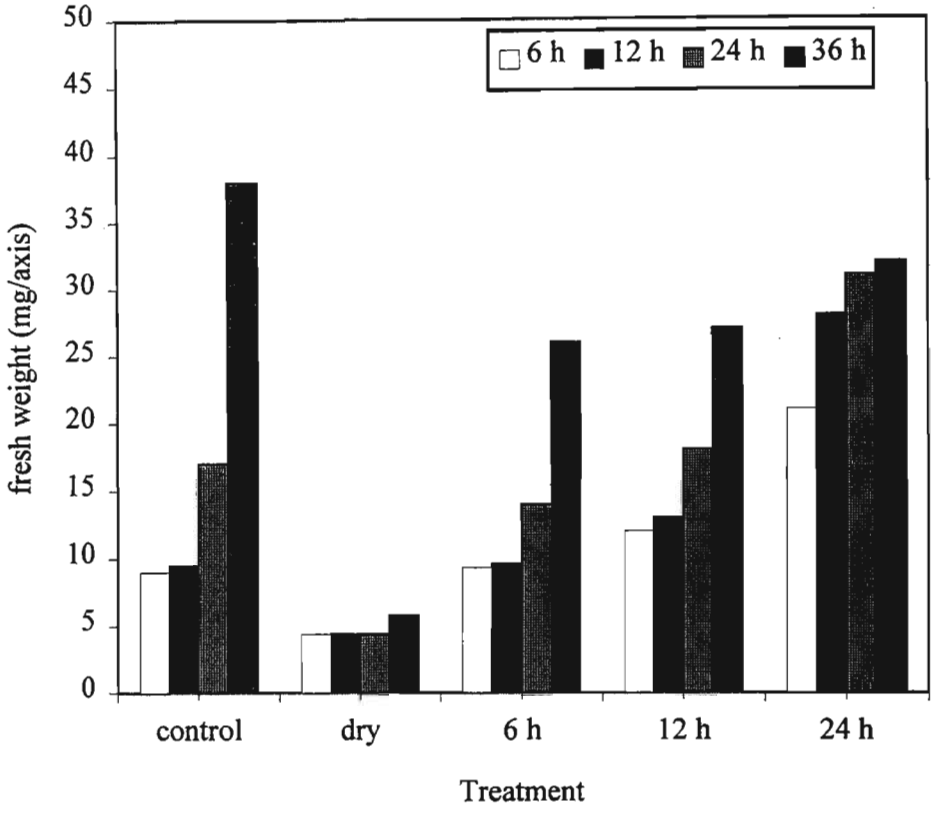

Figure 5.1. Changes in the fresh weight of axes from seeds allowed to hydrate for 6, 12, 24 and 36 hours (legends), respectively, (controls). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours (x-axis), and the fresh weight of axes measured. For convenience, this protocol is described as a H-D-R treatment in subsequent Figures. Values are the mean of three experiments.

5.3.2 Leakage of phosphorus and protein

Analysis of leakage from axes of seeds dried to their original weight revealed that the amount of phosphorus lost increased during the first hour in axes dried after 6, 12, and 24 hours of hydration, after which no marked changes in the amount of phosphorus lost were observed (Figure 5.2). In contrast, axes of seeds dried after 36 hours of hydration not only released higher amounts of phosphorus to the bathing medium, but also continued to release phosphorus to the bathing medium for the duration of the leakage experiment. After an initial increase was seen after the first hour (Figure 5.2), phosphorus leakage increased almost exponentially for up to 12 hours.

Leakage of protein was also quite rapid during the first hour of rehydration in all the dehydrated axes (Figure 5.3), after which only small changes were observed in the rate and amount of protein released in axes dried after 6 and 12 hours of hydration. In axes of seeds dried after 24 hours, however, the increase in protein leakage was observed over a period of 4 hours before declining. This could possibly have been a result of re-absorption by the axes. The magnitude of protein leakage was highest in axes dried after 36 hours of hydration, where protein leakage was extensive during the first hour, increased gradually and progressively between 1 and 8 hours, and increased sharply thereafter.

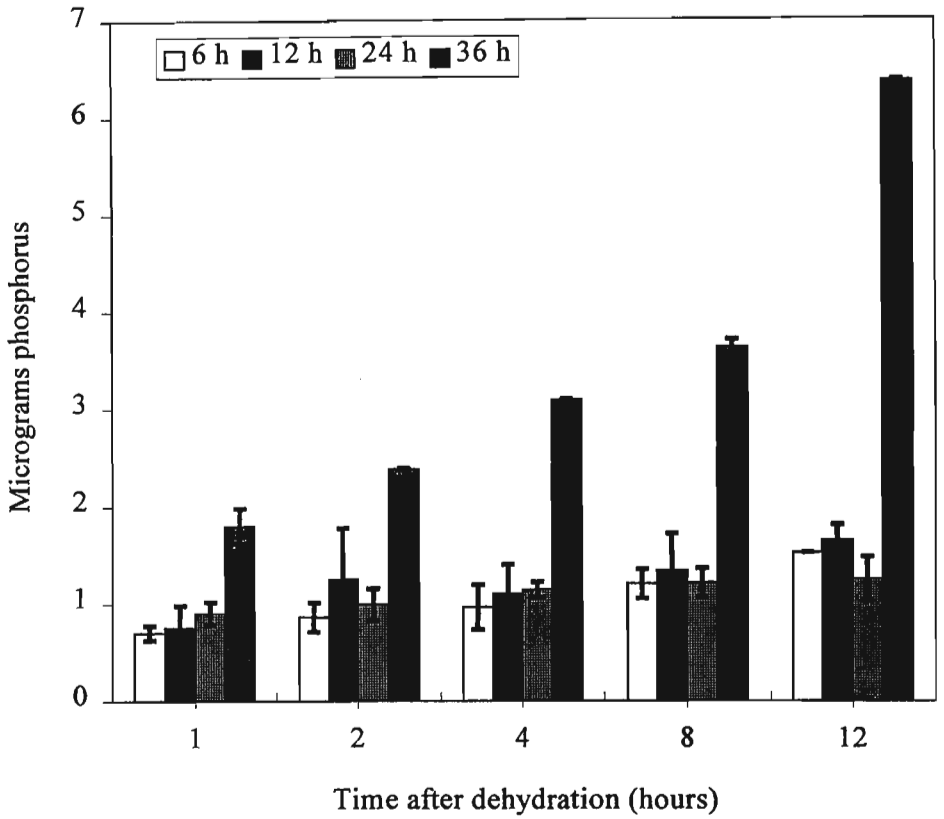

Figure 5.2. Leakage pattern of phosphorus from soybean axes after H-D-R treatments. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (legends), before drying in an air stream for 36 hours to their original weights. Isolated axes were then places in vials with 5 ml of water, and levels phosphorus measured in the bathing medium at the times indicated. Values are the mean ± SE of three experiments.

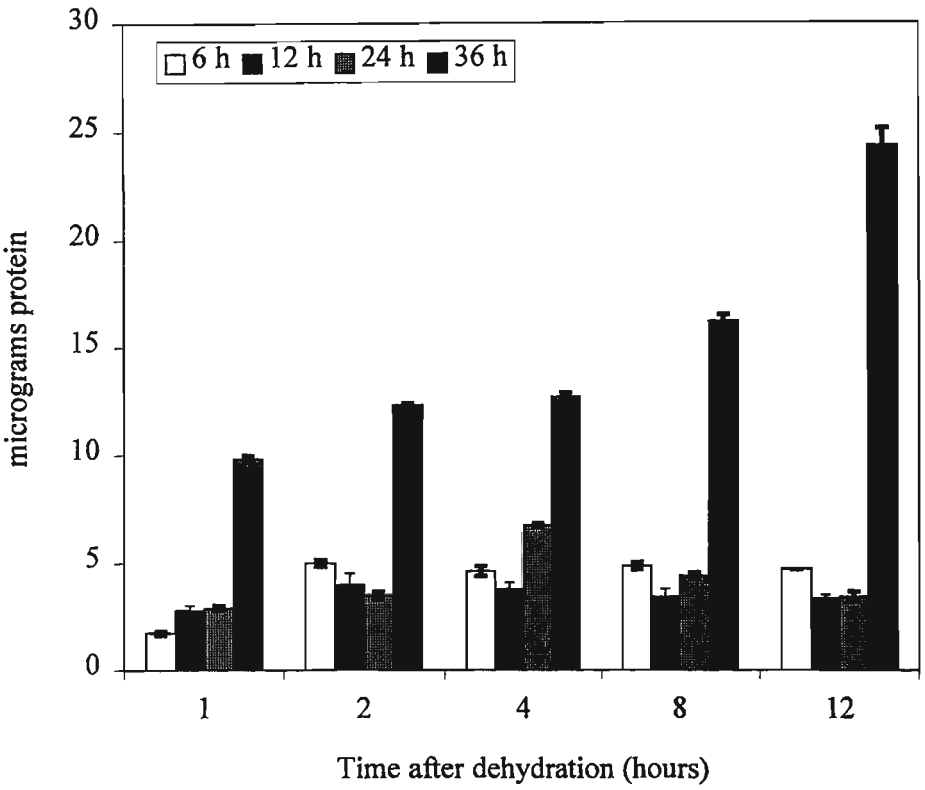

Figure 5.3. Leakage pattern of protein from soybean seed axes following H-D-R treatments. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (legends), before drying in an air stream for 36 hours to their original weights. Isolated axes were then places in vials with 5 ml of water, and levels protein released were measured in the bathing medium at the times indicated. Values are the mean ± SE of three experiments.

5.3.3 Changes in phospholipids

6 hour treatment

Overall, the phospholipid classes of axes from seeds dried after 6 hours of hydration declined following the first 6 hours of seed rehydration (Figure 5.4). Major phospholipids, PC and PE, decreased by 60% and 70%, respectively, whereas PA increased by 41%. A progressive rise in the phospholipids was observed after 6 hours of rehydration. Between 6 and 24 hours of rehydration, PC, PE and PA increased by 4.5-fold, 18-fold and 2.5-fold, respectively. At 24 hours of rehydration PC was 1.8-fold higher than control value, PE was 1.6-fold higher, and PA was 3.5-fold higher.

12 hour treatment

In axes dried after 12 hours of hydration, PC and PE decreased by 29% and 49%, respectively, whereas PA decreased by 63% (Figure 5.5). PC declined by a further 25% when the seeds were subsequently rehydrated for 6 hours whereas PE and PA recorded increases. The phospholipids showed a gradual rise at 12 hours of rehydration before increasing sharply at 24 hours of rehydration to reach maximum.

24 hour treatment

The phospholipids, in axes of seeds dried after 24 hours of hydration, declined progressively following 36 hours of dehydration, and the first 6 hours of rehydration (Figure 5.6). At 6 hours of rehydration, PA, PC, and PE had declined by 63, 68 and 62%, respectively, relative to the control values, after which the phospholipids increased gradually. However, only PA reached levels that were higher than the control levels at 24 hours of rehydration. Overall, PL levels were lower than for the 6 and 12 hour treatments.

36 hour treatment

All four phospholipid classes showed a decline in the axes of seeds dried after 36 hours of hydration (Figure 5.7). At 6 hours of rehydration, only PA was detected. Thereafter, low levels of all four phospholipids were recorded after 12 hours of seed rehydration, before PE disappeared totally at 24 hours of seed rehydration whilst PC decreased by 72%. At the end of the 24 hour rehydration period, the axes had lost 100% PE, 90% PC and 50% PA relative to the control axes. Again, overall PL levels were lower than for preceeding treatments.

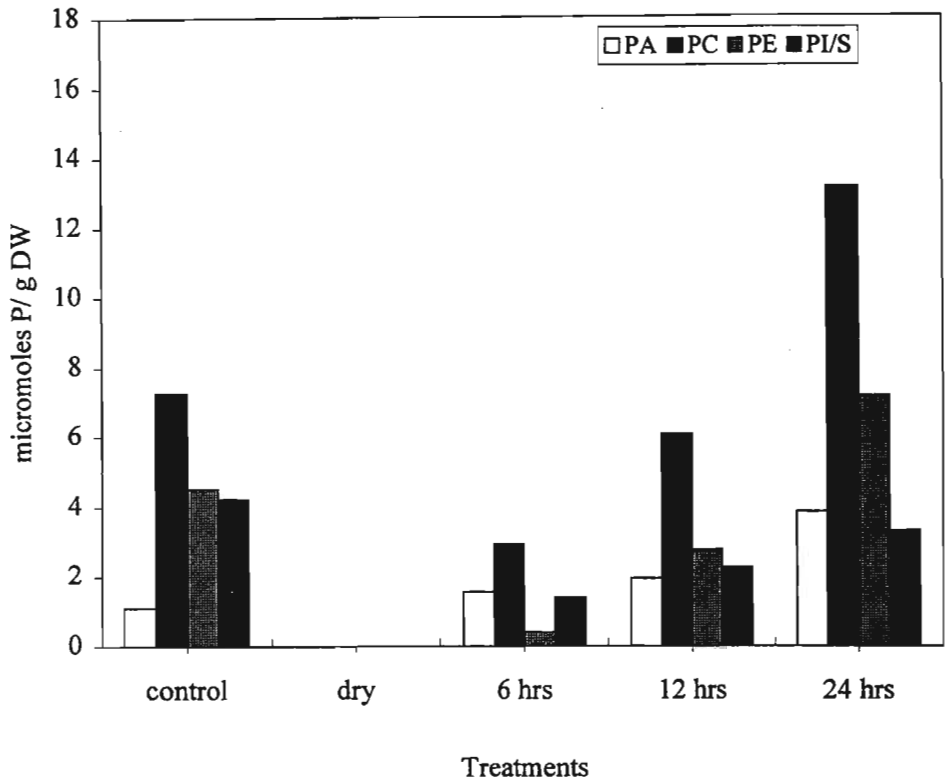

Figure 5.4. Changes in phospholipid classes PC, PE, PI/S, and PA during the hydration-dehydration-rehydration of 6 hour-hydrated axes. Seeds were allowed to hydrate for 6 hours (control). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours, and the phospholipid classes of axes were quantified. No phospholipid measurements were made for dried axes. Values are the mean of three experiments.

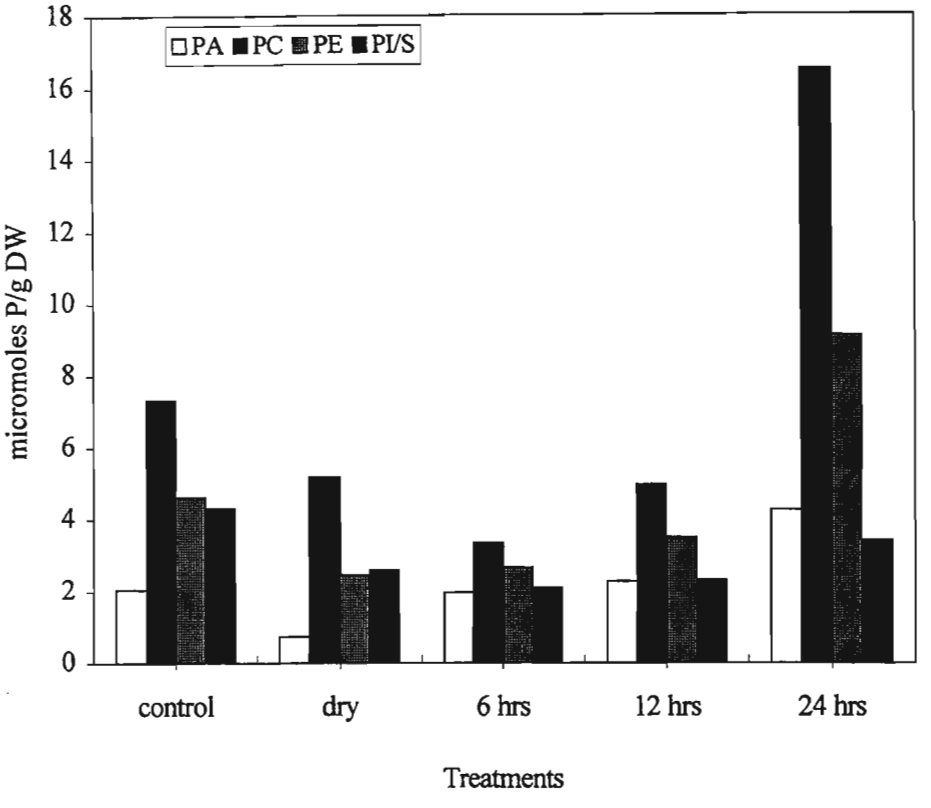

Figure 5.5. Changes in phospholipid classes PC, PE, PI/S, and PA during the H-D-R of 12 hour-hydrated axes. Seeds were allowed to hydrate for 12 hours (control). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours, and the phospholipid classes of axes were quantified. Values are the mean of three experiments.

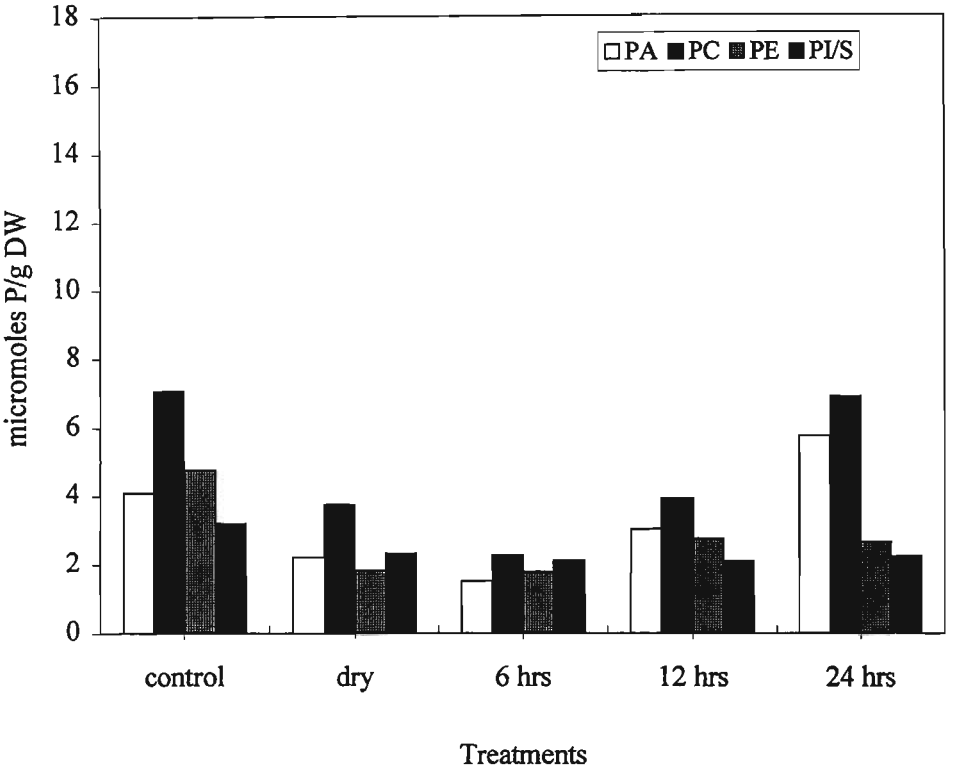

Figure 5.6. Changes in phospholipid classes PC, PE, PI/S, and PA during the H-D-R of 24 hour-hydrated axes. Seeds were allowed to hydrate for 24 hours (control). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours, and the phospholipid classes of axes were quantified. Values are the mean of three experiments.

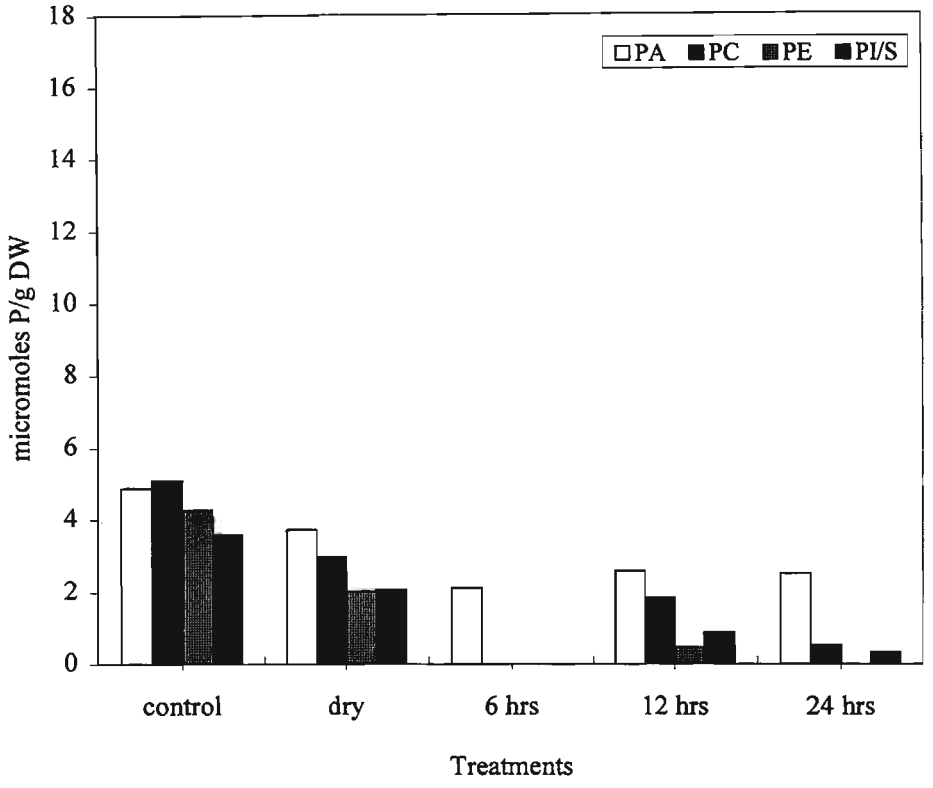

Figure 5.7. Changes in phospholipid classes PC, PE, PI/S, and PA during the H-D-R of 36 hour-hydrated axes. Seeds were allowed to hydrate for 36 hours (control). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours, and the phospholipid classes of axes were quantified. Values are the mean of three experiments.

5.3.4 Changes in lipid hydroperoxides

The results of the previous experiments suggested a progressive loss of PC and PE, and an increase in PA during the H-D-R experiments, and one of the best-documented changes associated with desiccation-sensitivity in seeds is the accumulation of peroxidative damage following drying. It would be expected that phospholipid decline could be brought about by uncontrolled LOOH, and free-radical mediated reactions. Changes in LOOH levels during H-D-R treatments were therefore studied to gain further insight into the effects of dehydration on the levels of lipid peroxidation.

Figure 5.8, control, illustrates that the longer the seeds were allowed to hydrate in water, the higher the levels of LOOH that accumulated in axes. Overall, axes of seeds imbibed for 6 and 12 hours had the lowest levels of LOOH compared to the levels of LOOH seen in axes of seeds at 24 and 36 hours of hydration. Following dehydration for 36 hours, the largest increases of 6-fold and 6.7-fold in the levels of LOOH were recorded in the axes of seeds dried after 24 and 36 hours, respectively, relative to the hydrated controls. The change in LOOH levels was negligibly small in the axes of seeds dried after 6 and 12 hours of hydration, compared to the control.

When dried seeds were rehydrated for 6, 12 and 24 hours and the changes in the levels of LOOH in axes were measured (Figure 5.8), LOOH levels decreased dramatically during the first 6 hours of rehydration in the axes of seeds dehydrated after 24 and 36 hours of hydration, after which small changes in LOOH were observed. In spite of this, LOOH levels were still very much higher than the normal, hydrated controls. In contrast, the increase in LOOH levels was gradual and progressive during rehydration in the axes of seeds dehydrated after 6 and 12 hours of hydration, but the levels were much lower than those of axes of seeds dehydrated after 24 and 36 hours of hydration. Of note in the latter treatments is that LOOH levels varied negligibly relative to hydrated controls at different hydration/imbibition periods.

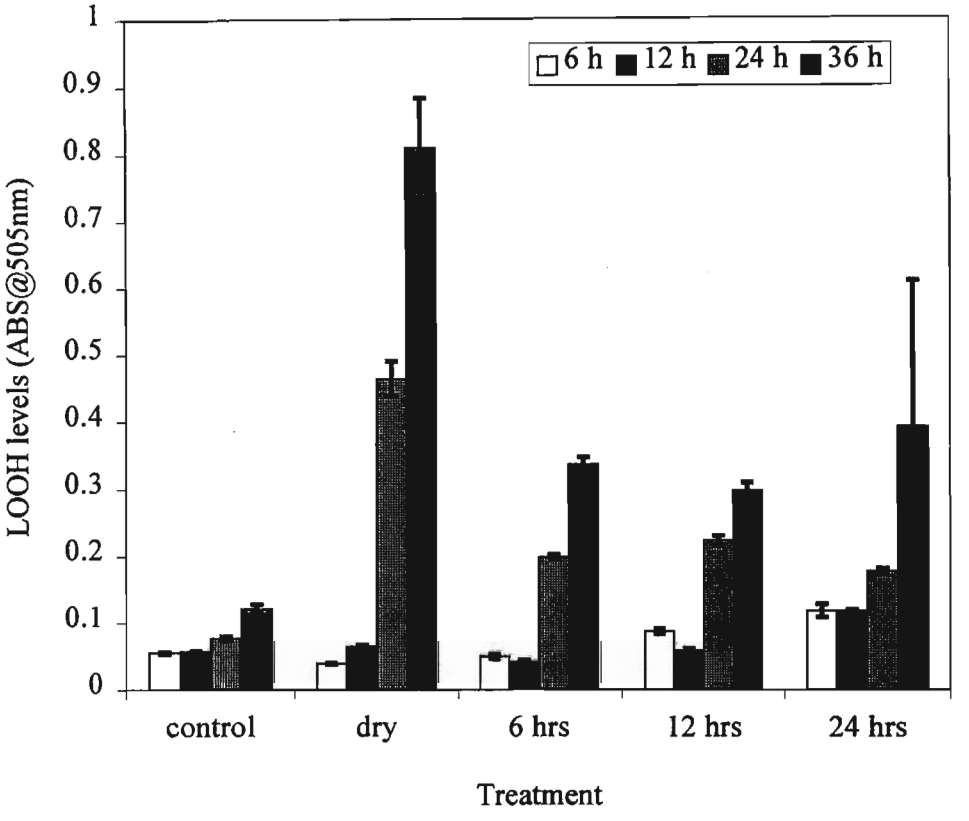

Figure 5.8. Lipid hydroperoxide (LOOH) changes in the axes of soybean seeds during the H-D-R treatment of seeds. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (legends), respectively (controls). After drying in an air stream for 36 hours, dry seeds were then rehydrated for 6, 12, and 24 hours (x-axis), and the LOOH levels of axes were measured. Values are the mean ± SE of three experiments.

5.3.5 Changes in total glutathione

6 hour treatment

Total glutathione content declined substantially in the axes of seeds dried after 6 hours of hydration (Figure 5.9). The decline was as a result of a 39% decline in the reduced glutathione (GSH) content compared to hydrated control, whilst the oxidised form of glutathione increased, but only marginally. When the dried axes were subsequently rehydrated, total glutathione increased gradually, and progressively at 6, 12 and 24 hours of rehydration, respectively. The increase in total glutathione was due not only to increased levels of GSH, but the doubling of GSSG content at 24 hours of rehydration also contributed to the high total glutathione content recorded at this period. Similarly, the decrease in GSH/GSSG ratio from 23.6 after 12 hours of rehydration to 13 at 24 hours of rehydration was a further confirmation of the high levels of GSSG at 24 hours. Of note in the 6 hours treatment were the reduced levels of total glutathione coupled with the reduction in the GSH/GSSG ratio from 40.1 to 20.8 at 6 hours of rehydration relative to control, hydrated axes.

12 hour treatment

Soybean axes from seeds dried after 12 hours of imbibition also recorded a 34% decline in total glutathione as a result of a decline in GSH (Figure 5.10). Upon rehydration of seeds, a marked increase in total glutathione content in the axes, especially GSH, was noted during the first 12 hours of rehydration. GSSG, on the other hand, was lowered during the first 12 hours of rehydration, but increased again after 24 hours of rehydration. As a result, the GSH/GSSG ratio was reduced from 31.7 at 12 hours of rehydration to 15.6 at 24 hours.

24 hour treatment

Total glutathione content decreased only slightly in axes of seeds that were dehydrated after 24 hours of hydration, but the change in the proportion of GSH and GSSG was especially noticeable (Figure 5.11). GSSG increased 7.9-fold, and GSH decreased by 24% compared to the hydrated, control values. When the dried axes were rehydrated, total glutathione content peaked at 12 hours of rehydration before decreasing somewhat at 24 hours of rehydration. The GSH/GSSG ratio increased progressively from 3.4 in the dried axes, to 21.9 at 12 hours of rehydration, as a result of increased levels of GSH, and declining levels of GSSG. In 24 hour rehydrated axes, higher levels of total glutathione were seen relative to the 24 hour hydrated axis, but because of higher levels of GSSG the GSH/GSSG ratio was a third of that recorded in 24 hour hydrated axes.

36 hour treatment

The axes of seeds dried after 36 hours of imbibition recorded a 36% decline in GSH and a 300% increase in GSSG (Figure 5.12). Total glutathione declined further when the axes were rehydrated for 6 hours, due to substantial declines in both GSH and GSSG. The GSH levels were the lowest recorded for this experimental series. An apparent recovery of total glutathione levels was noted at 12 hours rehydration, and the levels were intermediate between control and dried axes values.

It is worth noting that the overall trend of increasing total glutathione after drying and during rehydration concurred with increasing initial hydration trend.

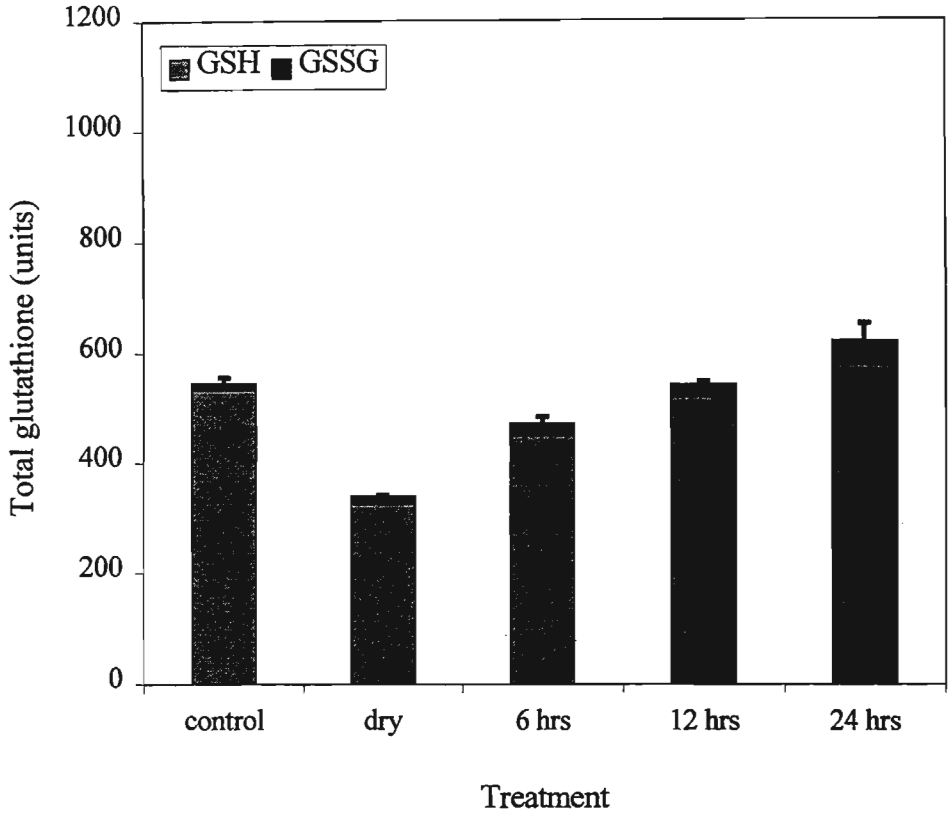

Figure 5.9. Reduced glutathione (GSH) and oxidised glutathione (GSSG) contents of axes following H-D-R treatment of 6 hour hydrated soybean seeds. Seeds were allowed to hydrate for 6 hours (control). After drying in an air stream for 36 hours, dry seeds were then rehydrated for 6, 12, and 24 hours, and the total glutathione content of axes was measured. Units expressed as nmol per grams FW per axis. Values are the mean ± SE of three experiments.

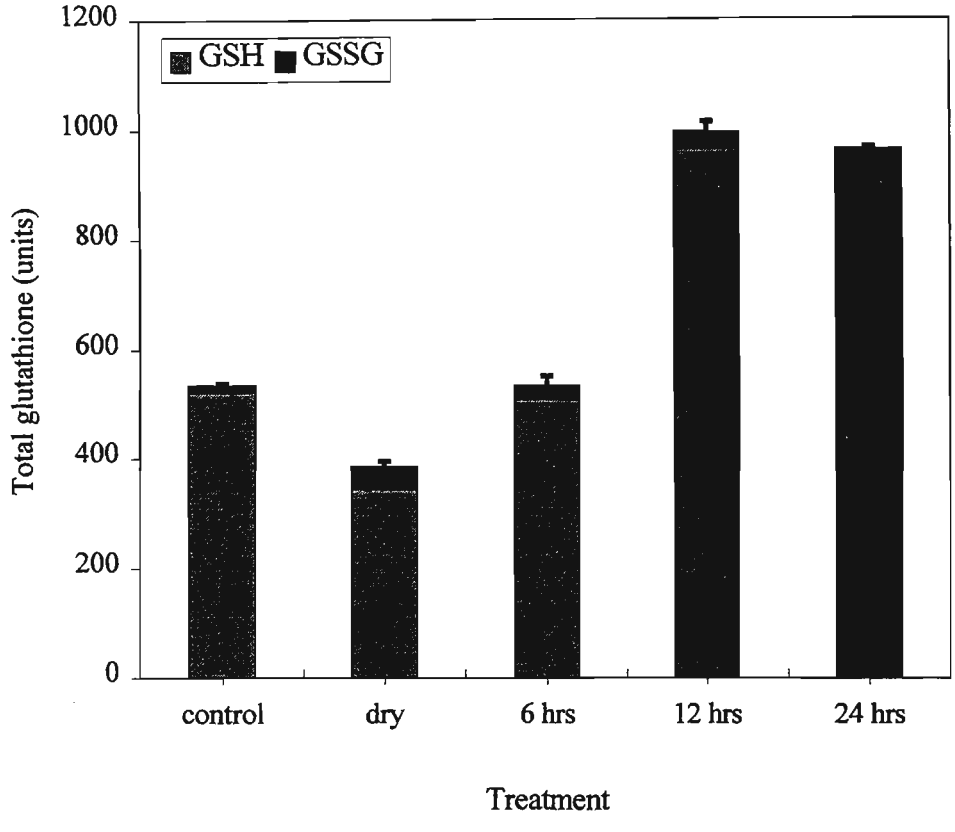

Figure 5.10. Reduced glutathione (GSH) and oxidised glutathione (GSSG) contents of axes following H-D-R treatment of 12 hour hydrated soybean seeds. Seeds were allowed to hydrate for 12 hours (control). After drying in an air stream for 36 hours, dry seeds were then rehydrated for 6, 12, and 24 hours, and the total glutathione content of axes was measured. Units expressed as nmol per grams FW per axis. Values are the mean ± SE of three experiments.

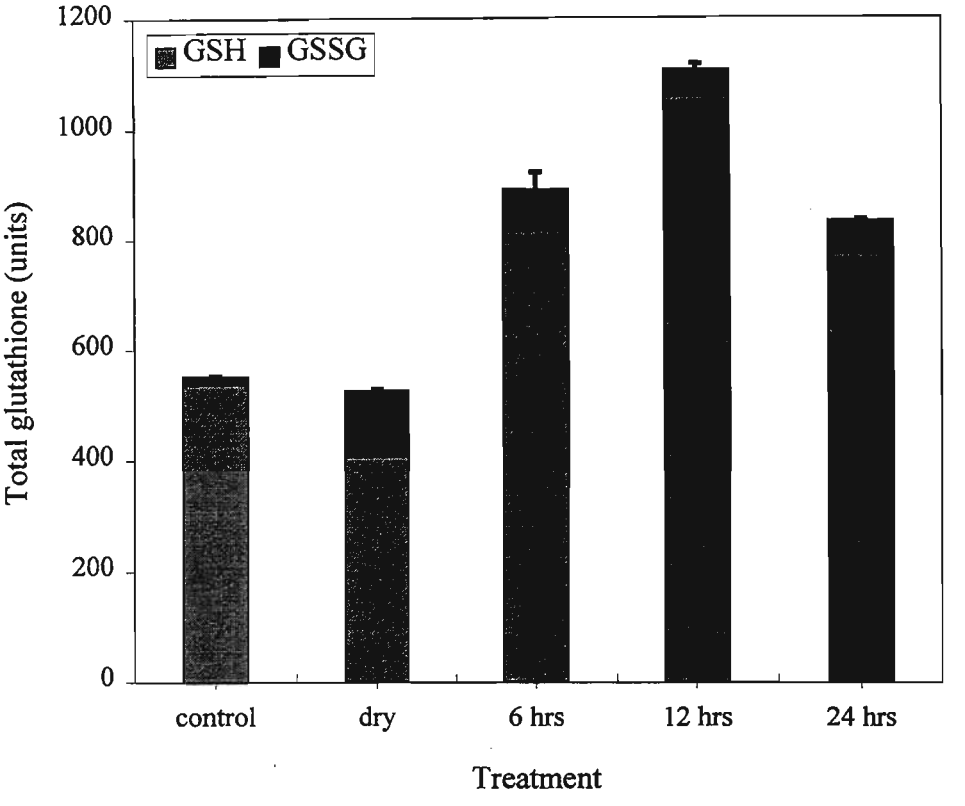

Figure 5.11. Reduced glutathione (GSH) and oxidised glutathione (GSSG) contents of axes following H-D-R treatment of 24 hour hydrated soybean seeds. Seeds were allowed to hydrate for 24 hours (control). After drying in an air stream for 36 hours, dry seeds were then rehydrated for 6, 12, and 24 hours, and the total glutathione content of axes was measured. Units expressed as nmol per grams FW per axis. Values are the mean ± SE of three experiments.

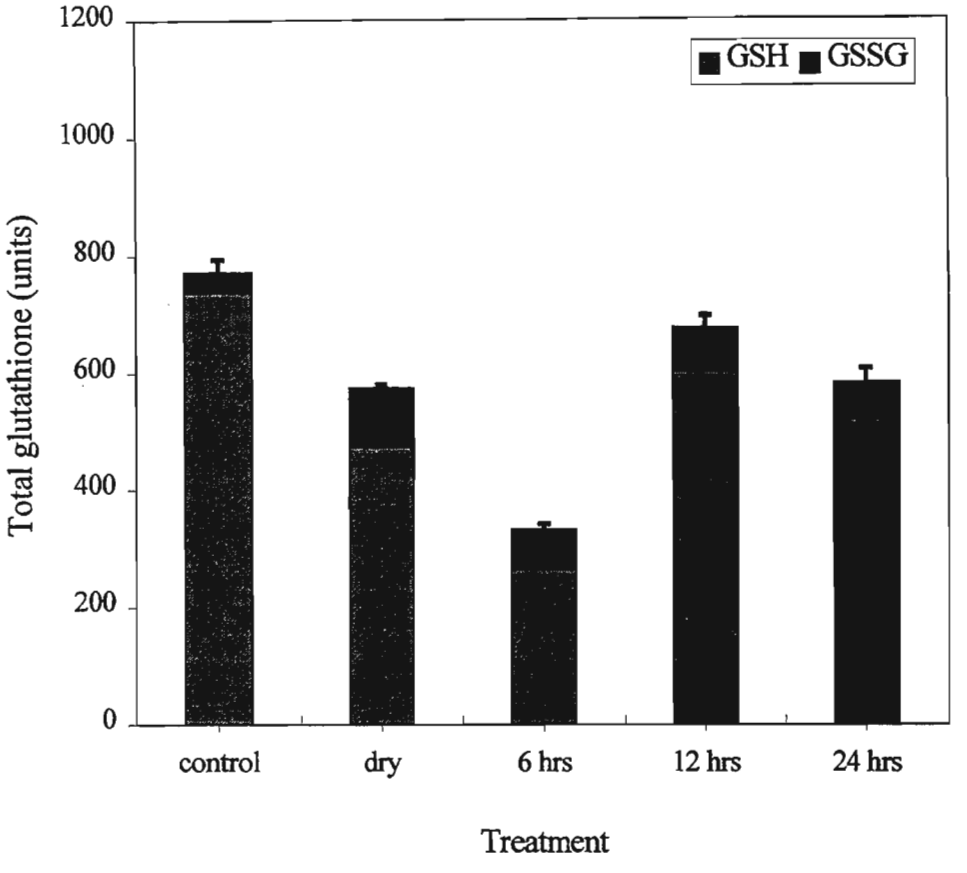

Figure 5.12. Reduced glutathione (GSH) and oxidised glutathione (GSSG) contents of axes following H-D-R treatment of 36 hour hydrated soybean seeds. Seeds were allowed to hydrate for 36 hours (control). After drying in an air stream for 36 hours, dry seeds were then rehydrated for 6, 12, and 24 hours, and the total glutathione content of axes was measured. Units expressed as nmol per grams FW per axis. Values are the mean ± SE of three experiments.

5.3.6 Changes in total ascorbate

6 hour treatment

Total ascorbate content of axes declined by approximately 57% relative to the hydrated control when the seeds were dried back after 6 hours of hydration (Figure 5.13). Both ASC and DHA were affected by the dehydration treatment, recording a 50% and 67% decline, respectively, on drying. Subsequent rehydration of seeds resulted in a marginal increase in total ascorbate content during the first 12 hours of rehydration, while at 24 hours total ascorbate increased 5-fold. Most of the increase was attributed to increased levels of ASC, whilst DHA levels increased only marginally.

12 hour treatment

In axes of seeds that were dried back after 12 hours of hydration, total ascorbate decreased only slightly after 36 hours of dehydration relative to the hydrated control (Figure 5.14). Only ASC levels were reduced by the dehydration treatment, whereas the DHA levels were increased slightly. After 6 hours of rehydration, DHA increased further and constituted 37% of the total ascorbate pool. At 12 hours of rehydration, DHA decreased drastically and accounted for only 4% of the total ascorbate pool. A 4-fold increase in total ascorbate content was recorded at 24 hours of seed rehydration as a result of a sharp increase in ASC in the axes, this resulting in an increase in the ASC/DHA ratio.

24 hour treatment

Following hydration of seeds for 24 hours, ASC levels of axes were 33 units, while DHA levels were 6 units (Figure 5.15). When the seeds were dehydrated for 36 hours, the ASC levels increased only slightly whereas DHA levels increased 5-fold, this resulting in an increase in total ascorbate pool. On rehydration, ASC increased progressively with time, while DHA decreased. At 24 hours of rehydration, the total ascorbate pool in the axes was approximately 3-fold higher than the 24 hour-hydrated, control axes.

36 hour treatment

Axes of seeds dried after 36 hours of hydration showed a 44% decline in the total ascorbate pool (Figure 5.16), as result of 54% decrease in ASC. DHA, on the other hand, increased on drying and constituted 45% of the total ascorbate pool. The total ascorbate pool declined further after the first 6 hours of rehydration, but increased levels at 12 and 24 hours of rehydration. At the same time, DHA content decreased gradually from 14 units at 6 hours of rehydration to 4 units at 24 hours of rehydration.

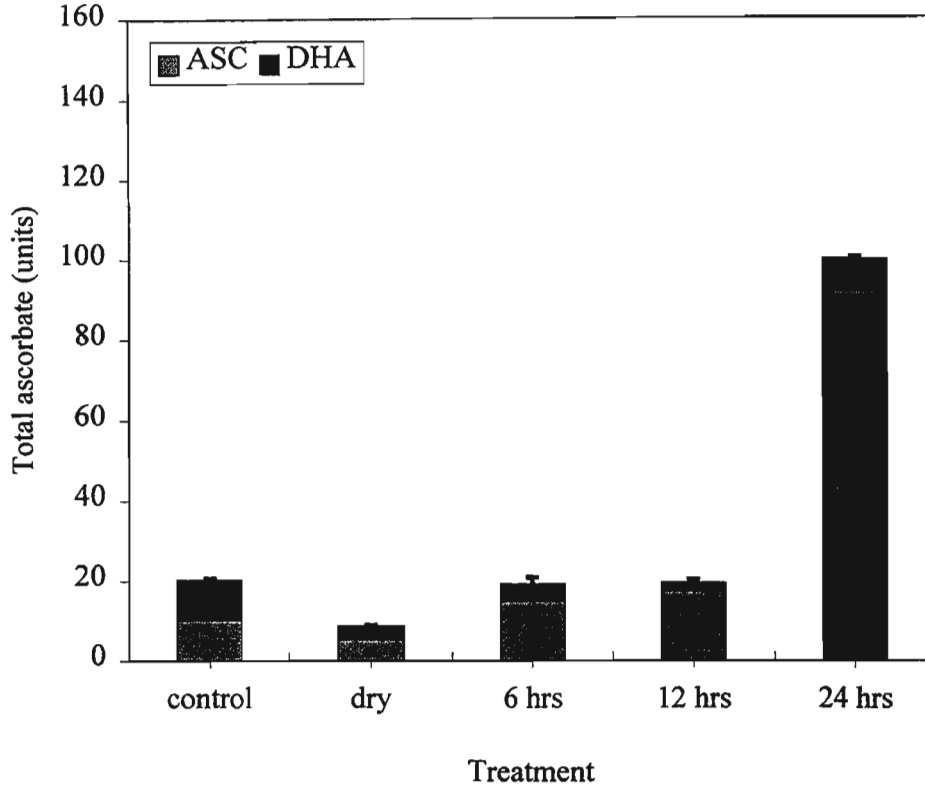

Figure 5.13. Ascorbate (ASC) and dehydroascorbate (DHA) contents of axes following H-D-R treatment of 6 hour hydrated soybean seeds. Seeds were allowed to hydrate for 6 hours (control). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours, and levels of ASC and DHA were determined. Units expressed as nmol per grams FW per axis. Values are the mean ± SE of three experiments.

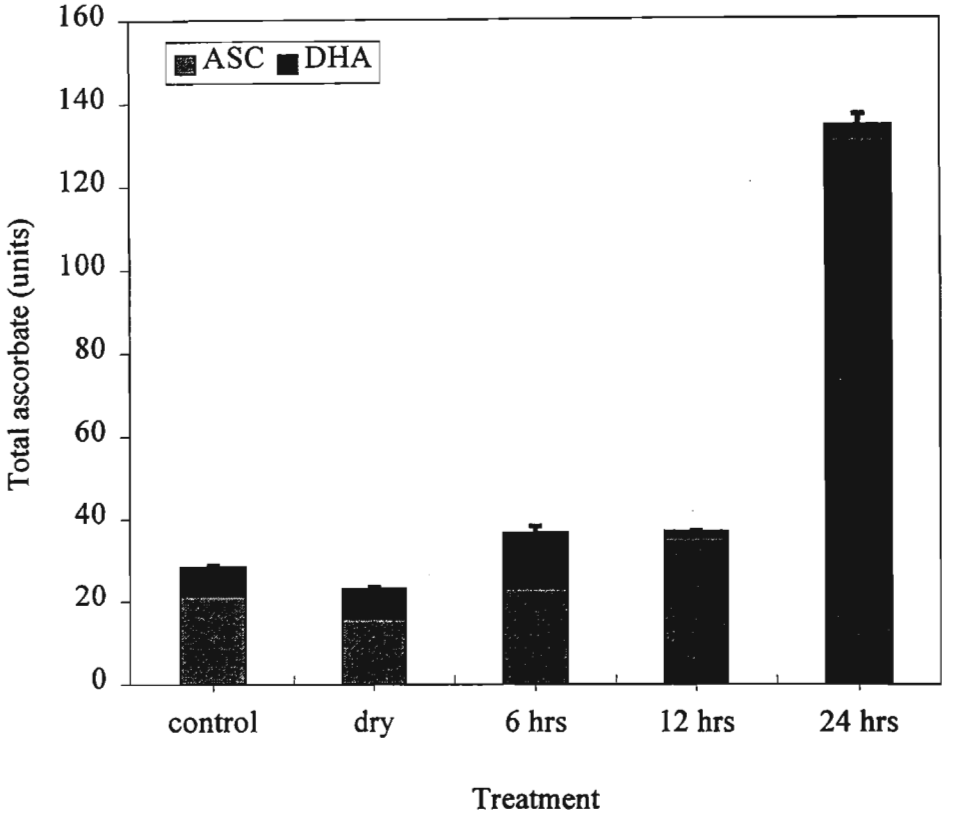

Figure 5.14. Ascorbate (ASC) and dehydroascorbate (DHA) contents of axes following H-D-R treatment of 12 hour hydrated soybean seeds. Seeds were allowed to hydrate for 12 hours (control). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours, and levels of ASC and DHA were determined. Units expressed as nmol per grams FW per axis. Values are the mean ± SE of three experiments.

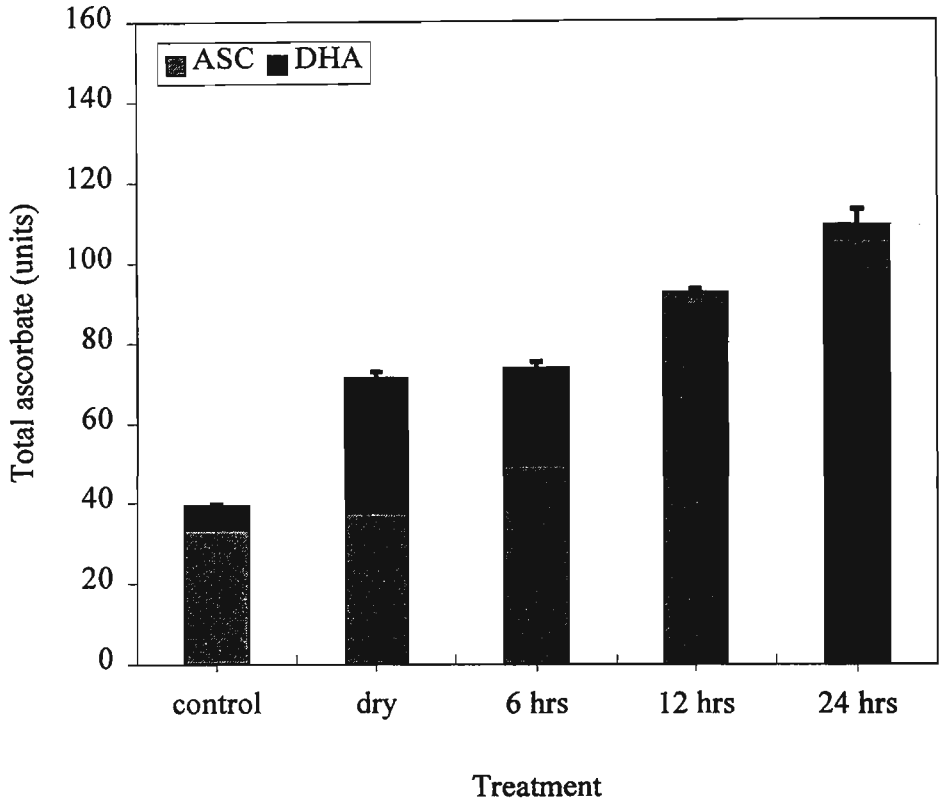

Figure 5.15. Ascorbate (ASC) and dehydroascorbate (DHA) contents of axes following H-D-R treatment of 24 hour hydrated soybean seeds. Seeds were allowed to hydrate for 24 hours (control). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours, and levels of ASC and DHA determined. Units expressed as nmol per grams FW per axis. Values are the mean ± SE of three experiments.

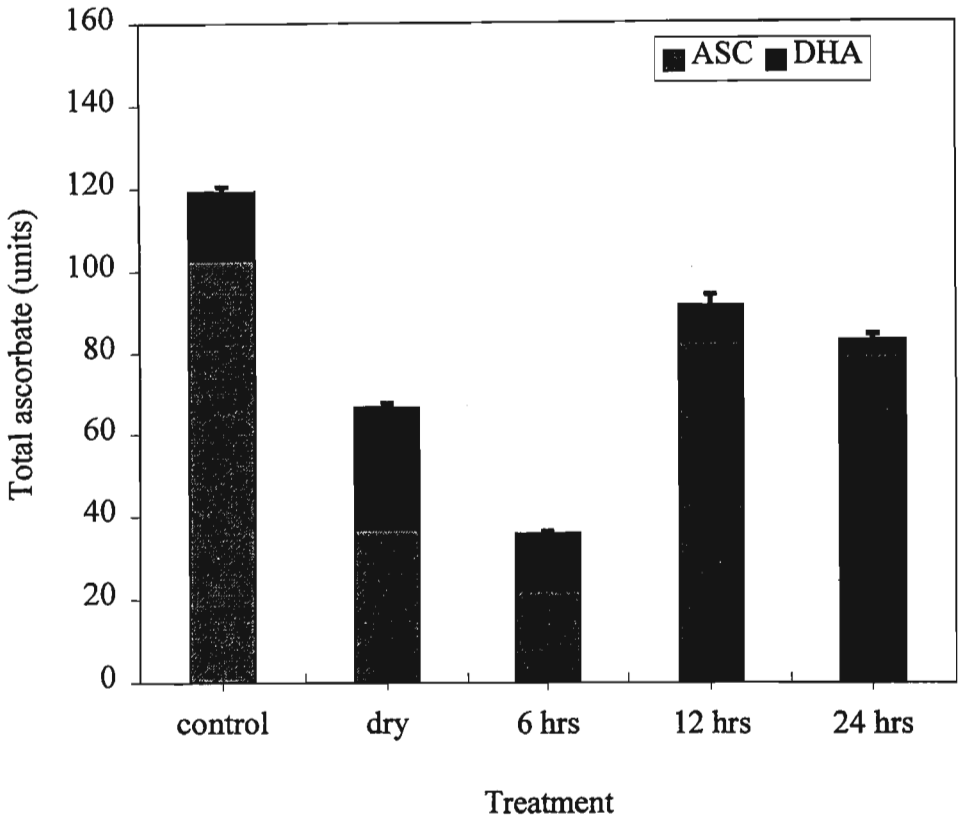

Figure 5.16. Ascorbate (ASC) and dehydroascorbate (DHA) contents of axes following H-D-R treatment of 36 hour hydrated soybean seeds. Seeds were allowed to hydrate for 36 hours (control). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours, and levels of ASC and DHA determined. Units expressed as nmol per grams FW per axis. Values are the mean ± SE of three experiments.

5.3.7 Changes in DHA reductase

The observed changes in the total ascorbate pool might have been due to changes in the activity of DHA reductase, an enzyme catalysing the reduction of DHA to ASC. As such, the changes in the activity levels of the enzyme were followed to determine its contribution to the total ascorbate pool. Axes from seeds hydrated for 6 and 12 hours recorded higher levels of DHA reductase than those hydrated for 24 and 36 hours (Figure. 5.17). Following dehydration for 36 hours, DHA reductase levels declined in all the dehydrated axes. However, axes dried after 6 and 12 hours of hydration lost more enzyme units (*i.e.* absolute amounts) than the axes dried at 24 and 36 hours of hydration. The percentage decline of DHA reductase activity was, however, more than 50% in all the dried axes. Subsequent rehydration of the seeds for 6 hours resulted in a rise in the levels of DHA reductase relative to dry levels. Axes dried after 6 and 12 hours of hydration recorded the highest values of DHA reductase compared to those dried at 24 and 36 hours of hydration. However, when the rehydration period was extended to 24 hours, a decline in the enzyme activity was noted in all the treatments. The decline, in absolute amounts, was more marked in the axes dried in their early stages of hydration (*e.g.* 6 and 12 hours) than those dried after 24 and 36 hours of hydration.

The presence of DHA reductase in the axes of seeds exposed to a H-D-R treatment was further confirmed using PAGE (Figure 5.17). High enzyme activity associated with control tissues, and axes rehydrated for 6 hours, is denoted by high staining band intensities in Figure 5.17 A and C, respectively. In keeping with the *in vitro* assays, DHA reductase activity was markedly reduced after 12 hours of rehydration (Figure 5.18 D).

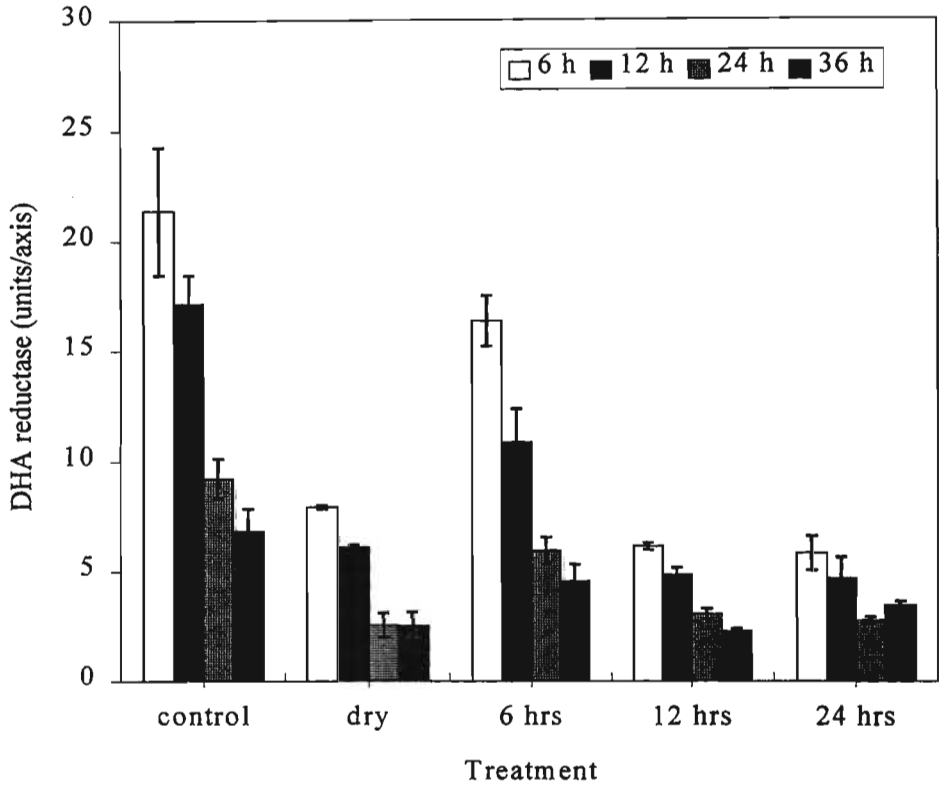

Figure 5.17. Changes in DHA reductase in soybean axes from seeds subjected to H-D-R treatments. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (legends), respectively (controls). After drying in an air stream for 36 hours, dry seeds were then rehydrated for 6, 12, and 24 hours (x-axis), and the enzyme assayed. One unit of DHA reductase activity was defined as one nmol DHA reduced mg^{-1} protein min^{-1}. Values are the mean ± SE of three experiments.

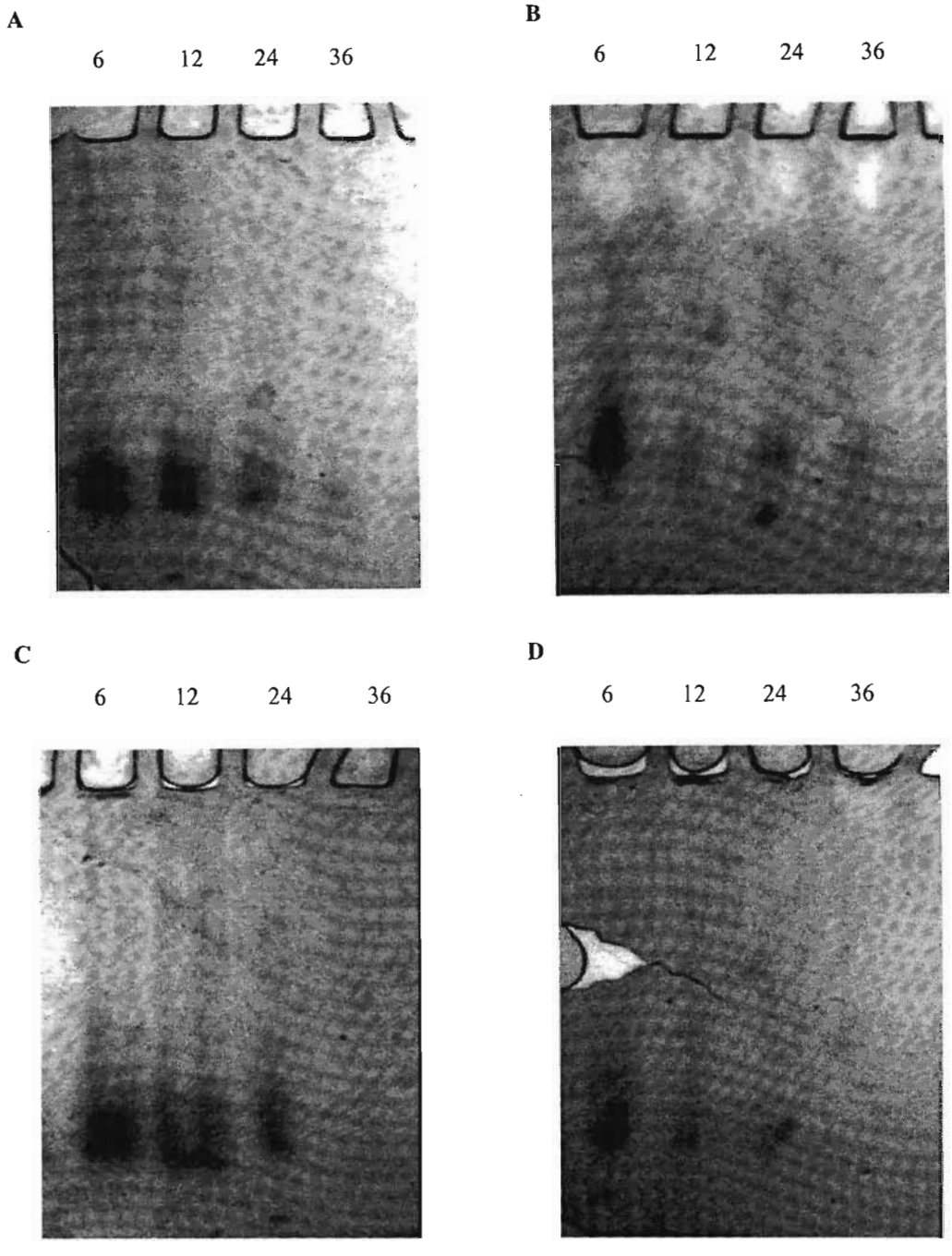

Figure 5.18. PAGE separation of DHA-reducing proteins in soybean axes following H-D-R treatment of seeds. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (control, A). After drying in an air stream for 36 hours (B), dry seeds were rehydrated for 6 (C) and 12 (D) hours. 50 μg protein was loaded in each lane.

5.3.8 Changes in AFR reductase

The levels of DHA reductase did not appear to show a clear relationship to ASC and DHA levels in the H-D-R treatments, suggesting a possible influence of other factors, such as AFR reductase. Recorded levels of AFR reductase were higher in axes of seeds hydrated for 24 and 36 hours in comparison to those hydrated for 6 and 12 hours (Figure 5.19). No measurements of AFR reductase activity were made in dehydrated embryonic axes. At 6 hours of rehydration, the levels of AFR reductase were, overall, lower than the control levels. A progressive increase in AFR reductase activity was recorded in axes of seeds dried after 6 and 12 hours of hydration when the rehydration period was extended to 24 hours. Axes of seeds dried after 24 hours of hydration, on the hand, showed a sharp rise in AFR reductase activity between 6 and 12 hours of rehydration, after which the enzyme levels decreased slightly. In the axes of seeds dried after 36 hours of hydration, AFR reductase levels were the lowest after the 24-hour rehydration period and were equal to those recorded after 6 hours of rehydration. It is noteworthy that, the low levels of AFR reductase in the latter axes were not as a result of a decline in the enzyme levels between 6 and 24 hours of rehydration, but that the enzyme level at 6 hours of rehydration was maintained throughout the 24-hour rehydration period.

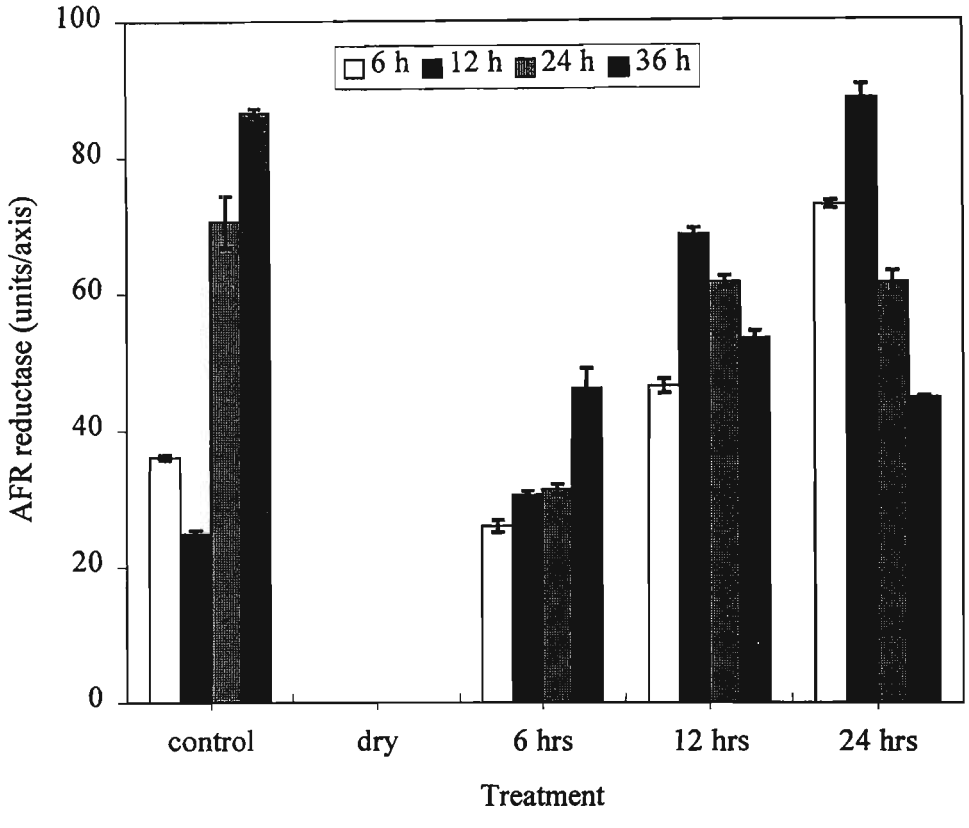

Figure 5.19. The activity levels of AFR reductase in soybean seed axes subjected to H-D-R treatments. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (legends), respectively (controls). After drying in an air stream for 36 hours, dry seeds were then rehydrated for 6, 12, and 24 hours (x-axis). One unit of AFR reductase activity was defined as one nmol AFR reduced mg^{-1} protein min^{-1}. Values are the mean ± SE of three experiments. Values for dried axes (dry) were not determined (ND).

5.3.9 Changes in SOD

Axes from seeds hydrated for 24 and 36 hours recorded relatively higher levels of SOD activity than those from seeds hydrated for 6 and 12 hours (Figure 5.20). Subsequent dehydration of hydrated seeds resulted in sharp decline in SOD levels of axes. Although the percentage decline was well over 90% for all the treatments, axes from seeds dried after 24 and 36 hours of hydration lost the most units (*i.e.* absolute amounts) of SOD. Superoxide dismutase increased more than 10 fold during the first 6 hours of rehydration relative to the dried axes, at which time the SOD levels equalled control, hydrated levels. Further increases in SOD levels were recorded at 12 and 24 hours, such that the enzyme levels surpassed hydrated control levels at these periods. What was perhaps most significant is that axes from seeds initially hydrated for 24 and 36 hours before dehydration showed larger increases than the other treatments.

The polyacrylamide gel electrophoresis (PAGE) pattern of SOD isozymes is illustrated in Figure 5.21, and this further confirmed the presence of the enzyme following a H-D-R treatments. Seven bands characteristic of SOD isoenzymes were identified after the gels were stained. Bands 2, 3, and 4 appeared to represent most of the SOD activity and showed no marked change in all the treatments. Lower band staining intensities were obtained from extracts of dehydrated axes, before the band intensities increased progressively upon rehydration. However, the increase in staining intensity was not as dramatic as the increase in assay activity.

5.3.10 Changes in CAT

A 36-hour dehydration treatment of seeds hydrated 6, 12, 24 and 36 hours resulted in a drastic decline in CAT activity, especially in axes of seeds dried at 24 and 36 hours of hydration (Figure 5.22). Approximately 77 and 87% of the enzyme activity was lost in the axes of seeds dried after 24 and 36 hours of hydration, respectively. For axes of seeds dried after 12 and 6 hours of hydration, CAT activity declined by 50% and 32%, respectively. When the dried seeds were rehydrated, the levels of CAT activity in the axes increased progressively with time in all the treatments. Of note was the fact that axes from seeds that were initially hydrated for 12 and 24 hours before drying showed larger increases in CAT compared to other treatments.

Visual observation of the gels confirmed that catalase was present following H-D-R treatment, and appeared as a diffuse band at the top of the gel in all the treatments (Figure 5.23). The changes observed for *in vitro* enzyme activity paralleled the electrophoresis results for CAT, especially in showing the gradual rise in the enzyme levels during rehydration.

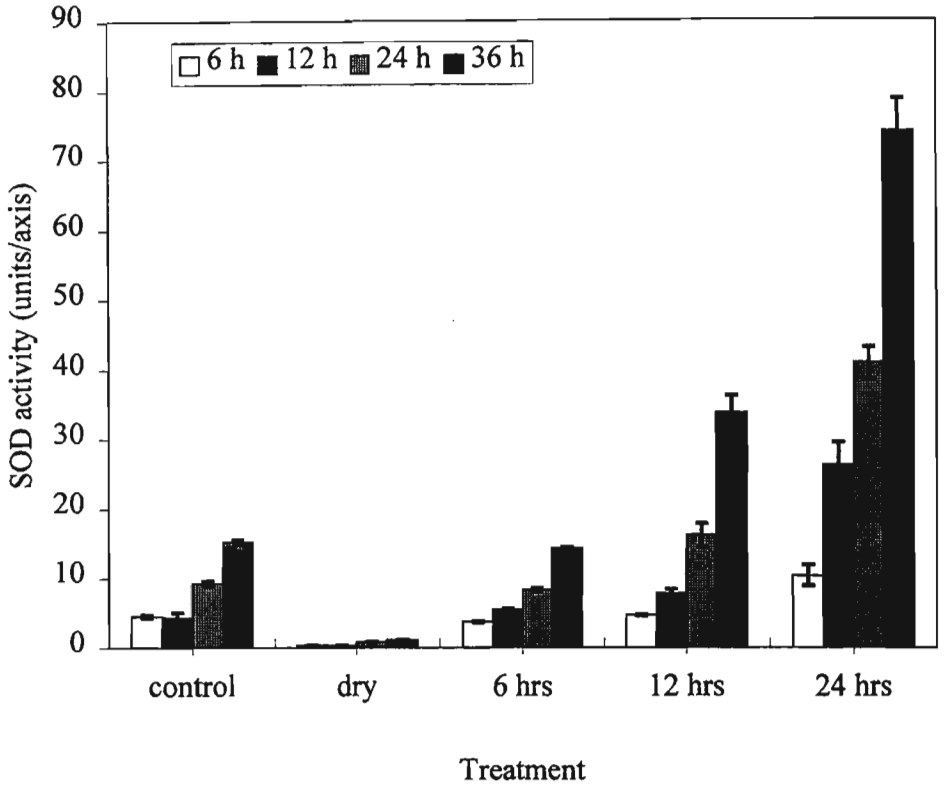

Figure 5.20. The activity levels of superoxide dismutase (SOD) in soybean axes subjected to H-D-R treatments. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (legends), respectively (controls). After drying in an air stream for 36 hours, dry seeds were then rehydrated for 6, 12, and 24 hours (x-axis), and enzyme levels determined. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction at 560nm. Values are the mean ± SE of three experiments.

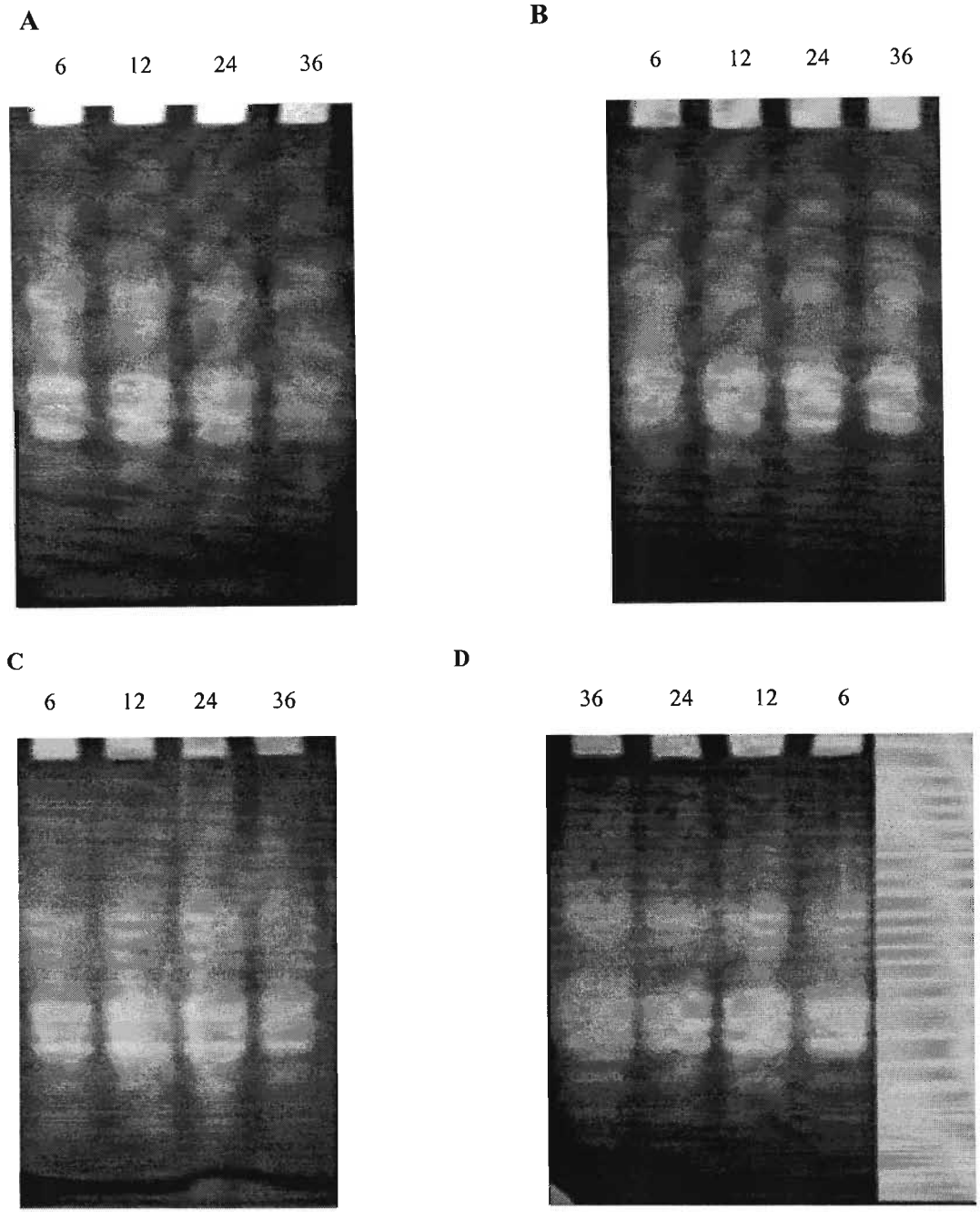

Figure 5.21. Superoxide dismutase isozyme profiles of soybean axes. Following seed hydration for 6, 12, 24 and 24 hours, seeds were dehydrated for 36 h (A), and subsequently rehydrated for 6 (B), 12 (C) and 24 (D) hours, and axes analysed for SOD activity. Fifty micrograms protein was loaded in each lane.

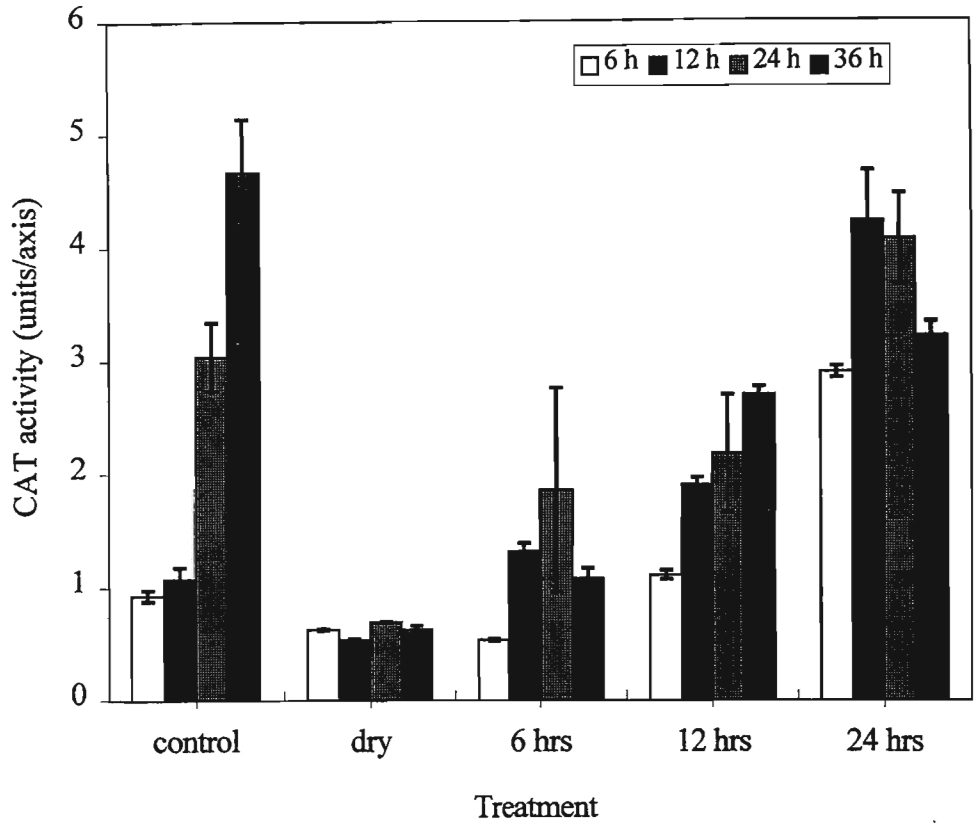

Figure 5.22. Changes in the activity levels of catalase (CAT) in soybean axes from seeds subjected to H-D-R treatments. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (legends), respectively (controls). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours (x-axis), and enzyme levels determined. One unit of CAT activity was defined as the amount one μmol H_2O_2 decomposed mg^{-1} protein min^{-1}. Values are the mean ± SE of three experiments.

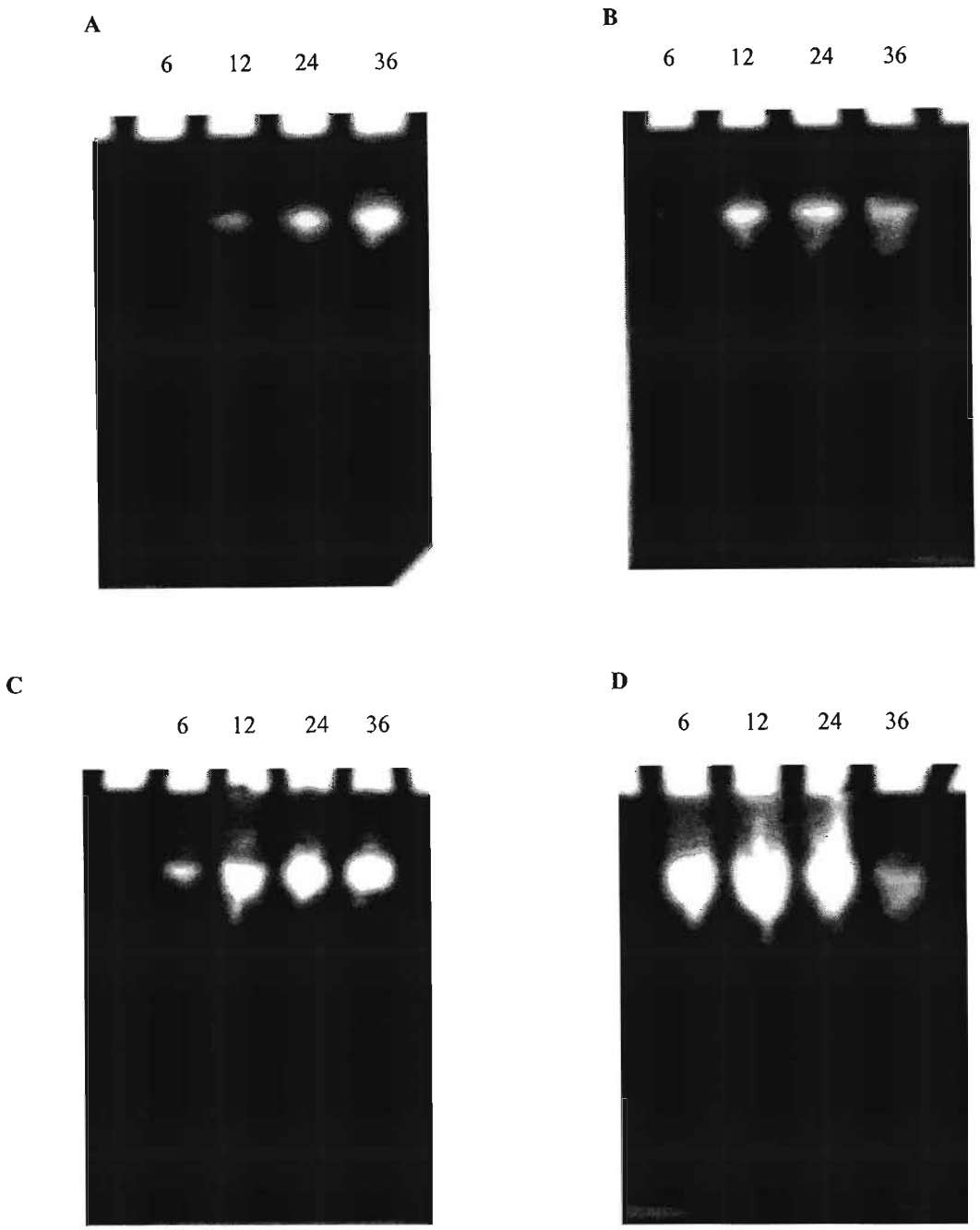

Figure 5.23. Catalase enzyme profiles of soybean axes. Following seed hydration for 6, 12, 24 and 36 hours, seeds were dehydrated for 36 hours (A), and subsequently rehydrated for 6 (B), 12 (C) and 24 (D) hours, and axes analysed for SOD activity. Fifty micrograms protein was loaded in each lane.

5.3.11 Changes in guaiacol POD

Guaiacol POD levels were high in axes of seeds hydrated for 36 hours, lower in axes of seeds hydrated for 24 hours, and lowest in axes hydrated for 6 and 12 hours (Figure 5.24, control). Subsequent dehydration of the hydrated seeds resulted in a decline in the activity levels of the enzyme, with more than 58% and 84% of guaiacol POD activity being lost in axes dried after 24 and 36 hours of hydration, respectively. Axes from seeds dried after 6 and 12 hours of hydration, on the other hand, recorded the smallest decline guaiacol POD levels. The levels of the enzyme in the axes increased progressively with time when the dehydrated seeds were subsequently rehydration. Control levels were slightly exceeded in the 12 hour H-D-R treatment as early as 6 hours of rehydration, whereas in axes of seeds dried after 6, 24 and 36 hours of hydration, the enzyme levels were still below their respective control values at 12 hours of rehydration. Sharp increases in guaiacol POD were recorded in the axes between 12 and 24 hours of seed rehydration, except for axes of seeds dehydrated after 36 hours of hydration, which recorded a decrease in the enzyme levels between 12 and 24 hours of rehydration.

5.3.12 Changes in ASC POD

Ascorbate POD activity in soybean axes could only be detected at low levels after 12 hours of seed hydration, and thereafter increased progressively at 24 and 36 hours of hydration where it reached maximum (Figure 5.25). The levels of the enzyme were reduced dramatically in the axes when the hydrated seeds were subsequently dehydrated, where more than 80% of the enzyme activity was lost. The decrease, in absolute amounts, in ascorbate POD was more pronounced in axes of seeds dehydrated after 36 hours of hydration. In contrast, no major changes in ascorbate POD were noted in the axes of seeds dried after 6 and 12 hours of hydration. The first 6 hours of seed rehydration resulted in a 4-fold increase in ascorbate POD in the axes of seeds dried after 24 and 36 hours of hydration, after which the activity of the enzyme decreased sharply. In contrast, ascorbate POD levels increased gradually with time in rehydrating axes of seeds dried after 6 and 12 hours of hydration.

Electrophoretic patterns of ascorbate POD activity revealed that the enzyme was present in detectable levels during hydration (Figure 4.15) but declined to undetectable levels in the axes of dehydrated seeds (Figure 5.26 A). After 6 hours of rehydration ASC POD activity was only detected in axes of seeds dried after 24 and 36 hours of hydration (Figure 5.26 B). However, only axes of seeds dried after 6 and 12 hours of hydration recorded ASC POD after 24 hours of rehydration (Figure 5.26 D). These results parallelled *in vitro* assay results.

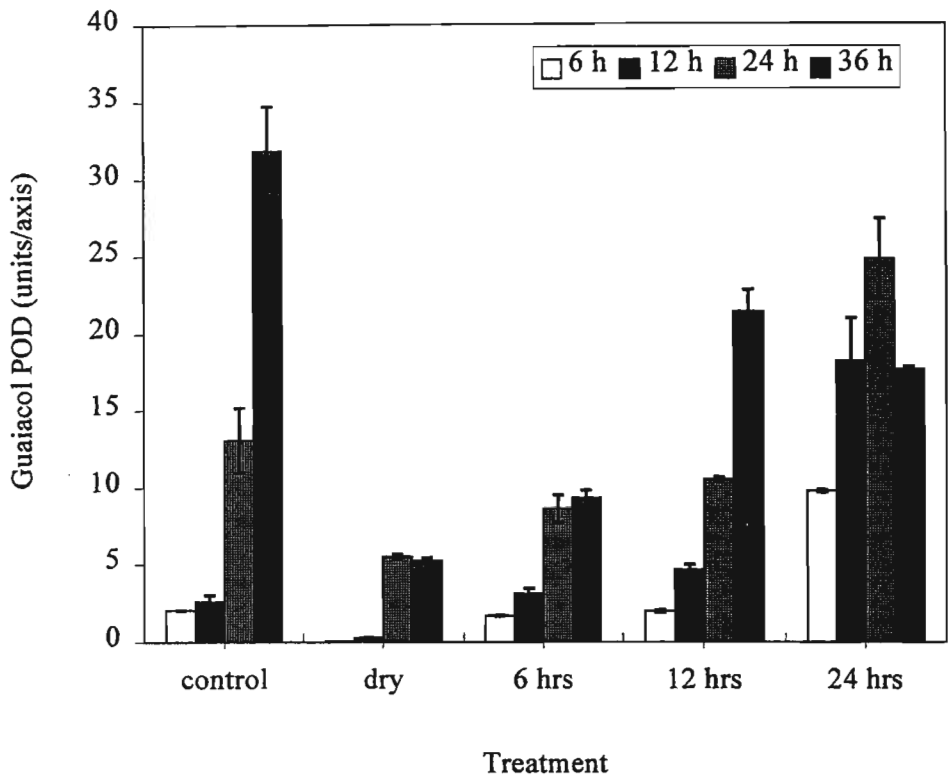

Figure 5.24. The changes in guaiacol POD activity in soybean axes from seeds subjected to H-D-R treatments. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (legends), respectively (controls). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours (x-axis), and enzyme levels determined. One unit of guaiacol POD activity was defined as one nanomole guaiacol oxidised mg^{-1} protein min^{-1}. Values are the mean ± SE of three experriments.

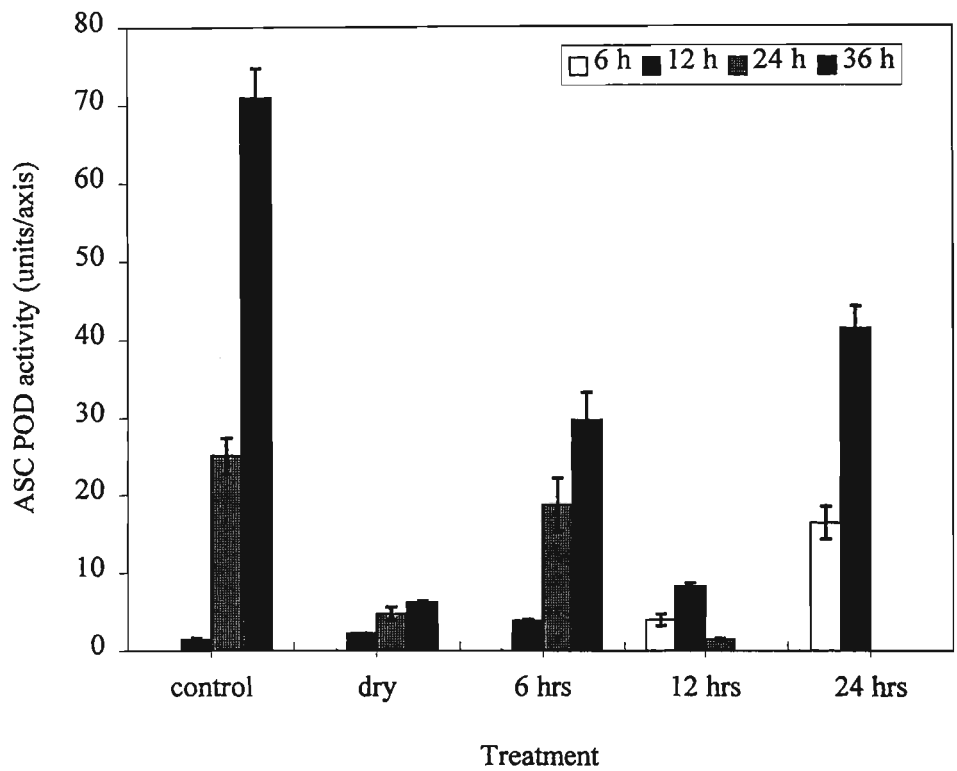

Figure 5.25. Changes in the ascorbate POD levels in soybean axes from seeds subjected to H-D-R treatments. Seeds were allowed to hydrate for 6, 12, 24 and 36 hours (legends), respectively (controls). After drying in an air stream for 36 hours, dry seeds were rehydrated for 6, 12, and 24 hours (x-axis), and enzyme levels determined. One unit of ascorbate POD activity was defined as one nmol ASC oxidised mg^{-1} protein min^{-1}. Values are the mean ± SE of three experiments.

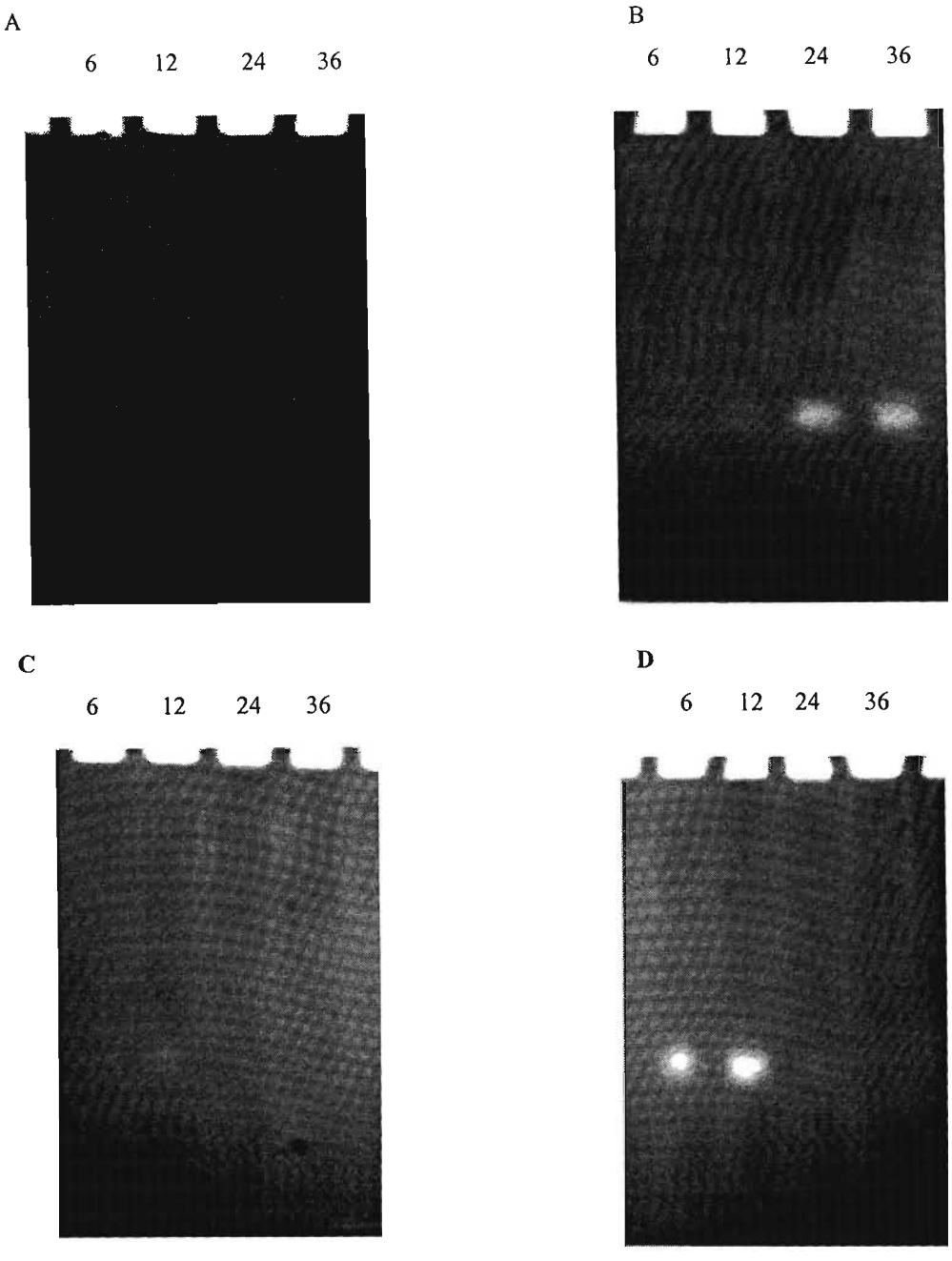

Figure 5.26. Electrophoretic patterns of ascorbate POD enzyme in soybean axes. Following seed hydration for 6, 12, 24 and 24 hours, seeds were dehydrated for 36 hours (A), and subsequently rehydrated for 6 (B), 12 (C) and 24 (D) hours, and axes analysed for ASC POD activity. Fifty micrograms protein was loaded in each lane.

5.4 DISCUSSION

The length of the hydration period prior to dehydration was seen to be critical to the maintenance of seed viability. The proportion of seeds capable of tolerating desiccation treatment was observed to decrease when drying was imposed after 12 hours of hydration, and declined to zero in seeds dried after 36 hours of hydration. Similar changes in sensitivity to dehydration have been reported during germination of oats (Akalehiywot and Bewley, 1980), corn (Deltour and Jacqmard, 1974), and rye (Sen and Osborne, 1974) and would appear to be a general phenomenon associated with germination.

Dehydration-induced damage was less severe in axes of seeds dried after 24 hours of hydration compared to axes of seeds dried after 36 hours of hydration. This was inferred from the observation that the former axes still displayed evidence of cell turgidity during rehydration, and the fact that 30% of the seeds completed their germination. If cellular collapse had induced a rupturing of the plasmalemma in such a manner as to destroy its continuity, it would be anticipated that cell turgidity would not be achieved at all, and instead a massive solute leakage might follow. This, however, is not supported by leakage studies in Figures 5.2 and 5.3, which depict axes of seeds dried after 36 hours of hydration to be the only axes that were severely affected by the dehydration treatment. The fact that some aspects of selective permeability were maintained suggested that a large-scale rupture of the membrane had not occurred.

It has long been recognised that the extent of electrolyte leakage from tissues can be used to indicate the effectiveness of membranes as barriers to solute diffusion (Leopold, 1980; Simon, 1974). Whereas relatively low levels of leakage are taken to indicate that cellular membranes are semipermeable or more intact, high levels of leakage indicate damage to membranes. In this study, the inverse relationship between stage of hydration at which drying was imposed and leakage suggested that at some point during dehydration, the membranes of desiccation-sensitive soybean axes were altered and viability was lost. Because increased leakage was apparent during the first hour of rehydration of the tissues (Figure 5.2 & 5.3), membrane alterations, however small, apparently occurred during the dehydration period. The possibility that the differential rates of leakage may have been a response to different rates of water uptake and hydration of cellular compartments was not fully supported by the data presented in Figures 5.1, 5.2 and 5.3. The high leakage of protein and phosphorus from the axes of seeds dried after 36 hours of hydration compared to that of other treatments was the only case which suggested a high incident of membrane damage and/or disorganisation as a result of dehydration and subsequent rehydration of seeds.

Lethal desiccation of soybean axes that have been imbibed for 36 hours has been shown to induce a decline in the total phospholipid content, and this decline has been associated with an increase in the free fatty acid in the membranes (Senaratna *et al.*, 1984). In the present study, phospholipids in axes also declined to below control levels during dehydration and recovered on rehydration of seeds dried after 6, 12 and 24

hours of hydration. The decrease in total phospholipid content in soybean axes following dehydration was strongly suggestive of a possible degradation of phospholipids. Phospholipid degradation appeared to have been more severe in the axes of seeds dried after 36 hours of hydration, as none of the major phospholipid classes (PC and PE) could be detected after 6 hours of seed rehydration, and very low levels were seen at 24 hours of rehydration (Figure 5.7).

The PC and PE contents of a cell have been considered to be an approximate measure of total membrane content (Williams and Chapman, 1970), since they make up major proportions of non-photosynthetic membranes. The loss of these phospholipids could have been responsible for the disorganisation, with a concomitant loss of function, of membranes in the desiccation-sensitive axes upon subsequent rehydration. Unlike phospholipids from animal sources, plant PC and PE do not differ appreciably with respect to fatty acid composition (Greinier and Willemot, 1974; Smolenska and Kuiper, 1977). Thus, the observed decrease in membrane integrity was, in part, probably the result of reduced levels of PC in membranes of the desiccation-sensitive axes. Accompanying the decline in these phospholipid classes was an increase in proportion of PA, indicating that phospholipases were apparently active during desiccation and subsequent rehydration. Phosphatidic acid is one of the major end-products of membrane degradation. An increase in the activity of phospholipase D, which is universally present in plant tissues (Quarles and Dawson, 1969), can be assumed to be responsible for the accumulation of PA. Phospholipase D has been proposed to be a structural protein of membranes containing PC or PE (Roughan and Slack, 1976). Furthermore, the enzyme may be rather inactive under physiological conditions, but is activated when the hydrophobic bonding between the structural protein and the phospholipids is disrupted (Roughan and Slack, 1976).

Phillips *et al.* (1972) and Michaelson *et al.* (1974) have indicated that the differences between the molecular architecture of the polar-head groups of PC and PE permit the development of a more fluid membrane, without changing the overall fatty acid composition. The packing density of PE head groups has been observed to be very high relative to that of PC, and thus a predominance of PE tends to form rigidly packed membranes (Phillips *et al.*, 1972; Michaelson *et al.*, 1974). Vertucci and Farrant (1995) have also indicated that PE can undergo lethal hexagonal phase changes with drying in recalcitrant seeds. The high levels of PE in the pollen grains of Gramineae may be responsible for their recalcitrant behaviour (Hoekstra *et al.*, 1989). Thus, the high proportion of PE in desiccation-sensitive axes (Figure 5.7) may permit the presence of gel-phase domains in cellular membranes which could conceivably contribute to their loss of functional integrity, and consequently the loss of cell viability in response to desiccation. The formation of these domains would indicate a lateral phase separation of phospholipids in the bilayer. Membrane proteins are "squeezed out" of phospholipids as they form gel phase lipid domains (Shinitzky and Inbar, 1976). This alters the pattern of protein organisation, and also affects enzyme activity (Sandermann, 1978) and transport (Baldassare *et al.*, 1979). The presence of gel phase lipid also renders the lipid bilayer leaky

(Barber and Thompson, 1980), which may contribute to the increased leakage observed in the dehydration-sensitive tissues (Figure 5.7).

Data have accumulated supporting the involvement of free radical processes in desiccation damage of sensitive seeds (Finch-Savage *et al.*, 1996), thus confirming the importance of the concept of unregulated metabolism, particularly respiratory metabolism. Parish and Leopold (1978) have suggested that deteriorative changes in membranes occur via peroxidation reactions. The present study has shown that lipid peroxidation is a significant event that can be associated with membrane damage. Dehydration treatment of seeds hydrated for 24 and 36 hours was sufficient to bring about a significant increase in lipid peroxidation in the dehydrated tissues (Figure 5.8). On rehydration, lipid peroxidation declined, but the levels were nonetheless still several-fold higher than the hydrated controls. These data (Figure 5.8) demonstrate that desiccation treatment results in peroxidative damage to lipids in soybean axes, and potentiates the rehydrated desiccation-sensitive axes to further damage. Dhindsa and Matowe (1981) have also reported an increase in lipid peroxidation in desiccation-intolerant mosses.

Initiation of radical-mediated peroxidation of lipids is usually ascribed to the hydroxyl radical, although other forms of activated oxygen may be implicated (Halliwell and Gutteridge, 1990). It is suggested that the increase in the levels of LOOH in the axes of seeds dehydrated after 24 and 36 hours of hydration was a result of damage from oxygen radicals, generated as a consequence of impaired electron transport chain in fully functional mitochondria undergoing dehydration. With increased oxygen uptake, there is more opportunity for electrons to be leaked to oxygen (Halliwell, 1987). In axes of seeds dried after 6 and 12 hours of hydration, the LOOH levels were quite low, probably because the respiratory system was not reconstituted, and the electron transport chain was probably not in place. The delay in respiratory activity in the latter seeds has often been ascribed to a need for repair or reconstitution of full membrane and enzymatic functionality (Bewley and Black, 1994; Bewley, 1997).

Interestingly, the LOOH levels in the axes of the seeds dried after 24 and 36 hours of hydration declined when the seeds were subsequently rehydrated (Figure 5.8). This is in contrast to the observation made by Leprince *et al.* (1990), who noted that lipid peroxidation was compounded when dried maize seeds were rehydrated. The decline in LOOH levels, in the present study, could be partly due to the failure of the rehydrating axes to resume respiration as a result of damage to part of the respiratory apparatus (Leprince *et al.*, 1992). Rehydrating axes of seeds dried after 6 and 12 hours of hydration, on the other hand, showed a small but gradual rise in LOOH with time, and suggested that the metabolic events and the respiratory chains set during the first 12 hours of hydration were conserved during drying.

Nucleophilic addition of superoxide has been shown to cause de-esterification of phospholipids, releasing free fatty acids and thereby causing a change in membrane composition (McKersie *et al.*, 1990). The

accumulation of free fatty acids has been reported in conditions of drought, and other environmental stresses (Navari-Izzo *et al.*, 1990, 1991) and is responsible, at least partly, for increased membrane microviscosity and appearance of gel-phase lipid in the liquid-crystalline domains (Senaratna *et al.*, 1987). In this way there is an increased rigidity of the bilayer, and the presence of gel-phase lipid makes the membrane leaky (Barber and Thompson, 1980; Paulin *et al.*, 1986; Droillard *et al.*, 1987). Results from the present study provide strong support for phospholipid de-esterification, as shown by the decrease in polar lipids (Figures 5.5 & 5.6), and also possibly through oxidation of unsaturated fatty acids as seen in the accumulation of LOOH (products of this oxidation).

If large quantities of LOOH were produced during the desiccation-sensitive stage, then it would be expected that there might be effects on lipid composition, if repair does not keep pace with the damage. A major function of GSH in protecting cells against the toxic effects of free radicals is to keep the free radical-scavenging ascorbate in its reduced, and hence active, form (Noctor and Foyer, 1998). In imbibed controls, at the most, 5% of the GSH pool was in the form of oxidised glutathione (GSSG) (Figures 5.9, 5.10, 5.11 and 5.12), presumably because there was a balance between oxidation rate and reduction by glutathione reductase (GR) (Law *et al.*, 1983; Foyer *et al.*, 1989). If the regeneration capacity of this enzyme is exceeded, the GSH pool will be oxidised. In the present study, the proportion of GSSG increased in axes of seeds dehydrated after 24 and 36 hours of hydration, and this increase was more pronounced in axes dried in the desiccation-sensitive stage (36 hours). However, all the axes showed the ability to reduce their GSSG content after 24 hours of rehydration, suggesting that GSSG accumulation, *per se*, was not a primary determinant in the loss of viability.

On the other hand, the ascorbate pool appeared to be more sensitive to dehydration than GSH. Ascorbate indicated a potential for loss or oxidation during dehydration as early as the first 6 hours of rehydration. This response was more obvious in axes dried after 24 and 36 hours of hydration in which fully functional ascorbate synthesising machinery is supposedly operational (DeGara *et al.*, 1997). Ascorbate oxidation must have taken place at the same time as ascorbate synthesis during the dehydration of axes of seeds hydrated for 24 hours. This is inferred from the observation that ASC levels remained unchanged after drying, and the fact that the increase in total ascorbate pool was a result of DHA accumulation (Figure 5.15). ASC synthesis probably did not take place in the axes of seeds dried after 36 hours of hydration since almost 50% of the total ascorbate pool was lost after drying, and the resulting total ascorbate pool was made up of 50% DHA (Figure 5.16). In rehydrating axes of seeds dried at 6 and 12 hours of hydration, a lag phase was observed during the first 12 hours of rehydration before active ASC synthesis was evident. These seed were probably dried before *de novo* ascorbate biosynthesis was completely restored, and thus the observed delay was necessary for repairs to be effected. In axes of seed dried after 24 hours of hydration, DHA decline provided the axes with ASC during the first 12 hours rehydration, since in these axes total ascorbate pool did not change greatly although DHA levels were reduced (Figure 5.15).

Also involved in the regeneration of ASC are the enzymes AFR reductase and DHA reductase (Foyer *et al.*, 1992; Noctor and Foyer, 1998). Activity of neither AFR reductase nor DHA reductase was completely eliminated in the H-D-R treatments, and as such ASC levels did not decline completely. The roles of these enzymes were more evident when the dried seeds were subsequently rehydrated, and although DHA reductase activity varied (Figure 5.17), the levels were probably sufficient to reduce DHA. On the other hand, the decline in DHA content, especially in axes that recorded low levels of DHA reductase, provided a likely explanation for the high levels of AFR reductase. It appeared as if the dismutation of AFR, the initial product of ascorbate oxidation, to DHA was not taking place. Instead it is suggested that AFR may have been converted directly to ASC via an AFR reductase-catalysed reaction.

Among the enzymes responsible for the scavenging of active oxygen species, SOD activity in axes was clearly decreased by the intial dehydration treatment (Figure 5.20, dry), irrespective of the stage of seed hydration. However, SOD activity in axes from seeds dried after 24 and 36 hours of hydration showed the greatest decline following the unscheduled dehydration treatment, this being indicative of the severity of the challenge imposed by dehydration at these late stages of imbibition (Figure 5.20, dry). Dhindsa and Matowe (1981) also reported a decline in SOD activity in drought-sensitive moss undergoing slow desiccation. A decline in SOD may favor the accumulation of O_2^- and contribute to membrane damage. Upon rehydration of seeds, SOD activities in the axes increased progressively with time, irrespective of the stage at which dehydration was imposed. The increased SOD activity could have been a reflection of enhanced O_2^- production, or have indicated that the mechanism of synthesis/induction of SOD is quite resilient to potential disruption and responsive to increases in O_2^-.

SOD catalyses the disproportionation of O_2^- resulting in the production of H_2O_2 and oxygen (Allen, 1995; Scandalios, 1993). The breakdown of H_2O_2 is catalysed by CAT and ASC POD, and the activities of these enzymes declined following a desiccation treatment. Even though ASC POD was inactive in the dried axes, it can be argued that its activity was probably vital in limiting oxidative damage during the drying process or the early stages of water loss by the axes. Although the reduced activity of SOD following desiccation may have minimised the generation of H_2O_2 from O_2^-, the decrease in CAT and ASC POD activities could have favoured the increased accumulation of H_2O_2 in soybean axis following desiccation. Further increases in H_2O_2 could have taken place upon rehydration, as consequence of increased SOD activity, and thus the observed progressive increase in ASC POD and CAT in axes dried after 6 and 12 hours of hydration (Figures 5.22 & 5.25) was probably in response to the accumulation of H_2O_2. In contrast, the loss of ASC POD in rehydrated axes of seeds dried after 24 and 36 hours of hydration, and the lack of any appreciable increase in CAT, could have impaired the H_2O_2-scavenging systems of axes. Also, CAT on its own is not efficient in breaking down H_2O_2, as this enzyme has low affinity for its substrate.

Elevated production of both $O_2^{\cdot}$, as shown by high SOD activity, and H_2O_2, as result of a non-functional ASC POD, may have led to production of highly reactive hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen radicals ($^1O_2$) as a result of mutual interactions (Kellogg and Fridovich, 1975). These free radicals can eventually bring about lipid peroxidation with consequent membrane damage (Pederson and Aust, 1973; Kellogg and Fridovich, 1975; Dhindsa *et al.*, 1982). Water stress-induced damage in soybean seeds might well be mediated through generation of these free radicals. Increased membrane permeability, as a result of increased lipid peroxidation, has been demonstrated by Dhindsa and Matowe (1981) and Dhindsa *et al.* (1982) during both water stress and senescence.

Evidence that oxygen radicals are likely to be the cause of, rather than the result of, damage following desiccation cannot be secured directly. Evidence that desiccation fundamentally alters the properties of membranes (Senaratna *et al.*, 1984) is accumulating. In soybean axes this is seen in the rise of lipid peroxidation and increased solute leakage. In the hydrated tissues, compounds with scavenging functions normally regulate free radical production. It is possible that water loss by cells alters the equilibrium between free radical production, and enzymatic defense reactions, in favor of the first. Reduced enzyme activity during dehydration of hydrated seeds is a characteristic trend seen previously in seeds (Brandy and Davidson, 1990; Salin, 1991; Leprince *et al.*, 1992), and this could be attributed to the lack of water necessary for antioxidant enzymes to function. The ability of the cell to recover enzymatic functions that curb free radical activities upon rehydration then becomes critical to its viability. Failure to restore some, and fully co-ordinate, enzyme activity upon rehydration of the axes of seeds dried in the desiccation-sensitive stage could be a major factor in determining desiccation intolerance.

Chapter 6

GENERAL CONCLUSIONS

6.1 Seed development and germination

During seed development, the acquisition of desiccation tolerance appeared to be a continuous, rather than an all-or-none process. Two stages appeared to be critical in the ability of the seed to develop into a viable and vigorous seedling on completion of the development process. The early stages of development (0-55 DAF) were characterised by high moisture content and a rapid accumulation of dry weight, and thus signalled intense metabolic activity within the seed. The high levels of lipid peroxidation during this first stage of seed development were suggestive of oxidative stress and free radical attack. That free radicals are produced in response to high respiratory rates, and metabolic activity is well documented (Halliwell, 1987; Puntarulo *et al.*, 1991; Leprince *et al.*, 1992, 1994). The high moisture content of the seeds, and the presence of respirable sugars, such as glucose and fructose, also provided an ideal environment for this to occur.

However, the levels of lipid peroxidation appeared to be controlled by the synthesis and/or accumulation of protective systems. SOD was responsible for the dismutation of superoxide radicals, and yielded as a reaction product H_2O_2, which in turn was broken down by ASC POD and CAT. ASC POD was probably the more effective of the two H_2O_2-scavenging enzymes, since CAT is known to have low affinity for H_2O_2. The increase in ASC POD was mirrored by increases in ASC, an indication that the functioning of this enzyme was dependent on the availability of ASC as suggested by De Gara *et al.* (1997). The recycling of ASC from its oxidised form was catalysed by both DHA reductase and AFR reductase. It is suggested that GSH also contributed, indirectly, to the recycling of ASC. The increased activity of ASC POD would lead to reduced levels of H_2O_2, which could react with $O_2^{\cdot-}$, thus leading to the formation of hydroxyl radicals. The latter radical is thought to be responsible for initiating lipid peroxidation (Smirnoff, 1993; Winston, 1990). The accumulation of sucrose during the first stage of seed development signalled the onset maturation drying and was, possibly, a pre-programmed event associated with the protection of cellular structure from collapsing during the desiccation stage, as suggested by Vertucci and Farrant (1995). Also, a possible role of sucrose in free radical quenching cannot be overlooked.

The second stage of seed development (50-80 DAF) was marked by a cessation of dry weight accumulation, and by a dramatic, overall reduction in seed moisture. Also, during these last stages of seed development, both respirable substrates and the levels of LOOH were reduced, this showing the close relationship between respiratory activity and LOOH levels. These observations provided further evidence for a possible role of metabolism and respiration in the production of free radicals. Also of note was that there was no appreciable damage or change in the phospholipids, and most of the antioxidant defense systems were maintained at relatively high levels throughout this stage. However, the decrease in ASC POD could have led to an increase in H_2O_2. The decrease in this enzyme paralleled the decrease in ASC content, but at this time it is not certain whether the decrease in ASC content caused the decline in ASC POD or whether ASC POD activity was reduced as a result of reduced hydration levels of the seed.

That seeds are able to retain cellular integrity during the desiccation stage of seed development has been attributed, in part, to sucrose. An association between sugars and late embryogenic abundant proteins (LEA's) has been suggested to control and optimise the rate of water loss during maturation drying (Blackman *et al.*, 1991; Carpenter *et al.*, 1987, 1990; Wolkers *et al.*, 1998). During this period of rapid water loss, structural damage to the cells was probably limited or reduced by sucrose, which is believed to fill up the spaces left by water, thus cushioning the internal organelles, biomolecules and membranes from collapsing. Sucrose has been suggested to be an important protectant and/or stabiliser of macromolecular structures when water is removed (Crowe *et al.*, 1992, 1997; Leopold *et al.*, 1994), and thus the conservation and/or stabilisation of some of the antioxidant enzymes could be attributed to sugars, although this remains to be substantiated. Ideally, these enzymes would not be expected to function in dry systems, and their conservation in storage will depend on the storage conditions of the seed.

Re-introduction of water to the seed during imbibition is sufficient for metabolic activities to resume, and one of the earliest changes is the resumption of respiratory activity (Bewley and Black, 1994; Bewley, 1997). Many of the structures and enzymes necessary for this initial resumption of metabolism upon hydration were probably conserved during the desiccation phase that signalled seed maturation. The triphasic pattern of LOOH changes was similar to the patterns of both water and oxygen uptake patterns by the seed (Bewley and Black, 1994; Bewley, 1997). The initial rise in LOOH levels occurred at a stage where most of the imbibing seeds increased their water and oxygen uptake. At this stage, mitochondria are reported to be poorly differentiated, as a result of maturation drying, and are unable to resume respiratory activities immediately (Bewley, 1997). As a result, the electron transport chain may be incomplete resulting in the production of active oxygen species (AOS). Water and oxygen uptake slow down drastically during the second phase of water uptake (Bewley, 1997), at which time no further increases in LOOH were noted. Another increase in LOOH concurred with the final period of rapid water and oxygen uptake.

In spite of the observed peroxidative reactions associated with imbibition, the seeds were able to germinate and develop into seedlings. This was, in part, ascribed to the ability of the seeds/axes to reactivate or develop their protective systems rapidly enough to conteract oxidative reactions or curb excessive peroxidative reactions. It might be expected that the supporting and cushioning of internal cellular structures was gradually "transferred" from sucrose and/or other compounds to water, and that sucrose was broken down to respirable sugars. The breakdown of superoxide was achieved by SOD, whilst CAT and ASC POD were responsible for H_2O_2 detoxification. The dependence of ASC POD on the supply of ASC with increasing duration of imbibition, and on the hydration levels of tissues was again evident during germination. Also, there seemed to be a threshold value of ASC that had to be present in the seed tissues before ASC POD became operational. AFR reductase appeared to be responsible for most of ASC recycling, since DHA reductase levels decreased progressively with germination.

6.2 Hydration-dehydration-rehydration (H-D-R) treatments

The results of H-D-R experiments showed that soybean seeds could be hydrated for up to 12 hours and then dried back to their original moisture without losing their ability to resume germination on rehydration. The fact that seeds were able to withstand drying back to original moisture without losing viability is an indication that metabolically-derived damage was probably sufficiently controlled or negligibly small as LOOH measurements showed no appreciable changes. The seeds were able to resume normal metabolism and germination upon rehydration, at which stage a typical LOOH increase was paralleled by early increases in protective systems as seen in normal germination. That subsequent germination performance was enhanced upon rehydration confirmed the concept that the embryo can be held to a water potential that precludes germination but permits certain metabolic processes to proceed unhindered, which is the basis of 'priming' (Bray, 1995). It can be postulated that the reduction in the lag phase of enzyme activation might form the basis of seed priming, and further studies will have to be conducted in this regard.

Seed dried after 24 hours of hydration (desiccation-sensitive stage) not only failed to germinate upon subsequent rehydration, but also recorded the highest levels of LOOH. Drying at this desiccation-sensitive stage might have resulted in the perturbations in the electron transport chains, this leading to the formation of active oxygen species. Tissues at this stage of hydration are actively respiring (Bewley, 1997), and the production of free radicals can be expected. The rise in LOOH is a possible indication that phospholipid de-esterification may have occurred. The loss of major phospholipids, PC and PE, and increased leakage indicated loss of membrane integrity (Simon, 1974).

Most of the damage to tissues dried in the desiccation-sensitive stage was probably incurred during the drying treatment. This is inferred from the observation that lower levels of LOOH were recorded in rehydrated seeds than in dehydrated seeds. Peroxidative damage to cellular structure and/or components

during drying in the desiccation-sensitive stage must have proceeded unoppossed since the protective enzymes were probably operating at very low levels, if at all. Although some of the enzymes involved in peroxidation control appeared to increase upon subsequent rehydration, the damage incurred during dehydration was probably too extensive to repair. It seemed that the imbalance between peroxidative reactions and the protective systems within seed tissues during the unscheduled dehydration was critical in the loss of viability in seeds dried in the desiccation-sensitive stage.

There appeared to be a good correlation between the ability of the seeds to elevate the activities and functioning of the antioxidant defense systems, the ability to limit peroxidation, and the maintenance of membrane integrity. Uncontrolled lipid peroxidation, such as observed in seed tissues dried in the desiccation-sensitive stage must have led to extensive membrane disruption, loss of cellular integrity, and ultimately seed death. The critical switch from tolerance to intolerance of desiccation appeared to be linked to the lack of integrated or synergistic operation of the protective systems against active oxygen species. The need for protection, and the failing response in one or more of the enzymatic systems upon dehydration, was a key element leading to deterioration of the lipid components of rehydrating tissue, which was further compounded by the physical disruption of cellular integrity by the rapid inrush of water. It is concluded that the capacity to limit membrane damage to repairable levels by controlling lipid peroxidation may be an important facet of desiccation tolerance in seeds.

6.3 Perspectives and future insights

The results of the present study indicate that the theory proposed by Smith and Berjak (1995) on possible changes in lipid peroxidation during the course of seed imbibition does not apply to imbibing soybean seeds and axes. According to that proposal, in seeds of higher viability, lipid peroxidation increases during the first few hours of seed imbibition, reaching a peak at some point midway through imbibition, after which lipid peroxidation declines. In seeds of low viability, on the other hand, the progressive increase in lipid peroxidation overwhelms the antioxidant defence of the seed, and viability is lost. It was clear from this study that high levels of LOOH ***can*** be tolerated by tissues, as long as the requisite antioxidant systems ***are*** functional. However, before the theory proposed by Berjak and Smith (1995) can be revised, further studies should examine the effects of extending the imbibition period, and also seek to establish its application to a wide variety of other plant species.

A puzzling result arising from the H-D-R studies was the unexpected rise in some of the antioxidant systems in the axes of seeds during rehydration after being dried in the desiccation-sensitive stage. It might have been expected that the levels of these systems would be low at that stage of drying back, concomitant with the loss of desiccation tolerance. Some possible events that could have resulted in this apparent discrepancy, and possible scope for future studies, might be as follows:

a) The production of AOS is believed to take place within several subcellular compartments (mitochondria, glyoxysomes, microsomal fractions, etc). Their production may vary in response to different adverse conditions. Similarly, the subcellular distribution of the antioxidant enzymes is known to vary. It would, thus, be worthwhile to establish if there are any differences in the enzyme activity between the different subcellular compartments, as this could be a major cause for desiccation susceptibility in soybean. It would be expected that a synergy and/or co-ordination in the functioning of the antioxidant systems and oxidative activities between the various compartments is important, overall, in desiccation tolerance.

b) O_2^- and H_2O_2 are two of the most commonly produced species of AOS although neither is particularly harmful at physiological concentrations. However, their toxicity *in vivo* is intensified by a metal ion-dependent conversion to hydroxyl radicals ($OH^·$), which is capable of reacting indiscriminately to cause lipid peroxidation, protein denaturation and DNA mutation and/or damage. A detailed study on the composition, and actual levels of the AOS within the tissue, in addition to the antioxidant defenses themselves, should give clarity on or, a rationale, for the differential responses between lipid peroxidation on the one hand, and the AOS-scavenging systems on the other. Also, a modulation of AOS levels should present an interesting route to characterize the signal transduction pathway(s) involved in hydration, dehydration and oxidative stress.

c) Ultrastructural studies might help elucidate possible compartments or sites of damage during oxidative stress, and provide possible insights into the extent of damage and subsequent repair to cellular structures during the H-D-R treatment.

d) It is not yet clear what lesions to DNA and/or DNA repair mechanisms lead to its loss of transcriptional function, and thus it is proposed that that failure of desiccation-sensitive tissues to survive H-D-R treatments might be due to irreparable damage to DNA. As such, the tissues might be unable to initiate some of the maturational drying responses (LEAs, oligosaccharides, devacuolation, etc). Also, severe DNA damage would likely lead to an inability of cells to divide, or improper metabolic regulation.

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