PHYSIOLOGICAL, BIOCHEMICAL AND CHEMICAL STUDIES ON DESICCATION TOLERANCE PRIMARILY IN DEVELOPING WHEAT SEEDS

by

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SUMMARY

This thesis is the result of my investigations into the phenomenon of desiccation tolerance in developing seeds of sweet corn and wheat. Methods employed include the HPLC of sugars, predominantly sucrose and raffinose, thin layer chromatography together with diffuse reflectance using infrared dispersion spectra (DRUIDS), confocal microscopy of lipids, solid state ^{13}C NMR CP/MAS spectroscopy and embryo culture.

The major results obtained were :

1) Neither sugar concentration nor the mole%ratio of raffinose/sucrose were found to be good predicters of desiccation tolerance. Sugar increases in embryos take place due to an alteration of atmospheric conditions affecting of detached seeds.

2) Diffuse Reflectance Using Infrared Dispersion Spectra (DRUIDS) and thin layer chromatography (TLC) have established a substantial rise of the ester carbonyl of triacylglycerols from a low amount at the desiccation-sensitive stage (15 DAA) to a high amount at the desiccation-tolerant stage (20 DAA). Subsequent desiccation leads to corresponding diminution in the ester carbonyl signal and the appearance of free fatty acids as indicated by a carboxyl carbonyl peak at 1710 cm^{-1} and an associated loss of the TAG ester carbonyl at 1743 cm^{-1}.

3) ^{13}C NMR spectra are predominantly the signals corresponding to carbohydrates and has enabled the comparison of the spectra from desiccated embryos and fresh embryos of the same corresponding age. After subtraction of one spectrum from the other, there were clearly large differences in the subtracted spectra for the desiccation-tolerant stage when compared to the subtracted spectra for the desiccation-sensitive stage.

4) A confocal microscope study of the neutral lipids stained with Nile red established that although there was virtually a complete loss of TAG's as measured by infrared ester carbonyl diminution, there was no corresponding decrease in Nile red fluorescence suggesting that ester bond cleavage did not lead to complete loss of Nile red staining. This was interpreted as mostly un-ionised residual free fatty acid.

The literature on desiccation tolerance has been reviewed and my results have been discussed in the context of this literature. I conclude that both the carbohydrate and lipid content of embryos exhibit major changes during desiccation. The independent methods and novel observations from this thesis suggest that future workers should carefully examine the role of triacylglycerols in desiccation tolerance and the nature and role of the carbohydrate species characterised in the ^{13}C NMR of the difference spectra of embryos at the desiccation-tolerant stage.

STATEMENT

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

SIGNED: **DATE:** 1/2/96

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CHAPTER 1

Introduction and Literature Review

1.1 Introduction

Most agricultural crop seeds are 'orthodox' i.e. desiccation is a necessary feature of their complete life cycle. This evolutionary advantage permits the seeds to survive dry storage (Leopold 1990). Roberts (1973) has indicated that a low moisture content (<10% wet weight basis) lengthens the storability of orthodox seeds. In contrast, seeds that do not tolerate low moisture content (recalcitrant seeds) lose viability if their moisture contents are reduced below a relatively high critical value (12-31%).

Desiccation tolerance has been a subject of interest in recalcitrant seeds ever since attempts have been made to dry and store these mature seeds. No successful method has been found to dry recalcitrant seeds to a low moisture content and allow the seeds to maintain their viability, whereas the mature orthodox seeds can tolerate extremely low moisture contents (1-5% wet weight basis) and still survive (King and Roberts 1980). Orthodox seeds do exhibit a sensitivity to desiccation at during early development (Blackman *et al.* 1991, 1992, Bochicchio *et al.* 1988, 1994; Dasgupta *et al.* 1982) and during germination (Koster and Leopold 1988; Senaratna and McKersie 1983). Thus, there is considerable interest in whether there are differences in the mechanisms of desiccation tolerance and desiccation sensitivity of seeds.

To elucidate the mechanism of desiccation tolerance and desiccation sensitivity, much comparative research has been done on orthodox and recalcitrant seeds. In orthodox species, both desiccation-tolerant and the desiccation-sensitive stages

have been identified and examined in developing and germinating seeds (Blackman *et al.* 1991, 1992, Bochicchio *et al.* 1988, 1994; Dasgupta *et al.* 1982; Koster and Leopold 1988; Senaratna and McKersie 1983). In recalcitrant species, to date, study has focused on the seed during the development or storage phase (Berjak *et al.* 1984, 1989, 1990, 1992; Farrant *et al.* 1986).

There are at least three factors involved in desiccation tolerance. These are sugars, i.e. sucrose and oligosaccharides, late embryogenesis abundant (LEA) proteins and free radical scavenging systems (Leprince *et al.* 1993). Unfortunately, many findings have shown that the role of these factors is not consistent across species or varieties and no single mechanism appears to play a dominant role in conferring desiccation tolerance (Leprince *et al.* 1993).

Because of inconclusive results on the basis of desiccation tolerance, the aims in this thesis are to study further the role of sugars in desiccation tolerance in developing seeds, and to investigate other possible mechanisms which could be involved in desiccation tolerance and desiccation sensitivity. Spectroscopic and microscopic methods have been employed to provide more information on these topics.

1.2 Desiccation tolerance during seed development

The mechanisms of desiccation tolerance and desiccation sensitivity have been studied in a numbers of different stages of development and plants/tissue. This review, therefore, includes some of these different study which are : 1) desiccation of orthodox seeds early in development when compared to later in development 2) desiccation of recalcitrant seeds later in seed development when compared to orthodox seeds 3) desiccation of germinating orthodox seeds 4) desiccation of moss (a vegetative structure) and 5) desiccation of somatic embryos. However, most of the review will focus on desiccation tolerance and sensitivity in orthodox and recalcitrant seeds.

'Desiccation tolerance' is the ability of seeds to tolerate drying and resume germination following rehydration (Bewley 1979). 'Desiccation tolerant' refers to orthodox seeds and 'desiccation sensitive' is the term for recalcitrant seeds (Hanson 1984). The difference between these two groups is their response to desiccation, i.e. orthodox seeds tolerate desiccation whereas recalcitrant seeds do not (Ellis 1987). In orthodox species, the degree of desiccation tolerance increases during seed development (Leprince *et al.* 1993). Seeds are desiccation sensitive at the stage of histo-differentiation and become desiccation tolerant at the stage of maturation (Kermode 1990).

1.2.1 Classification of seed developmental stages

In orthodox species, seed development can be described in 3 general phases : 1) histo-differentiation phase, where fertilisation, rapid cell division and differentiation takes place; 2) cell expansion and storage accumulation in the cell; and 3) maturation drying (or desiccation), when water is lost from the seeds which then pass into a quiescent or dormant stage (Kermode *et al.* 1986; Goldberg *et al.* 1989; Kermode 1990). The loss of moisture in the last phase is necessary to complete the

life cycle of orthodox seeds. It also ensures that the seeds are able to withstand a severe environment (Leopold 1990).

During development, physiological and morphological changes have been observed. In cereal plants such as wheat, visual observation of the colour of the seed coat or the softness of the endosperm are useful criteria to predict the stages of development (Peterson 1965; Percival 1921). In maize, a black layer at the basal endosperm is a visual sign of maturity when maximum dry weight is obtained (Daynard and Duncan 1969).

Developmental stages can be classified by examining endosperm development (Evers and Bechtel 1988). In this method, the tissue is examined microscopically and the developmental stage of the endosperm cell noted. Embryo development can also be used as a criterion for predicting the developmental stage. For these criteria, Rogers and Quatrano (1983) classified seed development in wheat into 5 stages :

Stage 1, the tissues of the embryos are not differentiated, and the embryos cannot be removed from the surrounding tissue.

Stage 2, embryos can be dissected easily from the surrounding tissues and differentiation is apparent. The embryos are clear and gelatinous but they become opaque (except scutellum) at the end of this stage.

Stage 3, lateral roots develop at the early part of this stage. The scutellum is square and maintains this appearance through to stage 5.

Stage 4, primary leaves appear and caps are found on the lateral roots. Embryos are fully developed.

Stage 5, embryos adhere to the surrounding tissue, and dissecting embryos is difficult.

Considering the three general phases of seed development described by Kermode (1990), stages 1-2 could correspond to the histo-differentiation phase whereas

stages 3-4 could correspond to the reserve accumulation phase and stage 5 is maturation drying.

1.2.2 Acquisition of desiccation tolerance and desiccation sensitivity during seed development

Seeds of most agricultural crops are desiccation tolerant at maturity (Kermode 1990). However, at the early stages of development, when cell division and expansion take place, they are desiccation sensitive (Kermode *et al.* 1986; Kermode 1990). Drying the seeds at this stage results in the loss of ability to germinate because of loss of cellular integrity may occur due to the disruption of membranes (Kermode 1990).

The onset of desiccation tolerance differs between plant species; for example it is approximately 22-26 days after flowering (DAF) in *Phaseolus vulgaris* (Kermode *et al.* 1986; Dasgupta *et al.* 1982) and 30 DAF in soybean (Adams and Rinne 1981). It has been reported that desiccation tolerance develops at about mid-pod fill in soybean (Rosenberg and Rinne 1986) and roughly half-way through the development in castor bean and French bean (Kermode *et al.* 1989*a*, *b*).

1.2.3 Effect of drying on germination of developing seeds

Drying has been reported to enhance germination of many seeds (Robertson and Curtis 1976; Ellis and Filho 1992; Rasyad *et al.* 1990). In wheat, mature seeds should be dried for 3 days prior to a germination test, because dry seeds have a higher %germination than fresh seeds (Robertson and Curtis 1967). Ellis and Filho (1992) reported that seeds of spring and winter cultivars of barley and wheat were able to germinate following desiccation at the stage they attained physiological maturity. Fresh immature wheat seeds (12 days after anthesis, ~~27%~~ 72% moisture content) did not germinate until they were air-dried (Rasyad *et al.* 1990). Likewise, immature maize seeds must be dried to approximately 25% moisture content to elicit

germination (~~Spraque~~ Sprague 1936). Adams and Rinne (1981) reported that soybean seeds (30 DAF) harvested and dried in intact pods for 3 weeks exhibited 86% germination whereas fresh green seeds (35 DAF) failed to germinate. In cereal seeds, it has been suggested that drying may change either chemical or physical properties of the seed coat to allow a higher rate of gaseous exchange (Wellington 1956; Radley 1976; Symons *et al.* 1983). Kermode and Bewley (1985*a*) have reported that drying seeds causes a transition from the developmental stage to the germination stage.

However, there is some confusion about the use of slow drying and rapid drying to test for desiccation tolerance. This is because the tolerance to either slow drying or rapid drying depends on seed maturity (Kermode and Bewley 1985*a*). Immature seeds tolerate slow drying while mature seeds tolerate rapid drying (Robertson and Curtis 1967; Kermode and Bewley 1985*a*). Furthermore at early developmental stages, the tolerance to slow drying of seeds coincides with the transition from development to germination. For example, Kermode and Bewley (1985*a*) exposed immature ungerminated castor bean seeds (25 DAP) to slow drying (seeds were dried over a series of saturated salt solutions of known relative humidity) and found that the 0% germination of fresh 25 DAP seeds increased to 75% after slow drying. Thus, drying the 25 DAP castor bean seeds terminated the developmental stage and switched the seeds to the germinative stage (Kermode and Bewley 1985*a*). Under these circumstances, it is difficult to decide whether it is a case of desiccation tolerance of the seeds or a transition from seed development to germination. Some authors (Kermode and Bewley 1985*a*; Ellis *et al.* 1987; Koster and Leopold 1988; Leprince *et al.* 1990*a*, Blackman *et al.* 1992) use rapid drying to test for desiccation tolerance. In principle, slow drying should approximate to conditions during normal seed maturation and rapid drying under minimal RH approximates to maximal moisture stress, and hence can assist in the detection of desiccation tolerance in developing seeds.

Seeds switch from the developmental to germinative mode due to physiological and biochemical changes as they are dried slowly (Kermode 1990). Slow drying reduces the sensitivity of the embryos to abscisic acid (ABA) resulting in germination (Kermode *et al.* 1989*a*; Oishi and Bewley 1992). It also induces the seeds to produce post-germination-specific enzymes, e.g. malate synthase and isocitrate lyase in the lipid rich soybean and castor bean seeds, whereas fresh ungerminated seeds do not contain these enzymes (Adams *et al.* 1983; Kermode and Bewley 1985*b*; Kermode and Bewley 1988). Slow drying also results in the production of enzymes involved in protein reserve breakdown (e.g. leucyl ß-naphthylamidase; LeuNAase). Rosenberg and Rinne (1986) also found an accumulation of polypeptides of 31 and 128 kD in mass during drying.

While slow drying has been reported to elicit germination of many immature seeds, rapid drying has a deleterious effect; thus, castor bean seeds at 30-50 days after pollination show 100% germination after slow drying over various saturated salt solutions but 0% germination if they are dried rapidly over silica gel (Kermode and Bewley 1985*a*). In maize, excised embryos at 16 days after pollination have 100% germination, but after rapid drying at 35°C for 48 hours, there is zero germination (Bochicchio *et al.* 1988). Soybean seeds (26 days after flowering) shelled and dried outside the pods have lower %germination than the seeds slow-dried within the whole pods (Adams *et al.* 1983). This inability of seeds to withstand rapid drying is useful to quantify the degree of desiccation tolerance during seed development (Leprince *et al.* 1993).

The onset of tolerance to rapid drying is associated with moisture loss from the seeds (maturation drying) and physiological maturity (Ellis *et al.* 1987). Ellis *et al.* (1987) studied the desiccation tolerance of seeds of six grain legumes, during development by drying the seeds in a cabinet at 20-30°C and 15-25%RH for 6 days. They found that seeds tolerated rapid drying when they reached maximum dry weight and moisture content of maturing seeds had declined to approximately

55-60%. In tomato seeds, desiccation tolerance occurs when embryos reach maximum protein content, size and dry weight (Berry and Bewley 1991). The review by Kermode (1990) indicates that the ability of seeds to withstand rapid desiccation differs between species. Gramineae tend to survive an extremely low moisture content (around 5%) at the early stages of development (Kermode 1990) but the immature *Ricinus communis* (Kermode and Bewley 1985*a*) and *Phaseolus vulgaris* (Long *et al.* 1981) do not tolerate such a low moisture content. The reasons why different species exhibit different levels of tolerance are not known.

1.2.4 Transition from developmental stage to germination stage and desiccation tolerance

In the cases where the germinability of seeds increases after slow drying, it seems that germinability and desiccation tolerance take place simultaneously. Many authors have suggested that germination of fresh seeds is inhibited by several factors such as hormones (Misra *et al.* 1985; Kermode *et al.* 1986; Bewley *et al.* 1989; Kermode *et al.* 1989*a*, *b*) or the tissue surrounding the embryos (Neil *et al.* 1987; King 1976 and Le Page-Degivry and Garello 1991). Drying may overcome this inhibitory effect while removal of the embryos from surrounding maternal tissues will affect the flow of germination-suppressing substances (e.g. ABA) to the embryos or alter the sensitivity of the embryos to suppressive or promotive substances (Kermode *et al.* 1986). Leprince *et al.* (1993) points out that if the germinability of the seeds at a given stage of development is inhibited, the desiccation tolerance might also be as well. Thus, when inhibitory factors are present it is neither possible to measure the levels of desiccation tolerance by the loss of germination after drying, nor is it possible to classify the seeds as being desiccation tolerant because the seeds will not germinate before drying.

1.2.5 Biological changes during acquisition of desiccation tolerance

There is no clear explanation why the seeds are desiccation sensitive at the young stage and are desiccation tolerant at the mature stage of development. It seems that immature seeds require a certain time to become desiccation tolerant. There is a transition between the desiccation-sensitive stage and desiccation-tolerant stage (Kermode 1990). In an early study, Klein and Pollock (1968) studied the difference between the fine structure of cells and the metabolic activity of young and mature lima bean seeds. Loss of polysomes and the appearance of free ribosomes (polysome degradation) and loss of golgi bodies and mitochondria matrix (metabolic activity cessation) were evident in mature axes.

For *Phaseolus vulgaris*, Dasgupta *et al.* (1982) report that at 22 days after anthesis (DAA), seeds are at a desiccation-sensitive stage. Drying the seeds at this stage results in the damage of the cell fine structure upon subsequent rehydration. Damage to the cells is not evident after rehydration of seeds which are at a desiccation-tolerant stage. Klein and Ben-Shaul (1965) also found no damage to the cellular fine structure of dried mature seeds after imbibition for 1 to 4 hours. Perner (1965) pointed out that natural slow drying results in no damage to cell organelles. When seeds gradually lose moisture during maturation drying, they have a ~~steadily decrease~~ steady reduction in metabolic activity and so tolerate desiccation (Perner 1965).

1.3 Desiccation tolerance of seeds during germination

When mature seeds imbibe in an optimum environment. The germination process takes place. Bewley and Black (1983) described the germination process as consisting of three phases : 1) imbibition 2) active metabolism and 3) ~~mobilisation~~ radical elongation. During the last phase, there is cell expansion and cell division.

Germinating seeds have been extensively used for the study of desiccation tolerance and desiccation sensitivity because they exhibit both characteristics. Desiccation-tolerant and desiccation-sensitive stages during germination are well documented in many seeds (Deltour and Jacqmard 1974; Dell'Aquila *et al.* 1978; Senaratna and McKersie 1983; Senaratna *et al.* 1985*a*, *b*; Koster and Leopold 1988; Leprince *et al.* 1992). Germinating soybean seeds tolerated desiccation within 6 hours of imbibition; however, after 36 hours of imbibition, the seeds became desiccation sensitive (Senaratna and McKersie 1983; Senaratna *et al.* 1985*a*, *b*). In maize, 72 hours of imbibition corresponds to the desiccation-sensitive stage (Leprince *et al.* 1990*b*). Koster and Leopold (1988) reported that the desiccation-sensitive stage is at 18 hours of imbibition in soybean, 24 hours in pea and 36 hours in maize. The conditions as well as the plant species used for the germination test also appear to influence the time of desiccation sensitivity.

In the case of soybean and maize seeds some authors (Senaratna and McKersie 1983; Senaratna *et al.* 1985; Koster and Leopold 1988) have reported that the desiccation-sensitive stage coincides with radicle protrusion and membrane damage. It has been hypothesised that at times of high metabolic activity, moisture is required to maintain this activity, and hence, the drying of seeds at this time is deleterious to the germinating seeds (Berjak *et al.* 1984).

Radicle protrusion at the desiccation-sensitive stage is not a universal indicator of tolerance for all germinating seeds. In Zizania, there is no protrusion of the radicle, but the seeds still do not tolerate desiccation (Probert and Brierley 1989). Using germinating soybean seeds, Senaratna and McKersie (1983) prevented radicle protrusion by either osmotic treatment with polyethylene glycol or treating the seeds with cycloheximide solution to prevent cell elongation; however, the seeds still lost their desiccation tolerance.

To summarise, in orthodox species, developing seeds exhibit different degrees of tolerance to drying; mature seeds tolerate rapid drying whereas immature seeds tolerate slow drying. The tolerance of drying coincides with the ability to maintain their cellular structure from damage during drying. This mechanism is similar to those in germinating seeds.

1.4 Subcellular changes induced by desiccation

Subcellular changes after drying have been reported in desiccation-sensitive seeds. In recalcitrant species, such as rubber trees, drying the seeds to a moisture content below a critical moisture level (15-20% wet weight basis) results in damage to the cell wall, nuclear membrane, tonoplast and chromatin of cells of root tips (Chin *et al.* 1981). In *Avicennia marina* at the time of shedding, cells contain large nuclei and fairly long rough endoplasmic reticulum, and polysomes are common. However, after drying the seeds to approximately 18% (wet weight basis), the cells collapse and wall fragmentation occurs (Berjak *et al.* 1984; Berjak *et al.* 1989).

Berjak *et al.* (1990) reported that if recalcitrant seeds are dried very rapidly, the ultrastructure of cell still maintains its integrity. In this case, the seeds survive desiccation even though their moisture content is decreased to only 16%. This is achieved by flash-drying (a stream of compressed air passing though two columns of silica gel and then through the container containing Petri dishes of seeds stored over silica gel). Seeds can be dried to 13% moisture content (dry weight basis) within 60 minutes. In the study of Berjak *et al.* the experiment on *Landolphia kirkii* shows that embryonic axes dried by flash drying survive desiccation to 16% (dry weight basis) after drying for one hour. These flash dried seeds exhibit 81% germination (with initial viability 89%). In contrast, the seeds dried over silica gel for 20 days still have a high moisture content (49% dry weight basis) but viability decreases to 7%. The results from ultrastructure studies on the embryonic axes show that flash dried axes maintain membrane integrity even though seed moisture

content is reduced to 16%. In contrast, seeds dried over silica gel for 20 days show a deterioration of subcellular components, including a loss of plastids, a break down of polysome structure and vacuolation of the cell. Further drying over silica gel (28 days) results in a rupture of the cell wall and the loss of seed viability.

Cellular damage also occurs in germinating orthodox seeds at the desiccation-sensitive stage after drying. For example, Crèvecoeur *et al.* (1976) found that after 72 hours of imbibition (desiccation-sensitive stage), the ultrastructure of the radicle tip of the maize embryo is damaged following desiccation. Damage was apparent in the mitochondria, plasmalemma and spherosomes. In the desiccation-sensitive stage, when the seeds are rehydrated after desiccation, chromatin condensation is irreversible, while in the desiccation-tolerant stage, condensed chromatin of seeds returns to normal after 24 hours of imbibition .

In developing orthodox seeds, an inability of the subcellular organelles to recover from the damage following rehydration has also been reported (Dasgupta *et al.* 1982). At the desiccation-sensitive stage (22 DAF) in *Phaseolus vulgaris*, desiccation results in the coalescence of protein bodies, mitochondrial damage and disruption of the nucleus. This subcellular damage is not repaired after rehydration. In contrast, desiccation of 32 DAF embryos (which are in the desiccation-tolerant stage) is not deleterious to the ultrastructural components and the organelles completely recover within 24 hours of rehydration.

One indirect method for detection of damage due to desiccation is to measure the electrolyte leakage upon rehydration (Senaratna and McKersie 1983). In soybean seeds, the rate of electrolyte leakage from the embryonic axes of the seeds dried at the desiccation-sensitive stage is higher than that in the desiccation-tolerant stage (Senaratna and McKersie 1983). In birdsfoot trefoil seeds, the contents of electrolyte leakage i.e. potassium, phosphate, sugar, amino acid and protein are low at the desiccation-tolerant stage but they are high at the desiccation-sensitive

stage (McKersie and Stinson 1980). Simon (1974) has suggested that desiccation alters membrane permeability.

To summarise, subcellular damage provides evidence for desiccation sensitivity in germinating and developing orthodox seeds, and mature recalcitrant seeds. For the former, when the seeds are in a desiccation-tolerant stage, drying does not damage the cell. Even if there is some subcellular damage, the cell returns to normal after rehydration. However, in a desiccation-sensitive stage the cell appears unable to repair damaged subcellular structures after rehydration. For recalcitrant seeds, drying severely damages the subcellular structures resulting in an inability of seeds to germinate upon rehydration. If the seeds are dried rapidly, no substantial damage occurs to the cell, and seeds can germinate after reimbibition.

1.5 Possible factors conferring desiccation tolerance

1.5.1 Sugars

Sugars are present in many kinds of seeds and have frequently been reported to play a role in conferring desiccation tolerance. The mechanisms by which sugars in confer desiccation tolerance have been studied in detail, but the role of sugars in conferring desiccation tolerance remains a matter of controversy (Leprince *et al.* 1993; Ooms *et al.* 1993; Bochicchio *et al.* 1994).

Sucrose has been reported to accumulate in seeds at all stages of development in many plant species (Amuti and Pollard 1977). Raffinose accumulates in many seed species at the later stage when the seeds are mature. During seed development glucose and fructose increase in some plant species, but these monosaccharides can no longer be detected at maturation (Amuti and Pollard 1977; Lowell and Kuo 1989). In mature seeds during storage, changes in sugar content have been reported to correlate with seed viability. For example, in maize, sucrose and raffinose are present in the embryos of viable seeds but are absent from non-viable

seeds. In contrast to these di and trisaccharides, monosaccharides (glucose and fructose) are absent from the embryos of viable seeds but they are present in those embryos of non-viable seeds (Ovcharov and Koshelev 1974).

In germinating
~~During germination of~~ soybeans, sucrose and other oligosaccharides in the embryonic axes ~~have been reported to~~ decline concomitant with an increase in monosaccharides such as glucose and fructose (Hsu *et al.* 1973). It has been suggested that in germinating maize seeds, an increase in monosaccharides before the desiccation-sensitive stage is attained; it induces a rapid respiration rate and finally contributes to the total loss of the respiratory system (Leprince *et al.* 1992).

Many authors have proposed that other oligosaccharides, i.e. raffinose and stachyose, in the presence of sucrose play a role in conferring desiccation tolerance to developing and germinating seeds (Rogerson and Matthews 1977; Leopold and Vertucci 1986; Koster and Leopold 1988; Chen and Burris 1990). It has been reported that sucrose and oligosaccharide contents are low at the desiccation-sensitive stage in germinating seeds such as soybean, pea, maize (Koster and Leopold 1988), and cauliflower (Haigh 1990; Hoekstra *et al.* 1994). In the developing tissues of *Brassica campestris* , sucrose and stachyose contents increase in the radicle tips of the embryos at the time that the seeds tolerate desiccation (Leprince *et al.* 1990*a*). In germinating cauliflower seeds, a decrease of sucrose and stachyose in the hypocotyl and root tissues correlates with the differences in desiccation tolerance of the tissues (Haigh 1990). It has been suggested that the supply of metabolisable sucrose and oligosaccharides to the cellular membranes as a protective factor is necessary for desiccation tolerance (Leprince *et al.* 1990*a*; Haigh 1990). This will be discussed in more detail later in this section.

In developing seeds, Chen and Burris (1990) found that maize seeds harvested at moisture contents of 550, 450 and 400 g H_2O kg^{-1} fresh weight, will tolerate high temperature drying (50°C, 17%RH) after the seeds have been preconditioned by

incubating at 35°C, 22%RH for 6, 12, 24, 36 and 48 hours before drying. During preconditioning periods, the ratio of raffinose/sucrose increases; the raffinose/sucrose ratio correlates highly with conductivity tests, germination tests and sugar leakage levels.

The importance of sugars in desiccation tolerance has been implied from the beneficial effects of the exogenous application of sugars. Somatic embryos of *Medicago sativa* L. will tolerate desiccation when they are germinated in a medium containing 3% sucrose; the embryos are even more tolerant when the medium contains 6% sucrose (Anandarajah and McKersie 1990).

1.5.1.1 Mechanisms by which sugars may confer desiccation tolerance

Since the ratio of raffinose/sucrose increases in mature maize embryos during preconditioning is concomitant with an improvement of germination and membrane integrity, it has been suggested that sugars may play a role in desiccation tolerance by membrane stabilisation (Herter and Burris 1989; Chen and Burris 1990). Koster (1991) suggested that the role of sugars in conferring desiccation tolerance on seeds may relate to the prevention of cell damage by cytoplasmic glass formation. Glass formation refers to the non-crystalline solute state when water is removed. In this state, the cell solute is very viscous and the diffusion of water is restricted. Because of their viscous property, glasses may fill spaces in the cell and prevent cellular collapse during drying or stop chemical reactions that require diffusion (Burke 1986; Williams and Leopold 1989). In the glassy state, hydroxyl groups of sucrose may replace water by hydrogen-bonding to the phospholipid head groups of membranes (Crowe *et al.* 1984*b*). However, during dehydration sucrose tends to crystallise, the intermolecular hydrogen bonding between sucrose molecules in the crystalline state resulting in fewer hydroxyl groups being available for bonding to the membranes. Thus, sucrose alone may not provide a good membrane

protection. The interaction of oligosaccharides such as raffinose and stachyose may help to prevent crystallisation of sucrose (Caffrey *et al.* 1988) and maintain sucrose in a glassy state, thus resulting in the desiccation tolerance of the membrane (Koster and Leopold 1988; Williams and Leopold 1989).

Williams and Leopold (1989) found that a glassy state forms in the maize embryo at room temperature (20°C) when the moisture content is below 12% g/g dry weight. These investigators suggest that maize embryos which contain 17% sucrose and 3% raffinose of embryo dry weight exhibit a strong tendency to glass formation at a low temperature.

The proportion of sucrose and oligosaccharides induced in plants that survive desiccation is similar to that of anhydrobiotic animals and some lower plants (Barrett *et al.*, 1970; Crowe and Madin 1975; Womersley and Smith 1981; Womersley *et. al.* 1982; Drennan *et al.* 1993). Oligosaccharides such as trehalose have been reported to be excellent glass formers at the low moisture contents (Clegg *et al.* 1982). Thus, it has been suggested that the presence of sucrose and raffinose contributes to glass formation, which helps the survival of the desiccated seeds (Williams and Leopold 1989).

More evidence has been reported by Koster (1991) in the presence of both sucrose and oligosaccharides at the desiccation-tolerant stage. The sugar mix in the ratio representative of desiccation-tolerant embryos (i.e. 85% w/w sucrose and 15% w/w raffinose) is found to form glasses at ambient temperatures. In contrast, the sugar mix at 75% w/w glucose and 25% w/w sucrose representative of non-desiccation tolerant embryos only forms glasses at subzero temperatures. It is possible that tolerant embryo cells form sugar glasses at storage temperatures and water contents, whereas intolerant embryo cells do not (Koster 1991).

Santarius (1973) studied the role of sugars in stabilising membranes in isolated chloroplasts from mature leaves of spinach. Addition of sugars (prior to water

stress) prevents inactivation of cyclic phosphorylation with phenazine methosulphate and of dichlorophenolindophenol (DCIP) reduction. The effectiveness of protection depends on the molecular weight and sugar concentration. It was found that the activity of DCIP reduction by isolated thylakoids increases as sucrose concentration increases from 0.1 M to 1.0 M and raffinose is more effective than sucrose and glucose in stabilising cell membranes.

Hoekstra *et al.* (1989) reported that *Pennisetum* pollen will tolerate a lower moisture content than maize pollen. *Pennisetum* pollen will survive 3% moisture content while maize pollen will only tolerate desiccation to a 7-8% moisture content. It has been found that the sucrose content increases to 17% of dry weight in *Pennisetum* pollen during drying but it increases only to 12% of dry weight in maize pollen, which is consistent with the higher sucrose content of *Pennisetum* pollen contributing to its tolerance to lower moisture content.

1.5.1.2 Negative evidence for sugars in desiccation tolerance

Although many investigators have reported a role for sugars in conferring desiccation tolerance (Burke 1986; Williams and Leopold 1989; Koster 1991), others have recently reported that there is no relationship between desiccation tolerance and sugar content. For examples, raffinose was undetectable in maize embryos during development even though at 25 days after anthesis (DAA) the seeds were in the desiccation-tolerant stage (Bochicchio *et al.* 1994). Moreover, it has been found that immature soybean axes which are in the desiccation-sensitive stage form 'glasses' upon drying (Sun *et al.* 1994). Thus, an accumulation of sugars alone cannot explain desiccation tolerance in soybean seeds (Sun *et al.* 1994). In *Arabidopsis thaliana* seeds, there was also no obvious link between the acquisition of desiccation tolerance and the amount of oligosaccharides present. Furthermore, the desiccation tolerant types have the lowest amount of sucrose and accumulate sugars in the later part of seed development (Ooms *et al.* 1993).

Farrant *et al.* (1993) have reported a high concentration of sucrose and oligosaccharides in the seeds of recalcitrant species during development in spite of the fact that they do not tolerate desiccation at all. Likewise, Finch-Savage and Blake (1994) found a high amount of sucrose, raffinose, glucose and fructose in the embryos of the recalcitrant *Quercus robur* L., and Sun *et al.* (1993) found an accumulation of sucrose, raffinose and stachyose in the recalcitrant developing seed of red oak. The fact that these species which are desiccation sensitive still contain supposedly sufficient sugars to make them desiccation tolerant implies that they do not play an important role in the desiccation tolerance. However, there is no information on what happens to these sugars during desiccation. It is possible that sugars may decline during desiccation and insufficient sugars remain to stabilise the membranes. Further investigations on recalcitrant seeds are required to confirm or refute the proposal that sucrose, raffinose and stachyose are essential features of the desiccation tolerant-stage.

1.5.2 Proteins

During seed development and germination, specific proteins such as storage proteins and germination proteins are synthesised at a specific periods (Hill and Breidenbach 1974; Lalonde and Bewley 1986; Sánchez-Martínez *et al.* 1986; Rosenberg and Rinne 1986). For example, during germination and early seedling growth, pea axes exhibited 6 sets of proteins at different time after imbibition (Lalonde and Bewley 1986). In developing soybean seeds, proteins were separated into three major fractions, they were 2.2S, 7.5S and 11.8S fractions. These protein fractions accumulated at different stages of development (Hill and Breidenbach 1974). Laroche-raynal *et al.* (1984) have reported that in radish seeds, a subset of mRNAs decay in immature seeds whereas they accumulate in late embryogenesis. Rosenberg and Rinne (1986) have found that 'maturation polypeptides' are synthesised during precocious maturation and in natural matured soybean seeds; however, their roles are unknown.

One set of proteins synthesised during maturation is of considerable interest. They are reported to accumulate at the desiccation-tolerant stage in barley (Bartels *et al.* 1988), maize (Bochicchio *et al.* 1988) and soybean seeds (Blackman *et al.* 1991) and have been hypothesised to play a role in desiccation tolerance (Blackman *et al.* 1991). The range in molecular mass from 25.5 to 170 kD and are termed 'maturation proteins' or 'late embryogenesis abundant (LEA) proteins'. The periods when the LEA proteins are detectable is at the desiccation-tolerant stage of seed development. The loss of these proteins occurred at the desiccation-sensitive stage of seed germination (Blackman *et al.* 1991).

The function of LEA proteins is unknown but they are hydrophilic, heat soluble, ABA-responsive and tend to accumulate in vegetative tissues during water stress (Gómez *et al.* 1988; Close *et al.* 1989; Blackman *et al.* 1991; Hsing 1992). It has been suggested that LEA proteins play a role in desiccation tolerance by bonding with macromolecules and preventing cellular damage during desiccation (Galau *et al.* 1986; Baker *et al.* 1988; Dure *et al.* 1989; Blackman *et al.* 1995). Dehydration caused a loss of group 3 LEA proteins in roots of germinating wheat. After rehydration, the roots did not resume growth and subsequently died whereas shoot and scutellum survive desiccation (Ried and Walker-Simmons 1993).

The presence of LEA proteins does not correlate with desiccation tolerance in all cases. In recalcitrant species that do not tolerate desiccation, such as English oak (*Quercus robur* L.) and European chestnut (*Aesculus hippocastanum* L.), LEA proteins are also detectable in the seeds (Finch-Savage *et al.* 1994). In Arabidopsis, the presence of LEA proteins does not correlate with desiccation tolerance (Ooms *et al.* 1993). It has been suggested that LEA proteins alone may not be sufficient to induce desiccation tolerance (Blackman *et al.* 1992; Bradford and Chandler 1992) or that these proteins confer desiccation tolerance to only a limited extent (Finch-Savage *et al.* 1994). An accumulation of sugars such as sucrose and oligosaccharides (i.e. raffinose and stachyose) as well as the LEA

proteins may be necessary for desiccation tolerance (Blackman *et al.* 1991, 1992). Blackman *et al.* (1992) have suggested that the LEA proteins are an early response system before sugars reach high concentrations and they may also function in conjunction with oligosaccharides in the development of desiccation tolerance .

1.5.3 Lipids

Lipids are the first source of energy reserves to accumulate in wheat embryos (Evers and Bechtel 1988). In wheat, during the early stages of development, when the endosperm cell division phase is drawing to a close, lipids accumulate as droplets in the scutellum (Evers and Bechtel 1988; Hargin *et al.* 1980). These droplets become much more numerous from the grain filling stage to embryo maturation (Evers and Bechtel 1988).

1.5.3.1 Origin and structure of lipid droplets

Seeds store reserve lipids in the form of triacylglycerols (TAG) which are packed in subcellular organelles called lipid droplets (spherosomes, oleosomes, oil bodies or lipid bodies) (Huang 1985). Aleurone layer and scutellum tissue contain the highest level of TAG in seeds of cereals (Barnes 1983). The origin of lipid droplets has been a matter of controversy. Some investigators have reported that lipid droplets form directly in the cytoplasm and are then surrounded by a membrane which may be derived from the endoplasmic recticulum (ER) (Bergfeld *et al.* 1978; Rest and Vaughan 1972). Other investigators have suggested that lipid droplets arise from the ER and then they are bound by a half-unit membrane (Yatsu and Jack 1972; Wanner and Thiemer 1978; Wanner *et al.* 1981).

Although lipid droplets are distributed within the cytoplasm of cells (Nir *et al.* 1970*a*, *b*; Bolduc 1983), they are usually located near the ER (Murphy *et al.* 1993; Wanner and Thiemer 1978; Sitte 1977) or plastids (Bergfeld *et al.* 1978). Consequently the suggestion has been made that the origin of the membrane of lipid

droplets is from either the ER or plastids (Wanner *et al.* 1981). Recently, Murphy *et al.* (1993) suggested that fatty acids synthesised in the plastids are exported to the ER for triacylglycerol (TAG) synthesis.

Sitte (1977) has pointed out that the spherical shape of lipid droplets is a consequence of minimisation of the surface area. The size of lipid droplets also varies substantially in different plant tissues. In general, endosperm and embryo lipid droplets are less than 1.0 μm in diameter. However in the oleogenic fruits such as olive, oil palm or avocado; the mesocarp tissue accumulates the large storage lipid droplets, and the fruit lipid droplets are approximately 20 μm in diameter (Murphy *et al.* 1993). This difference in lipid droplet size has been ascribed by Murphy *et al.* (1993) to a difference in function in the plant. Seeds which must survive desiccation have lipid droplets which are small and hence present a larger surface area which makes for rapid lipolysis after germination. Fruit lipid droplets are in an aqueous environment, and are not used for plant metabolism as are the smaller droplets of germinated seeds (Murphy *et al.* 1993).

Trelease (1969) also found that lipid droplets in the scutellum of maize embryos are larger than those in the embryonic axis. In olive seeds, lipid droplet diameters are in the range of 0.5-2.0 μm but they are 10-20 μm in the mesocarp (Ross *et al.* 1993). Recently, Tzen *et al.* (1993) measured the size of lipid droplets in the mature seeds of rape, mustard, cotton, flax, maize, peanut and sesame, the average size of the lipid droplets is in a narrow range of 0.6-2.0 μm. The size of these lipid droplets is in accord with the size-range of lipid droplets in the maize scutellum reported by Wang and Huang (1987).

Lipid droplets containing TAG are surrounded by a monolayer of phospholipids (PL) embedded ~~with~~ within the basic protein "oleosin" (Tzen *et al.* 1993). From the model of lipid droplets in maize, a surface view of the structural unit has been proposed which consists of a ratio of 13 PL molecules to 1 oleosin molecule. Lipid

droplet surfaces possess a net negative charge at neutral pH with an isoelectric point of 5.7-6.6. The negative charge surface is due to the net charge of the surface oleosins, phospholipids and a small quantity of free fatty acids. Thus, the droplets do not fuse or clump to one another at neutral pH due to the net negative charge repulsion on their surfaces. Both the phospholipid molecules as well as the oleosins are required to stabilise the lipid droplets and prevent coalescence (Huang 1992; Tzen and Huang 1992). Tzen *et al.* (1993) also noted that triacylglycerols and oleosins accumulate during seed maturation. Cummins *et al.* (1993) studying rape seed have also suggested that oleosins accumulate in mature seeds for the purpose of stabilising lipid droplets during desiccation. Dehydration of lipid droplets extracted from young embryos of the rape seed resulted in a breakdown of lipid droplets and coalescence into large clumps of oil. However, the oleosin-rich lipid droplets from mature embryos were stable to dehydration.

1.5.3.2 The degradation of lipid droplets

When degradation of lipid droplets takes place following germination, the enzyme lipase must attach to them prior to lipolysis. Huang (1992) suggested that the oleosins provide the binding sites for the lipases. In castor bean, normally maximal activity for lipase was established at pH 5.0 and decreased to zero at pH 7.0. However after germination, lipase activity was active at pH 7.0 (Huang 1992). In the scutellum of the maize kernel, lipase activity appeared after 2 days of imbibition and reached a maximum at about 5-6 days of imbibition (Wang and Huang 1987).

In wheat, Swift and O'Brien (1972*b*) reported that during germination, lipid droplets were degraded in the scutellum. This was made visible by microscopy and the lipid droplets were observed to gradually disperse from the membrane of the cells. Complete dispersion of lipid droplets occurred within 24 hours of imbibition. In the later stages, there was a decline in the number of lipid droplets. Most of the lipid droplets disperse within the cytoplasm and only a few are seen

near the cell wall. Within 6 hours of imbibition, there is an increase of starch grains but later in germination they too are not evident. None of these changes in cell fine structure are detectable in the air-dry seeds or after 3 hours of imbibition; only the appearance of ER and dictysomes and a dispersion of the nuclear chromatin (Swift and O'Brien 1972*a*) are observable. Marinos and Fife (1972) also reported the dispersion of lipid droplets in the shoot apical dome of wheat embryos at 12 hours of imbibition.

In the radicles of germinating rye (*Secale cereale* L.), Hallam *et al.* (1972) found a decrease in number and migration of lipid droplets from the plasmalemma into cytoplasm after 24 hours of imbibition. In wheat, the mobilisation of TAG occurs in the embryonic axis before in the scutellum. In the first 12 hours of imbibition, the mobilisation of TAG proceeds rapidly but after 2 days it continues more slowly.

The degradation of lipid droplets during desiccation has only been reported in certain plants. In maize tissue undergoing desiccation, Nir *et al.* (1970*a*, *b*) found that prior to lipid degradation, numerous lipid droplets disperse in the cytoplasm of the dehydrated cells of maize roots. They postulated that lipid droplets were displaced from their position in the mitochondrial and other cellular components. Desiccation also caused chromatin aggregation, cell wall distortion, disruption of polysomes and changes in cellular membranes.

1.5.3.3 The role of lipids in desiccation tolerance

The role of neutral lipids other than as an energy store remains uncertain, but lipid accumulation in the embryos has been shown to play a significant role after germination commences. After germination, rapid lipid metabolism is well documented (Kornberg and Beevers 1957; Longo and Longo 1970; Doig *et al.* 1975; Newman and Briggs 1976; Beevers 1980). Triacylglycerols are hydrolysed into glycerol and fatty acids. These fatty acids are then known to be degraded to acetyl coenzyme A and incorporated as such into the glyoxylate cycle in the

glyoxysome, thus providing a mechanism to convert lipid to carbohydrate mainly in the form of sucrose. Rost (1972) showed that in *Setaria lutescens* seeds wherein the hydrated dormant embryo did not metabolise lipids, whereas there was rapid lipid metabolism in the germinated embryos.

The importance of lipids in the tolerance by nematodes to desiccation (a feature known as anhydrobiosis) is well documented (Barrett *et al.* 1970; Madin and Crowe 1975). These species contain high contents of lipid in their bodies. Marked changes in lipid and carbohydrate content have been observed when the nematodes are desiccated (Madin and Crowe 1975; Womersley and Smith 1981) and during egg development (Barrett *et al.* 1970). It has been suggested that glycerol in the presence of trehalose help preserve cellular structure in the animals during desiccation (Crowe *et al.* 1984*a*).

There is not much evidence regarding a role for lipids in desiccation tolerance in seeds. For developing plants, Dakhma *et al.* (1995) have found that in one-month-old rape plants, drought-stress induces neutral lipids content in leaves. Attree *et al.* (1992, 1994) have reported an increase of triacylglycerols (TAG) in somatic embryos of white spruce when they are cultured in a medium containing abscisic acid and polyethylene glycol for seven to eight weeks. The treatments that promoted TAG biosynthesis also prevented precocious germination and promoted desiccation tolerance.

1.5.4 Free Radicals and free radical scavengers

Free radicals are molecular species containing an unpaired electron which can be induced by many factors including aging (artificial aging or natural aging), water stress, light, temperature etc. (Benson 1990; Hendry 1993). Free radicals need to pair their single electrons with other molecules, and this cause a propagation of many different types of highly reactive free radicals in the cell. The major cellular source of electrons for free radicals is membrane lipid (Benson 1990). One

example of a free radical is superoxide radical ($O_2^{\cdot -}$), which is very reactive and forms hydrogen peroxide (H_2O_2) leading to a formation of singlet oxygen and the hydroxyl radical ($HO^{\cdot}$) which is the most damaging species. The $HO^{\cdot}$ combines with nucleic acids, proteins and lipids causing mutation, DNA damage, inactivation of enzymes and destruction to membrane lipids (Bewley 1979; Leprince *et al.* 1993).

To summarise, sugars and LEA proteins are well documented as factors in desiccation tolerance. Suggested mechanisms for sugars include membrane stabilisation during drying by means OH groups of sucrose replacing lost water hydroxyls which were hydrogen-bonded to the phospholipid polar head groups of membranes. However, the sugar content in developing seeds (orthodox and recalcitrant seeds) does not correlate well with desiccation tolerance in all cases. The mechanism of LEA proteins are not well understood, but they have been correlated with the prevention of subcellular damage. For lipids, most data are derived from somatic embryos, the role of lipids in desiccation tolerance has not clearly been elucidated.

Transfer to page 24 as paragraph 4 prior to Section 1.5.4

1.5.4.1 Free radicals and their roles in damaging membranes

Free radical formation in dry stored seeds causes denaturation of proteins, DNA, RNA and inactivation of enzymes. The mechanism of damage by free radicals is mainly through lipid peroxidation (Benson 1990). In germinating seeds, free radicals have been reported to play a role in damaging membranes during the desiccation-sensitive stage in soybean seeds (Senaratna *et al.* 1985*a*, *b*, 1987). It has been reported that in the desiccation-sensitive stage, there is an increase in free fatty acid/phospholipid ratio when the microsomal membranes are exposed to a free radical source (Senaratna *et al.* 1985*b*). Desiccation increases the amount of free radical injury to the cell membranes by breaking the linkage between glycerol and fatty acid side chains of the phospholipid molecules. Thereafter, the saturated free

fatty acids increase rigidity of the membrane resulting in the alteration of membrane properties (Senaratna *et al.* 1985*b*; 1987).

In recalcitrant species, *Quercus robur* L., there is a 200-fold increase in the amplitude of the electron para-magnetic resonance (EPR) which indicates an increase of radical species in the axes after drying. Furthermore, thiobarbituric acid-reactive products from lipid peroxidation have been reported to accumulate in the axes upon drying (Hendry *et al.* 1992). An increase of lipid peroxidation is also evident in germinating maize seeds when the seeds are desiccated at the desiccation-sensitive stage (Leprince *et al.* 1990*b*). Priestley *et al.* (1985) have demonstrated that free radicals increase during natural and accelerated aging and result in loss of viability in soybean seeds.

1.5.4.2 The roles of the radical scavengers

'Scavenging' systems will protect cells from free radical damages (Benson 1990). Such scavenging systems include the antioxidants carotenoids, vitamin E (α-tocopherol), ascorbate and glutathione. Tocopherols are chain-breaking antioxidants, and therefore block lipid peroxidation. Enzymic free radical-processing systems include superoxide dismutase (SOD) which converts superoxide ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2), and this in turn can be detoxified by catalase, glutathione reductase, ascorbate, and other peroxidases (Bewley 1979; Benson 1990; Leprince *et al.* 1993). It has been suggested that desiccation sensitivity in plants results from a loss of balance between free radical producing and scavenging systems (Bewley 1979).

The mechanisms of action free radical scavenging systems in desiccation tolerance have been reported to be different among species and the mode of protection is still unclear (Leprince *et al.* 1993). Protection in one species may be by antioxidants whereas in others it appears to be by enzymic free radical-processing systems. In the case of desiccation sensitivity in germinating soybean axes, α-tocopherol

decreases at the desiccation-sensitive stage. The α-tocopherol content decreases from 52 mg g^{-1} lipid at the desiccation-tolerant stage to only 0.1 mg g^{-1} lipid at the desiccation-sensitive stage (Senaratna *et al.* 1985*a*), and it has been postulated that the high amounts of α-tocopherol in the soybean axes prevent membrane damage during desiccation at the desiccation-tolerant stage. In germinating maize seeds, desiccation at the sensitive stage suppresses the activity of the enzymes superoxide dismutase, peroxidase and glutathione reductase. However, the amounts of free radical scavengers, i.e. α-tocopherol, γ-tocopherol and glutathione increase during dehydration. It has been pointed out that the switch from desiccation tolerance to desiccation sensitivity in this species appears to be a failure of the protective enzymic systems, especially superoxide dismutase and peroxidase, rather than antioxidants (Leprince *et al.* 1990*b*).

In *Quercus robur* L., a recalcitrant species, desiccation sensitivity is associated with a decrease in enzymic protection (ascorbate peroxidase) against lipid peroxidation which coincides with a decrease in free radical scavengers (ascorbic acid and α-tocopherol) in embryonic axes (Hendry *et al.* 1992).

In sand-dune moss (*Tortula ruraliformis*), Seel *et al.* (1992) have reported that desiccation tolerance results from antioxidant accumulation rather than activated oxygen-processing enzymes removing chloroplastic hydrogen peroxide. In *Tortula ruralis*, Dhindsa (1991) demonstrated an increase of the glutathione metabolism stimulated by desiccation.

To summarise, desiccation sensitivity in seeds has been associated with membrane damage by free radicals. The damage occurs through lipid peroxidation and de-esterification. In most reported cases, which include germinating orthodox seeds, recalcitrant seeds and moss, the mechanisms involved in desiccation sensitivity have been associated with an increase in lipid peroxidation and a decrease in scavenging systems. The latter include antioxidants as well as and enzymic

protection against lipid peroxidation. There seems to be no universal protective mechanism for germinating orthodox seeds, and different species exhibit different systems of protection.

1.5.5 Abscisic acid (ABA)

ABA has been reported to increase in concentration in seeds of many species during development and then decrease during maturation, especially in those seeds that do not become dormant (Walton 1980/81; King 1982; Xu and Bewley 1991). The amounts of ABA in embryos increase during the grain filling period in wheat (McWha 1975; King 1976), maize (Jones and Brenner 1987; Smith *et al.* 1989), *Phaseolus vulgaris* L. (Hsu 1979), castor bean (Kermode *et al.* 1989*a*, *b*), sunflower (Le Page-Degivry and Garello 1991) and in recalcitrant species such as *Quercus robur* L. (Finch-Savage *et al.* 1992). These authors have demonstrated that while ABA accumulates during the period of reserve deposition, it declines during maturation drying. In *Quercus robur* L., where there is no maturation drying before shedding, but ABA decreases when the seeds are mature (Finch-Savage *et al.* 1992).

1.5.5.1 The role of ABA during seed development

During seed development, ABA has been reported to be involved in the regulation of many processes such as preventing precocious germination, induction of reserve protein accumulation, induction of LEA proteins, development of desiccation tolerance and induction of seed dormancy (King 1976, 1982; Nikolaeva 1977; Hsu 1979; Black 1980/81; Walton 1980/81; LePage-Degivry and Garello 1991; Hilhorst and Karssen 1992). The exact role of ABA in the above processes is still unknown.

Changes in sensitivity to ABA may be more important in preventing precocious germination than absolute concentration of ABA (Oishi and Bewley 1992; Walker-

Simmons 1987; McCarty *et al.* 1989). A study using an ABA insensitive mutant of maize indicates that embryos germinated precociously because they have reduced sensitivity to ABA (McCarty *et al.* 1989). In wheat, sprouting-susceptible and sprouting-resistant cultivars do not have much difference in ABA contents. The difference between these two cultivars is that ABA inhibited embryo germination in sprouting-resistant cultivars more effectively than in sprouting-susceptible cultivars (Walker-Simmons 1987).

1.5.5.2 The role of ABA in conferring desiccation tolerance

The role of ABA by itself in conferring desiccation tolerance is not clear and demands further explanation. It appears that during seed development, ABA influences synthesis of storage proteins and that slow desiccation stimulates protein synthesis (Skriver and Mundy 1990; Gómez *et al.* 1988) as well as TAG synthesis (Attree *et al.* 1992, 1994). It has been suggested that the presence of ABA in the embryos at maturation leads to an accumulation of maturation proteins which assist the seed to survive desiccation (Gómez *et al.* 1988).

In germinating seeds of soybean, Blackman *et al.* (1991) have reported that ABA extends the period of desiccation tolerance. After 18 hours of imbibition, seeds do not survive desiccation; however, the seeds imbibed in the presence of 100 mM ABA will then survive desiccation even at 36 hours of imbibition. This is coincident with the presence of LEA proteins until 36 hours of imbibition. Without ABA, maturation proteins are absent from the embryos after 18 hours of imbibition.

Recently, the use of fluridone (which is an ABA synthesis inhibitor) has been employed to study the role of ABA during seed development (Xu and Bewley 1995). They showed that fluridone suppressed ABA synthesis in developing alfalfa seeds. These seeds with suppressed ABA synthesis did not synthesise two proteins (with molecular mass between 29-36 kDa) and were found to be

desiccation sensitive, whereas control seeds containing these two proteins were found to be desiccation tolerant (Xu and Bewley 1995).

1.5.5.3 Desiccation tolerance of somatic embryos

Much research has been done to induce desiccation tolerance in somatic embryos by application of exogenous ABA. The induction of desiccation tolerance appears to depend on many factors such as ABA contents, stage of development, rate of drying and plant species (Senaratna *et al.* 1989; Anandarajah and McKersie 1990; Attree *et al.* 1991; Tetteroo *et al.* 1995).

The concentration of ABA to induce desiccation tolerance in somatic embryos differs among species. For carrot embryoids (Tetteroo *et al.* 1995) and white spruce somatic embryos (Attree *et al.* 1995), the addition of 20 μM ABA to a culture medium was sufficient. However, concentrations of 40-60 μM ABA resulted in the formation of abnormal white sprout somatic embryos (Dunstan *et al.* 1991). Misra *et al.* (1993) has suggested that in white sprout somatic embryos, an application of ABA should always be in combination with an optimal osmoticum concentration. When white spruce somatic embryos were cultured on a medium containing ABA and a low osmoticum level, they lacked major crystalloid and matrix polypeptides.

In alfalfa somatic embryos, the inclusion of 10^{-5} M ABA with 6% sucrose in the medium induced desiccation tolerance. The optimum time for ABA application was when the somatic embryos were at the cotyledonary-stage (Anandarajah and McKersie 1990; Senaratna *et al.* 1989).

When white spruce somatic embryos were treated with ABA, there was an increase in storage lipids and proteins which included triacylglycerols (Attree *et al.* 1995) and polypeptides (Misra *et al.* 1993). The amounts of storage lipid also depended upon the concentration of ABA, kind of media and the osmotic pressure. It has

been suggested that embryos tolerate desiccation because of the storage reserves induced by ABA (Finkelstein and Crouch 1986; Anandarajah and McKersie 1990).

However, the role of ABA in desiccation tolerance can apparently be replaced by other factors such as sucrose. Anandarajah and McKersie (1990) reported that in alfalfa somatic embryos (14 to 22 days after sieving) that with a relatively high sucrose concentration in the medium (9% w/v sucrose), desiccation tolerance can be induced without the use of exogenous ABA.

Although ABA is associated with desiccation tolerance, the mechanisms involved require further investigation. Exogenous ABA treatments which regulate the acquisition of desiccation tolerance also stimulate LEA protein synthesis (Leprince *et al.* 1993; Blackman *et al.* 1995) and the decreased cellular damage after soybean seeds are treated with ABA correlates well with decreased electrolyte leakage from the axes and less visible cellular damage as seen under microscope (Blackman *et al.* 1995). Exogenous ABA also extends the time of desiccation tolerance in germinating soybean embryos. Thus, the role of ABA in desiccation tolerance is complex, and it appears that it works in conjunction with other factors (Blackman *et al.* 1995).

In summary, desiccation tolerance and sensitivity occur in a number of different developmental stages, i.e. germinative stage, developmental stage, vegetative stage and different kinds of seeds, i.e. recalcitrant and orthodox seeds. However, the physiological and biochemical changes indicating the tolerance or the sensitivity to desiccation of these seeds appear to be similar. These are :

1) The sensitivity to desiccation is due to subcellular and cellular damage caused by drying. This is evident in developing and germinating orthodox seeds as well as recalcitrant seeds.

2) The tolerance to desiccation has been correlated with the presence of sucrose and oligosaccharides which appear to assist in preventing membrane damage caused by drying. This mechanism has been well documented for some developing and germinating orthodox seeds. However, it is not universal for all orthodox seeds and does not correlate well in recalcitrant species.

3) The tolerance to desiccation has also been ascribed to the presence of scavenging systems e.g. antioxidants and protective enzymes which act against membrane damage by free radicals. This mechanism has been studied in germinating orthodox seeds, recalcitrant seeds and moss.

4) The tolerance to desiccation has also been correlated with the presence of LEA proteins having a role in membrane protection. This mechanism has been studied in developing and germinating orthodox seeds. However, the presence of LEA proteins does not confer desiccation tolerance in recalcitrant seeds. The synthesis of these LEA proteins is associated with the presence of ABA.

5) The tolerance to desiccation has been associated with an increase in storage proteins and TAG synthesis stimulated by ABA. The mechanism has been studied in somatic embryos, and its mode of action remains unclear.

From this literature review, it is apparent that more information is required on the mechanisms of desiccation tolerance. In this thesis, desiccation tolerance and desiccation sensitivity were studied in developing orthodox seeds. The aims of this thesis are:

1) to study the role of sucrose and oligosaccharides in conferring desiccation tolerance

2) to employ spectroscopic methods, such as IR and NMR, to investigate other possible mechanisms which can be involved in desiccation tolerance and desiccation sensitivity

3) to use confocal microscopy to monitor biochemical changes in lipid metabolism in the embryos at the the desiccation-sensitive and the desiccation-tolerant stages.

CHAPTER 2

Materials and Methods

2.1 Plant materials

2.1.1 Sweet corn (*Zea mays*)

Sweet corn seeds (kernels) cultivar 'Illini Gold' were planted in soil and grown in a glasshouse at the Waite Agricultural Research Institute, one plant per pot, and twenty plants per replication. The temperature in the glasshouse ranged from 15°C-38°C. Pollination was by hand when the silk length was approximately 1 cm. Two cobs from each replication were collected at each harvesting date. Seeds from the middle of the cobs were removed using a scalpel and pooled. Samples were drawn from the pool for germination tests, desiccation tolerance tests and moisture content determination.

2.1.2 Wheat (*Triticum aestivum*)

Wheat plants were grown either in the glasshouse or in a growth room at 18°C with 16 h light and 8 h dark. Each pot (25 cm diameter) contained 16 plants grown on recycled soil. The plants were fertilised with Aquasol (Hortico; N:P:K = 23:4:18) 2.6 g/pot at 1 month after germination. Ears were tagged at the time of anthesis and seeds (caryopses) collected at the required harvesting date.

2.2 Germination test

2.2.1 Filter paper germination test

Seeds were surface sterilised by soaking in NaOCl (1% available chlorine) for 1 minute (sweet corn) and 20 minutes (wheat) followed by three washes in distilled

water. The seeds were placed in 12-cm-diameter Petri dishes containing 2 layers of filter paper (Whatman No.1) moistened with 6 ml of distilled water then covered with 1 layer of moist filter paper. The Petri dishes were sealed with parafilm to prevent moisture loss and then stored at 25°C in darkness for 7 days. Distilled water was added to the Petri dishes as required to maintain a high moisture content during germination test. Seeds with at least 2 mm of radicle protrusion were counted as germinated.

2.2.2 Embryo germination on artificial media

Embryos were removed aseptically from the seeds then transferred to an artificial medium containing (g/L of H_2O) : NH_4NO_3 (0.6), $MgSO_4.7\ H_2O$ (0.4), KH_2PO_4 (0.4), K_2HPO_4 (0.16), Fe citrate.5 H_2O (0.006), $CaHPO_4$ (0.4), sucrose (20) and agar (8) (Bochicchio *et al.* 1988), supplemented with 1.4 mM GA_3. Each 5 cm diameter Petri dish contained 5 embryos and 5 ml of medium. The germination test was carried out at 25°C in the dark for 5 days. Seedlings with well developed roots and shoots or with slight defects were scored as normal whereas those with albino, twisted, or undeveloped coleoptiles and roots were scored as abnormal. Embryos which were not able to produce roots or shoots or those developing only calli were scored as non-germinated embryos.

2.3 Moisture content determination

To determine the moisture content, the mass difference between fresh and dry embryos was calculated. The latter mass was obtained by drying the embryos at 85°C for 48 hours. Moisture content was expressed as % moisture content (wet weight basis).

2.4 Desiccation tolerance test

Seeds were placed in either nylon plastic bags or on 5-cm-diameter Petri dishes and stored in jars (12 cm diameter; 22 cm height) containing 100 g of silica gel. The bags

or Petri dishes were supported on a wire frame approximately 9 cm above the silica gel. The jars were kept in darkness at 25°C for 6 or 7 days. The silica gel was replaced when the color changed from blue to pale blue.

2.5 Sugar analysis

2.5.1 Sugar extraction

Embryos
~~Embryonic axes~~ were removed from the seeds using a scalpel and then weighed. Samples of embryos ~~axes~~ (the number varied depending on age) within the range 0.01-0.06 g fresh weight were ground in 2 ml of boiling 80% (v/v) ethanol and boiled at 85°C in a water bath for 3 minutes. A further one ml of 80% ethanol was added to the crude extract which was again ground and boiled for 2 minutes. After cooling to room temperature, the sample was centrifuged at 3,000 rpm for 3 minutes. The supernatant was decanted into a 5 ml volumetric flask. The pellet was re-extracted with 1 ml of 80% ethanol and centrifuged 3 times. The supernatant was decanted into the same 5 ml flask and the volume made up to 5 ml with 80% ethanol. A two ml aliquot of the supernatant was evaporated to dryness in a rotary evaporator (Savant 'Speed Vac') and stored at -20°C prior to analysis.

2.5.2 Preparation of samples for high performance liquid chromatography (HPLC)

The dry sample prepared by the sugar extraction method was redissolved in 0.5 ml of HPLC water in a water bath at 85°C for 3 minutes and then vortexed. The solution was then passed through a column packed with acid washed sand and 0.4 ml of resin [a mixture of Amberlite CG 120 Na^+ form and Amberlite CH 4B OH^- form; 1 : 2 (w/w)]. Three washes of 0.5 ml of HPLC water were then passed through the column and the elute volume was made up to 2 ml. One ml of solution was then passed through a 0.45 µm nylon syringe tip filter into a glass HPLC vial. Samples (100 µl) were injected by an automatic injector (Waters, model WISP 710 B) with a

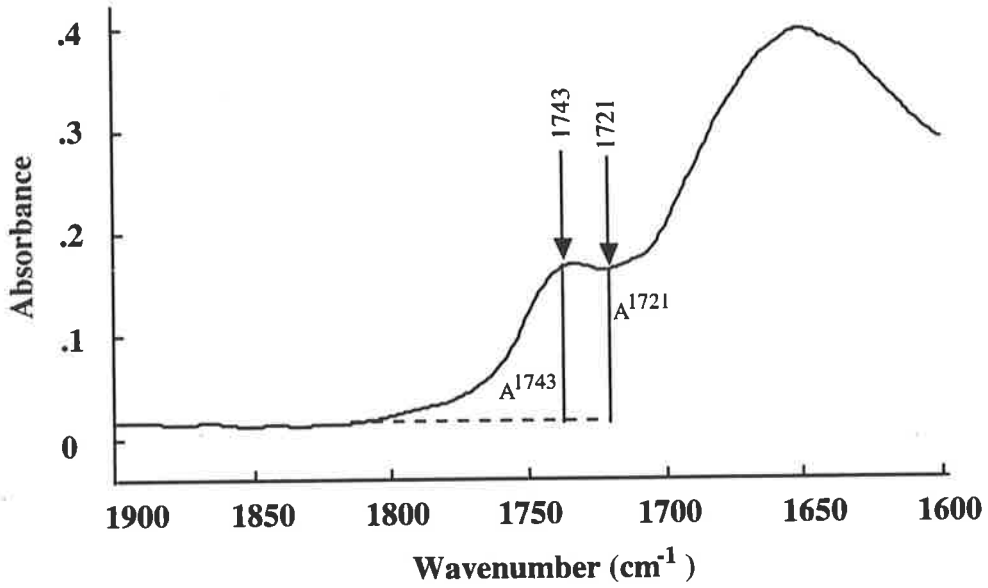
run time of 62 minutes/sample at a constant temperature of 90°C. The mobile phase was ultrapure HPLC grade water heated at 85°C for 24 hours and then sonicated in an ultrasonic bath for 20 seconds before use. The flow rate was 0.5 ml min^{-1}.

Sucrose and raffinose were analysed using a Waters Dextro-Pak (100 x 80 mm) column packed with silica particles bonded with octadecylsilane and detected with the Waters 410 RI detector. A standard solution (100 µl), containing 20 µg sucrose and 20 µg of raffinose was injected to calculate the peak response data for the samples. The amounts of the sugars were expressed as µmol/g dry weight or nmol/embryo.

2.6 Diffuse Reflectance Using Infrared Dispersion Spectra (DRUIDS)

One embryo from either fresh or dried seed was ground with solid KCl (1 mg sample : 5 mg KCl). Deionized water (100 µl) was added and the sample then lyophilised. After drying, the sample was ground into a fine powder in a small glass tube and examined with a Perkin-Elmer 983 G IR spectrometer fitted with a diffuse reflectance attachment model DRA-3SP (Harrick). The infrared absorbance spectra of the reflected light from the samples were scanned in the range 4000-625 cm^{-1}, and functional groups interpreted according to Williams and Fleming (1989). The absorbance ratio of the 1743 cm^{-1} peak to the 1721 cm^{-1} trough (the ester carbonyl peak position) was employed as a sensitive indicator for changes in the peak at 1743 cm^{-1} (Fig. 2.1). Absence of a significant peak gives a ratio of ≤ 1.0, strong peaks have ratios >1.0. Individual samples exhibiting major changes at the time of development of desiccation tolerance were extracted with hexane, both the residue and the extract spectra were recorded and a portion of the extract was examined by thin layer chromatography.

Fig. 2.1 The infrared spectrum from 1900-1600 cm^{-1} of wheat embryos at 15 DAA showing position of the initially weak ester carbonyl peak at an aborbance 1743 cm^{-1}. The ratio of the absorbance of the 1743 cm^{-1} peak to the 1721 cm^{-1} peak trough has been used to detect changes in the triacylglycerol content of embryos during development and after drying. The A^{1743}/A^{1721} in this instance = 1.02. During development there is a major increase in the absorbance at 1743 leading to absorbance ratios$\geq$ 1.2. During desiccation it diminishes towards $\leq$ 1.



$$\text{Absorbance ratio} = \frac{A^{1743}}{A^{1721}}$$

2.7 Microscopic methods for lipid droplets

2.7.1 Sample preparation

Seeds were cut in half longitudinally and impregnated in a mixture of 15% (w/v) gelatin + 4% (v/v) glycerol at 37°C for 2 hours (Knox 1970; High 1984; Evans *et al.* 1992). Seeds were embedded in the mixture and allowed to cool and set at 4°C for 8 hours and the blocks were trimmed. After trimming, the blocks were soaked in a solution of 4% (w/v) formaldehyde+2% (w/v) Ca acetate at 4°C for 16 hours for hardening (adapted from High 1984). Samples were frozen by placing the blocks of samples in a metal can in an insulated box containing an ethanol+dry ice mixture. Frozen blocks were then cut at -30°C with a cryostat (Cryocut 1800; Reichert-Jung). Sections (10 µm) were picked up on slides coated with 1% gelatin and allowed to dry at room temperature for 1 hour before staining with Nile red. Delipidated controls were prepared by washing sections with 3 ml chloroform, followed by 3 ml ethanol. Sections were then dried at room temperature for at least 20 minutes before staining. Autofluorescence controls were prepared by mounting unstained sections with a few drops of 75% (v/v) glycerol.

2.7.2 Preparation of dye

Nile red (Molecular Probes, Inc., USA) staining mixtures were prepared from a stock solution containing 1 mg/ml in acetone. For staining lipid droplets, a mixture of Nile red, 5 µg/ml in 75% (v/v) glycerol was prepared and kept in the dark at 4°C until use. The sections were stained at room temperature for 20 minutes and mounted in Nile red solution (Evans *et al.* 1992).

2.7.3 Fluorescent microscopy

The sections were viewed with a laser confocal system Bio-Rad MRC-1000 (U.K.) coupled to an inverted Nikon Diaphot-300 (Japan) microscope. Excitation was

provided by an Ion Laser Technology (USA) Krypton Argon laser. The excitation filter was 488 (± 5) nm, beamsplitter 510 nm and emission filter 522 (± 16) nm. A Nikon x 10 (numerical aperture 0.2) objective was used. Each image, sample was scanned at the centre of the tissue. The same settings for neutral density filter, confocal aperture, photomultiplier voltage and intensity threshold for the emitted light were used to allow a quantitative comparison of the fluorescent intensities among treatments. The lipid fluorescence of each part of the embryo, i.e. scutellum, shoot (in this experiment the 'shoot' includes coleoptile, the first leaf and shoot apex) and radicle, was counted in the sampling areas of 4451 μm^2. Counting was performed randomly 3 times for each part of a section. Means for each treatment were obtained from 18 observation (6 seeds, 3 sections for each seed). Images in each Fig. represented longitudinal sections of half embryos, either shoot+scutellum ot radicle+scutellum.The invaluable assistance of Dr. Peter Kolesik, Department of Horticulture Viticulture and Oenology, with this technique is gratefully acknowledged.

2.8 Solid-state ^{13}C CP/MAS NMR analysis

^{13}C CP/MAS techniques were employed to study changes in the absorbance intensitiy of different types of carbons in wheat embryos during development and during desiccation. The 50.309 MHz solid-state ^{13}C CP/MAS NMR spectra were obtained on a Varian Unity 200 spectrometer with a 4.7 Tesla wide-bore Oxford superconducting magnet. Samples were spun at 5 kHz in 7 mm diameter zirconia rotors with Kel-F caps in a Doty Scientific MAS probe. The damage caused to the embryo samples by the centrifugal forces was minimised for all samples as follows. The rotor was initially filled with loosely packed fumed silica, and then spun at 5.0 kHz for approximately five minutes. Five wheat embyos were then placed along the center axis of the rotor (with the silica spread against the inner walls of the rotor).

All spectra were attained with a one millisecond contact time and a three second recycle delay time. Using the standard Varian cross-polarisation pulse sequence, the free

induction decays were accquired over a sweep width of 40 kHz, an acquisition time of a point database. All spectra were obtained with 16 k zero-filling, 20 Hz Lorentzian line broadening and 0.010 s Gaussian broadening. Chemical shift assignments were externally referenced to the methyl resonance of hexamethyl benzene at 17.36 ppm.

The proton spin-lattice relaxation time constants, T1(H), were determined for the first two samples (22d fresh, 15 d 5d) to ensure that the recycle delay was sufficient for the magnetisation of the H-spins to have fully relaxed between each acquisition pulse.

The three second recycle delay time was more than 7 times T1(H). The T1(H) constants were determined by a standard 180° -delay -90° inversion-recovery pulse sequence with cross-polarization. The integral intensities of the following spectral regions were used to calculate the relaxation decay time constants for each carbon chemical environment; carbonyl (200 to 165 ppm), aryl (165 to 110 ppm), O-alkyl (110 to 60 ppm) and alkyl carbons (60 to 0 ppm).

Interrupted decoupling (ID) ^{13}C CP/MAS NMR spectra were attained using the above conditions with an addition of a 42 μs interruption in the proton decoupling. A delay of 42 μs was selected (Wilson, 1987) in order to minimise signals from methine and methylene carbons, and maximise the signal from non-protonated carbon and carbon undergoing rapid fluxional motions such as methyl groups spinning around the central axis. The signal contribution of the carbonyl region of the spectra of the samples were normalised for the standard CP spectrum and the ID spectrum. The invaluable assistance of Dr. Philip Clarke of the Soil Science Department with this technique is gratefully acknowledged.

CHAPTER 3

Desiccation tolerance in developing seeds and the role of sugars

3.1 Introduction

The ability of seeds to tolerate desiccation has been investigated for many species such as bean (*Phaseolus vulgaris* L.) (Dasgupta *et al.* 1982; Misra *et al.* 1985), castor bean (Kermode and Bewley 1985*a*, *b*), maize (*Zea mays* L.) (Chen and Burris 1990) and soybean (*Glycine max* L.) (Rosenberg and Rinne 1986; Blackman *et al.* 1991, 1992). Biochemical changes in embryos have been reported to be associated with desiccation tolerance. Examples of these changes include an accumulation of late embryogenesis abundant proteins, sugars and antioxidants at the desiccation tolerant stage (Blackman *et al.* 1991, 1992; Koster and Leopold 1988; Leprince *et al.* 1992; Hendry 1993).

Sugars form the basis of many experiments carried out on desiccation tolerance in seeds. This is because of the ability of their hydroxyl groups to hydrogen bond to the polar head groups of membrane lipids thus preventing membrane injury as water is lost from the seeds (Crowe *et al.* 1984*b*; Burke 1986). In developing seeds of many species, sugars (sucrose and raffinose) present in embryos at seed maturity (Amuti and Pollard 1977) have been hypothesised to play an important role in conferring desiccation tolerance (Koster and Leopold 1988). A high content of sucrose (13% of embryo dry weight) in the presence of raffinose (7%) in maize embryos has also been reported as contributing to 'glass' formation which results in the desiccation tolerance of the seeds (Williams and Leopold 1989). In other work, a mass ratio of 9% raffinose to sucrose in maize embryos has been reported to protect the membrane from the damage due to high temperature drying (50°C) during seed processing (Chen and Burris 1990).

In germinating seeds of many species (i.e. pea, soybean and maize), the embryo content of sucrose and raffinose decreases rapidly during imbibition. When the initial contents of sugars is low, the germinating seeds did not tolerate desiccation (Koster and Leopold 1988).

Although as noted above, many authors have hypothesised a role for sugars in conferring desiccation tolerance, some recent investigations (Bochicchio *et al.* 1994; Sun *et al.* 1994; Ooms *et al.* 1993) have questioned the validity of this hypothesis. Consequently, the role of sugars in conferring desiccation tolerance requires reassessment by independent methods. The experiments in this chapter were carried out to investigate the role of sugars based on the hypotheses that:

1) Sucrose and raffinose accumulate in the embryos during seed development
2) Drying immature seeds results in an increase of sucrose and raffinose
3) Oligosaccharides such as raffinose confer desiccation tolerance.

Experiment 1: Sugar concentrations and desiccation tolerance during seed development I Germination, sugar concentrations and desiccation tolerance of sweet corn seeds.

3.2 Materials and methods

3.2.1 Plant materials

Two replicates of sweet corn cultivar 'Illini Gold' were grown in the glass house in March 1992-June 1992 (see 2.1.1). Two cobs from each replicate were collected from two sets of plants :

Set 1 : 15, 20, 25, 30, 35, 40, 45, 50 and 70 DAP.

Set 2 : 20, 25, 30, 35, 40, 45, 50, 55 and 70 DAP.

At each harvesting time, embryonic axes of fresh seeds from each replicate were dissected for sugar analysis (see ~~2.4~~ 2.5). Seed moisture content and germination were tested ~~(see 2.1.3)~~ (see 2.2 and 2.3).

3.2.2 Drying regimes

Seeds were incubated in jars (12 cm diameter, 22 cm height) containing 200 ml of appropriate agents. Saturated solution of $MgCl_2$ and LiCl were prepared in distilled water at 25°C and at this temperature, the relative humidity of the atmosphere was 33% over the solution for $MgCl_2$ and was 11% for LiCl (Rockland 1960). For drying over $MgCl_2$ and LiCl, the jars were divided into 2 groups (2 jars/group), each jar contained a sample of 100 fresh seeds taken from the pool (75 seeds for a germination test and 25 seeds for moisture content determination). For drying over silica gel (at 1%RH., Schonbeck and Bewley 1981), 100 seeds were placed over 100 g of activated silica gel (self indicating) which was replaced when the colour changed. Silica gel was regenerated after each use by heating to 95°C for several hours. The jars were incubated at 25°C in darkness for 5 or 7 days. For moisture determination, and germination test see 2. 2.1 and 2.3.

3.3 Results

3.3.1 Influence of stage of development on germination and moisture content of sweet corn

Fig. 3.1 shows that freshly harvested seeds do not germinate until 35 days after pollination (DAP). The percent germination of fresh seeds was found to be low at this time with only 32% in set 1 and 23% in set 2. From 35 DAP to 45 DAP, germination of fresh seeds actually decreased, but seeds later regained germinability at 55 DAP. From 55 DAP to 70 DAP, germination increased rapidly from 35% to 92% in set 2 and high germination was obtained at 70 DAP. Fig. 3.1

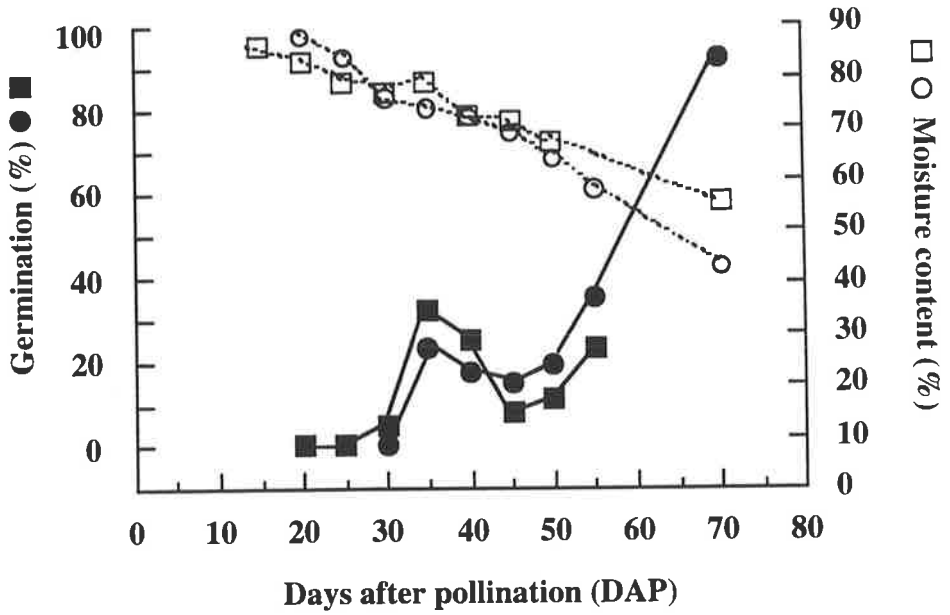


Fig. 3.1 Influence of developmental stage on seed moisture content and fresh germination of sweet corn seeds. Data from set 1 (■, □) and set 2 (●, ○).

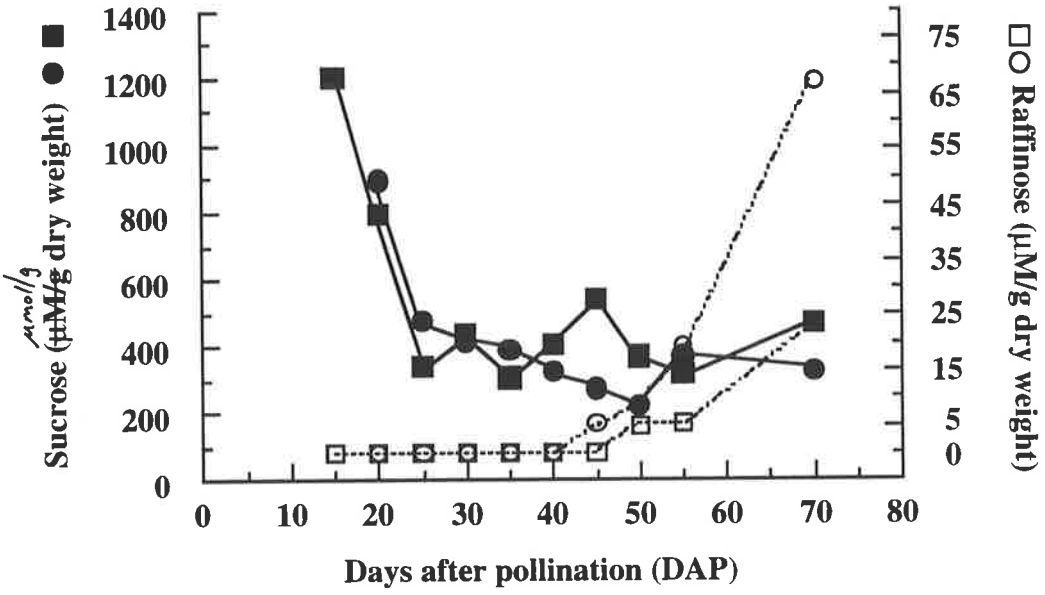


Fig. 3.2 Sucrose and raffinose concentrations in sweet corn embryos during development. Data from set 1 (■, □) and set 2 (●, ○).

also shows that moisture content of developing seeds slowly declines from approximately 85% at 20 DAP to about 50% at 70 DAP (Fig. 3.1).

3.3.2 Sucrose and raffinose concentrations in sweet corn embryos during the corresponding development period

Fig. 3.2 shows that during development there is a rapid rise in raffinose content from zero at 40 DAP to a range of 25-70 µmol/g dry weight at 70 DAP, in marked contrast to sucrose concentration which falls rapidly from 15-25 DAP to a plateau of approximately 400 µmol/g dry weight at 70 DAP.

3.3.3 Germination and seed moisture content

The effect of drying on germination is clearly shown in Fig. 3.3. Drying 55 DAP seeds over various relative humidities (33%, 11% and 1%) markedly enhances germination. At this stage of development, germination of fresh seeds was only 23% (set 1) and 35% (set 2); however, after a drying regime, germination increased and varied over a wide range from 27% to 98%. Drying the seeds over silica gel (1% RH) for 5 or 7 days resulted in higher germination than all other drying regimes. In set 2, the germination was 98% (10% moisture content) and 96% (6% moisture content) after drying over silica gel for 5 and 7 days respectively.

3.3.4 Effect of drying on seed moisture and germination of developing seeds

Table 3.1 shows poor % germination (32-15%) of fresh seeds at 35 to 50 DAP. It was again observed that germination of these less mature seeds can be substantially enhanced by drying the seeds over silica gel for 7 days, and the effect of drying on germination was more pronounced on seeds which were more mature.

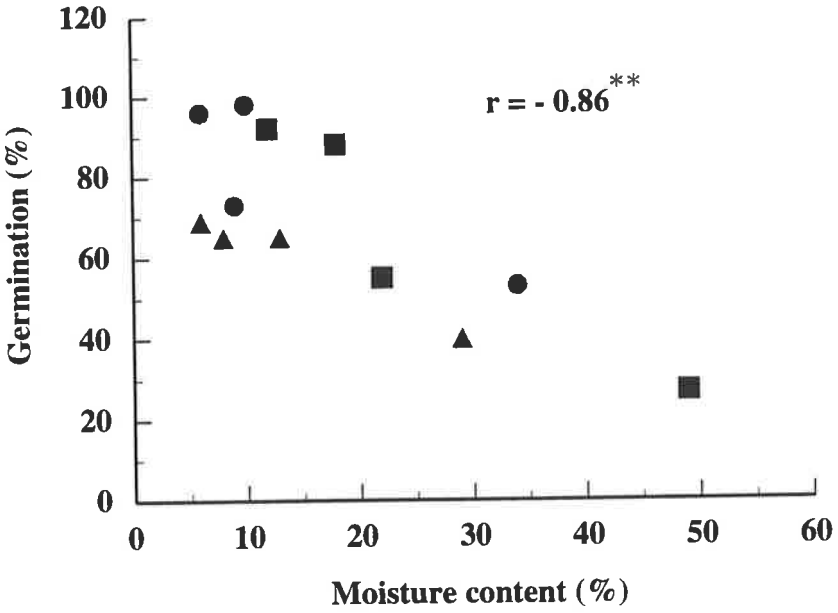


Fig. 3.3 Negative correlation between moisture content (%) and germination (%) of sweet corn seeds. Data from 55 DAP after drying over $MgCl_2$ (■) (33% RH), LiCl (▲) (11% RH) and silica gel (●) (1% RH) for 5 and 7 days.

Table 3.1 Germination of fresh developing sweet corn seeds and effect of drying on seed moisture content and germination.

Days after pollination	Seed conditions	Germination (%)	Moisture content (%)
35	fresh	32	79
	dried*	48	48
40	fresh	17	72
	dried	59	34
45	fresh	15	69
	dried	73	8
50	fresh	19	58
	dried	92	12

* Seeds were dried over silica gel for 7 days.

3.4 Discussion

3.4.1 Germinability of sweet corn seeds during development and effect of drying on germination

The results from these experiments (Fig. 3.1) show that fresh sweet corn seeds are capable of germination during early seed development (35 DAP). However, between 35 DAP and 45 DAP, ~~the seeds apparently lose their germinability.~~ the germinability of the seeds decreases. The bi-modal germination pattern in fresh sweet corn seeds which exhibit two rises in germination with an intervening trough is similar to that reported for wheat seeds by King (1976). However, this pattern may differ among plant species and varieties. For example, germination of fresh wild-type corn seeds was not observed until 50 DAP by Neil *et al.* (1987), while Oishi and Bewley (1992) reported that freshly harvested corn seeds did not germinate until 77 DAP.

It is noticeable that there is a trough of germination in both sets of experiments at 45 DAP (Fig. 3.1). This may be associated with changes in abscisic acid (ABA) concentration as observed for wheat (King 1976). In wheat, when a trough of germination of immature seeds is observed, ABA concentrations have reached their peak (King 1976). However, the concentration of ABA was not investigated in the sweet corn experiment shown in Fig. 3.1. In wheat, Walker-Simmons (1987) has reported that germination takes place when embryos are not sensitive to ABA. In addition, the ability of embryos to resume growth after germination depends upon the ability of aleurone layer to respond to gibberellin produced by the embryos (Oishi and Bewley 1990). In developing sweet corn seeds in this experiment (Fig. 3.1), an early period of development could conceivably elicit many metabolic changes in the embryos. Thus, it can be hypothesised from the literature, that there is a transient increase in ABA concomitant with an increase in sensitivity of embryos to ABA resulting in a trough of germination at 45 DAP. King (1976) also reported a trough of germination in wheat cultivars WW 15 and Gabo at 40 DAA. Further work is required to examine this phenomenon.

Fig. 3.3 confirms that the percent germination of sweet corn seeds can readily be increased by drying the seeds to an appropriate moisture content. However, Table 3.1 also indicates that the improvement in germination produced by drying depends on the stage of development. Although, drying immature seeds (35-40 DAP) to a low moisture content increases germination, the %germination achieved (48-59%) is still low compared to mature seeds (70 DAP, >90% germination). When embryos are mature (70 DAP), and the moisture content is below 60% further desiccation has little effect on germination and most of the seeds germinate without further drying (Fig. 3.1).

3.4.2 Desiccation tolerance during seed development

Bewley (1979), defined desiccation tolerance of seeds as the ability to survive desiccation and resume germination upon subsequent rehydration. Thus those seeds which show more than 50% germination after rapid drying (regardless of what initial % germination is) are interpreted as being desiccation tolerant. If this definition is applied in this study (Table 3.1), the 40, 45 and 50 DAP seeds, which exhibited 59%, 73% and 92% germination after rapid drying would be classified as desiccation tolerant.

In these sweet corn experiments, it was not possible to detect ~~at~~ which stages were desiccation-sensitive, and precisely when the onset of the desiccation-tolerant stage occurred, because immature corn seeds do not germinate and will only show higher germination percentages after dehydration in the desiccation tolerance test (Table 3.1). Germination of corn seeds was suppressed due to an ~~inhibited~~ inhibitive effect of endosperm (Neil *et al.* (1987). To overcome this effect , excised embryos should be used for germination test. Neil *et al.* (1987) found that isolated corn embryos germinate nearly 100% at 20 DAP whereas whole kernels germinate 0%. Bocchichio *et al.* (1988) also reported 100% germination of corn embryos at 16 DAA.

The results of sugar measurements in Fig. 3.2 show that the sugars, sucrose and raffinose are present in the seeds during development as previously observed (Amuti and Pollard 1977). However, the patterns of the concentration (µmol/g dry weight) versus time of these two sugars are quite different, sucrose concentrations tend to decrease to a plateau during development while raffinose concentrations increase at maturity (Fig. 3.2). The data in Figs. 3.1 and 3.2 also suggest that there is no obvious relationship between the initial amounts of sucrose, raffinose and germination, because fresh seeds with either high (20 DAA) or low (25 DAA) amounts of sucrose do not germinate. In addition, the 35-40 DAP seeds are

capable of some germination (20-30%), even though there is no measurable raffinose present.

It has been reported that the high concentration of oligosaccharides detectable in embryos may help desiccation tolerance by stabilising the membranes during drying (Koster and Leopold 1988; Chen and Burris 1990; Leprince *et al.* 1990*a*). Thus, the observed (Fig. 3.2) rapid increase of raffinose at 70 DAP seems to support the role of raffinose in conferring desiccation tolerance at maturity. However, in immature seeds high concentrations of raffinose before drying may not be necessary because Bochicchio *et al.* (1994) found no measurable raffinose in fresh maize embryos (27 DAP) that tolerated desiccation. In the experiment shown in Fig. 3.2 and Table 3.1, similar results have been observed. The initial raffinose content in 45 DAP embryos (which tolerate desiccation) is zero and raffinose concentrations are always low except at maturity (70 DAP) (Fig. 3.2). This leads to the question as to whether or not high concentrations of embryo raffinose prior to drying are really essential for desiccation tolerance. Chen and Burris (1990) have reported that corn embryos had a mass %raffinose/sucrose ratio of 9% which was induced during pre-conditioning at 35°C for 6-48 hours and that this induced sugar ratio correlated with desiccation tolerance. As the sugar concentrations were not measured after drying in the current experiment (Table 3.1), it was still possible that the raffinose concentrations may have been induced during desiccation. Further investigations on the increase in sugar concentrations in embryos during drying are reported in Experiment 2.

In summary, the % germination of fresh immature developing sweet corn seeds is low because of a suppressive effect of endosperm. This suppressive effect can subsequently be derepressed to some extent either by drying or maturation. An increase in sucrose concentration is detectable earlier than the increase in raffinose, but the sucrose concentration declines as the seeds mature. In contrast raffinose concentrations are low (at or near zero) in the early stages, but increase rapidly at

maturity. It appears that in fresh developing sweet corn embryos, high initial concentrations of embryo raffinose are not a good measure of desiccation tolerance since seeds can still tolerate desiccation even when the initial content is zero. The possibility of an increase of sugar concentrations occurring in embryos during desiccation and consequently leading to desiccation tolerance, will be examined in the next experiment (2).

Experiment 2: Sugar concentrations and desiccation tolerance during seed development II Effect of drying on sugar concentrations in sweet corn embryos.

Because the results of the first experiment were not consistent with the view that sucrose and raffinose are essential for conferring desiccation tolerance to embryos, the following experiment was carried out to investigate the role of sugars in more detail. The sugar composition of the embyros were followed using the 'Illini Gold' cultivar of sweet corn, and a variety of desiccation methods were used to manipulate them. The predictive relationship between the mole %ratio of raffinose/sucrose and desiccation tolerance was examined.

3.5 Materials and methods

3.5.1 Plant materials

Three replicates of sweet corn cultivar Illini Gold were grown in the glass house in January 1993-April 1993. Three cobs from each replicate were collected at each harvesting time at 25, 35, 45, 55 and 70 DAP. Seeds were carefully removed from the cobs and pooled. Then they were tested for moisture content, germination and sugar concentrations (see 2.2.1, 2.3 and ~~2.4~~ 2.5).

3.5.2 Drying regimes

3.5.2.1 Desiccation tolerance test (DT)

Ninety fresh seeds (1 replicate) were dried at 25°C for 6 days in a jar (12 cm diameter, 22 cm height) containing 150 g of silica gel (0-4% RH). Fifty seeds were used for moisture determination (25 seeds) and germination (25 seeds), 40 seeds were used for sugar analysis (20) and dry weight determination (20). Seeds were placed on a wire frame approximately 9 cm above the silica gel. The silica gel was replaced during drying when its colour changed from blue to pale blue or white.

3.5.2.2 Slow drying over saturated salt solutions (SD)

Freshly harvested seeds from the middle of the cobs in each replicate were pooled. Two sets of ninety seeds (1 replicate) from the pool were placed in nylon bags and stored in a jar (17 cm diameter, 24 cm height). The seeds were on a wire frame approximately 9 cm above 500 ml of unstirred saturated salt solutions. The jars were incubated in the dark at 25°C for 6 days. The descending RH series of saturated salt solutions were : KCl (87% RH); $Mg(NO_3)_2$ (55% RH) and $MgCl_2$ (32% RH) (Blackman *et al.* 1991). Seeds were carefully transferred from one jar to the next in the series every two days.

3.5.3.3 High relative humidity conditions (HRH)

For high relative humidity conditions (HRH), 2 sets of ninety seeds were stored in a jar (see 3.5.2.2) containing 500 ml of distilled water. Wet filter paper (Whatman No. 1) was placed into each jar to maintain the HRH throughout the incubation period. The jars were incubated in the dark at 25°C for 6 days.

After 6 days of incubation for SD and HRH seeds, one set of seeds was tested for germination, seed moisture content, embryo dry weight and sugar concentrations

(see 2.2.1, 2.3 and 2.4). Another set was tested for desiccation tolerance by transferring the seeds to another jar (12-cm-diameter, 22-cm-height) (see 3.5.2.1). After the test, the seeds were tested for germination, seed moisture content, embryo dry weight and sugar concentrations (see 2.2.1, 2.3 and 2.4).

3.5.3.4 Statistical analysis

Data from fresh developing seeds at 25, 35, 45, 55 and 70 DAP and for seeds after incubation were analysed by a completely randomised design using the 'Genstat' program (Genstat 5 Committee 1987). For the incubation treatments; the two factors consisted of 1) 5 stages of development : 31, 41, 51, 61 and 76 days ; 2) 3 incubation treatments : high relative humidity (HRH) conditions, slow drying (SD) and desiccation tolerance test (DT).

For the desiccation tolerance test on HRH and SD seeds, the same design was used. Two factors were 1) 5 stages of development : 37, 47, 57, 67 and 82 days ; 2) 2 incubation treatments : HRH and SD.

3.5.3.5 Calculation of %ratio raffinose/sucrose

Sucrose and raffinose were measured and expressed as µmol/g dry weight (DW). The mole %ratio of raffinose/sucrose was calculated as follows :

$$\text{mole \% ratio} = \frac{\text{µmol/g DW raffinose x 100}}{\text{µmol/g DW sucrose x 1}}$$

Mass %ratio was also calculated from the sugar measured as mg/gDW to facilitate comparison with the work of Chen and Burris (1990).

3.6 Results

3.6.1 Sugar composition and raffinose/sucrose ratio (%) in sweet corn embryos during seed development and after incubation treatments

As previously observed during seed development (Fig. 3.2), the sucrose concentrations of fresh embryos (Table 3.2) from 25 DAP to 70 DAP were in the range 390-583 µmol/g dry weight (Table 3.2). Raffinose again appears to accumulate in the later stages of development at 45 DAP. At the earlier stage, from 25 to 35 DAP, embryo raffinose concentrations are undetectable in fresh seeds (Table 3.2). Mole % ratio of raffinose/sucrose were in a range from 0%-12.3% (Table 3.2). The ratios are zero at 25-35 DAP but increase rapidly to 7.2% at 45 DAP. The maximal mole ratio (12.3%) is evident at maturity (70 DAP).

Marked changes in sugar concentrations were observed when seeds were subjected to various RH treatments (Table 3.2). High concentrations of embryo sucrose are evident after immature seeds at 25 and 35 DAP are slowly dried (SD) for 6 days. The amounts of sucrose increased to 16 and 18 fold in 31 and 41 day embryos respectively, when compared with those stored under HRH conditions at the same corresponding ages. The induction of sucrose and raffinose after such SD treatments is less in the more mature seeds at 51, 61 and 76 days (Table 3.2). It is noticeable that the abnormally high concentrations of sucrose and raffinose induced by SD treatments in 31 and 41 day embryos are much higher than those accumulating in fresh embryos at all stages of development.

Table 3.2 Effect of drying on moisture content, germination and sugar concentrations of sweet corn embryos. Data shown for seeds at different stages of development and after each was dried for 6 days as described in 3.5.2. Abbreviations : DAP, days after pollination; HRH, high relative humidity conditions; SD, slow drying over saturated salt solutions; DT, desiccation tolerance test.

DAP/DAP +6 days of drying	Seed conditions		Moisture content (%)	Germination (%)	Sucrose (µmol/gDW)	Raffinose (µmol/gDW)	Mole ratio (%) raffinose/ sucrose
	Fresh seeds	Stored seeds					
25/	F		73.0 A[1]	4.0 D	413 ns	0	0 (0)[3]
31		HRH	76.7	20.0	334	4	1.2 (1.7)
		SD	52.8	42.7	5516	131	2.4 (3.5)
		DT	11.7	61.3	504	35	6.9 (10.2)
35/	F		72.0 A	18.7 C	389	0	0 (0)
41		HRH	74.6	21.3	240	15	6.2 (9.2)
		SD	57.3	36.0	4522	370	8.2 (12.0)
		DT	10.4	61.3	719	38	5.3 (7.9)
45/	F		64.8 B	22.7 C	423	30 C	7.2 (10.6)
51		HRH	62.2	58.7	396	44	10.9 (16.2)
		SD	25.3	94.7	575	46	8.2 (11.8)
		DT	3.9	93.3	434	54	12.4 (18.3)
55/	F		54.8 A	65.3 B	583	44 B	7.6 (11.2)
61		HRH	55.4	62.7	275	37	13.4 (19.7)
		SD	10.0	90.7	616	61	9.9 (14.6)
		DT	4.2	96.0	613	48	7.9 (11.6)
70/	F		36.8 A	97.3 A	482	60 A	12.3 (18.2)
76		HRH	37.5	98.7	498	24	4.8 (7.1)
		SD	6.9	97.3	508	60	11.9 (17.5)
		DT	3.4	98.7	574	64	11.2 (16.5)
L.S.D.$_{(.05)}$[2]			5.3	-	502	44	-

1. Means within a column for 25-70 DAP followed by the same capital letters are not different at p≤0.05 content; ns = not significantly different among DAP.
2. L.S.D.$_{(.05)}$ for all incubation treatments from 31 to 76 DAP.
3. Mass %ratios of raffinose/sucrose in brackets were calculated from mg/g DW units.

At 25-35 DAP under HRH for 6 days, low concentrations of raffinose (3.9 and 14.8 μmol/g DW) accumulate in the embryos even though the seeds do not lose moisture content (Table 3.2). This is in marked contrast to the corresponding fresh sweet corn embryos from 25 to 35 DAP, where there is no accumulation of raffinose at all.

The mole %ratio raffinose/sucrose in HRH-treated embryos (Table 3.2) increases from 1.2% at 31 days to 13.4% at 61 days. Although very high concentrations of sucrose and raffinose are evident at 31 and 41 days in SD-treated seeds, the mole %ratio raffinose/sucrose is only 2.4% in 31 day embryos and 8.2% in 41 day embryos.

3.6.2 Desiccation tolerance test (DT) on fresh developing seeds and sugar concentrations in the seeds after the test

After the desiccation tolerance test (DT) with 6 days drying, the amount of raffinose in 31 day embryos increases up to 35 μmol/g dry weight while the content in fresh developing 35 DAP embryos is 0 μmol/g dry weight (Table 3.2). The raffinose concentrations increased in 31 day embryos undergoing DT is ultimately only approached at a much later stage (45 DAP) by fresh developing embryos (30 μmol/g dry weight). By contrast, the sucrose concentrations in the embryos after DT show only a slight increase; and the concentrations are not significantly different from those of controls (HRH) at the same stage of development (Table 3.2).

The mole %ratios of raffinose/sucrose increase in the embryos after DT (Table 3.2) from 0% in both 25 and 35 DAP embryos to 6.9% and 5.3% in 31- and 41-day embryos. An increase of the ratio in 31- and 41-day embryos coincides with an increase of desiccation tolerance, e.g. 31 and 41 day seeds survive DT and exhibit 61.3% germination.

3.6.3 Germinability of seeds and acquisition of desiccation tolerance during seed development

Table 3.2 shows that the steady increase in % germination of fresh seed from 4.0% to 97% from 25 DAP to 70 DAP developmental period coincides with steady decrease in seed moisture content from 73.0% to 36.8% at 70 DAP and the termination of dormancy. It is well documented that immature corn seeds do not germinate (Sprague 1936; Kiesselbach 1939; Oishi and Bewley 1992), however, seeds increase in their germinability as they are mature toward 70 DAP.

Germination of seeds always increased from 25-55 DAP independent of whether the seeds were subjected to slow or rapid drying for 6 days (Table 3.2). This table also shows that in 25 and 35 day seeds, rapid drying in the desiccation tolerance test (DT) enhances seed germination to a higher percentage than does slow drying.

To summarise, the increased germination of 25 DAP seeds after rapid drying indicates that these seeds will tolerate desiccation. Furthermore, the ability of seeds to withstand the rapid desiccation of the DT test gradually increases during seed development, ~~and presumably reflects a decrease in dormancy effects~~.

3.6.4 Germination and embryo sugar concentrations of HRH and SD seeds after the desiccation tolerance test

Table 3.3 shows that moisture contents of HRH and SD seeds after DT were in a range of 2.9-7.8% and that germination ranged from 33.3% to 96.0%. As mentioned previously, a decrease of dormancy was observed after DT and when the seeds were mature (Table 3.3).

By comparison with the data (Table 3.2) before the DT test, it is noticeable that for HRH seeds, sucrose and raffinose concentrations increase after DT (Table 3.3). In contrast to SD treatments of 37 and 47 days, DT results in a massive decrease of sucrose and raffinose. The ratios of (%) raffinose/sucrose of HRH

and SD embryo before (Table 3.2) and after DT (Table 3.3) are similar except in 37 day embryos of HRH treatment which show an increase of the ratio after DT (Table 3.3).

Table 3.3 Effect of drying on seed moisture content, germination and sugar concentrations of sweet corn embryos. Data shown at different stages of development after desiccation tolerance test (DT) for 7 days. Abbreviation : HRH, seeds stored at high relative humidity conditions for 6 days before DT; SD, seeds were slow dried over saturated salt solutions for 6 days before DT.

Stages of development (days)	Seed conditions	Moisture content (%)	Germination (%)	Sucrose (µmol/gDW)	Raffinose (µmol/gDW)	Mole ratio (%) raffinose/ sucrose
37	HRH	7.8	33.3	504	30	5.9 (8.7)[2]
	SD	4.4	69.3	695	18	2.6 (3.8)
47	HRH	5.6	33.3	475	34	7.2 (10.6)
	SD	3.7	72.0	501	42	8.4 (12.4)
57	HRH	3.7	69.3	625	60	9.5 (13.9)
	SD	3.4	90.7	600	48	8.0 (11.7)
67	HRH	3.5	81.3	677	62	9.2 (13.6)
	SD	2.9	89.3	598	56	9.3 (13.8)
82	HRH	3.9	93.3	708	42	6.0 (8.8)
	SD	3.0	96.0	842	103	12.2 (17.9)
L.S.D. (.05)		1.0	-	ns[1]	33	-

1. ns = non significantly different among treatments.
2. Mass %ratios of raffinose/sucrose in brackets were calculated from mg/g DW units.

3.7 Discussion

This experiment shows that sucrose and raffinose concentrations can be increased by drying, but under HRH conditions raffinose can accumulate in embryos without moisture loss during storage. The results indicate that the initial concentrations of sucrose and raffinose as well as the %ratio raffinose/sucrose in fresh developing seeds are not good criteria for determining desiccation tolerance. Similarly for detached seeds subjected to slow drying, these parameters are poor predicters of desiccation tolerance.

The present findings confirm the results obtained in Experiment 1 that there is no accumulation of raffinose in embryos of the seeds at the onset of the desiccation tolerance test (25-35 DAP). Table 3.2 shows that most 25- and 35-day seeds survive the desiccation tolerance test (DT) and have a 61.3% germination. The %germination obtained here is presumably partially suppressed by dormancy. Thus, the fresh seeds at 25-35 DAP are desiccation tolerant but raffinose content in the embryos at this stage is 0 μmol/g dry weight (Table 3.2).

Fresh 25 and 35 day embryos have no raffinose, but synthesis of raffinose is observed in the embryos after drying for 6 days in the DT test. Although this is consistent with raffinose production having a role in desiccation tolerance, nevertheless Table 3.2 clearly shows that there is a tremendous increase of raffinose content in the embryos of the 25 and 35 DAP seeds after slow drying (SD) but the germination percentages are always lower than those in the DT test, where the embryos have much lower sugar concentrations. The high seed moisture contents after SD (52.8% and 57.3% at 31 and 41 day) compared to more mature seeds may also be a consequence of the hygroscopic nature of sucrose and raffinose solutions. In all these experiments there is again an inverse correlation between moisture content and germination as noted previously. Furthermore, seeds stored under high relative humidity (HRH) conditions at 31 days exhibit a

slight increase of embryo raffinose, although they do not lose moisture content. An explanation for the latter observation is that detachment the seeds from parent plants may trigger the mechanisms of raffinose synthesis. An increase of raffinose concentrations in the seeds after drying (SD, DT) or without drying in the case of 31 and 41 day HRH embryos suggests that metabolic changes are still taking place in the embryos after harvesting. Sugar induction could be the response of the seeds to stress conditions, i.e. detachment from the parent plants. Drying possibly stimulates sugar metabolism to proceed more rapidly, but increased sugar concentrations are neither necessary for desiccation tolerance nor for germination (Tables 3.2 and 3.3).

To test whether sugars play a role in desiccation tolerance in stored detached seeds, seeds with embryo sugar induced to different concentrations need to be tested for DT. If higher concentrations of sugars are found to coincide with higher %germination after DT, the role of sugars in desiccation tolerance would be more clearly established.

Blackman *et al.* (1992) were able to increase stachyose concentrations in immature soybean seeds (34 days after flowering) by slow drying and demonstrated that an increase in stachyose concentrations were associated with desiccation tolerance. Soybean seeds which were stored under HRH conditions had low concentrations of stachyose, and such seeds did not survive desiccation. In the experiment shown in Table 3.2, differences in sugar concentrations in SD and HRH embryos are observed at 31 and 41 days. However, the degree of desiccation tolerance is not comparable (Table 3.3) because of seed dormancy, as discussed previously in Experiment 1. Using the survival of seeds after rapid drying as the criterion for desiccation tolerance, the seeds exhibit >50% germination after drying were interpreted as being desiccation tolerant. The data in Table 3.3 suggest that % mole ratios of sugars do not consistently predict desiccation tolerance. To make the data in these experiments comparable to those previously reported by Chen and

Burris (1990), % mass ratios raffinose/sucrose ratios were also calculated on mg/g DW unit. However, the mass %ratios calculated in Table 3.2 and Table 3.3 for 31 days SD seeds before (3.5%) and after the DT test (3.8%) with 42.7% and 69.3% germination respectively were still markedly lower than the mass ratio of 9% suggested by Chen and Burris (1990) for desiccation tolerance in maize embryos, which casts doubt on the universality of such ratios as predictors for desiccation tolerance in different varieties.

It is noticeable that after DT testing of 31 and 41 HRH seeds, sucrose and raffinose increase in the embryos at 37 and 47 days (Tables 3.2 and 3.3). This is again supportive evidence indicating that drying induces sugar synthesis. However, in SD treatments (Table 3.2; 31 and 41 days) before DT, embryos have very large concentrations of sugars, and the DT actually decreases sugar concentrations (37 and 47 days) (Table 3.3). It is possible that in 31- and 41-day embryos, sucrose and raffinose concentrations are induced by SD to maximum concentrations. Further drying (DT) may not facilitate sugar synthesis because the synthesis starts to slow down and then is finally inhibited when seed moisture contents are low (<10%).

These experiments clearly show that an increase of sucrose and raffinose takes place in 31 and 41 day embryos after slow drying (Table 3.2). An increase of sucrose and raffinose concentrations after drying indicates that enzymes for the synthesis of the sugars must be present, and drying triggers the activity of these enzymes (Bewley and Black 1994). As an increase of sucrose concentrations is concomitant with an increase of raffinose after SD (Table 3.2), it suggests that embryos may have sucrose (being a raffinose substrate) transported from elsewhere for raffinose synthesis. Black *et al.* (1995) suggested that starch breakdown in embryos does not account for the increase of raffinose in wheat embryo during development. Thus, sucrose possibly moves directly from the endosperm into the embryos during desiccation.

In germinated seeds, the degradation of starch and lipid is well documented (Newman and Briggs 1976; Beevers 1980). The final step of such degradation is the conversion of glucose to sucrose which is then mobilised to the growing point, i.e. embryonic axis. However, in the case of desiccation, sucrose may be incompletely utilised for the growth of the embryonic axis, leading to an accumulation of sucrose in the tissue.

It is still possible for metabolic changes to occur during SD such as protein synthesis (Adams *et al.* 1983; Rosenberg and Rinne 1986). For example, high concentrations of low molecular weight carbohydrates sugars depend upon an increase of α-amylase activity (Nicholls 1979) due to a sensitivity of aleurone layer to gibberellin induced by drying (Oishi and Bewley). The high concentrations of sucrose and raffinose may simply represent a readily-available energy source for metabolic processes occurring in the embryos during slow drying.

To summarise, in fresh developing seeds raffinose is initially absent from sweet corn embryos at the stage when most seeds survive desiccation. Increased concentrations of sucrose, raffinose and mole %ratio of raffinose/sucrose can be induced by rapid drying. Thus, sugar may play a protective role during drying. However, in immature (25 and 35 DAA) stored detached seeds, very high sugar concentrations can be induced by slow drying and storing the seeds at high moisture conditions and yet not enhance the %germination. From these data it can be concluded that neither the absolute concentrations nor the raffinose/sucrose ratio are uniformly associated with either germination or desiccation tolerance, which is in accord with the conclusion of Leprince *et al.* (1993) that the ability to tolerate desiccation is not solely due to the presence of these saccharides.

Experiment 3: Wheat embryos, desiccation tolerance, and the effect of drying on sugar concentrations

3.8 Materials and methods

3.8.1 Plant materials

Wheat cultivar 'Halberd' was grown in the field at Waite Agricultural Research Institute during June 1992-December 1992. Plots were divided into three small plots (replicates). As one or two spikelets on the ears underwent anthesis, a number of ears were tagged and the dates thereafter were recorded as days after anthesis (DAA). The ears were taken from the plants at 20, 25, 30, 35, 40, 50 and 60 DAA. At each harvesting date, 40 ears of each replicate were harvested. Fifteen seeds from the spikelets in the middle of the ears were removed and then pooled. Samples were drawn from the pool of each replicate for germination tests, embryo moisture content determination, desiccation tolerance tests and sugar analysis (see 2.2.1, 2.3 and 2.4).

3.8.2 Drying regimes

3.8.2.1 Desiccation tolerance test (DT)

Eighty five seeds for each replicate (40 seeds for sugar analysis, 20 seeds for moisture content determination, 25 seeds for seed germination) were stored in a jar (12 cm diameter, 22 cm height) containing 100 gm of silica gel. The jars were stored in the dark at 25°C for 7 days. After the test, seeds were tested for sugar concentrations, moisture content and germination (see 2.2.1, 2.3 and 2.4).

3.8.2.2 High relative humidity conditions (HRH)

Eighty five seeds were stored in a jar (12 cm diameter, 22 cm height) containing 200 ml of distilled water. Wet filter paper (Whatman No.1) placed into the jar to

maintain the high relative humidity. The jars were stored at the same conditions (see 3.11.1). After 7 days of incubation, the seeds were tested for germination, embryo moisture contents and sugar concentrations (see 2.2.1, 2.3 and 2.4).

3.8.2.3 Statistical analysis

A completely randomised factorial design with three replicates was set up. The three factors consisted of:

1) 7 harvesting dates: 20, 25, 30, 35, 40, 50 and 60 days after anthesis (DAA)

2) 2 methods of incubation: 1) desiccation tolerance test (DT) and 2) incubation at high humidity conditions (HRH)

3) 2 methods of germination tests: 1) the outer pericarp of the seeds were removed before the germination test and 2) the seeds were germinated with an intact pericarp.

In order to reduce variation of data obtained from samples, %germination was transformed to arcsin$\sqrt{}$percentage before analysis of variance.

3.9 Results

3.9.1 Moisture content, germination and sugar concentrations during wheat seed development

Changes in moisture content, germination and sugar concentrations during seed development are shown in Figs. 3.4a, 3.5 and 3.6. At 20-30 DAA (Fig. 3.5), fresh germination (with outer pericarp removed) is approximately 20 %. The germination increases from 30% at 35 DAA to 95% at 60 DAA. Removal of the outer pericarp only slightly improves germination of fresh seeds. It was found that stages of development, rather than whether or not the outer pericarp has been removed, had the most pronounced effect on germination (Fig. 3.5).

Fig. 3.6 shows that sucrose and raffinose concentrations both show a peak near 35 DAA and diverge subsequently with sucrose reaching a maximum at 60 DAA whilst the raffinose concentration steadily declines.

3.9.2 Effect of desiccation on seed moisture content, germination and sugar concentrations

Figs. 3.4a, b show that after the desiccation tolerance test (DT), the moisture content of seeds is reduced below the 10% plateau of 50-60 DAA seeds which have developed normally.

Germination of seeds incubated under these alternative high and low humidity regimes for 7 days at HRH conditions or after DT is shown in Figs. 3.7a, b. In both cases germination is enhanced by pericarp removal. The pericarp effect is more pronounced in the early stages (27-42 days) and is suggestive of some inhibitory factor derived from the pericarp. Fig. 3.7b shows that desiccation tolerance is observed at 32 days. When the pericarp is removed seeds have approximately 80% germination after rapid drying (desiccation tolerance test), but tolerance is delayed for a further 10 days in its presence.

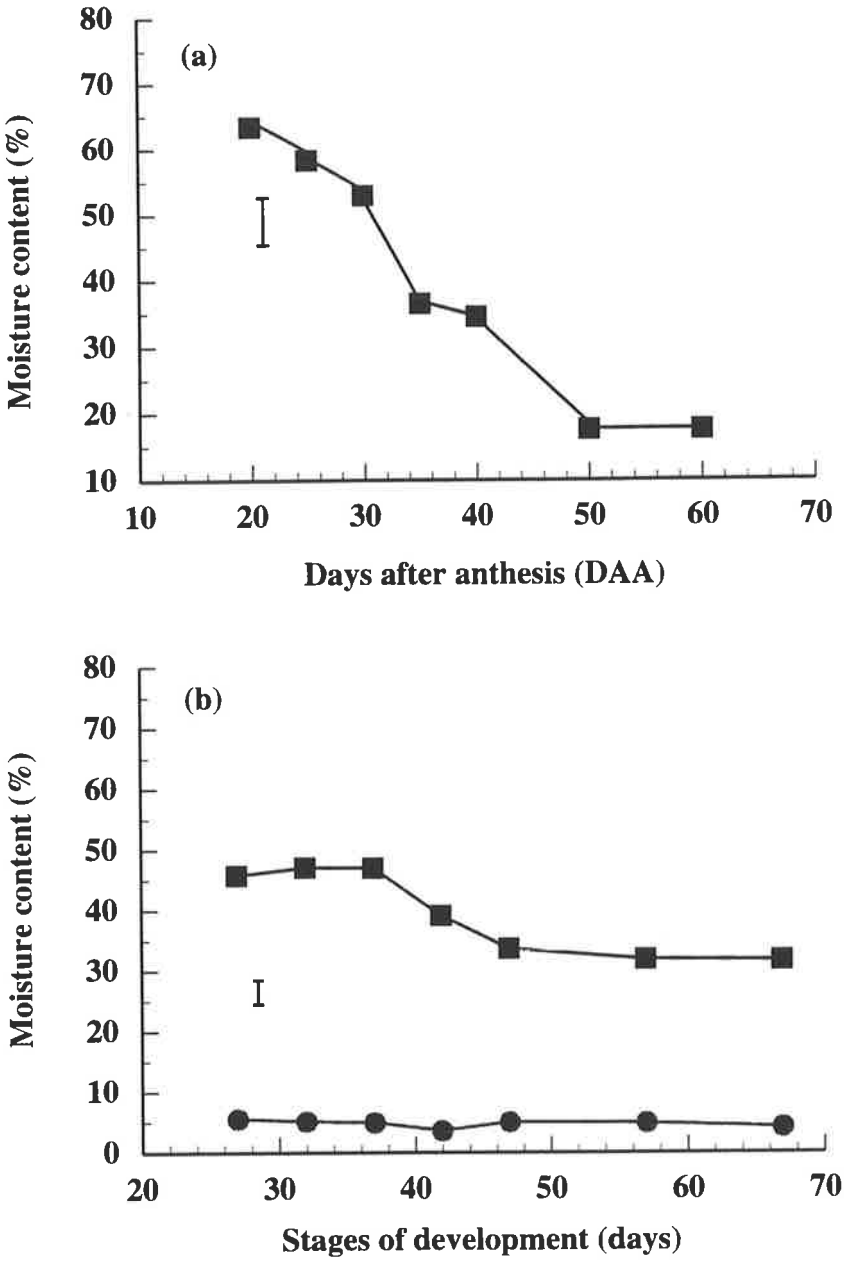


Fig 3.4 Moisture content (%) of wheat seeds **(a)** during seed development **(b)** after the seeds were stored under high relative humidity; HRH (■) or after the desiccation tolerance test; DT (●). Vertical bars are $l.s.d._{(.01)}$ for **(a)** mean difference of stages of development (days after anthesis) **(b)** the interactions between stages of development and drying methods.

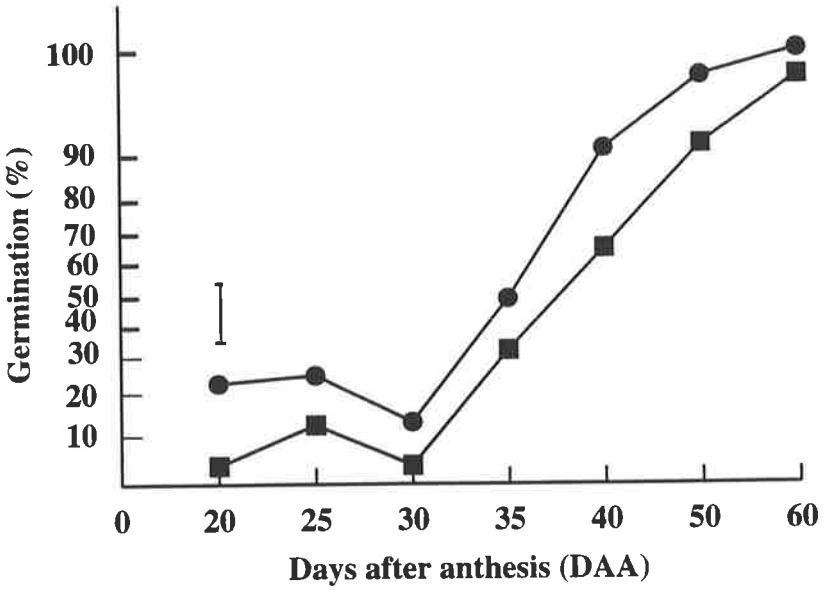


Fig. 3.5 Germination of fresh seeds during development, germination was from either intact seeds (■) or seeds with the outer pericarp removed (●). Vertical bar is the l.s.d.$_{(.05)}$ for the main effect of stages of development (days after anthesis).

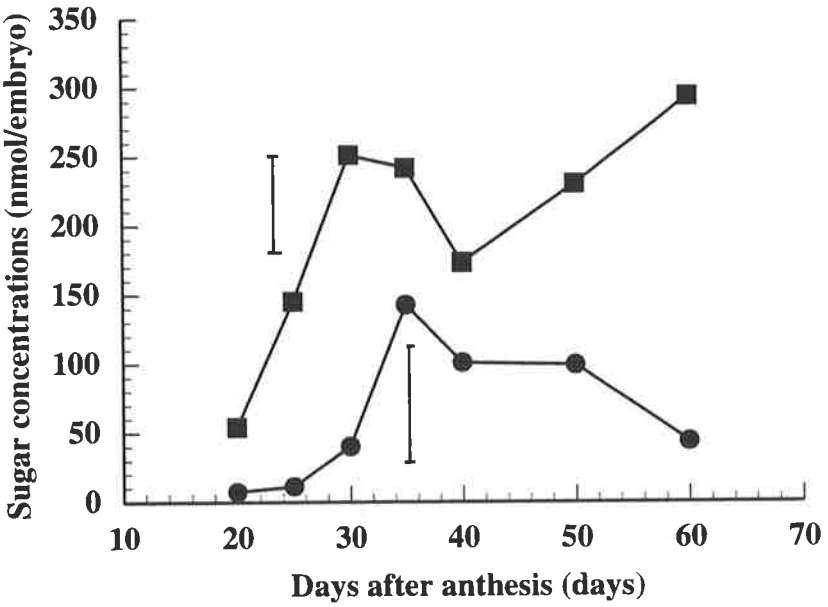


Fig. 3.6 Sucrose (■) and raffinose (●) concentrations in wheat embryos during seed development. Vertical bars are the l.s.d.$_{(.05)}$ for stages of development (days after anthesis).

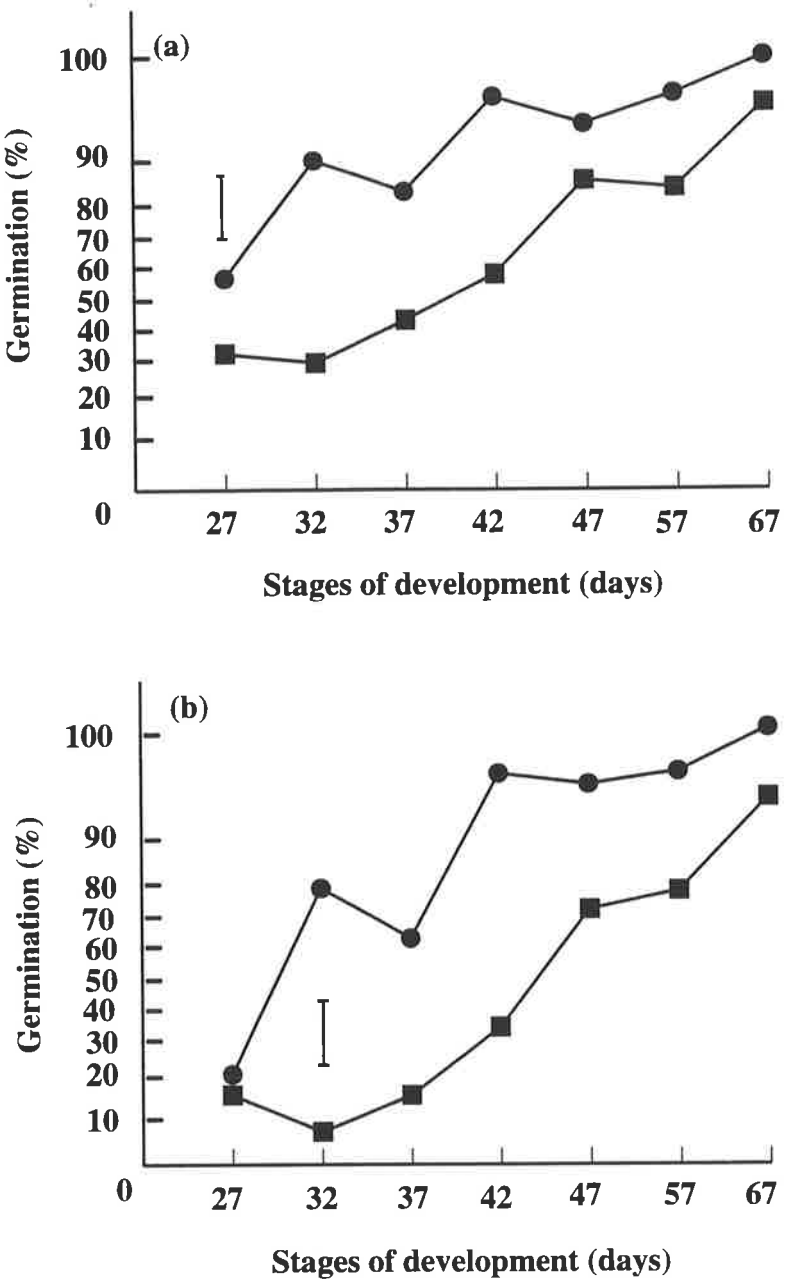


Fig. 3.7 Germination of wheat seeds with either outer pericarp removed (●) or intact (■) after the seeds were incubated at 25°C for 7 days (**a**) under high relative humidity conditions; HRH or (**b**) after desiccation tolerance test; DT. Vertical bars are the l.s.d.$_{(.05)}$ for the main effect of stages of development.

Figs. 3.8a, b show sugar concentrations in embryos of seeds stored at HRH conditions and DT for 7 days. Except at 42 and 46 days, there is not a significant difference in the increase in sucrose concentrations (Fig. 3.8a). Raffinose concentrations are high in 27 day seeds stored under HRH conditions (Fig. 3.8b), 3 fold higher than that of DT seeds (Fig. 3.8b). However, the final raffinose concentrations in 67 day embryos (60 days+7 days of drying) are not vastly different from those in fresh developing embryos at 60 DAA (Figs. 3.6, 3.8b).

Two features stand out in these high and low relative humidity experiments. First, there appears to be a repressive effect on germination of wheat embryos by the presence of the pericarp in the early stages of development. However, due to the large standard error in the current study, this was not statistically significant although other workers have made similar observations (Wellington 1956; Radley 1976; Symons *et al.* 1983). Second, whereas the sucrose content of the embryo reaches a final maximum towards maturity under conditions of either high or low humidity, the maximum raffinose content is induced under high humidity at 25 days and thereafter it declines rapidly. Under conditions of the desiccation tolerance test, the ultimate raffinose concentrations are not different from those of developing seeds.

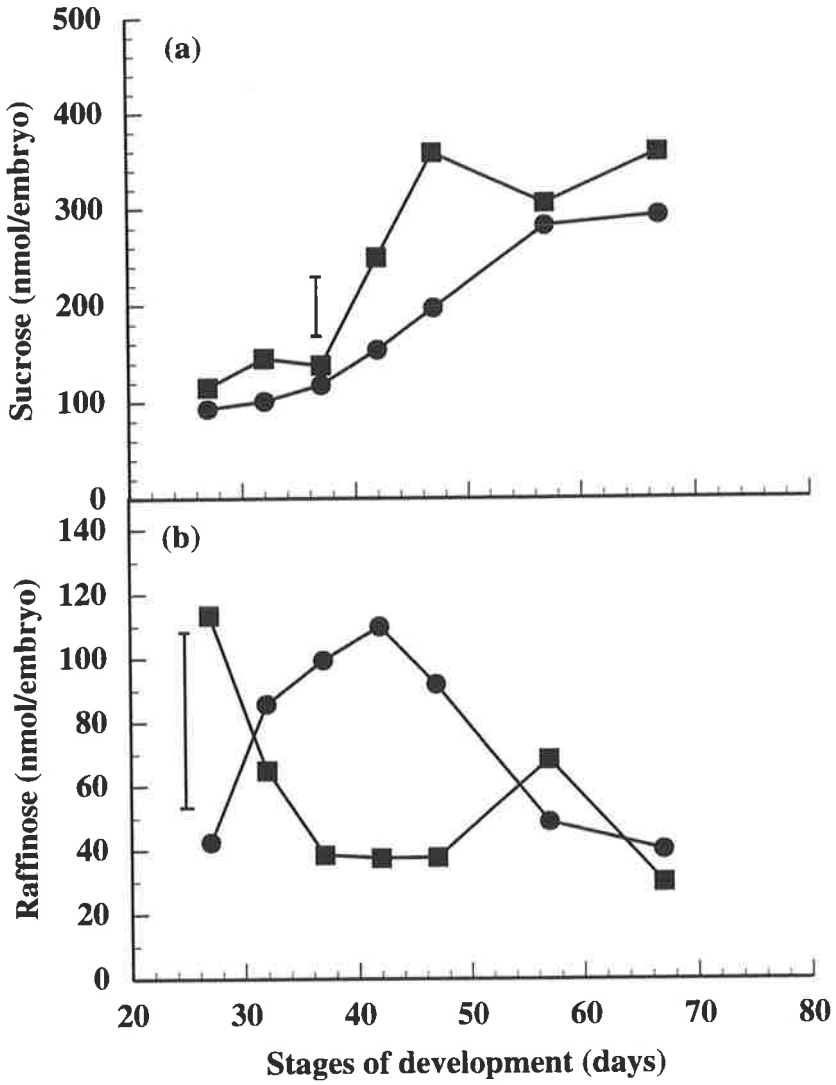


Fig 3.8 **(a)** Sucrose and **(b)** raffinose concentrations of wheat embryos after the seeds were incubated at 25°C for 7 days under high relative humidity conditions ; HRH (■) or after desiccation tolerance test; DT (●). Vertical bars are l.s.d.$_{(.05)}$ for **(a)** the main effects of stages of development and **(b)** the interactions of the effect of stages of development and drying methods.

Experiment 4: Sugar concentrations in wheat embryos at 20 and 25 DAA after the seeds have been stored under different relative humidity conditions

The results in experiment 3 showed that raffinose concentrations increased rapidly in immature embryos of wheat seeds at 27 days when stored under HRH conditions, just as in the case of the embryos of detached sweet corn seeds. To examine this response, raffinose and sucrose concentrations have been monitored in the embryos of seeds stored under different relative humidity conditions. Because detached seeds continue to develop during desiccation, care has been taken to compare data with fresh developing seeds of the same corresponding stage of development.

3.10 Materials and methods

3.10.1 Plant materials

Wheat ears were collected at 20 and 25 DAA from the plants grown in the glasshouse at Waite Agricultural Research Institute during May 1993-September 1993. For one replicate at each harvesting date, 6 ears were collected. Sixty seeds (40 seeds for sugar analysis and 20 seeds for embryo moisture content determination) were used for each incubation treatment.

3.10.2 Statistical analysis

The split-plot design with 3 replicate experiments was set up. Main plots consisted of 2 periods of drying: 3 and 7 days), subplots consisted of 5 methods of incubation [five relative humidity (RH) conditions to obtain different moisture contents]. Treatments consisted of:

1) distilled water+wet filter paper (98% RH)
2) distilled water (96% RH)

3) saturated salt solutions: $Mg(NO_3)_2$ (55% RH) for 3 days of incubation; a series of 3 days over $Mg(NO_3)_2$ (55% RH), 4 days over $MgCl_2$ (35% RH) for 7 days of incubation.
4) 98% glycerol (12% RH)
5) silica gel (0-2% RH)

Embryos from fresh developing seeds at the same corresponding ages of drying were incorporated for a comparison among treatments.

3.11 Results

3.11.1 Moisture content and sugar concentrations of seeds under different drying conditions

The moisture content of seeds stored under various RH conditions is shown in Figs. 3.9a, d. All lower humidity concentrations (55/35%, 12% and 0-2%) reduced actual seed moisture content to <10% after 7 days, whereas those of HRH treatments were in the range 65-75% (Figs. 3.9a, d).

During drying, sucrose concentrations of detached seeds increased in the 20 DAA (Fig. 3.9b) treatments but decreased in 25 DAA (Fig. 3.9e) treatments. In contrast, raffinose concentrations increased after 3 and 7 days of incubation in both 20 and 25 DAA embryos (Figs. 3.9c, f). It is noticeable that at 25 DAA, HRH conditions can enhance embryo raffinose content without a substantial loss of moisture from the seeds (Fig. 3.9d, f).

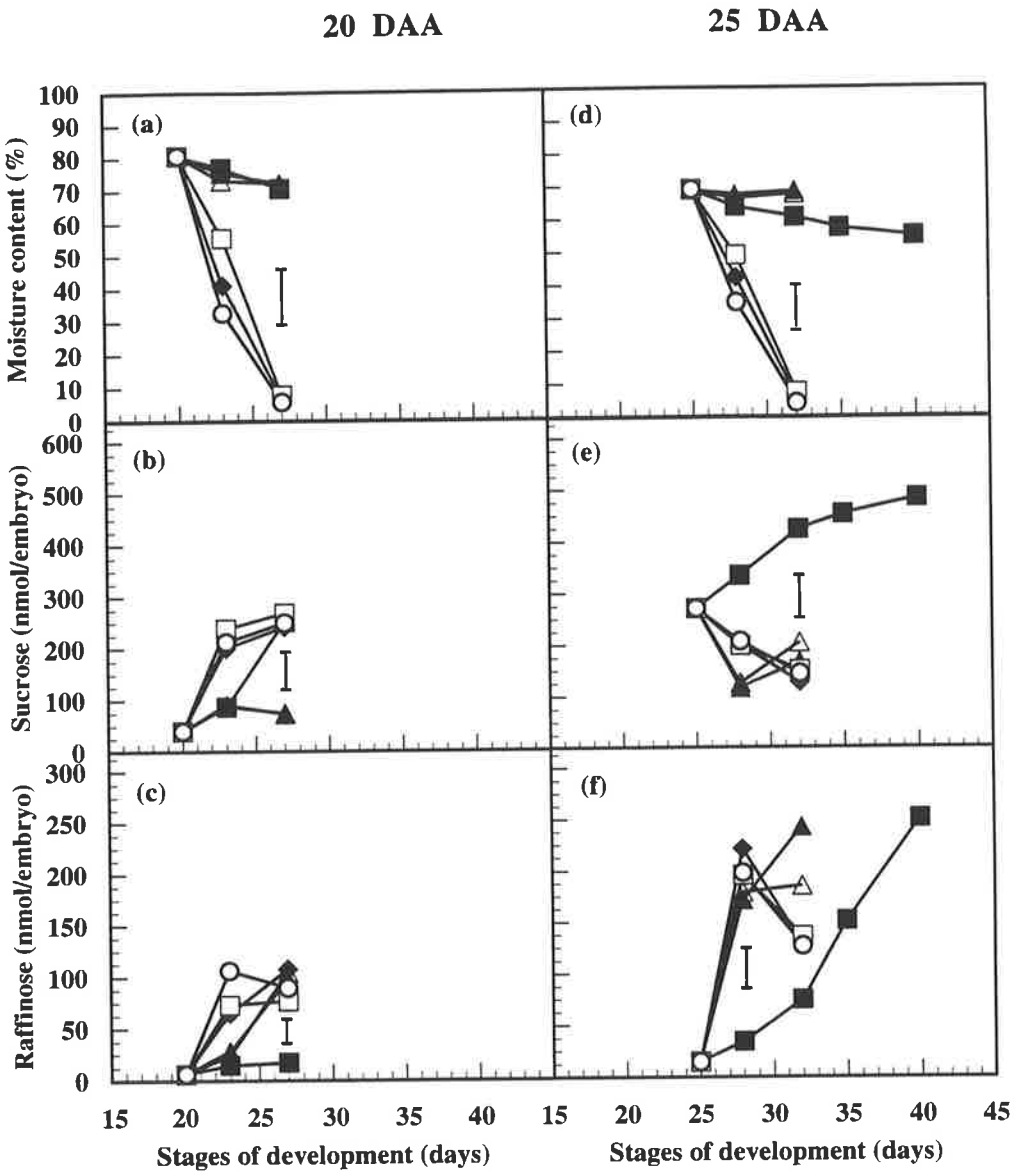


Fig. 3.9 Seed moisture content (%) (a, d), sucrose (b, e) and raffinose concentrations (e, f) (nmol/embryo) in embryos of wheat seeds at 20, 25 DAA during development on the plants (■) or after seed detachment and drying for 3 or 7 days under conditions as followed : 1) 98% RH (▲), 2) 96% RH (△), 3) 55/35% RH (□), 4) 12% RH (◆) and 5) 0-2% RH (○). Vertical bars are l.s.d.$_{(.05)}$ for mean difference among drying methods at the same period (3 or 7 days) of incubations.

The key observations from this experiment are that first the mole %ratios of raffinose/sucrose in 20 and 25 DAA embryos are 16.2 and 5.4 respectively (Fig. 3.10) and secondly, increased ratios can be induced under both HRH conditions and with slow drying. In particular, it was found that at both 20 and 25 DAA, the mole %ratios of raffinose/sucrose of seeds stored under HRH conditions were higher than either those of slow or rapid-dried seeds after 7 days of incubation.

Experiment 5: Desiccation tolerance and sugar concentrations in wheat embryos at 15 DAA

Experiment 4 showed that in wheat embryos raffinose concentrations can be adjusted by varying the RH conditions. Thus, to test the role of sucrose and raffinose in conferring desiccation tolerance in detached seeds, two conditions of drying were selected, i.e. 98% and 55/35% RH as these had previously shown markedly differing ratios of raffinose to sucrose (Fig. 3.10). These seeds, having different raffinose to sucrose ratio were tested for the degree of desiccation tolerance.

3.12 Materials and methods

3.12.1 Plant materials

Wheat plants were grown as previously described. At 15 DAA, ears were collected from the plants and seeds from the middle of the ears were removed and then pooled. Samples were then drawn from the pool for embryo moisture content and sugar analysis (see 2.3 and 2.4).

3.12.2 Embryo germination and desiccation tolerance test

Embryos were aseptically removed from the ears and they were germinated on artificial media (see 2.2.2). For the desiccation tolerance test, see 2.4.

3.12.3 Statistical analysis

Data were analysed as split plot design with 3 replicates.

Main plots are: 2 periods of incubation; 7 and 6 days

Sub plots are: 2 methods of incubation

1) incubation over distilled water+filter paper (98%RH)
2) incubation over saturated salt series i.e. 3 days over $Mg(NO_3)_2$ (55% RH) and 4 days over $MgCl_2$ (35% RH)

The first 7 days of incubation is a period to observe changes in sugars as a result of slow drying. The second 6-day incubation period was used for the desiccation tolerance test under conditions of rapid drying. The mole %ratios of sugars were calculated from raffinose and sucrose concentrations measured as µmol/embryo.

3.13 Results

3.13.1 Desiccation tolerance of 15 DAA seeds

Table 3.4 shows that when freshly excised 15 DAA embryos were used for the germination test, no inhibition of germination was observed and embryos showed 100% germination. Thus, at this 15 DAA stage a test for a degree of desiccation tolerance is feasible. After the desiccation tolerance test, germination decreases to 85% indicating that the seeds are already clearly in the desiccation-tolerant stage.

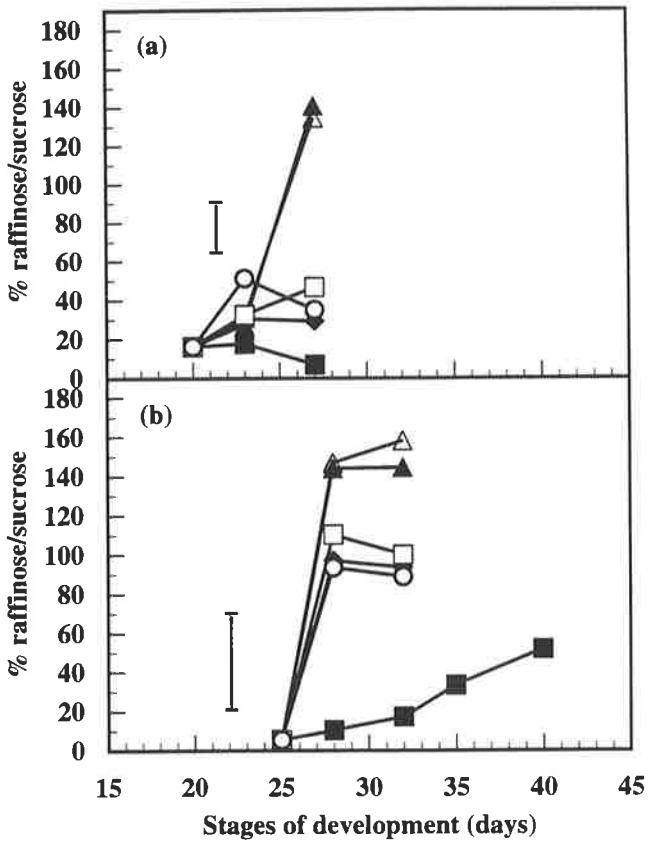


Fig. 3.10 Mole %ratio of raffinose/sucrose in embryos of wheat seeds at 20 DAA (**a**) and 25 DAA (**b**) and during development on the plants (■) or after the detached seeds were dried for 3 or 7 days under 1) 98% RH (▲), 2) 96% RH (△), 3) 55/35% RH (□), 4) 12% RH (◆) and 5) 0-2% RH (○). Vertical bar in (**a**) and (**b**) in l.s.d.$_{(.05)}$ for mean difference among treatments at the same periods of incubations.

Table 3.4 Effect of drying on sugar concentrations, embryo moisture content and embryo germination of wheat at 15 DAA. Data shown for fresh seeds at 15 DAA, after desiccation tolerance test (DT) for 6 days and fresh seeds at the same corresponding ages (21 DAA).

Stages of development (days)	Sucrose (nmol/ embryo)	Raffinose (nmol/ embryo)	Moisture content (%)	Germination (%)	Mole ratio (%) raffinose/ sucrose
15 D fresh	27.6	7.3	81.7	100	26.5
15 D+6DT	301.2	46.6	7.3	85	15.5
21 D fresh	253.1	12.0	69.4	100	4.7

3.13.2 Sugar concentrations in immature embryos at 15 DAA and after desiccation tolerance test

The mole %ratio of raffinose/sucrose in 15 DAA fresh embryos is high (26.5%) (Table 3.4). The ratio decreases rapidly to a low content (4.7%) in fresh seeds of the corresponding stage of development (21 DAA) and after the 15 D+6DT treatment, the ratio is 15.5%. Thus, Table 3.4 shows that after DT, sucrose, raffinose concentrations and the mole %ratio of raffinose/sucrose are all higher than those of fresh seeds of the same corresponding stage of development.

3.13.3 Moisture content during incubation and after desiccation tolerance test

Fig. 3.11 shows the seed moisture content after 7 days of controlled humidity incubation and again after another 6 days of rapid drying. Moisture content at HRH was approximately 70% while SD treatment (55/35% RH) reduced seed

moisture to <10% at day 7. After rapid drying, seed moisture contents were <5% (Fig. 3.11).

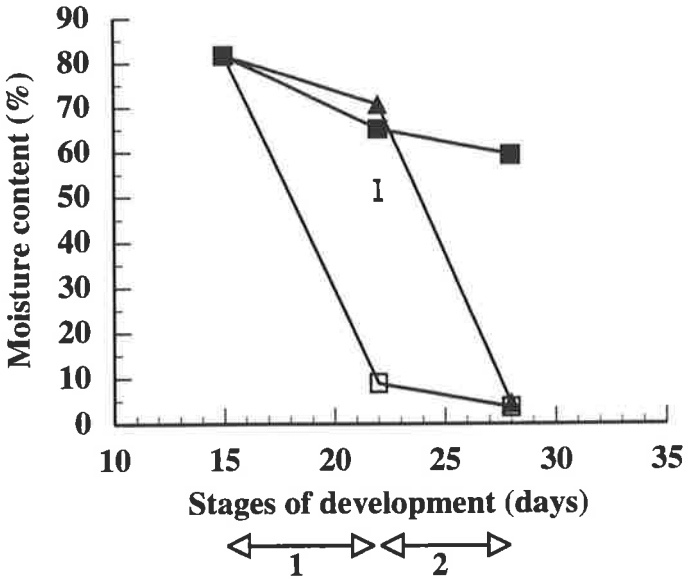


Fig. 3.11 Moisture content (%) of seeds at 15 DAA during development (■) and after pre-conditioning for 7 days (**1**) under 98% RH (▲) or 55/35% RH(□) and after desiccation tolerance test for a further 6 days (**2**). Vertical bar is $l.s.d._{(.05)}$ for mean difference among treatments at the same periods drying.

3.13.4 Sugar concentrations and %mole ratio of raffinose/sucrose in embryos of seeds stored at HRH, SD conditions and after desiccation tolerance test

Fig. 3.12a shows that there is a low concentration of sucrose at day 7 of incubation at HRH, but it is nearly 6 times higher in the SD treatment. Raffinose concentration increases in both the HRH and SD treatments at day 7 of incubation (Fig. 3.12b). The results confirm the observations of experiment 3 and 4 that an increase of raffinose takes place in detached seeds during incubation. Furthermore, raffinose synthesis can proceed without loss of moisture content (Fig. 3.11 and 3.12b). Raffinose synthesis is depressed by 6 days of rapid drying (Fig. 3.12b).

The mole %ratio of raffinose/sucrose of embryos at HRH and SD conditions are shown in Fig. 3.13. Under HRH conditions, the mole %ratio is approximately 4 times higher than under SD conditions. The very high ratio (~130%) in HRH embryos is mainly due to a slower increase ~~the fall~~ in sucrose content. After DT, the mole % ratio of seeds incubated at HRH and SD decreases to nearly initial %mole ratio of 15 DAA seeds (Fig. 3.13).

Germination data of after HRH and SD seeds were rapid dried (DT) for 6 days are not shown because some embryos (during the germination test) had fungal infections due to the HRH incubation conditions.

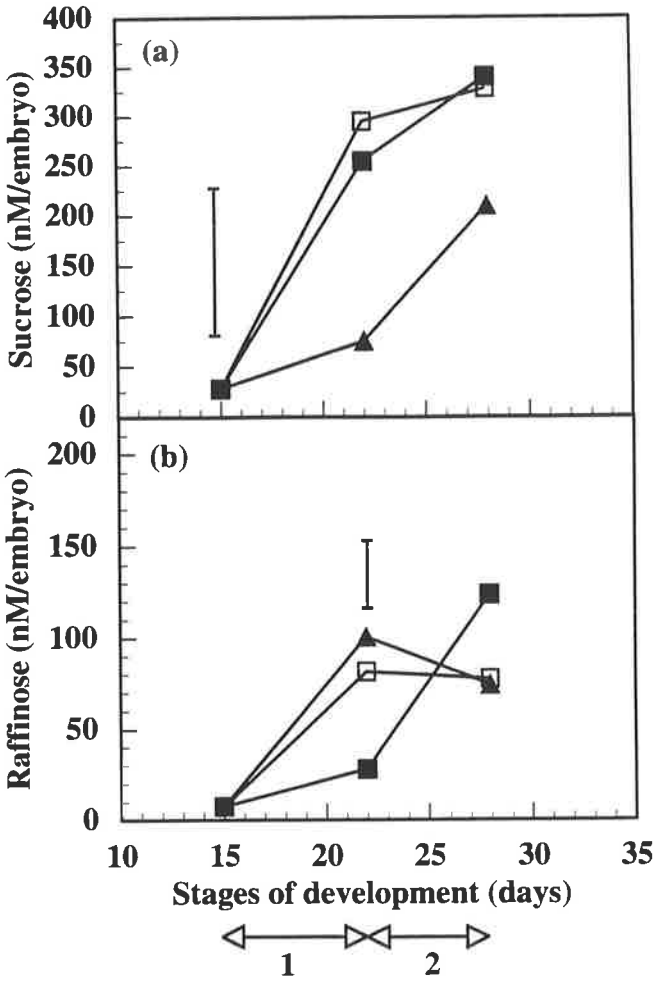


Fig. 3.12 Sucrose (**a**) and raffinose concentrations (**b**) in the embryos of fresh developing seeds (■) and after pre-conditioning for 7 days (**1**) under 98% RH (▲) or 55/35% RH (□) and after the desiccation tolerance test for a further 6 days (**2**). Vertical bars represent l.s.d.$_{(.05)}$ for mean difference among treatments at the same periods of drying.

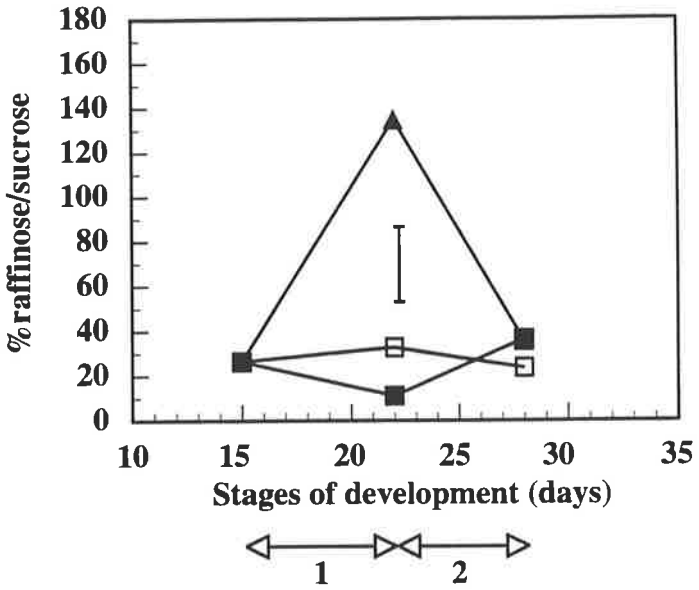


Fig. 3.13 Mole %ratio of raffinose/sucrose in embryos of fresh developing seeds (■) and after pre-conditioning for 7 days (**1**) under 98% RH (▲) or 55/35% RH(□) and after the desiccation tolerance test for a further 6 days (**2**). Vertical bar is l.s.d.$_{(.05)}$ for mean difference among treatments at the same periods of drying.

3.14 Discussion

3.14.1 Inhibitory effects on germination in developing wheat seeds

During development, germination of fresh wheat seeds is suppressed by many factors. Among these, seed maturity is a dominant parameter in their ability to germinate (Figs. 3.5 and 3.7). Previous workers (Wellington 1956; Radley 1976; Symons *et al.* 1983) have concluded that the outer pericarp is also an important

factor in the inhibition of germination and it has been suggested that the pericarp restricts gas exchange between embryo and environment. Although examination of Figs. 3.5 and 3.7 would seem to support this pericarp inhibition it was not supported by statistical analysis. The data in Figs. 3.5 and 3.7 must be deemed to be inconclusive.

The most significant factor inhibiting germination in experiments 3 and 5 was the presence of maternal tissue i.e. endosperm attached to the embryo. Full germination of immature seeds was not achieved if the endosperm was still intact (Figs. 3.5, 3.7 and Table 3.4). These results are fully in accord with the work of Kermode and Bewley (1989*b*) who have concluded that the presence of the endosperm inhibits germination during development.

Abscisic acid (ABA) has been implicated in the prevention of precocious germination (Oishi and Bewley 1992; King 1976; Hole *et al.* 1989). Germination ultimately takes place when embryos are no longer sensitive to ABA (Walker-Simmons 1987; Kermode and Bewley 1989*a*). Recently, it has been found that the suppressive effect of ABA is initiated when embryos express ABA responsive genes (Morris *et al.* 1989, 1991; Hilhorst and Karssen 1992) or the polypeptide 'e' (Noda and Kawakami 1993). According to these findings, it is possible that the endosperm produces signals controlling ABA-responsive gene expression in the embryos. As long as these ABA responsive genes are expressed, germination process is inhibited. In the case of isolated embryos, they are free from the controlling influence of the endosperm and its signals and hence germination process occurs in the isolated embryos (Table 3.4).

The question then arises as to why intact seeds stored at HRH conditions also have high germination especially when the outer pericarp is removed (Figs. 3.5a ~~3.4a~~ and 3.7). One possible explanation is that these endosperm signals are degraded ~~in time~~ when the seeds are detached from the parent plants, whether or not the seeds lose moisture

content. Furhermore, these detached seeds switch from developmental mode to germinative mode under HRH storage (Kermode and Bewley 1989); thereafter, biochemical changes as post-germinative events occur. Normally, the aleurone tissue of fresh immature developing wheat seeds is insensitive to gibberellic acid (Norman *et al.* 1982). Nicholls (1979) has found that under HRH conditions, immature wheat seeds increased in the ability of their aleurone tissue to respond to gibberellic acid; an induction of α-amylase activity was observed in these moist seeds. In this experiment (Fig. 3.7a), it suggests that these mechanisms occur in the HRH seeds. Thus, ~~detachment and~~ storing the seeds under HRH conditions switch the seeds from developmental mode to germinative mode, and then aleurone layer increases in its ability to respond to gibberellin. Removal of the outer pericarp could eliminate another source of ABA which reached the peak between 2-3 weeks after anthesis in wheat (Radley 1976). Taken together, these factors could contribute to germination process and embryos resume growth in the seeds stored under HRH conditions.

3.14.2 Embryo sugars in developing seeds show no clear relation to desiccation tolerance

During seed development, substantial amounts of sucrose are detectable in the embryo at the early stages (20-30 DAA) and are still high at maturity (Figs. 3.6 and 3.9e). Raffinose concentrations are very low when the seeds are immature (Figs. 3.6, 3.9c, f), but tend to accumulate at the later stage of development (Amuti and Pollard 1977). A loss of moisture is a normal occurrence during seed maturation (Kermode 1990). At 35 DAA, an increase of raffinose is evident (Figs. 3.6 ~~3.4a~~) concomitant with a rapid loss of seed moisture to <40% (Fig. 3.4a ~~3.6~~). This could be a natural response involving raffinose synthesis under stress conditions. The 'stress conditions' in this case not only refer to the moisture loss, but could include any treatments the seeds are exposed to, such as detachment from the parent plant or their storage under various conditions. Experiments 3-5 clearly

show that raffinose increases under such stress conditions (Figs. 3.8b, 3.9c, f and 3.12b). Consequently, the mole %ratio raffinose/sucrose also increases after various drying conditions (Figs. 3.10a, b and 3.13). It is noticeable (but not the rule) that, to a first approximation, moisture contents >30% facilitate raffinose synthesis (Figs. 3.4, 3.6, 3.9a, c, d and f). When seed moisture contents are <10%, raffinose synthesis is inhibited (Figs. 3.9a, c, d and f).

In fresh developing seeds, there is no clear relationship between sugar concentrations and desiccation tolerance (Figs. 3.6, 3.7, Table 3.4). For example the initial concentrations of sugars (before drying) is not an indicator of desiccation tolerance and seeds that can tolerate desiccation can have very low concentrations of embryo raffinose (Figs. 3.6, 3.7, Table 3.4). This is illustrated by 25 DAA seeds (Fig. 3.6) which have only ~10 nM raffinose/embryo, or 15 DAA seeds which have only ~7.3 nmol raffinose/embryo (Table 3.4). Chen and Burris (1990) have suggested a minimum of 9%mass ratio raffinose/sucrose is sufficient to confer desiccation tolerance in corn seeds. The 9%mass ratio raffinose/sucrose is equivalent to a 6.1%mole ratio. In the current wheat work, the mole ratios (%) in fresh seed embryos rapidly decrease at the early stage. From Table 3.4, it can be seen that seeds at 15 D fresh with a 26.5% ratio are desiccation tolerant. In 21 day fresh seeds the ratio is only 4.7% and they too exhibit desiccation tolerance. Thus, neither initial sugar concentrations nor %ratio raffinose/sucrose can be used as indicators of desiccation tolerance in fresh developing seeds. These results are similar to those observed in sweet corn, experiments 1 and 2.

Another possibility considered for the importance of sugars in conferring desiccation tolerance, was that the sugars may increase during the drying process. The induction of high concentrations of raffinose after drying is certainly evident in some cases (Figs. 3.9c, f, 3.12b); however, raffinose concentrations sometimes show only a transient response (Fig. 3.8b).

3.14.3 Embryo sugars in detached seeds stored at different %RH conditions

The relationship between sugar concentrations and desiccation tolerance in developing seeds is obscure. Most evidence indicates that an induction of raffinose occurs by storing the seeds at various RH conditions (Figs. 3.9 and 3.10). This suggests that an increase in raffinose concentrations is a response of detached seeds to stress conditions. A substantial increase of raffinose does not always coincide with a decrease of sucrose (Figs. 3.9b, c, e and f). In some cases, an increase of both sucrose and raffinose is observed (20 DAA seeds dried for 3 days; Figs. 3.9b, c). This suggests that during incubation, embryos gain sucrose from elsewhere, such as starch/lipid reserves or endosperm sucrose. However, as starch/lipid and sucrose concentrations in the endosperm were not measured in these experiments, no conclusions can be made as to whether embryos synthesise raffinose from the substrates in the embryo itself or from substrates translocated from the endosperm.

Black *et al.* (1995) have reported that in developing wheat embryos, an increase in raffinose coincides with a fall of embryo in starch content. Evers and Bechtel (1988) have reported that lipid is normally present at the early stage of development in wheat embryos. Thus, substrates for raffinose synthesis could come from embryo lipid degradation, sucrose transportation from the endosperm or even a breakdown of embryo starch. It is not known whether during desiccation sucrose is directly transported from the endosperm to the embryo or whether it is derived indirectly from starch degradation in the endosperm or embryo.

According to the review by Jenner *et al.* (1991), the grain enlargement stage in wheat (a period of water influx) commences at fertilisation and proceeds until 20 DAA while grain filling (a period of reserve deposition) commences 10-15 DAA.

Sucrose was directly transported in the phloem to the endosperm and starch had started to deposit in amyloplasts by the end of the first week after anthesis. Thus, it is possible that sucrose moves either directly from the endosperm to the embryo or that starch degradation in the endosperm/embryo occurs during desiccation and sucrose derived from the starch is translocated. However, the first case appears to be more reasonable, because endosperm starch would be expected to be present at a low content at the early stage of development. Furthermore, embryos are not a major source of starch during development or even at maturity (Evers and Betchel 1988).

At 25 DAA (Figs. 3.9e, f), raffinose increases nearly 3-4 fold during drying indicating a high rate of synthesis. However, a decrease of sucrose is observed during incubation. It is possible that the mobilisation of sucrose from the endosperm is not sufficient for the rapid rate of raffinose synthesis and under these conditions, embryo sucrose is used as the substrate for raffinose synthesis. The evidence also suggests that under incubation conditions, embryos become an important sink for sugars. Another possibility for the decline in sucrose is high energy requirements involving the oxidation of embryo sucrose during incubation.

To summarise, the evidence from all these experiments do not support the hypothesis that only drying induces raffinose synthesis and that raffinose and sucrose together confer desiccation tolerance. Neither raffinose concentrations nor the %raffinose/sucrose ratio appear to be sole determinant factors for desiccation tolerance.

3.15 Conclusions (Experiments 1-5)

1) In sweet corn and wheat, sucrose and raffinose concentrations in the embryos change markedly during seed development. Sucrose is observed at the early stage, while raffinose tends to accumulate at the later stage when the seeds are more mature.

2) In fresh developing seeds, sugar concentrations or the raffinose/sucrose ratio are not useful indicators of desiccation tolerance. After rapid drying, embryo sugar concentrations and raffinose/sucrose ratio tend to increase, but the results are variable.

3) In detached seeds, sugar concentrations and the ratio (%) of raffinose/sucrose do not uniformly correlate with desiccation tolerance.

4) An increase in embryo raffinose content appears to be a common feature when seeds are exposed to stress conditions including either detachment from the parent plants or storing the seeds at various %RH's. Raffinose synthesis is ultimately inhibited when seeds are dried to a low moisture content.

5) Detached embryos may utilise substrates for raffinose synthesis from the endosperm or in the embryo itself. Attached embryos can receive substrates form the parent plant.

6) In sweet corn and wheat, fresh immature seeds do not germinate. Immature sweet corn seeds require drying to induce germination whereas wheat seeds are able to germinate with no moisture loss. The most significant factor inhibiting germination in wheat is the presence of the endosperm.

7) Unfortunately, the general conclusion from all of these experiments is that although measurement of sucrose and raffinose concentrations is a relatively straight-forward procedure, such measurements are unlikely to throw useful light on the mechanisms of desiccation tolerance in these seeds, which is in accord with views recently expressed by Leprince *et al.* (1993).

CHAPTER 4

Spectroscopic (IR and NMR) and chromatographic (TLC) studies of desiccation tolerance in wheat embryos

4.1 Introduction

Desiccation tolerance of seeds is a phenomenon associated with physiological and biochemical changes in their cells (Bewley 1979). In attempting to elucidate the mechanisms involved, many aspects of the topic have been examined (Blackman *et al.* 1992, Sun *et al.* 1994; Chen and Burris 1990; Koster and Leopold 1988; Hendry *et al.* 1992; Leprince *et al.* 1990*a*; Senaratna *et al.* 1984, 1985*a*, *b*) and have recently been reviewed by Leprince *et al.* (1993).

Three main protective systems have been suggested to account for desiccation tolerance; 1) an accumulation of sugars; 2) an ability to protect against membrane damage by free radicals during desiccation; and 3) an induction of protective, late embryogenesis abundant (LEA), proteins by abscisic acid (Leprince *et al.* 1993).

The fundamental importance of water content and its state (bound or non bound) to either desiccation tolerance or sensitivity has been discussed by Leprince *et al.* (1993). Sorption isotherms and calorimetry studies have established the existence of 5 states of water in seed tissue, ranging from a tightly bound state (region 1) at 0-10% to the fully hydrated state (region 5) above 55% where germination proceeds without hindrance (Vertucci 1989, 1990; Berjak *et al.* 1990*b*, 1992; Pammenter *et al.* 1991). Three types of bound water have been described; type 1 water is tightly bound (ionic bonding) to macromolecules, type 2 water is weakly bound (condensed over the hydrophilic sites) to macromolecules, type 3 water is bound with negligible energy, this latter type of water forms bridges over the hydrophobic sites of macromolecules (Leopold and Vertucci 1989). Based on the water status in

seeds, it has been suggested that desiccation sensitivity is an inability of cells to maintain its integrity when nonfreezable bound water is removed. In desiccation-tolerant species, loss of this bound water does not cause critical cell damage (Pammenter *et al.* 1991).

In this chapter, diffuse reflectance using infrared dispersion spectra (DRUIDS), together with cross polarisation-magic angle spinning (CP/MAS) ^{13}C NMR nuclear magnetic resonance spectra have been enlisted to shed light on the functional group changes in embryo development and also during desiccation. Infrared spectra have previously been employed to study the role of sugars in desiccation tolerance by studying the hydrogen bonding of sugar -OH group with the polar head groups of phospholipid membrane (Crowe *et al.* 1984*a*, *b* , 1988). Previously, ^{13}C NMR techniques have been used to elucidate the molecular structure and identify chemical species (Gerasimowicz and Pfeffer 1989) which are present in samples, and they have been applied to the identification of seed component such as carbohydrates, proteins and lipids (O'Donnell *et al.* 1981; Rutar 1989). One perceived advantage of ^{13}C NMR is that it is a relatively nondestructive method of chemical analysis of seeds (O'Donnell *et al.* 1981). Advances in instrumentation since 1981 have warranted a reinvestigation by this technique.

The aims of this chapter are :

1) to examine whether there is any correlation between the presence of functional groups, detectable by their infrared absorbance and desiccation tolerance.

2) to investigate any significant changes in signals detectable in wheat embryos using CP/MAS ^{13}C NMR spectroscopy and correlate these changes with desiccation tolerance.

4.2 Materials and methods

4.2.1 Plant materials

Wheat plants (*Triticum aestivum* cultivars Halberd, WW15 and Gabo) plants were grown in a growth room at 18°C with 16 h light and 8 h dark (see section 2.1.2). Ears were tagged at the time of anthesis and collected at 15, 20 and 25 days after anthesis (DAA) in cultivars WW15 and Gabo and at 10, 13, 15 and 20 DAA in cultivar Halberd.

4.2.2 Preparation for germination test

Ears were surface sterilised by spraying with 70% (v/v) ethanol, and then wiping with tissue paper soaked in 70% ethanol. Seeds from the middle part of the ears were aseptically removed from the outer glumes and cut in half. The half parts with attached embryos were soaked in sterilised distilled water for 2 hours before the germination test. For the desiccation tolerance test, whole seeds removed from the ears were placed in jars (12 cm diameter, 22 cm height) containing 100 g of activated silica gel. After 3 or 7 days of drying, seeds were cut in half and then soaked in distilled water until the seed tissue became soft before the germination test.

Embryo germination on artificial media was described in section 2.2.2 and embryo moisture content determined as described in section 2.3. Seeds were tested for desiccation tolerance for 7 days (see 2.4) and the Diffuse Reflectance Using Infrared Dispersion Spectra (DRUIDS) is described in section ~~2.5~~ 2.6.

4.2.3 Thin Layer Chromatography

Lipid compound (s) of interest were extracted from samples used for infrared analysis by adding hexane (25 µl) and vortexing. The supernatant solution of hexane was transferred into a small glass tube and the sample re-extracted three times more with

25 µl of hexane. The combined hexane extracts (100 µl) were examined by thin layer chromatography (TLC). Samples were applied as spots 1 cm from the bottom edge of a 3x7 cm silica gel plastic sheet (Silica gel 60 F_{254}, Merck). The TLC sheet was placed in a 250 ml beaker containing 25 ml $CHCl_3$ as the mobile phase and removed when the solvent had moved 5 cm from the origin. After drying for 1-2 minutes, the samples were stained by exposing the TLC sheet in a 250 ml beaker containing iodine vapour until dark brown spots appeared against a paler background.

4.2.4 Argentation Chromatography

A silica gel plastic sheet (Silica gel F_{254} Merck) saturated with 5% $AgNO_3$ solution (Gurr and James 1971) was stored in the dark and then dried for 5 minutes before use. The samples were applied as described in 4.2.3. The silica gel sheet was chromatographed with a mixture of propan-2-ol and $CHCl_3$ in a ratio of 1.5 : 98.5 (v/v). After drying, compounds present were identified by spraying with a 3% (v/v) cupric acetate solution in 8% aqueous H_3PO_4 (v/v) (Fewster *et al.* 1969). The silica gel sheet was then heated on a hot plate at 150°C until the black stain was developed.

4.2.5 Column Chromatography

Ten embryos (at the desiccation tolerance stage) were ground with 100 µl hexane then passed through a 6-inch column packed with a mixture of silica gel H and hexane. The column was eluted with $CHCl_3$, fractions (31 fractions, 100 µl for each fraction) collected were vacuum dried onto KCl and examined by DRUIDS (see section 2.5).

4.2.6 CP/MAS ^{13}C NMR technique

Wheat seeds were collected and embryos examined using CP/MAS ^{13}C NMR (see section ~~2.7~~ 2.8). Treatments consisted of : 1) 15 DAA; 2) 15 DAA dried for 3 days; 3)

15 DAA dried for 7 days; 4) 20 DAA; 5) 20 DAA dried for 3 days; 6) 20 DAA dried for 7 days. Because detached seeds continue in their development during the drying period, fresh developing seeds at the same corresponding age were used for the comparison with those subjected to drying.

4.3 Results

Development, desiccation and DRUIDS studies

4.3.1 Developmental stage and germination of wheat embryos without drying and effect of desiccation on germination and moisture content

At 10 DAA, embryos are poorly developed; they are translucent and the endosperms are watery-like. At 15 DAA (Fig. 4.1), embryos are still translucent, but crushing the seeds shows they contain a white milky fluid indicating a start of milky stage. Embryo excision without major damage is easier at 15 DAA than at 10 or 13 DAA. From 15 to 20 DAA, changes in opacity, shape and size of an embryo are observed. At 20 DAA, embryos are fully developed and become white (Fig. 4.1). Fig. 4.2 shows that embryos at 15 DAA (which are translucent before drying) do not survive desiccation.

At 10 DAA, no excised fresh embryos germinate, but some develop calli. At 13 DAA, excised fresh embryos fail to develop normal seedling, but at 15 DAA nearly all excised embryos will germinate (Fig. 4.3). These freshly excised embryos are critically dependent on the days after anthesis for normal seedling development. Above 15 DAA normal germination of excised embryos is the rule. Fig. 4.3 also shows that desiccation tolerance is the quantitative characteristic.

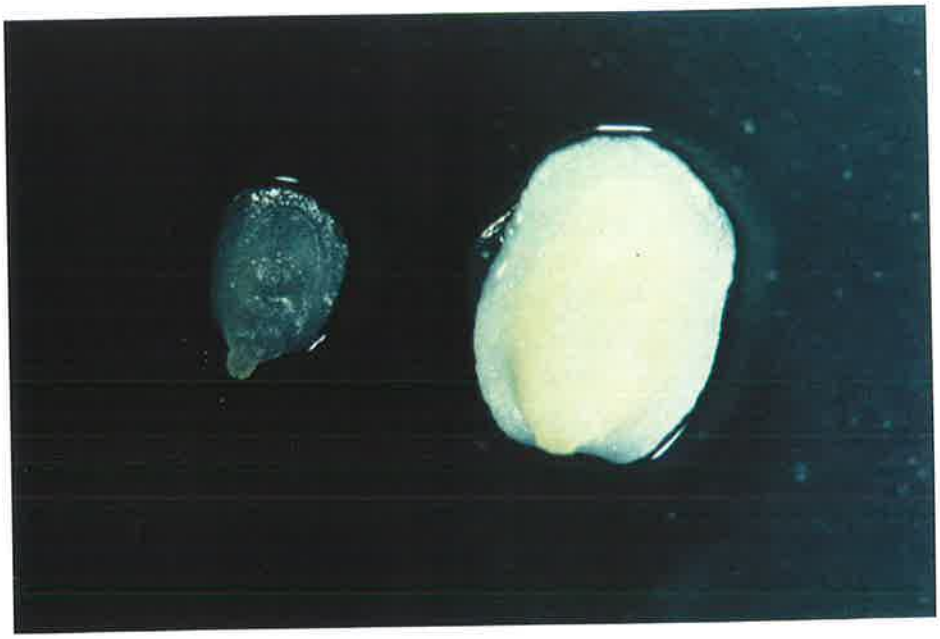
At 15 DAA, 3 or 7 days of desiccation steadily lowers the proportion of normal seedlings. At 10 or 13 DAA desiccation may or may not increase the proportion of normal seedlings. The uncertainty of these results at 10 or 13 DAA probably

reflects the greater difficulty in successful embryo excision without damage. Fig. 4.4 shows a comparison of cultival differences with respect to moisture loss from embryos under desiccation for 3 and 7 days. It is noticeable that after 15 DAA the variety Halberd consistently retains moisture more tenaciously than either WW15 or Gabo.

Fig. 4.1 Wheat embryos cultivar Halberd at 15 DAA (desiccation-sensitive stage) and 20 DAA (desiccation-tolerant stage). Note that embryos become opaque at 20 DAA.

Fig. 4.2 Embryo germination of wheat cultivar Halberd at 15 DAA (desiccation-sensitive stage) after desiccation tolerance test for 7 days (right). Note that normal germination of 22 DAA embryos was obtained (left).

a



b

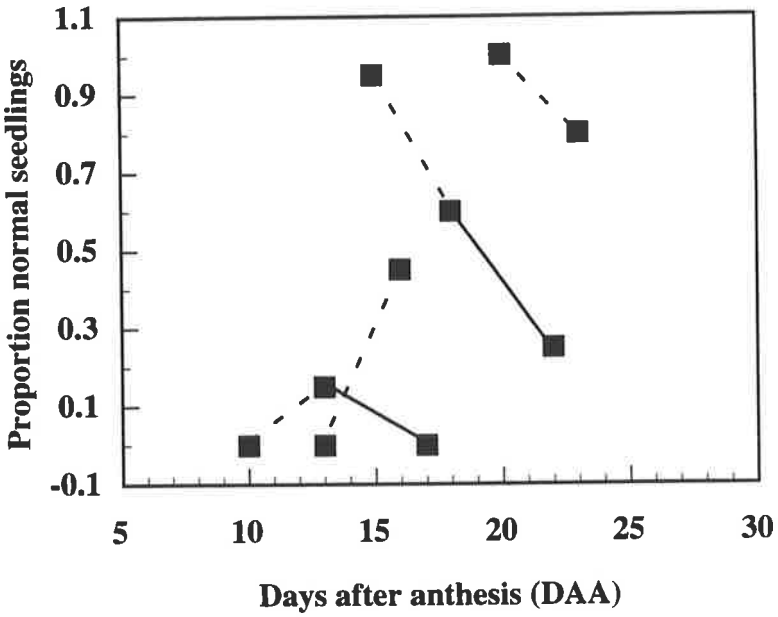


Fig. 4.3 Changes in proportion of normal seedlings of wheat cultivar. Halberd during drying for 3 or 7 days (---, —) of drying.

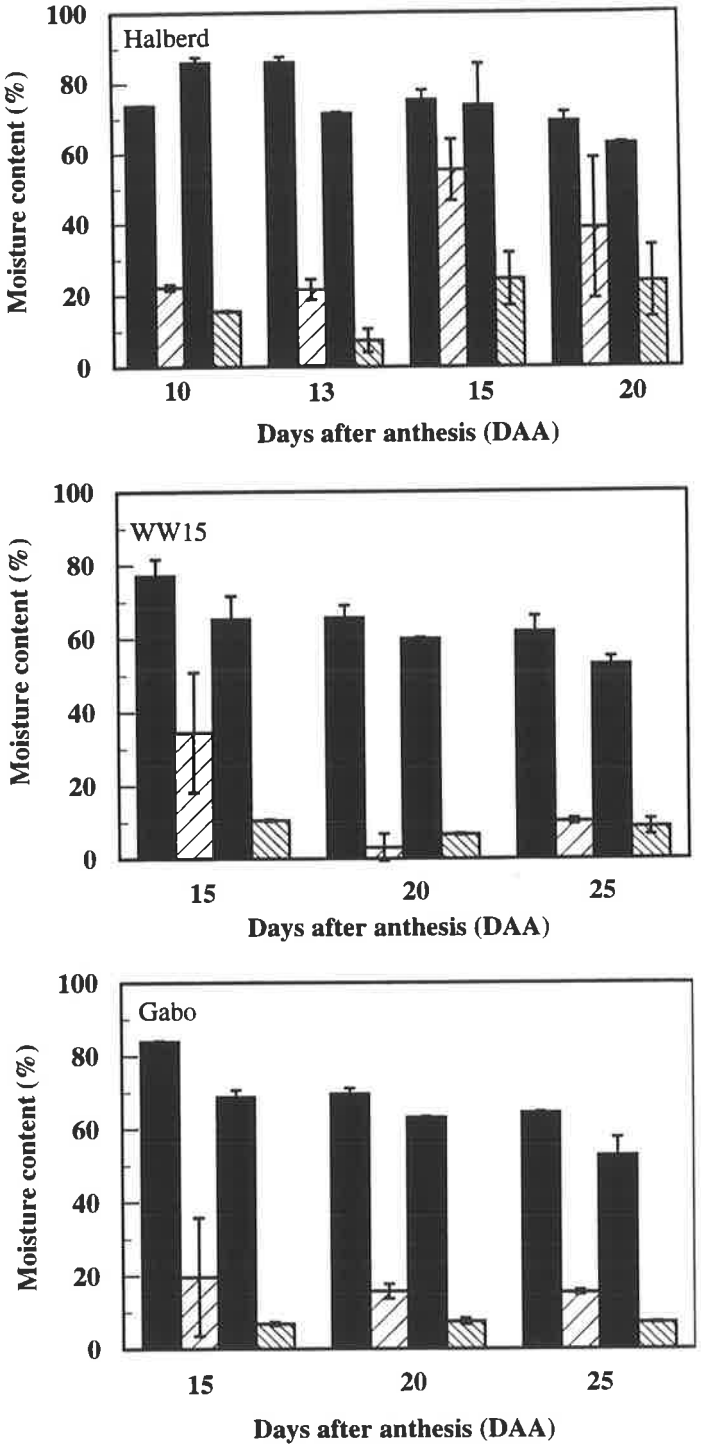


Fig. 4.4 Moisture content (%) of embryos after drying for 3 (▨) or 7 days (▧) compared with those of fresh developing seeds at the same corresponding ages (■). Vertical bars are standard deviation for each treatment.

4.3.2 Effect of desiccation on absorbance ratio (A 1743 cm^{-1}/A 1721 cm^{-1})

During development, there is an increase in the ester carbonyl absorbance at 1743 cm^{-1} (Fig. 4.5a, b, c). The size of this peak can be quantitated by measuring the absorbance peak (1743 cm^{-1}) to trough (1721 cm^{-1}) ratio (see section 2.5). During desiccation, as is discussed below, there is a diminution in this ratio for all varieties. This was noted for embryos at both the desiccation-sensitive stage (15 DAA) and the desiccation-tolerant stage (20 and 25 DAA).

Fig. 4.6 shows a correlation between the initial absorbance ratio and the proportion of normal seedlings from all varieties during seed development and during periods of drying (3 and 7 days). Spearman's correlation coefficients (r_s) were calculated for each variety. The initial absorbance ratio was highly correlated with the proportion normal seedlings in all cultivars, however, such pooling of the data from all drying treatments and developmental stages may hide an important effect on the proportion of normal seedlings. To examine the relationship of these two parameters in more detail, the 15 DAA experiment was repeated. Seeds of 'Halberd' were tested for desiccation tolerance using 10 replicates (Fig. 4.7) and the data for each replicate were derived from 4 absorbance readings and 2 germination assays. The results of this experiment clearly established that drying the seeds at the desiccation-sensitive stage (15 DAA) decreased both the absorbance ratio and the proportion of normal seedlings (Fig. 4.7).

Fig. 4.8 shows wheat cultivars WW15 and Gabo, at 15 DAA, also exhibit desiccation sensitive behaviour after 7 days of drying. Figs. 4.8 and 4.10 show that all varieties were desiccation tolerant at or after 20 DAA.

Figs. 4.9 shows that for wheat cultivar Halberd at 18 DAA, embryos exhibit more desiccation tolerance than at 15 DAA. At 20 DAA (Fig. 4.10), embryos are at the desiccation-tolerant stage. Again, the absorbance ratio of the ester carbonyl at 1743

cm^{-1} decreases after drying as previously observed at the 15 DAA desiccation-sensitive stage (Fig. 4.7). However, unlike the situation with 15 DAA material the ability of embryos to survive desiccation is not compromised by this decrease in ester carbonyl absorbance of the embryos in 18 and 20 DAA seeds. Thus, it suggests that the presence of this peak is necessary for the survival of seeds only at a certain stage, i.e. at the desiccation-sensitive stage (15 DAA). The critical absorbance ratio for the seeds to survive desiccation is approximately 1.03 (Fig. 4.7). After drying 15 DAA seeds for 3 or 7 days, the absorbance ratio of embryos decreases to less than 1.03 and this coincides with a decrease in germination. This absorbance ratio is less critical for the survival of the seeds at 18 DAA (Fig. 4.9). At the desiccation-tolerant stage (20 DAA), most of the seeds survive desiccation although the absorbance ratio of the embryos are less than 1.03 after drying (Fig. 4.10).

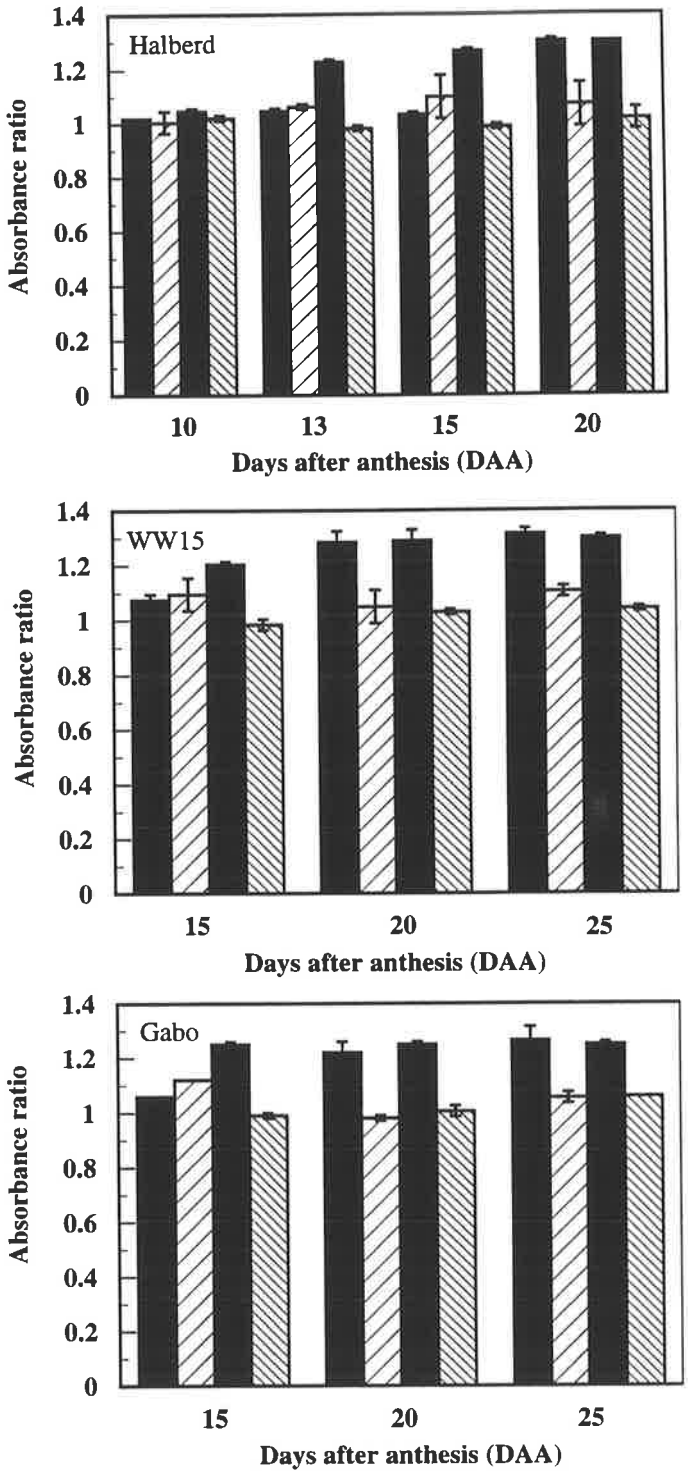


Fig. 4.5 The A1743 cm^{-1} /A1721 cm^{-1} absorbance ratio of wheat embryos after drying for 3 (▨) and 7 days (▧) compared with those of fresh developing seeds at the same corresponding ages (■). Vertical bars are standard deviation for each treatment. An absorbance ratio of ≤1.0 corresponds to a negligible ester carbonyl peak and ratios ≥1.2 correspond to a strong ester carbonyl peak.

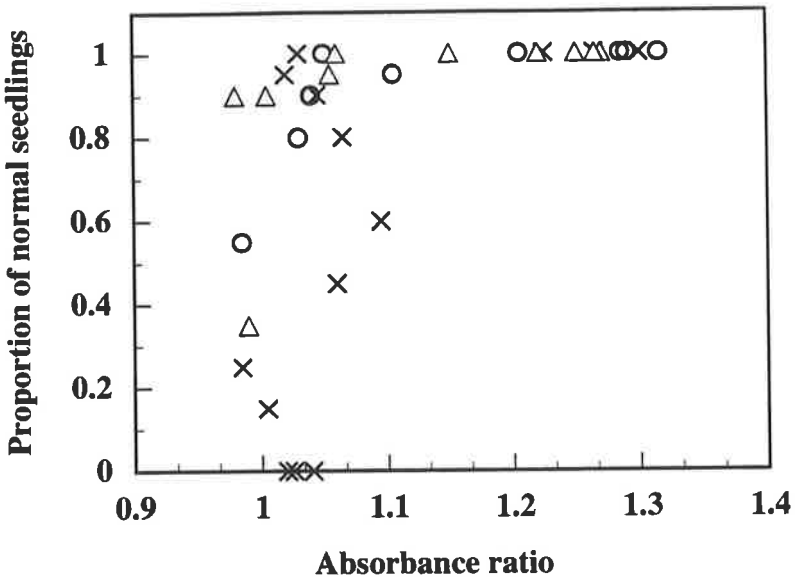


Fig. 4.6 Correlation between the initial absorbance ratio and proportion of normal seedlings of wheat cultivars Halberd (×), WW15 (○) and Gabo (△). Spearman correlation coefficient (r_s) = 0.68*, 0.69* and 0.71* respectively. Data from 15, 20, 25 DAA and after drying for 3 and 7 days.

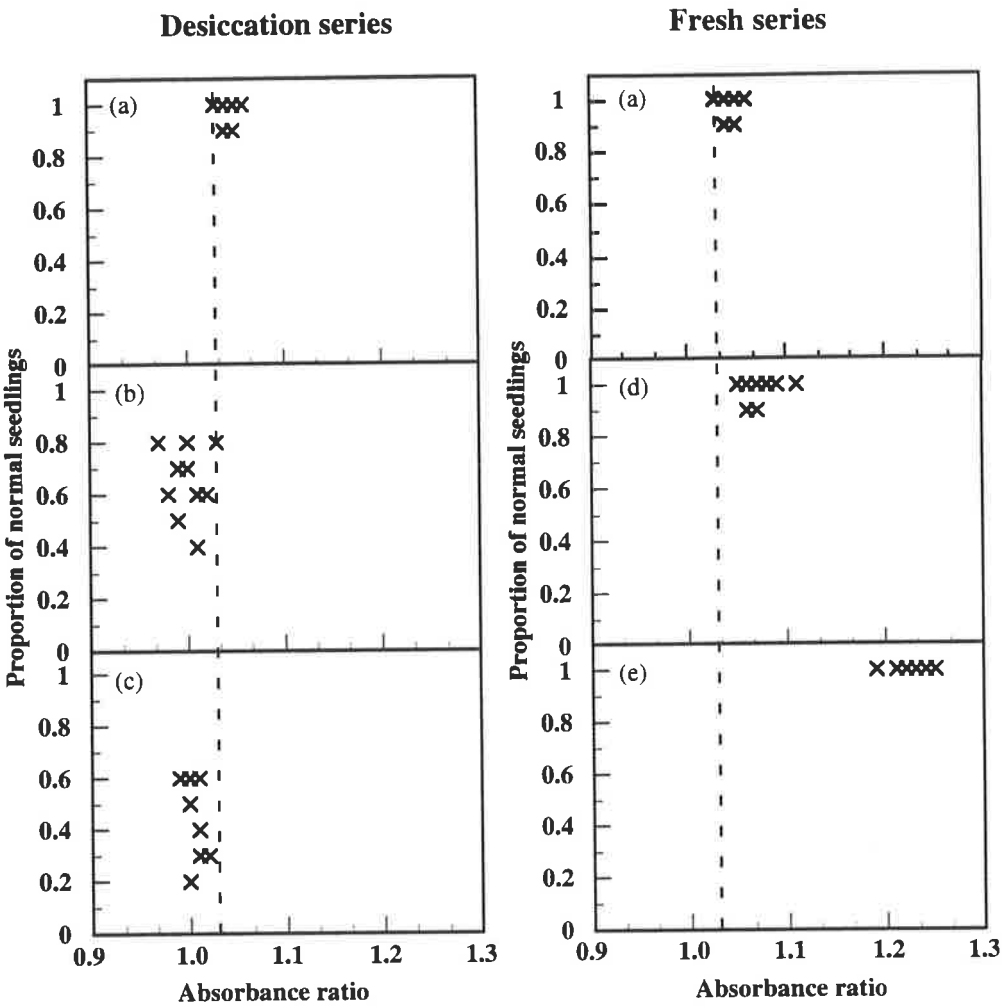


Fig. 4.7 Absorbance ratio and proportion of normal seedlings of wheat embryos cultivar Halberd of desiccation series: at (a) 15 DAA; (b) 15 DAA dried for 3 days or (c) 7 days; and fresh series: at (a) 15 DAA (d) 18 DAA and (e) 22 DAA. Panel (a) fresh series is the same data as panel (a) desiccation series. Each graph represents results of 10 replicates. Each replication (x) consists of mean of 4 absorbance readings and 2 germination assays.

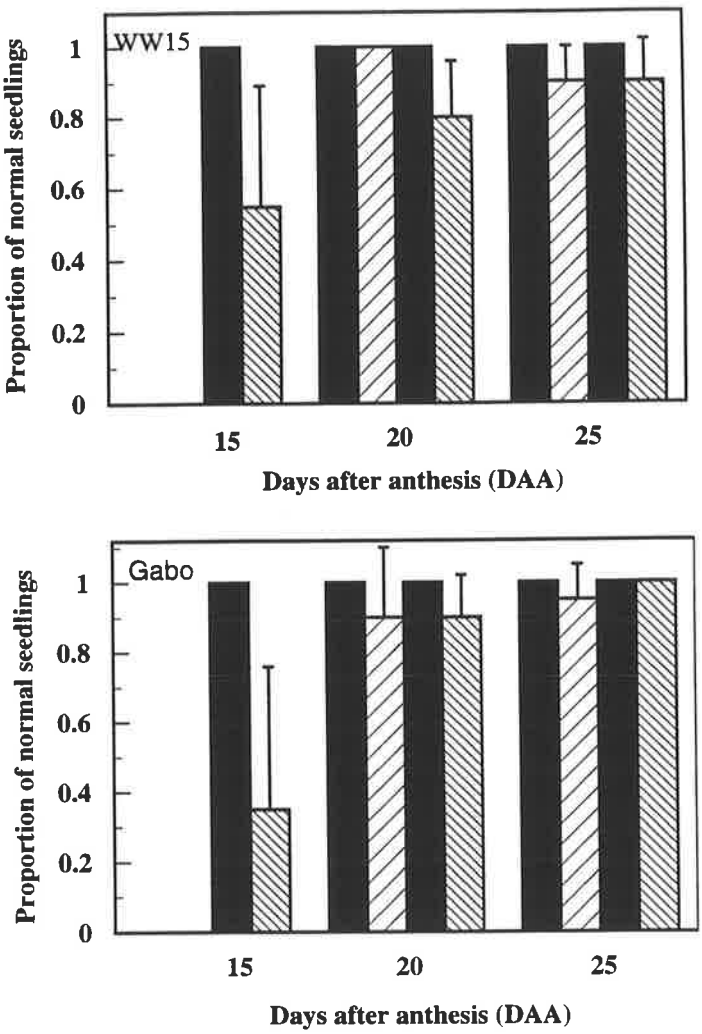


Fig. 4.8 Proportion of normal seedlings of wheat embryos var. WW15 and Gabo after drying for 3 (▨) or 7 days (▧) compared with those of fresh developing seeds at the same corresponding ages (■). Vertical bars are standard deviation for each treatment.

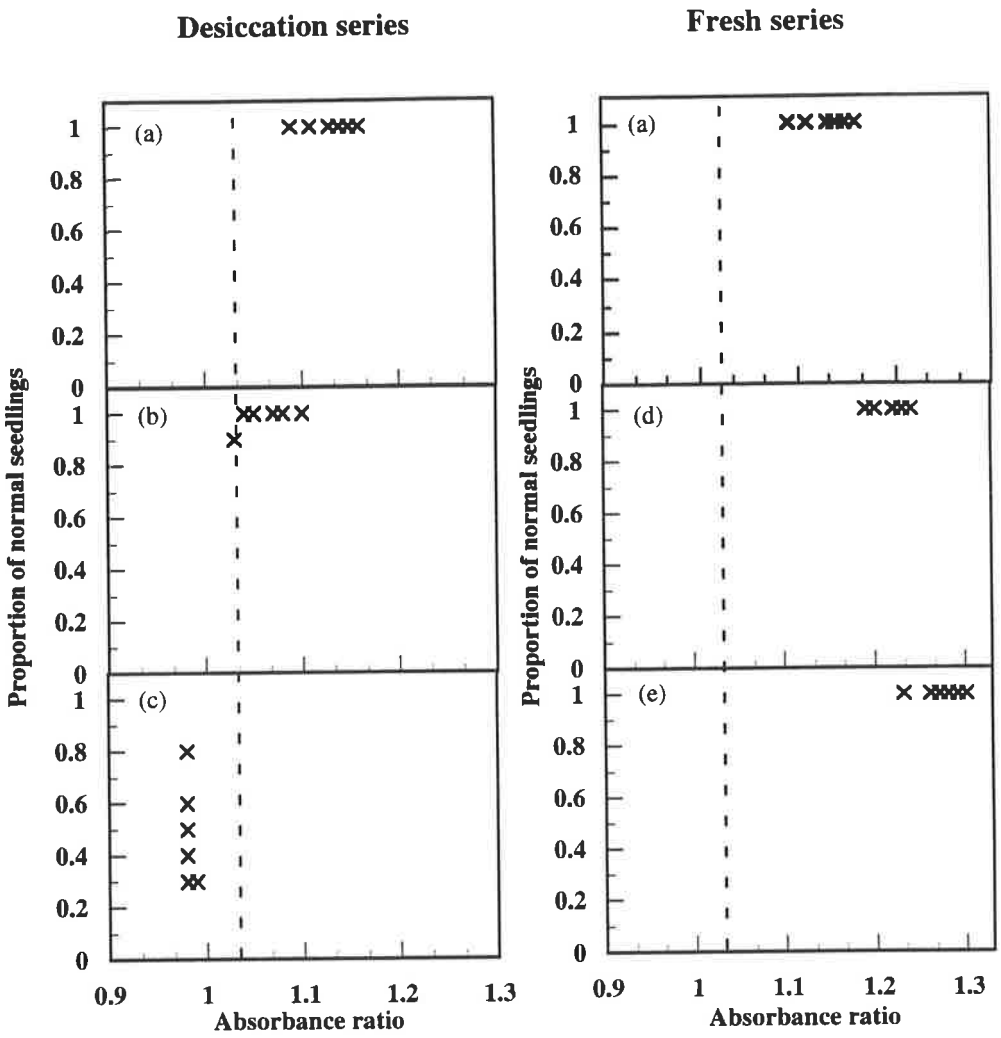


Fig. 4.9 Absorbance ratio and proportion of normal seedlings of wheat embryos cultivar Halberd of desiccation series: at (a) 18 DAA; (b) 18 DAA dried for 3 days or (c) 7 days; and fresh series: at (a) 18 DAA (d) 21 DAA and (e) 25 DAA. Panel (a) fresh series is the same data as panel (a) desiccation series. Each graph represents results of 10 replicates. Each replication (x) consists of mean of 4 absorbance readings and 2 germination assays.

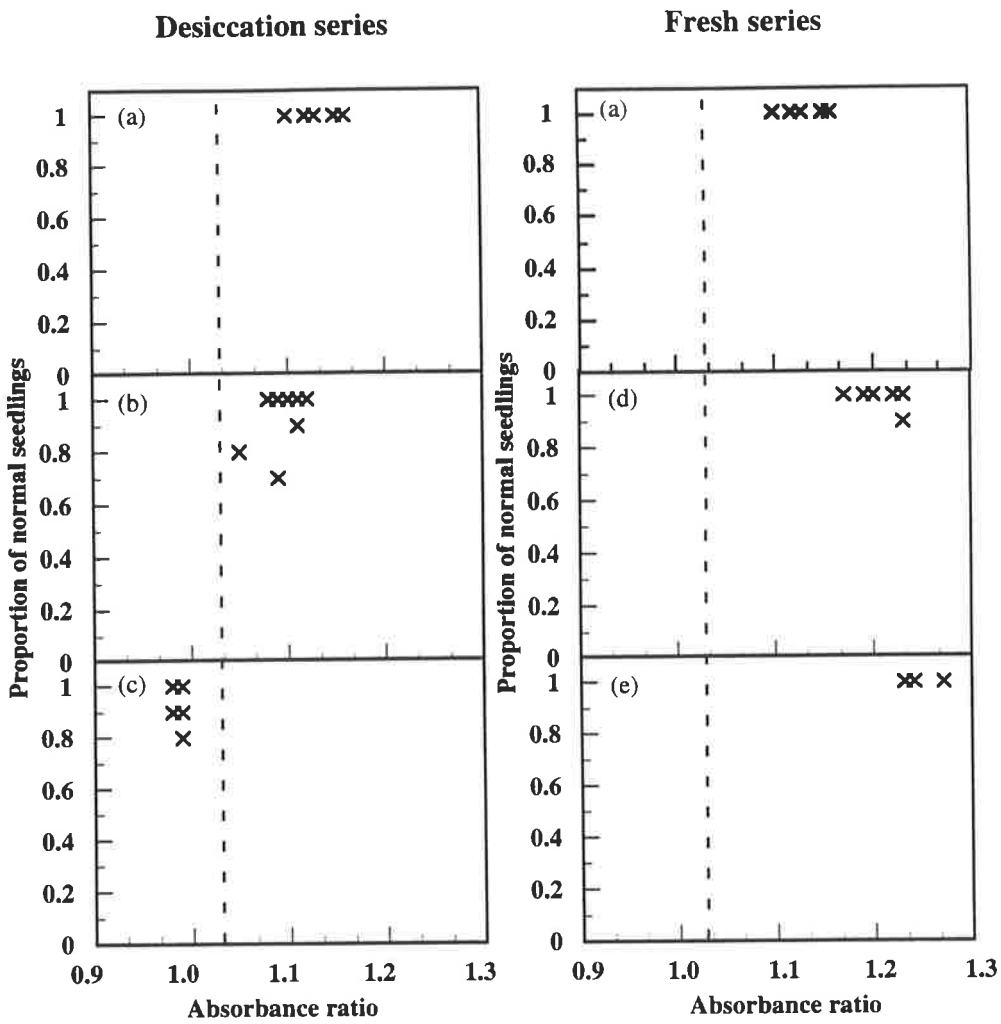


Fig. 4.10 Absorbance ratio and proportion of normal seedlings of wheat embryos cultivar Halberd of desiccation series: at (a) 20 DAA; (b) 20 DAA dried for 3 days or (c) 7 days; and fresh series: at (a) 20 DAA (d) 23 DAA and (e) 27 DAA. Panel (a) fresh series is the same data as panel (a) desiccation series. Each graph represents results of 10 replicates. Each replication (x) consists of mean of 4 absorbance readings and 2 germination assays.

4.3.3 The infrared spectra of the hexane soluble material from the developing embryo is similar to the olive oil triacylglycerols

Figs. 4.11a, b, c, d show an increase in the 1743 cm^{-1}, 2890 cm^{-1}, 2920 cm^{-1} and 3006 cm^{-1} peaks during embryo development. At 15 DAA, embryos are in the desiccation-sensitive stage while at 18 and 20 DAA, embryos are in the desiccation-tolerant stage. A strong 1743 cm^{-1} peak is apparent in the embryos from 18 DAA onwards (Figs. 4.11 b, c, d). Fig. 4.12 demonstrates the hexane extractability of the products associated with the 1743 cm^{-1} peak and Figs. 4.13 shows the strong similarity of these hexane soluble lipids from wheat to the triacylglycerols in olive oil. The strong saturated ester carbonyl (1743 cm^{-1}), the strong C-H stretching bands (2920 and 2890 cm^{-1}) together with a small ethylenic C-H stretch at 3006 cm^{-1} and the strong C-O stretching at 1100 cm^{-1} and its hexane solubility are consistent with the presence of a fatty acyl triacylglycerol containing some unsaturated esters.

Normal and argentation thin layer chromatography on silica gel (Figs. 4.14a, b) confirm the close similarity of the hexane soluble lipid fraction from wheat embryos to the saturated and unsaturated triacylglycerols present in olive oil.

4.3.4 Changes in functional groups after desiccation tolerance test

Figs. 4.15a, b show a strong 1743 cm^{-1} peak in fresh developing embryos at 22 and 27 DAA and a major decrease of this peak after drying. At the desiccation-tolerant stage (20 DAA), a small peak of 1743 cm^{-1} is still evident after drying (Fig. 4.15b). The existence of this residual desiccation resistant ester carbonyl at the desiccation tolerance stage is also apparent in the hexane lipid extracts (Fig. 4.14a). However, an almost complete loss of this peak is observed when 15 DAA seeds (the desiccation-sensitive stage) are dried for 7 days and an absorbance ratio is 0.8 (Figs. 4.7c, 4.15a).

Fig. 4.11 Infrared spectra of wheat embryos cultivar Halberd showing an increase of the ester carbonyl peak (1743 cm^{-1}) and C-H stretching bands (3006, 2920 and 2890 cm^{-1}) at 15 DAA (a), 18 DAA (b), 20 DAA (c) and 25 DAA (d). Each spectrum is the mean from 10 observations.

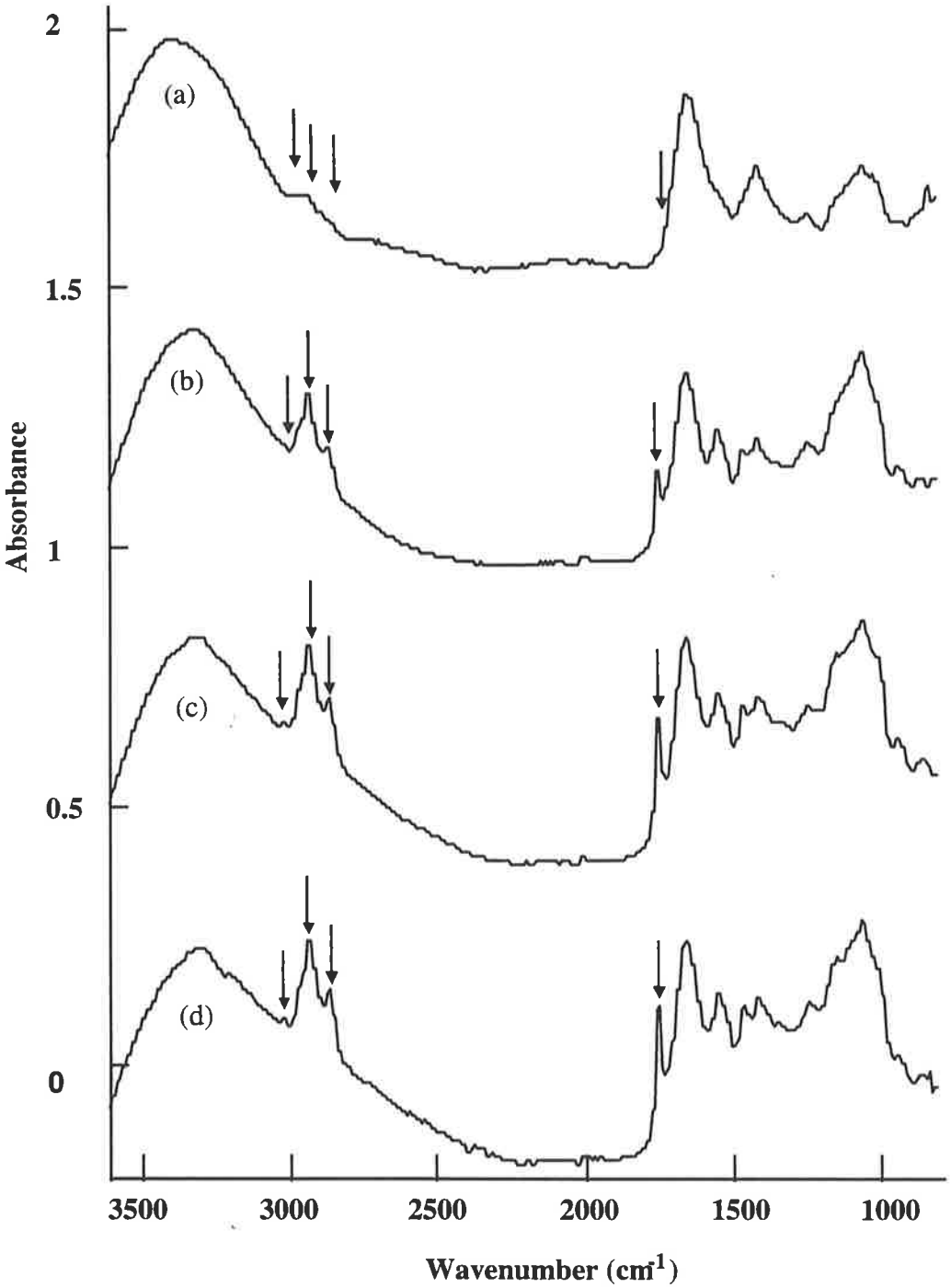
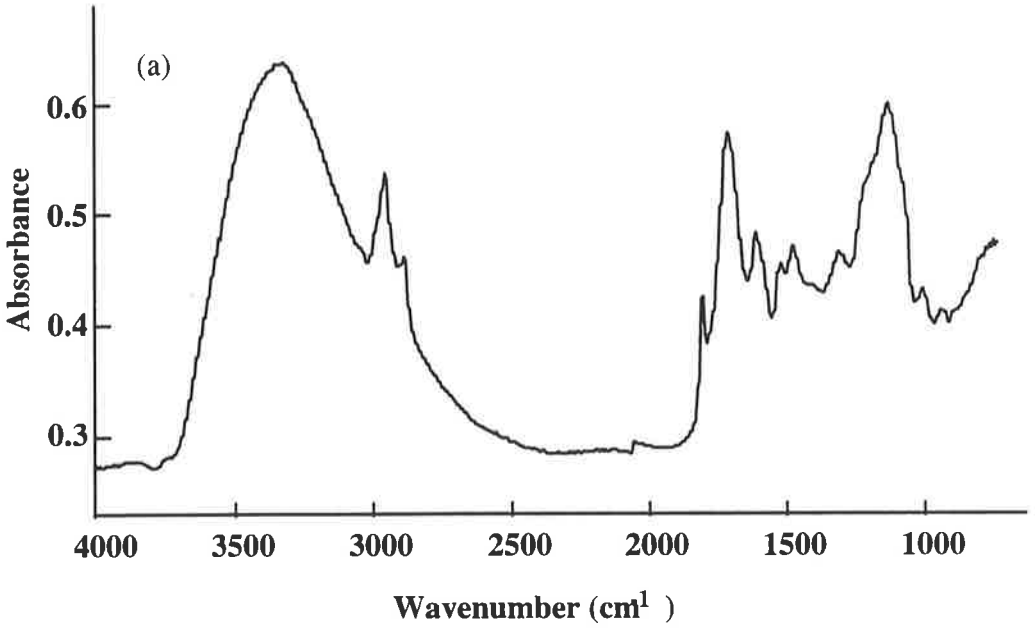

2
1.5
1
0.5
0
Absorbance
(a)
(b)
(c)
(d)
3500
3000
2500
2000
1500
1000
Wavenumber (cm-1)

Fig. 4.12 Infrared spectra of wheat embryos cultivar Halberd at 18 DAA showing that an embryo non-polar lipid containing the ester carbonyl peak (1743 cm^{-1}) was extractable by hexane. Fig. (a) shows the infrared spectrum of wheat embryos before an extraction with hexane; Fig. (b) shows the spectrum after an extraction with hexane.


(a)
Absorbance
0.6
0.5
0.4
0.3
4000
3500
3000
2500
2000
1500
1000
Wavenumber (cm1)

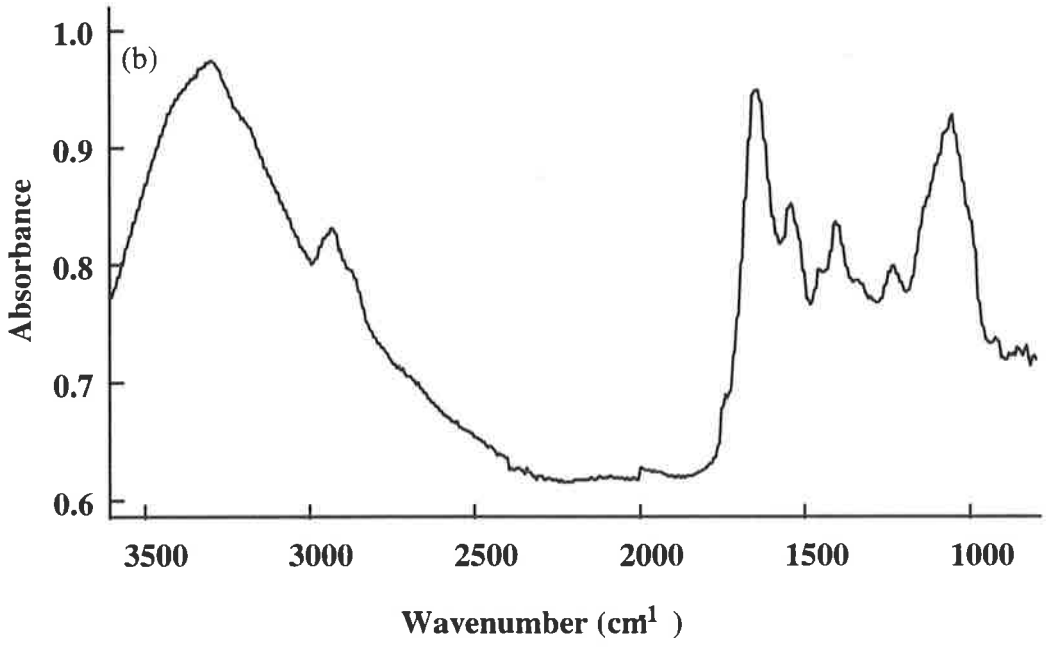

(b)
Absorbance
1.0
0.9
0.8
0.7
0.6
3500
3000
2500
2000
1500
1000
Wavenumber (cm1)

Fig. 4.13 Infrared spectra showing similarity of the spectra of olive oil triacylglycerols (a) and wheat embryo triacylglycerols extracted with hexane (b).

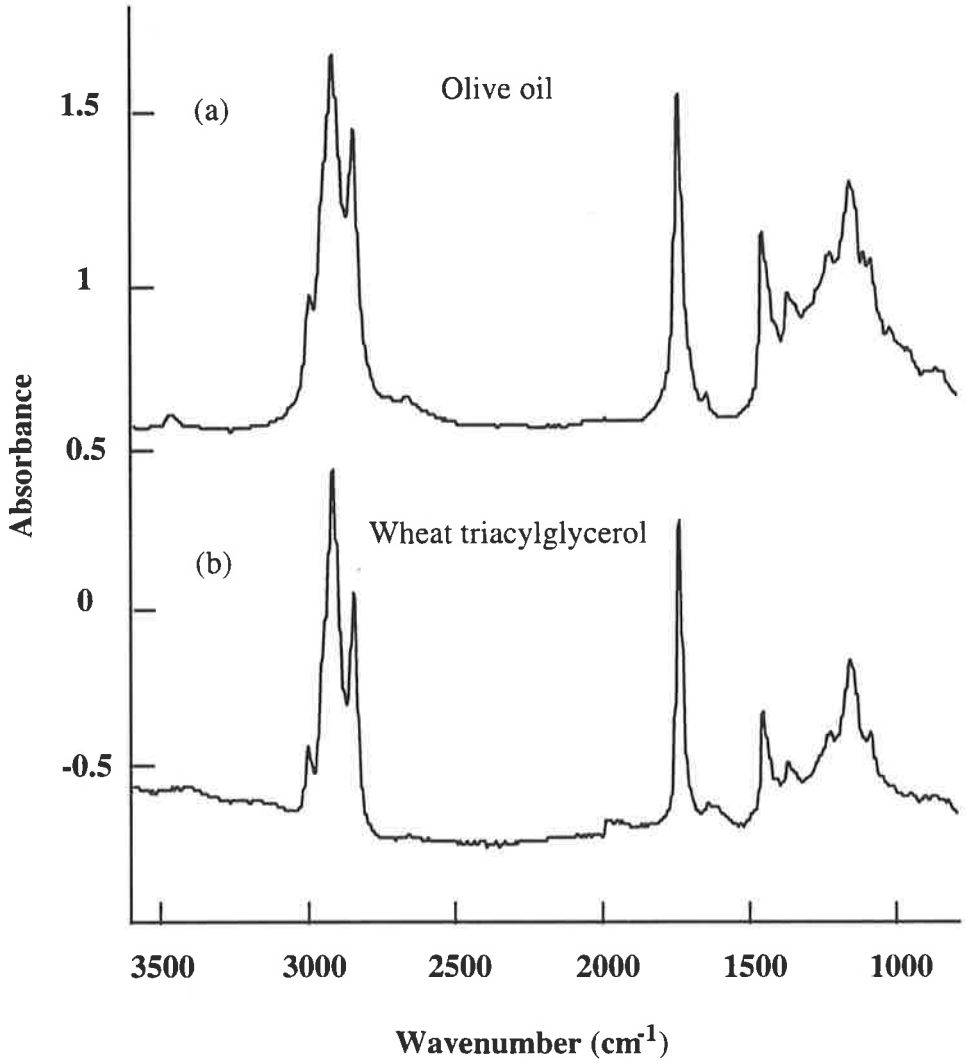

Absorbance
1.5
1
0.5
0
-0.5
(a)
Olive oil
(b)
Wheat triacylglycerol
3500
3000
2500
2000
1500
1000
Wavenumber (cm-1)

Fig. 4.14 Normal thin layer chromatography of triacylglycerols (TAG) in wheat embryos cultivar Halberd at 15 and 20 DAA compared with those after a desiccation tolerance test **(a)** and argentation chromatography of wheat triacylglycerols compared with olive oil triacylglycerols **(b)**

(a) Normal thin layer chromatography

Left: 1. Hexane 2. Olive oil 3. TAG of 15 day embryos 4. TAG of 20 day embryos

Right: 1. Hexane 2. Stearic acid 3. Olive oil 4. TAG of 22 day embryos 5. TAG of 15dSi7d 6. TAG of 27 day embryos 7. TAG of 20dSi7d

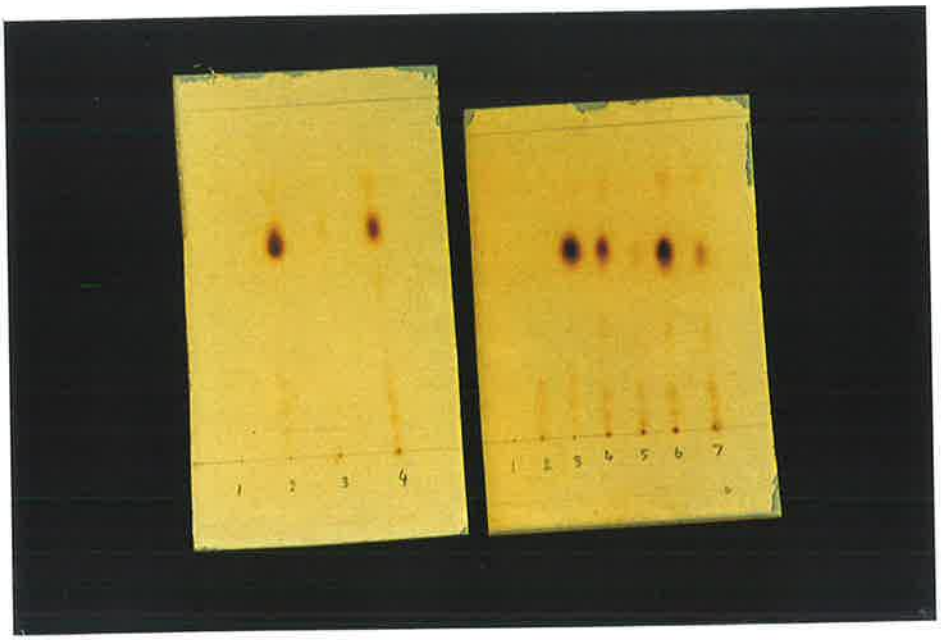
(b) Argentation chromatography

From left to right: 1. Hexane 2-3 Olive oil triacylglycerols 4-5 Triacylglycerols of wheat embryos

Note:

15dSi7d = 15 DAA dried over silica gel for 7 days
20dSi7d = 20 DAA dried over silica gel for 7 days

a



b

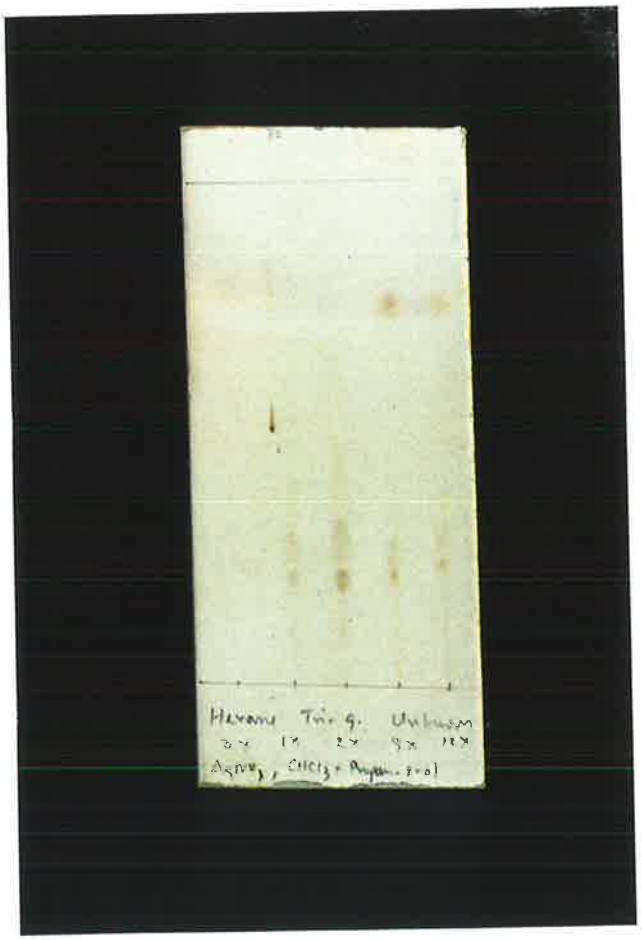


Fig. 4.15 Infrared spectra of wheat embryos cultivar Halberd at 15 DAA (a) and 20 DAA (b) after a desiccation tolerance test for 7 days and at the same corresponding ages. Note the diminution of the 1743 cm^{-1} ester carbonyl peak in the desiccated samples which also exhibit stronger O-H (3300 cm^{-1}) and C-O (1100 cm^{-1}) absorbances consistent with a higher carbohydrate content after desiccation. Each spectrum is the mean from 10 observations.

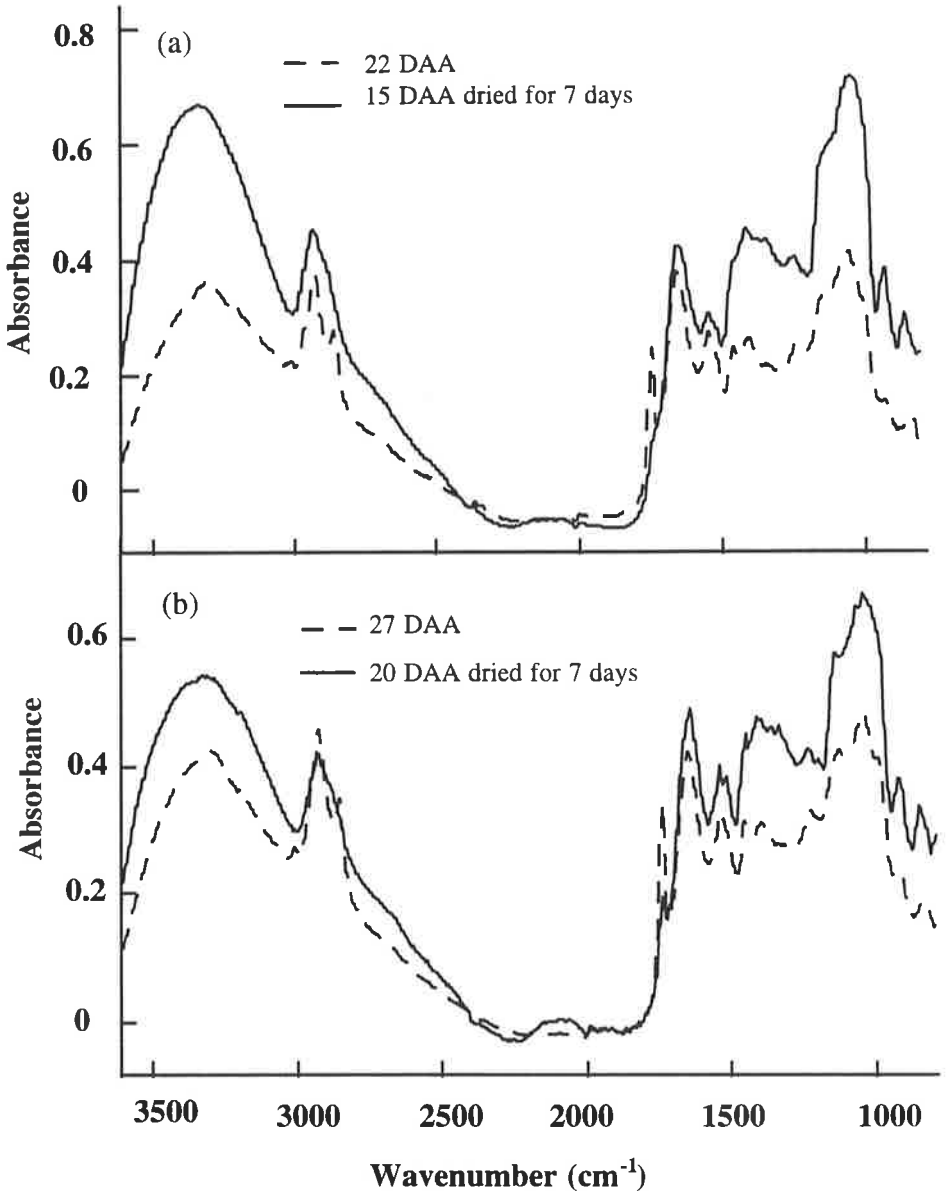
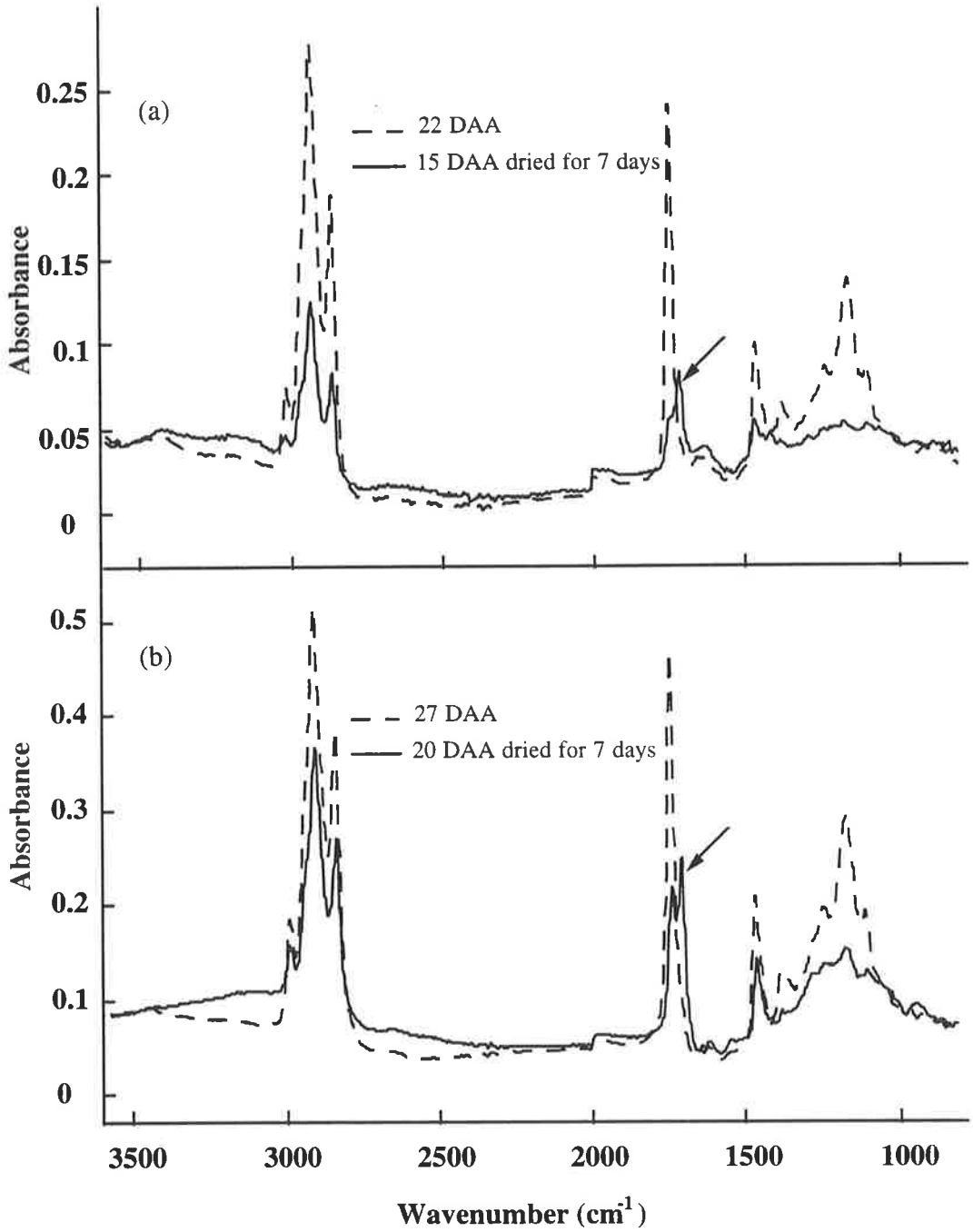

(a)
22 DAA
15 DAA dried for 7 days
Absorbance
0.8
0.6
0.4
0.2
0
(b)
27 DAA
20 DAA dried for 7 days
Absorbance
0.6
0.4
0.2
0
3500
3000
2500
2000
1500
1000
Wavenumber (cm-1)

Fig. 4.16 Infrared spectra of hexane soluble of wheat embryos cultivar Halberd showing the depletion of the ester carbonyl 1743 cm^{-1} peak after seeds were dried for 7 days and the shift to the carboxyl carbonyl 1710 cm^{-1} peak (indicated by arrows) in the dried embryos. The changes were observed at 15 DAA (a) and 20 DAA (b); spectra of fresh embryos at the same corresponding ages were shown in broken lines Each spectrum is the mean from 10 observations.


(a)
22 DAA
15 DAA dried for 7 days
Absorbance
0.25
0.2
0.15
0.1
0.05
0
(b)
27 DAA
20 DAA dried for 7 days
Absorbance
0.5
0.4
0.3
0.2
0.1
0
3500
3000
2500
2000
1500
1000
Wavenumber (cm-1)

However, the existence of residual 1743 cm^{-1} signal after drying at the desiccation-tolerant stage is not always apparent, as can be seen from the more comprehensive data in the desiccation series starting with 20 DAA embryos shown in Fig. 4.10c. In this case a decrease of the absorbance ratio to 0.9 was observed after drying for 7 days which corresponds to the loss of virtually all the 1743 cm^{-1} ester carbonyl signal of TAG.

Hexane soluble fractions (Fig. 4.16) confirm the loss of the ester carbonyl peak (1743 cm^{-1}) after drying. After drying the 15 DAA embryo, the ester carbonyl peak at 1743 cm^{-1} shifts to 1710 cm^{-1}. The shift of 1743 cm^{-1} peak is concomitant with the loss of ester C-O stretching at 1100 cm^{-1} (Fig. 4.16a). At 20 DAA, drying also results in the shift from 1743 cm^{-1} peak to 1710 cm^{-1} peak, however, small residual peaks of ester carbonyl (1743 cm^{-1}) and C-O stretching (1100 cm^{-1}) are still evident (Fig. 4.16b) in these samples. The formation of the 1710 cm^{-1} peak is consistent with the carbonyl absorbance for saturated carboxylic acids (William and Fleming 1989). Thin layer chromatography also confirms the loss of triacylglycerols after drying (lane 5, Fig. 4.14a). However, the intensity of the free fatty acid spot corresponding in Rf (relative distance of the free fatty acid front to chloroform front which is a mobile phase) to stearic acid is not commensurate with the decrease in triacylglycerols. This may reflect substantial catabolism of the free fatty acids, or differences in the solubility of iodine in the ester compared to the free fatty acid.

4.3.5 Signals detectable in wheat embryos using ^{13}C NMR spectroscopy

Fig. 4.17 shows signals attributed to carbohydrates (~110-57 ppm) and carbonyl groups (~190-165 ppm) in fresh wheat embryos at 15, 22 and 27 days. The %proportion of carbohydrate (area 110-57 ppm)/total spectrum (area 0-250 ppm) does not show significant changes during development (Table 4.1). The signals of aliphatic carbons were at ~0-50 ppm (Table 4.2).

Table 4.1 shows that after drying 15 DAA seeds for 7 days, a depletion of the proportion of carbohydrate is observed by comparison with the fresh 22 DAA seeds. This coincides with an increase of the proportion of carbonyl peak areas (Table 4.1).

Desiccation for 7 days showed no depletion of the relative proportion of the carbohydrate peak area. The proportion of carbonyl peak areas (20 DAA:7 day drying) also show a slight increase but at a lower level than that of 15 DAA dried for 7 days (Table 4.1). An increase of carbonyl groups after drying is supported by an increase of the peak at 176 ppm in Figs. 4.18 and 4.19. Changes in carbohydrate and carbonyl signals in fresh developing and after drying (at the desiccation-sensitive and -tolerant stages) are shown in Figs. 4.20a, b.

At 15 DAA, which is the desiccation-sensitive stage, the proportion of aliphatic areas (at 0-50 ppm) increases after drying (Table 4.2). This increase is shown by the signal peak at approximately 30 ppm (Fig. 4.20a). Drying 20 DAA seeds for 7 days (the desiccation-tolerant stage) only slightly increases the proportion of these aliphatic groups (Table 4.2 and Fig. 4.20b).

Table 4.1 % Proportion of peak area/total area (0-250 ppm) of ^{13}C NMR spectrum of carbohydrates of wheat embryos during development and after drying.

Seed conditions		Carbohydrate ~110-57 ppm	C-1 ~101 ppm	C-2,3,4,5 ~72.5-82.2 ppm	C-6 ~62.2 ppm	Carbonyl ~190-165 ppm
Fresh	15d	83.4	12.9	51.6	14.4	2.7
	22d	81.9	12.8	53.5	15.6	2.4
	27d	84.5	13.4	55.3	15.8	2.5
Dry	15d Si5d	76.8	12.4	50.2	14.2	3.9
	15d Si7d	75.4	11.5	48.6	15.2	4.0
	20d Si5d	75.8	11.9	49.2	14.7	3.6
	20d Si7d	84.3	13.4	55.7	15.2	3.2

Table 4.2 Changes in % proportion peak area/total area (0-250 ppm) of ^{13}C NMR spectrum of aliphatic carbons in wheat embryos before drying and after drying for 5 and 7 days

Seed conditions		$-CH_2-$/ $-CH_3$ ppm	Proportion of Integral areas (%)
Fresh	15 d	0-50	9
	22d	0-50	9
	27d	0-50	6
Dry	15d Si5d	0-50	10
	15d Si7d	0-50	14
	20d Si5d	0-50	13
	20d Si7d	0-50	8

Fig. 4.17 ^{13}C NMR spectra of carbohydrates in wheat embryos showing the predominant peaks (C_1-C_6) range from 57-110 ppm (a) at 15 DAA, (b) at 22 DAA and (c) at 27 DAA.

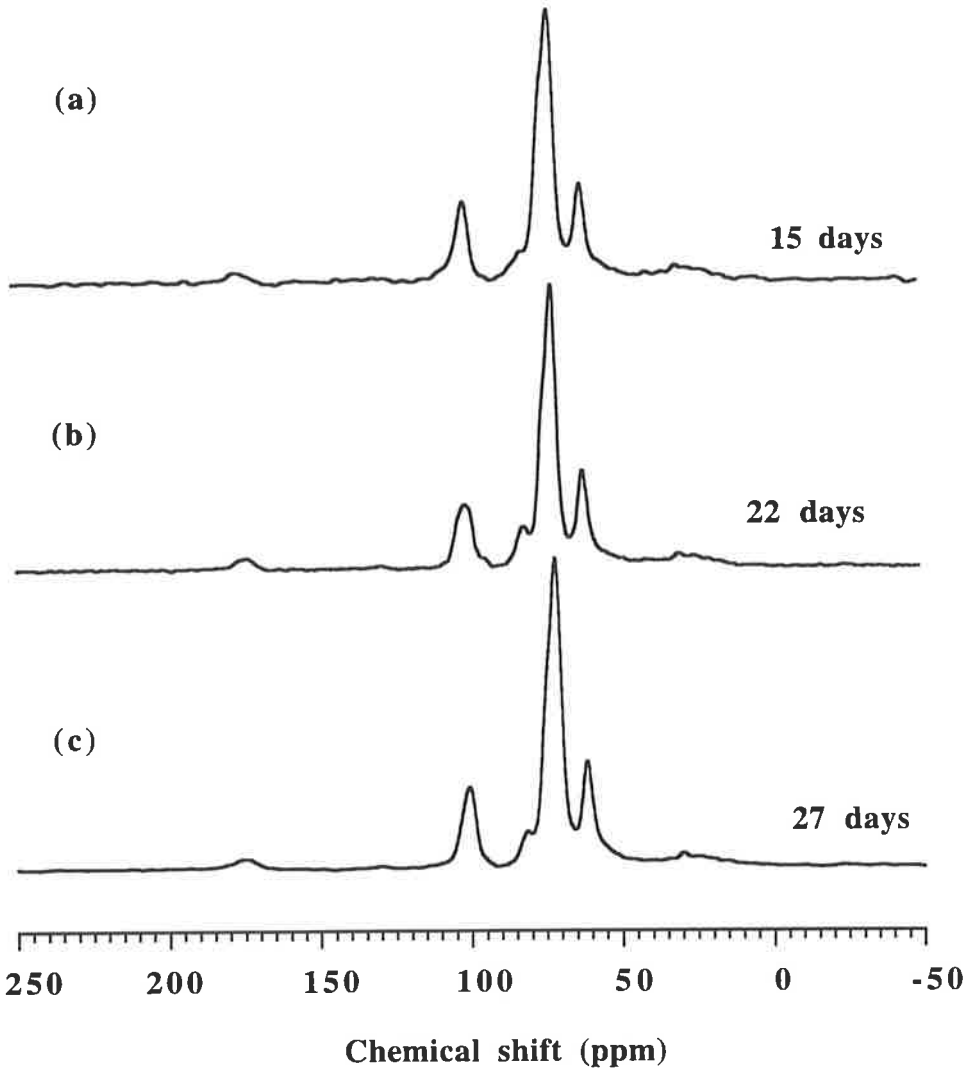

(a)
15 days
(b)
22 days
(c)
27 days
250
200
150
100
50
0
-50
Chemical shift (ppm)

Fig. 4.18 ^{13}C NMR spectra of carbohydrates in wheat embryos cultivar Halberd at 15 DAA after drying for 7 days (a) and 5 days (b). The spectra of developing seeds at 22 DAA and 15 DAA are shown in (c) and (d) respectively.

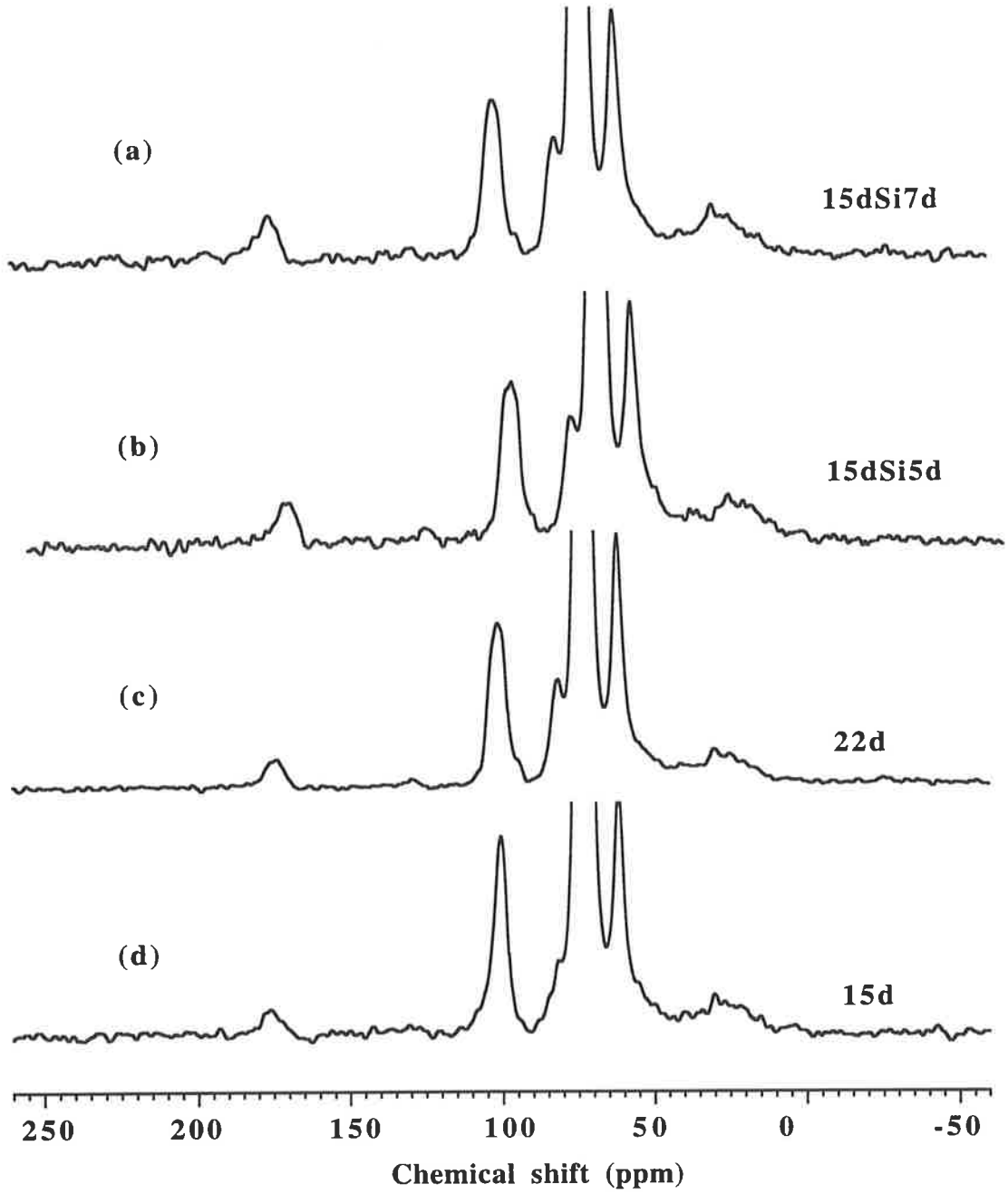

(a)
15dSi7d
(b)
15dSi5d
(c)
22d
(d)
15d
250
200
150
100
50
0
-50
Chemical shift (ppm)

Fig. 4.19 ^{13}C NMR spectra of carbohydrates in wheat embryos cultivar Halberd at 20 DAA after drying for 7 (a) and 5 days (b); spectrum at 27 days is shown in (c).

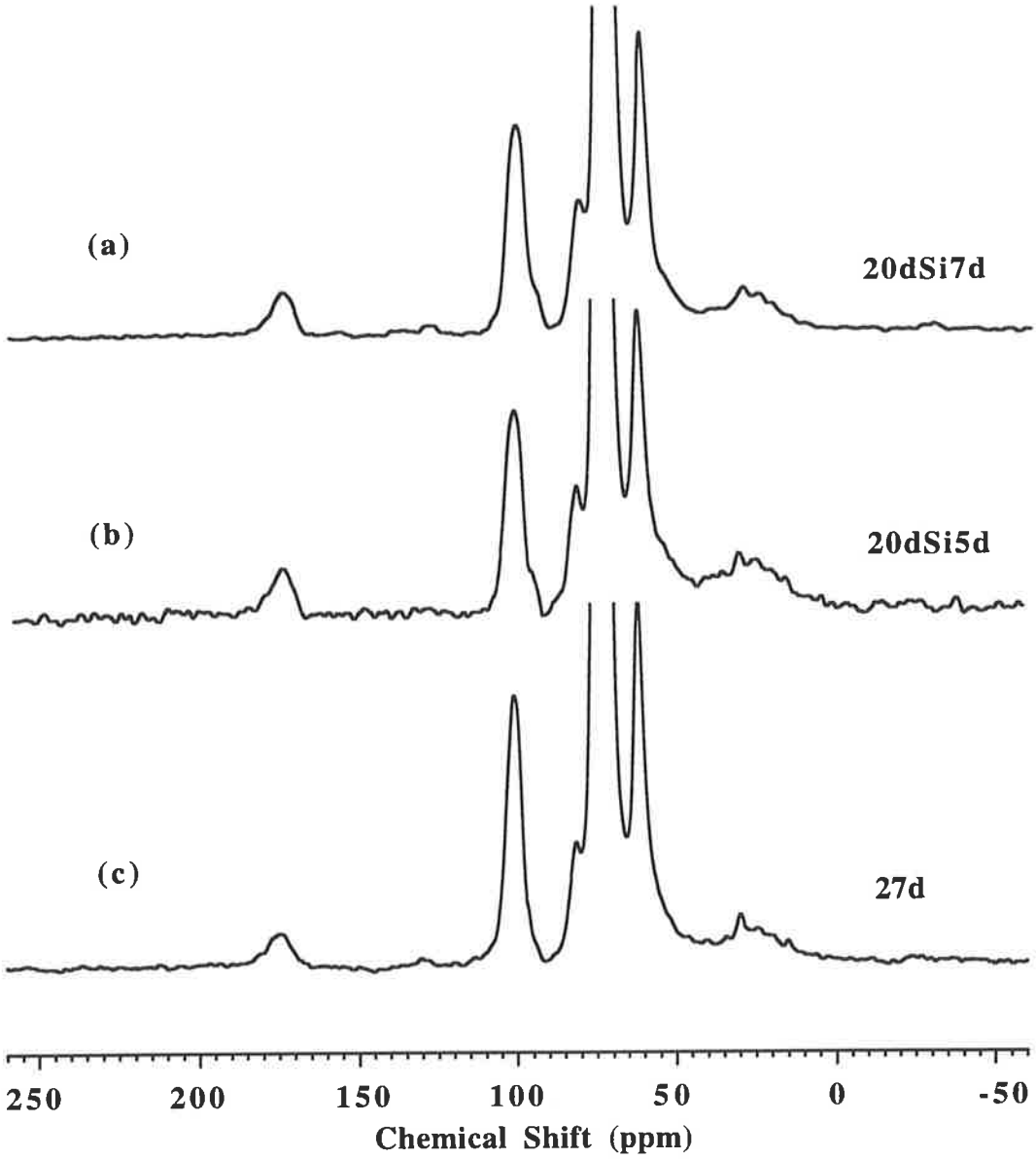
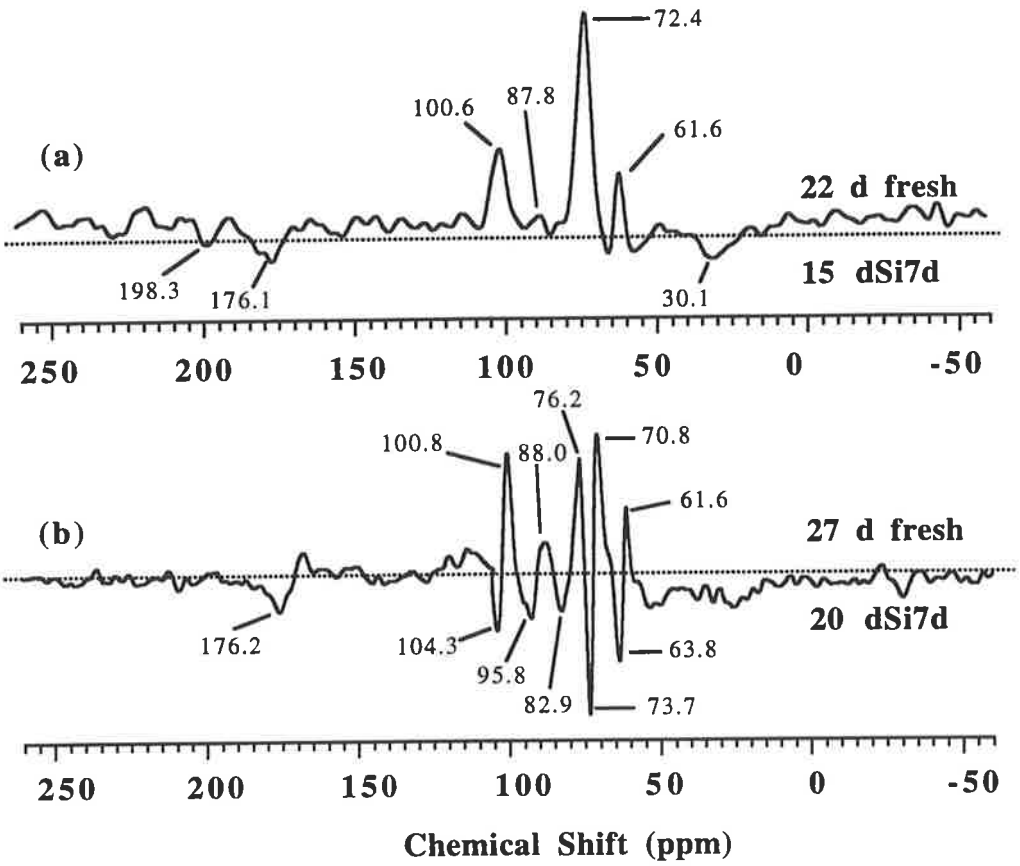

(a)
20dSi7d
(b)
20dSi5d
(c)
27d
250
200
150
100
50
0
-50
Chemical Shift (ppm)

Fig. 4.20 Subtract spectra of carbohydrates in wheat embryos (a) at 22 DAA vs 15 DAA dried for 7 days (b) at 27 DAA vs 20 DAA dried for 7 days; the spectra showed the difference in the carbohydrate residue in the dried embryos at the desiccation-sensitive stage (15 DAA) and the desiccation-tolerant stage (20 DAA). Positive proportion above broken lines show a higher proportion in fresh embryos, negative proportion below the line shows a higher portion in embryos after drying. Spectra were adjusted using mass balance difference.


(a)
72.4
100.6
87.8
61.6
22 d fresh
15 dSi7d
198.3
176.1
30.1
250
200
150
100
50
0
-50
(b)
76.2
100.8
88.0
70.8
61.6
27 d fresh
20 dSi7d
176.2
104.3
95.8
82.9
63.8
73.7
250
200
150
100
50
0
-50
Chemical Shift (ppm)

4.4 Discussion

4.4.1 Developmental stages and desiccation tolerance and use of infrared spectroscopy in studying the role of triacylglycerols in desiccation tolerance

The developmental sequence and the effect of maturation desiccation tolerance as shown in Figs. 4.1, 4.2 and 4.3 clearly shows that desiccation tolerance is a function of the developmental stage of the embryo. It is noticeable that the immature 10 DAA and 13 DAA embryos either simply do not develop or are too easily damaged at excision. Most of the embryos were translucent, having a club-shaped mass with a narrow elongated base and the endosperm was watery-like and occasionally some had passed into the milk stage. The milk stage is known to involve rapid cell division (Percival 1921; Peterson 1965).

Both the infrared absorbance ratio for the triacylglycerol ester carbonyl group and the proportion of normal seedlings decreased after 15 DAA 'Halberd' seeds were dried for 3 or 7 days. Thus it is possible that the triacylglycerol peak may be associated with the desiccation tolerance phenomenon. The presence of detectable triacylglycerol esters appears to be of importance for the germinability of these desiccation sensitive embryos, and would be consistent with the utility of TAG either as energy reserves or as undamaged membrane fatty acid depots for stress-related desiccation membrane damage-repair. In seeds at the desiccation-tolerant stage, there are frequently detectable residual triacylglycerols in the embryo even after 7 days of desiccation (Figs. 4.14a and 4.15b). At the desiccation-sensitive stage (15 DAA+7 days), the residual triacylglycerol signal at 1743 cm^{-1} is barely detectable and very faint in the TLC extracts (Figs. 4.14a, 4.15a).

Desiccation sensitivity has been reported to be associated with membrane injury due to free radicals causing phospholipid peroxidation (Senaratna *et al.* 1984, 1985*b*;

1987). During the desiccation-sensitive stage, these workers reported that there was an increase of a ratio of free fatty acid : phospholipid. This was associated with de-esterification of membrane phospholipids and free fatty acid accumulation (Senaratna *et al.* 1984, 1985*b*; 1987). The current observations are in accord with a substantial rise in free fatty acids, whereas the TLC data clearly indicate that the source of free fatty acid in this experiment (Fig. 4.16) is the triacylglycerols. Senaratna *et al.* (1984, 1985*b*; 1987) isolated microsomal membrane from soybean axes to detect changes during desiccation, but whole wheat embryos were used in this study. Thus, it is now known that free fatty acids could come from two sources i.e. membrane phospholipids and triacylglycerols. However, it is not clear whether the free fatty acids are derived from the de-esterification of TAG mediated by free radicals or the lipolysis of TAG by lipase.

Triacylglycerols are commonly detectable reserves in immature wheat embryos (Evers and Bechtel 1988). It is therefore reasonable to suggest that some of the importance of the triacylglycerols in the cell resides in their ability to act as a reserve depot of undamaged fatty acids to repair free radical damage in the critically important membrane phospholipids. Alternatively the structure and number of desiccation induced water 'bridges' across hydrophobic regions of triacylglycerol regions may be essential for membrane integrity.

At 15 DAA, the wheat embryo is at a critical stage for desiccation sensitivity and the endosperm is in the milky stage. Fig. 4.1 clearly shows that embryos were translucent, in some cases the embryonic axes were opaque. This stage of the embryo has been interpreted as the end of a pre-milk stage and the beginning of the milky stage (Peterson 1965). During this stage, embryos increased rapidly in both cell division and differentiation (Percival 1921). Thus, drying the seeds and thereby altering the lipid status as detected by the decrease in the 1743 cm^{-1} ester carbonyl (Figs. 4.7 and 4.15) has a very deleterious effect on membrane-associated

events in the germination of embryos (Kermode and Bewley 1985*a*; Kermode 1990; Senaratna and McKersie 1983; Koster and Leopold 1988).

At 15 DAA, a decrease in the ester carbonyl absorbance ratio to less than 1.03 is connected with a decrease in embryo survival (Figs. 4.7a, b, c and 4.11a). As suggested earlier, this could be because of insufficient initial triacylglycerols (Figs. 4.7a, 4.11a) to act as free fatty acid reserves for the repair of membrane damage during desiccation.

At 18 DAA, embryos are in an intermediate state of desiccation tolerance (Figs. 4.9a, b, c) and the ester carbonyl peak of triacylglycerols is also detectable in the embryos to a larger extent than that in 15 DAA embryos (Figs. 4.9a, 4.11b). At this intermediate stage, embryos presumably still have enough undamaged (unoxidised) free fatty acid reserves for the repair mechanisms. Thus, a decrease of absorbance ratio during desiccation to less than 1.03 exhibits a less deleterious effect on the survival of wheat embryos after desiccation (Figs. 4.9a, b, c) than at 15 DAA (Figs. 4.7a, b, c).

By the time the seeds are at 20 DAA, the embryos had reached the desiccation-tolerant stage and had large reserves of triacylglycerols as indicated in Figs. 4.10a, 4.11c. Again using the hypothesis of the essentiality of having a sufficiently large initial pool of unoxidised free fatty acid reserves for repairing membranes, a decrease of an absorbance ratio to less than 1.03 is not critical for the survival of 20 DAA embryos. This may be due to fully developed antioxidant mechanisms such as superoxide dismutase and α-tocopherol which prevent membrane oxidation. Furthermore, this is at the end of the milky stage, when the embryos are fully developed and white (Fig. 4.1). Moisture content begins to decline during normal development and drying at this stage is not harmful to germination.

In conclusion, the alterations in the 1743 cm^{-1} ester carbonyl groups during development and desiccation have now focussed attention on the triacylglycerols as

being hitherto unrecognised indicators of the status of the embryonic axis during development and desiccation. The precise role of triacylglycerols in desiccation tolerance in wheat remains to be elucidated.

4.4.2 Use of ^{13}C NMR to monitor the possible mechanisms of desiccation tolerance

During development, signals of ^{13}C NMR at 101.6, 81.8, 72.6 and 62.1 ppm were observed which represent the carbon positions in the carbohydrate polysaccharide structure, i.e. C_1, C_4, $C_{2,3,5}$ and C_6 respectively (O'Donnell *et al.* 1981; Wilson 1987; Jarvis and Apperley 1990). In the literature (Fyfe *et al.* 1983; Wilson 1987; Jarvis and Apperley 1990; O'Donnell *et al.* 1981; Haw and Maciel 1983), these signals have been intepreted as two different forms of polysaccharides, cellulose and amylose.

The first form is cell wall structural polysaccharide (Fyfe *et al.* 1983; Wilson 1987; Jarvis and Apperley 1990). Because cellulose is a major component of the cell wall, it is reasonable to suggest that immature embryos synthesise cellulose. The cell wall is synthesised during cell division in the meristematic tissue. At the early stage of cell division, primary cell wall is thin with only a few layers (Torrey 1967; Cutter 1977). Wilson (1987) stated that the number of glucose units when cellulose is being synthesised commonly varies from 2 to 4000. Because the developing embryos are soft, this suggests that the cellulose present must only have non-complex units or be present in thin walls. The immature embryo (15DAA) has an amorphous form of cellulose (C_4 at ~81.8 ppm) (Fig. 4.17a). Thereafter, these amorphous forms change their structure when embryos are more mature at 22 and 27 DAA (Figs. 4.20a, b). The chemical shift of C_4 to 88.0 ppm in 27 days fresh embryos (Fig. 4.20b) has been interpreted to mean that the amorphous form of cellulose (C_4 at 81.8 ppm) possibly becomes the crystalline form (C_4 at 88.0 ppm)

(Doyle *et al.* 1986; Jarvis and Apperley 1990) which is known to increase strength and rigidity of cell wall (Anderson and Beardall 1991).

The second major form of carbohydrate is the reserve carbohydrate amylose (O'Donnell *et al.* 1981; Haw and Maciel 1983). Signals at approximately 105, 75 and 62 ppm have previously reported to correspond to C_1, $C_{2,3,5}$ and C_6 in amylose (O'Donnell *et al.* 1981). The carbohydrate spectra obtained here (Fig. 4.17a, b and c) are identical with those observed in corn starch and starch of many seed species include wheat (O'Donnell *et al.* 1981).

O'Donnell *et al.* (1981) also suggested that the signals at 170-180 ppm together with those at 20-30 ppm can be attributed to seed proteins. In this interpretation, the signal ~175 ppm arises from the peptide carbonyl carbon in a protein, and the signal ~22 ppm comes from aliphatic side chains of the amino acids in the proteins. The investigators also observed the relationship between these two signals, i.e. when the protein signals were prominent, the starch signals at ~62 ppm were obscured. However, the regions at ~170-180 ppm and ~14-40 ppm are also known to correspond to the regions of carbonyl groups of carboxyl (the former) and aliphatic carbon (the latter) of fatty acids (Rutar *et al.* 1977; Wilson 1987; Rutar 1989). These carbonyl and aliphatic areas are consistent with those detectable using DRUIDS (Figs.4.16a, b). The results of DRUIDS suggested the presence of increased free fatty acids (Fig. 4.16a, b) in desiccated wheat embryos. Thus, the presence of carbonyl carbons in the ^{13}C NMR data (Figs. 4.20a, b) is also consistent with the presence of fatty acids as well as possible protein signals.

After drying, an increase of the carbonyl and aliphatic peaks is observed (Figs. 4.20a, b). This is coincident with the changes in carbohydrate regions (Figs. 4.20a, b). At 20 DAA, drying for 7 days elicits changes which can be interpreted as changes in carbohydrate structure from the crystalline to an amorphous form of cellulose (Fig. 4.20b). These changes are indicated by an appearance (in negative

proportion) of peaks at 176.2, 104.3, 82.9, 73.7 and 63.8 ppm after drying (Fig. 4.20b). The signals at 104.3, 82.9, 73.7 and 63.8 ppm are known to correspond to carbohydrate signals. In this interpretation, the signal of C_4 at ~83.0 ppm indicates the presence of the amorphous form in dried embryos (20dSi7d) rather than the crystalline form (C_4 at ~88.0 ppm) in fresh embryos (27d) (Figs. 4.20b). Changes to the crystalline form could be associated with desiccation tolerance because 15 DAA embryos do not exhibit a prominent peak for the crystalline form (C_4 at 88.0 ppm) until 22 and 27 DAA that a prominent crystalline cellulose signal at 88.0 ppm is apparent in fresh embryos which by that time are at the desiccation-tolerant stage (Figs. 4.17, 4.20a, b). It is possible that the development of the crystalline form of cellulose increases the strength of the cell wall, hence maintaining the integrity of the cell under the stress of desiccation.

Alternatively, the difference spectra in Figs. 4.20a, b show that changes occur in carbohydrate difference spectra which distinguish an embryo in the desiccation-sensitive stage (15 DAA+7D) from the desiccation-tolerant stage (20 DAA+7D). Fig. 4.20a shows that drying at 15 DAA results in virtually the complete absence of signals between 60-105 ppm which are very prominent in the 20 DAA+7 days desiccation spectra (Fig. 4.20b). These residual carbohydrate species 104.3, 95.8, 82.9, 73.7 and 63.8 at 20 DAA+7 days desiccation may play a vital role in maintaining cellular integrity at the desiccation-tolerant stages.

The corresponding difference data of total sugars (sucrose+raffinose) from the experiments in Chapter 3 (Table 4.3) indicate an excess of the total sugars (sucrose+raffinose) after drying in the desiccation tolerant embryos. Thus, difference spectral data in Fig. 4.20b could be due sucrose and raffinose. However, this difference spectrum is not consistent with the spectrum of pure sucrose because the signal at 95.8 ppm (the region of anomeric carbon C_1 of glucose) is broad with a shoulder. In particular spectra, the 104.3 signal is in the region for fructofuranosyl C_2 glycosides. The signals at 82.9, 73.7 and 63.8 ppm are

consistent with C_5, $C_{3,4}$ and $C_{1,6}$ in fructofuranosyl ring (Breitmaier and Voelter 1990; Pfeffer *et al.* 1979). Because the initial spectra of carbohydrate components of the embryos before and after drying at the desiccation-tolerant stage are not different (Fig. 4.19), the evidence is consistent with the presence of a small amount of some kind of fructofuranosyl glycan at the desiccation-tolerant stage.

Table 4.3 Total sugar concentrations in wheat embryos in fresh embryos and after the seeds were tested for desiccation tolerance for 6 or 7 days.

Seed age	Sugar (su+ra) fresh (nmol/embryo)	Sugar (su+ra) dry (nmol/embryo)	Difference (su+ra) dry-fresh (nmol/embryo)
15 DAA+6D*	265.1	347.8	82.7
27 DAA+7D	268.9	339.5	70.6

* These glasshouse 15 DAA seeds were already desiccation tolerant.

Abbreviation: su = sucrose; ra = raffinose.

Alternatively, signals of 104.3, 82.9, 73.7 and 63.8 ppm, could also be interpreted as representing free monosaccharides arising from saccharide degradation. However, it is unlikely that free monosaccharides are detectable in the desiccation-tolerant stage. This is because monosaccharides are normally only detectable in non-viable seeds (Ovcharov and Kosheler 1974) or in the embryos at the desiccation-sensitive stage (Koster and Leopold 1988). Furthermore, an increase of monosaccharides has been reported to regulate respiration; Leprince *et al.* (1992) have demonstrated that the monosaccharide-mediated increase in respiration also correlates to the loss of desiccation tolerance.

In summary, spectro-chemical changes in wheat embryos are detectable by both DRUIDS and ^{13}C NMR techniques. At the desiccation-sensitive stage, desiccation results in a decrease of the triacylglycerol ester carbonyl peak at 1743 cm^{-1} in the infrared coincident with a decrease of germination. Triacylglycerols appear to be associated with desiccation tolerance in immature embryos. Although polymethylene signals are of low intensity in the ^{13}C NMR and changes in this region are not easily measured by this technique, there are substantial differences in the residual carbohydrate signals in 7 day desiccated 15 DAA seeds compared to 7 days desiccated 20 DAA seeds. These signals may well be structural carbohydrate (polysaccharide) signals responsible for maintaining cellular function integrity during desiccation. Quantitation of the ^{13}C NMR signals points to a depletion of carbohydrates taking place during drying at the desiccation-sensitive stage.

4.5 Conclusions

1) The data suggest that triacylglycerols in developing embryos play a role in the tolerance to desiccation in the developing wheat (cultivar Halberd) embryo. It is possible that these triacylglycerols act as a storage depot for undamaged fatty acids to repair free radical damaged lipids and thereby maintain the integrity and critical functioning of crucial membranes in the developing embryo.

2) Drying induces both quantitative and qualitative changes in carbohydrates in embryos. The evidence from the ^{13}C NMR differential data suggests that desiccation sensitivity is associated with cell wall damage caused by ~~drying cell~~ the cell drying before their structural polysaccharides are fully synthesised and that some kind of fructofuranosyl glycan, such as a higher molecular weight fructan, play an important role in the desiccation tolerance of seeds.

CHAPTER 5

A confocal microscopic study of lipid droplet changes in wheat embryos during development and after desiccation

5.1 Introduction

Lipids have been reported as primary energy reserves in immature wheat embryos during seed development. Accumulation of lipids continues from early stages of cell division until the seeds are mature (Evers and Bechtel 1988). At maturity, lipids in the wheat embryos represent approximately 30% of the total lipid content of the whole grain (Steven 1959). The storage lipids are known to be triacylglycerols (TAG) which are packed within droplets bound by a monolayer membrane (Hargin *et al.* 1980). These lipid droplets are degraded by lipase hydrolysis following the germination process (Wang and Huang 1987).

After germination, lipids in the embryonic axis are mobilised. The utilisation of lipids can be observed as lipid droplet degradation. Microscopy indicates that lipid degradation involves dispersion of lipid droplets from the cell wall, followed by a rapid decrease in the numbers of the droplets (Swift and O'Brien 1972*b*). The degradation of lipids starts by hydrolysis of triacylglycerols (TAG) into glycerol and fatty acids (Huang 1992). Thereafter, mechanisms for the conversion of fatty acids into sucrose through the glyoxylate cycle occur. Sucrose is then mobilised to the embryonic axis growing point (Kornberg and Beevers 1957; Longo and Longo 1970; Doig *et al.* 1975; Newman and Briggs 1976; Beevers 1980).

Apart from Nir *et al.* (1970*a*, *b*), who reported the effect of desiccation on lipid degradation in germinating maize seeds, there are few reports on the degradation of lipid droplets during desiccation. According to these workers, desiccation

resulted in lipid droplet degradation as indicated by the dispersion of the droplets. These results are similar to the degradation and lipid mobilisation after seed germination (Swift and O'Brien 1972*b*).

Comai *et al.* (1990) measured the density of RNA polymerase complexes on a gene from the dry oil seeds of rapeseeds (*Brassica napus.*) They found that although the metabolic activity of the dry seeds is relatively low, isocitrate lyase and malate synthase genes are transcriptionally active. This suggests that the metabolic processes of lipid degradation are ready to take place following germination process.

Infrared studies of desiccated wheat embryos (Chapter 4) using Diffuse Reflectance Spectroscopy have clearly established an increase of the ester carbonyl peak (1743 cm^{-1}) of the triacylglycerols coincides with the loss of desiccation sensitivity at 20 days after anthesis (DAA). Thin layer chromatography results have established that the decrease in ester carbonyl is primarily associated with the loss of TAG. This suggests that triacylglycerols confer desiccation tolerance by providing a readily metabolisable energy source to the desiccated embryos for the subsequent resumption of germination after rehydration.

To test this hypothesis, confocal microscopy has been employed to make visible the accumulation and degradation of lipid droplets in wheat embryos during development and after desiccation. Nile red, a fluorescent dye suitable for neutral lipids in plant tissue (Evans *et al.* 1992), has been used to stain the triacylglycerols present as lipid droplets in the embryonic tissues. The desiccation sensitive (15 DAA) and desiccation tolerant (20 DAA) wheat embryos were examined after drying for 5 days. The fresh embryos of developing seeds at the same corresponding ages (20 DAA and 25 DAA) were tested for a comparison with the desiccated seeds

The aims of the study were :

1) to investigate the accumulation of lipids, focusing on both the desiccation sensitive and desiccation tolerant stages

2) to examine the visible changes of lipids detectable in the embryos after desiccation in both stages and

3) correlate these observations with the infrared data

5.2 Materials and methods

5.2.1 Plant materials

Wheat seeds cultivar Halberd were collected from plants grown at 18°C under 16 h light 8 h dark. Ears were collected at 15 DAA (the desiccation-sensitive stage) and at 20 DAA (the desiccation-tolerant stage) respectively. At each harvest, seeds from the middle of the ears were removed for drying treatments. They were placed in a 5-cm-diameter Petri dish in jars (12 cm diameter, 22 cm height) containing 100 gm of silica gel. The Petri dishes were supported on a wire frame approximately 10 cm above the layer of silica gel. Incubation conditions were 25°C in darkness for 5 days. After 5 days, the dried seeds were prepared for microscopy. As was observed in previous experiments described in Chapter 3, detached seeds continue their development during drying, and hence developing fresh seeds at the same corresponding age were used for comparison.

5.2.2 Preparation of seeds for microscopy

See 2.6.1 - 2.6.3.

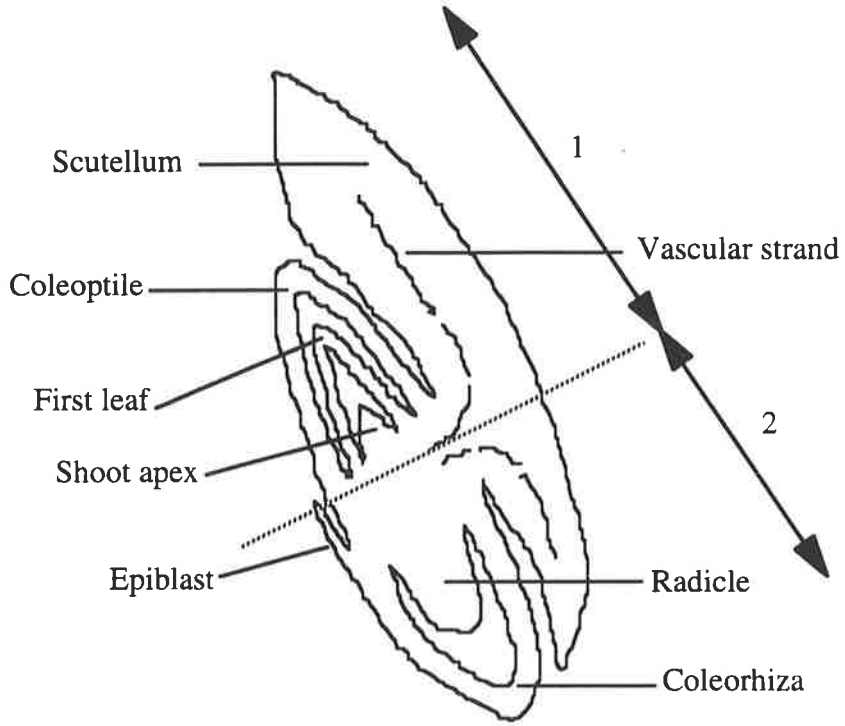


Fig. 5.1 Structure of a wheat embryo (longitudinal section)

1 = shoot side; 2 = radicle side.

5.3 Results

5.3.1 Distribution of lipid droplets in fresh embryos and in the embyros after drying

Wheat embryos at 15 DAA contain few lipid droplets and these are distributed mainly in the middle part of the scutellum (Fig. 5.2). The relatively weak fluorescence in the tissue of shoot and radicle of the embryonic axis (Figs. 5.2a-f) indicates that lipid deposition is low at this stage.

Figs. 5.3, 5.5, 5.7 and 5.9 shows lipid distribution in the sections of fresh developing embryos at 20 and 25 DAA. Lipid distribution in the sections of dried seeds are shown in Figs. 5.4, 5.6, 5.8 and 5.10. Lipid droplets become much more numerous in the fresh embryos at 20 and 25 DAA, as the embryos rapidly increase in size (Figs. 5.3, 5.5, 5.7 and 5.9). By comparison with the earlier developmental stage (20 DAA, Fig. 5.3), at 25 DAA (Fig. 5.7) vascular tissue with small lipid droplets in the scutellum becomes more evident. A myriad of spherical lipid droplets which strongly fluoresce with Nile red are present in the scutellum and the embryonic axis, i.e. shoot and radicle. Apart from the small droplets in the vascular tissue, lipid droplets in scutellum appear larger than those in the shoot and radicle (Figs. 5.3 and 5.7). These large lipid droplets are located near the tip of the scutellum (Figs. 5.3, 5.5, 5.7 and 5.9) whereas the numerous small droplets are located in the embryonic axis (Figs. 5.4, 5.5, 5.7 and 5.9).

After the 15 DAA and 20 DAA seeds have been dried for 5 days, small lipid droplets appear to be distributed throughout the scutellum and the embryonic axis (Figs. 5.4, 5.6, 5.8 and 5.10). The droplets maintain a spherical shape but the large droplets observable at the tip of the scutellum in fresh embryos (Figs. 5.3 and 5.7) are not evident. There is a very bright background fluorescence that is not in droplet form in the seeds after drying (Figs. 5.4, 5.6, 5.8 and 5.10).

Fig. 5.2 Lipid droplets in fresh wheat embryos at 15 days after anthesis (DAA). Images for 6 seeds (a-f) plus two controls, a delipidated sample (g) and an autofluorescence test (h). *sh*, shoot; *ra*, radicle; *sc*, scutellum; bar = 250 µm.

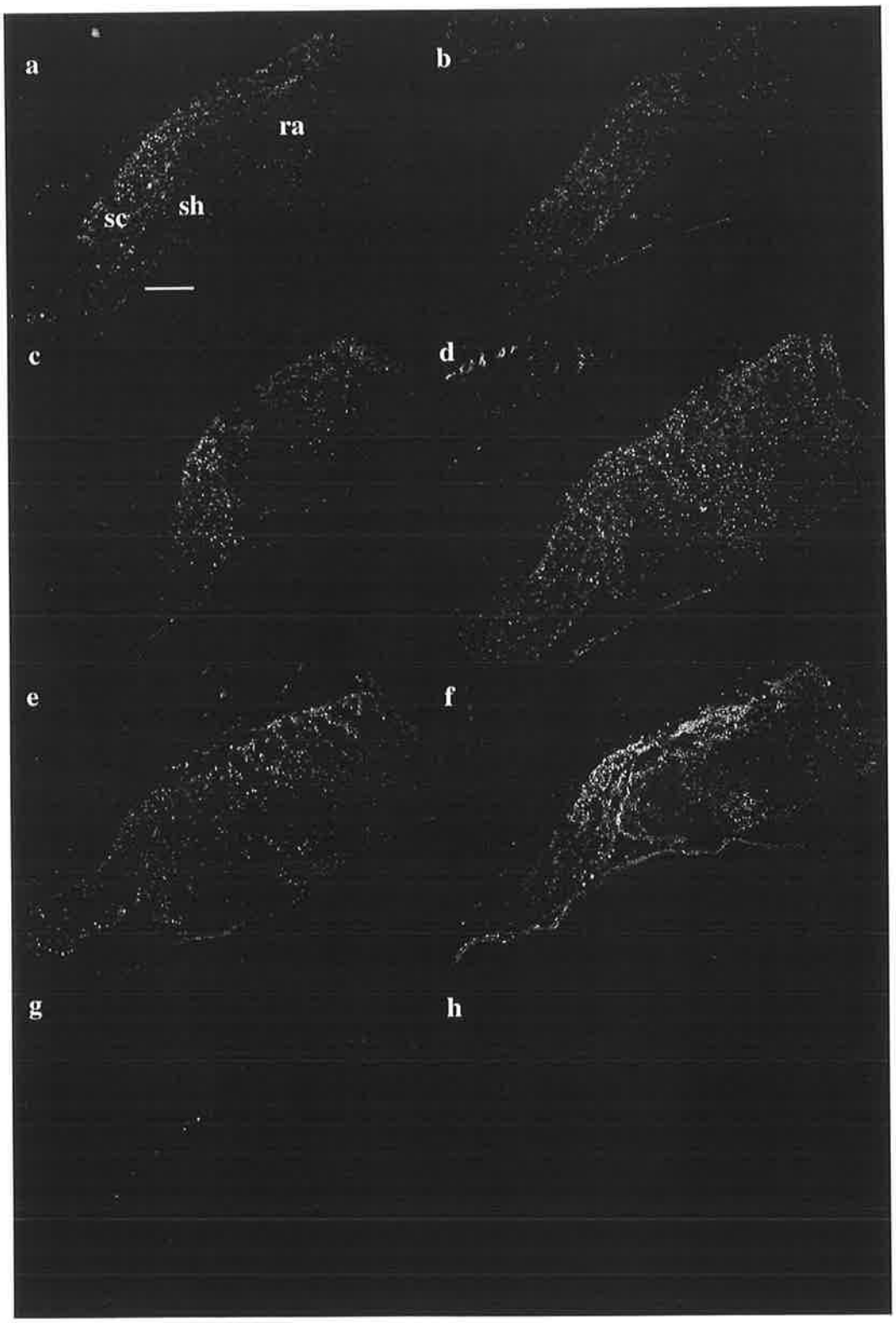

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Fig. 5.3 Lipid droplets in fresh wheat embryos at 20 days after anthesis (DAA). Images for 6 seeds (a-f) plus two controls, a delipidated sample (g) and an autofluorescence test (h). *sh*, shoot; *sc*, scutellum; bar = 250 μm. Arrows indicate a big droplet in the scutellum and a small droplet in the shoot. Each image represents shoot side of longitudinal section of embryo.

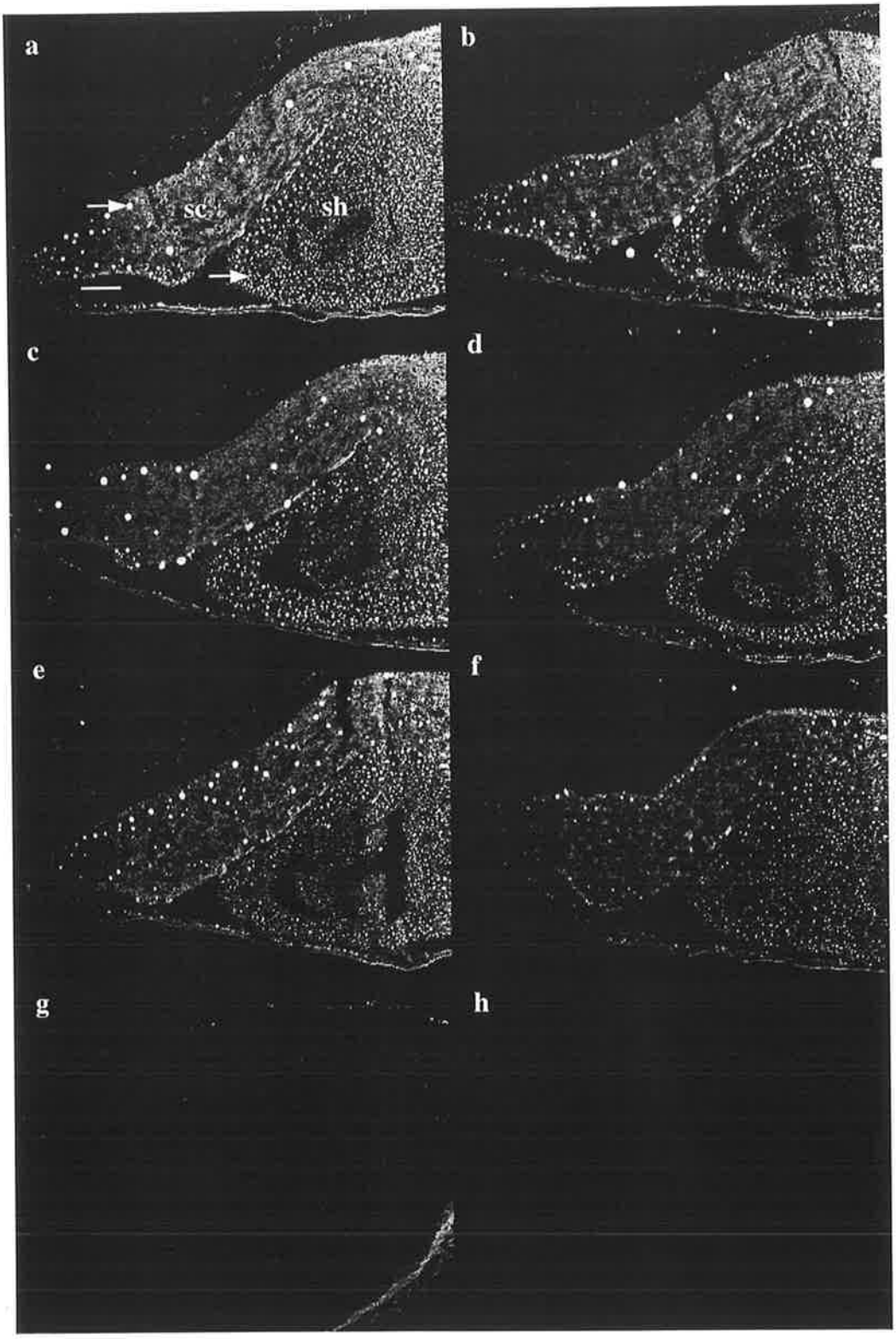
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Fig. 5.4 Lipid droplets in wheat embryos at 15 days after anthesis (DAA) after drying for 5 days. Images for 6 seeds (a-f) plus two controls, a delipidated sample (g) and an autofluorescence test (h). *sh*, shoot; *sc*, scutellum; bar = 250 µm. Each image represents shoot side of longitudinal section of embryo.

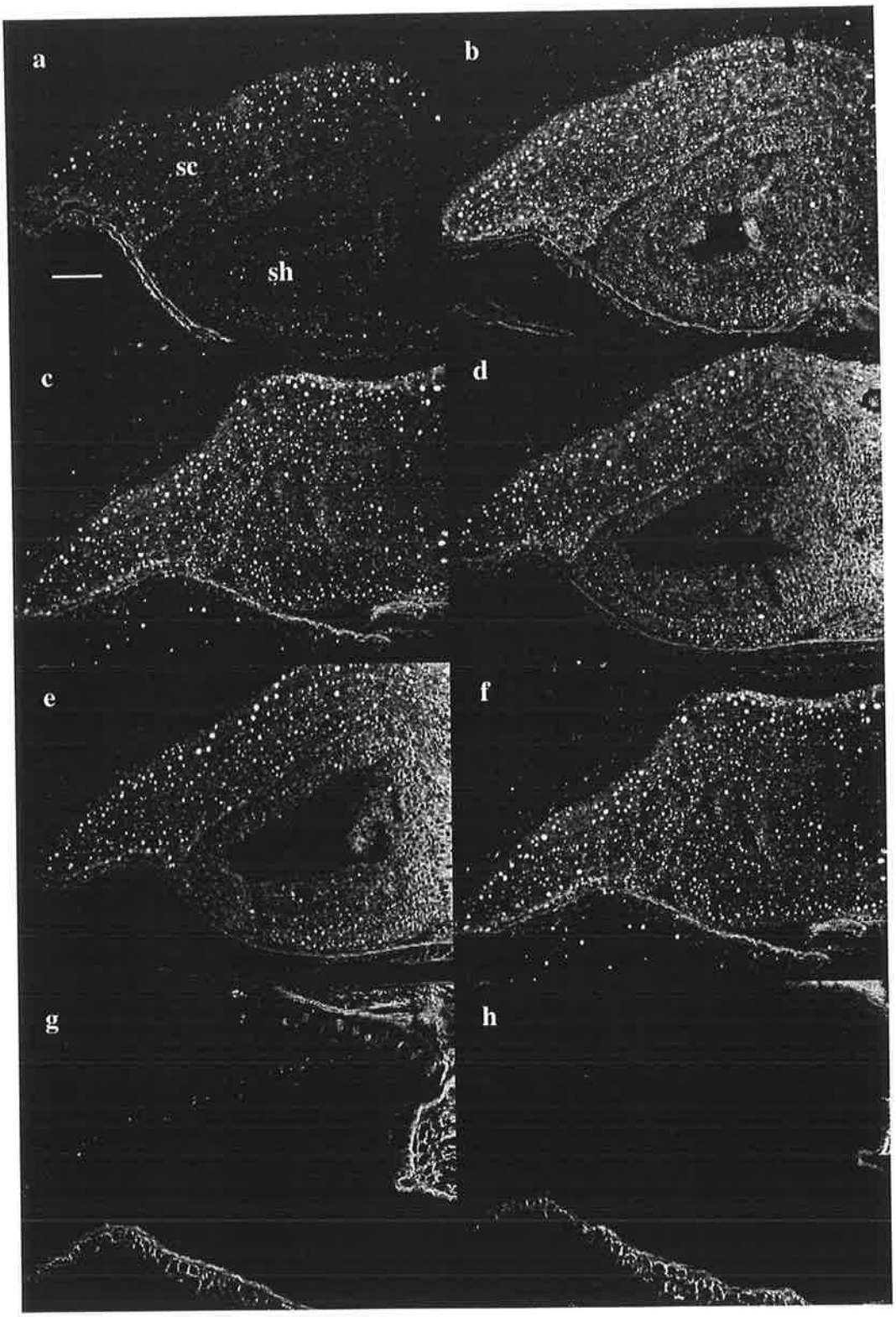
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Fig. 5.5 Lipid droplets in fresh wheat embryos at 20 days after anthesis (DAA). Images for 6 seeds (a-f) plus two controls, a delipidated sample (g) and an autofluorescence test (h). *ra*, radicle; *sc*, scutellum; bar = 250 µm. Each image represents radicle side of longitudinal section of embryo.

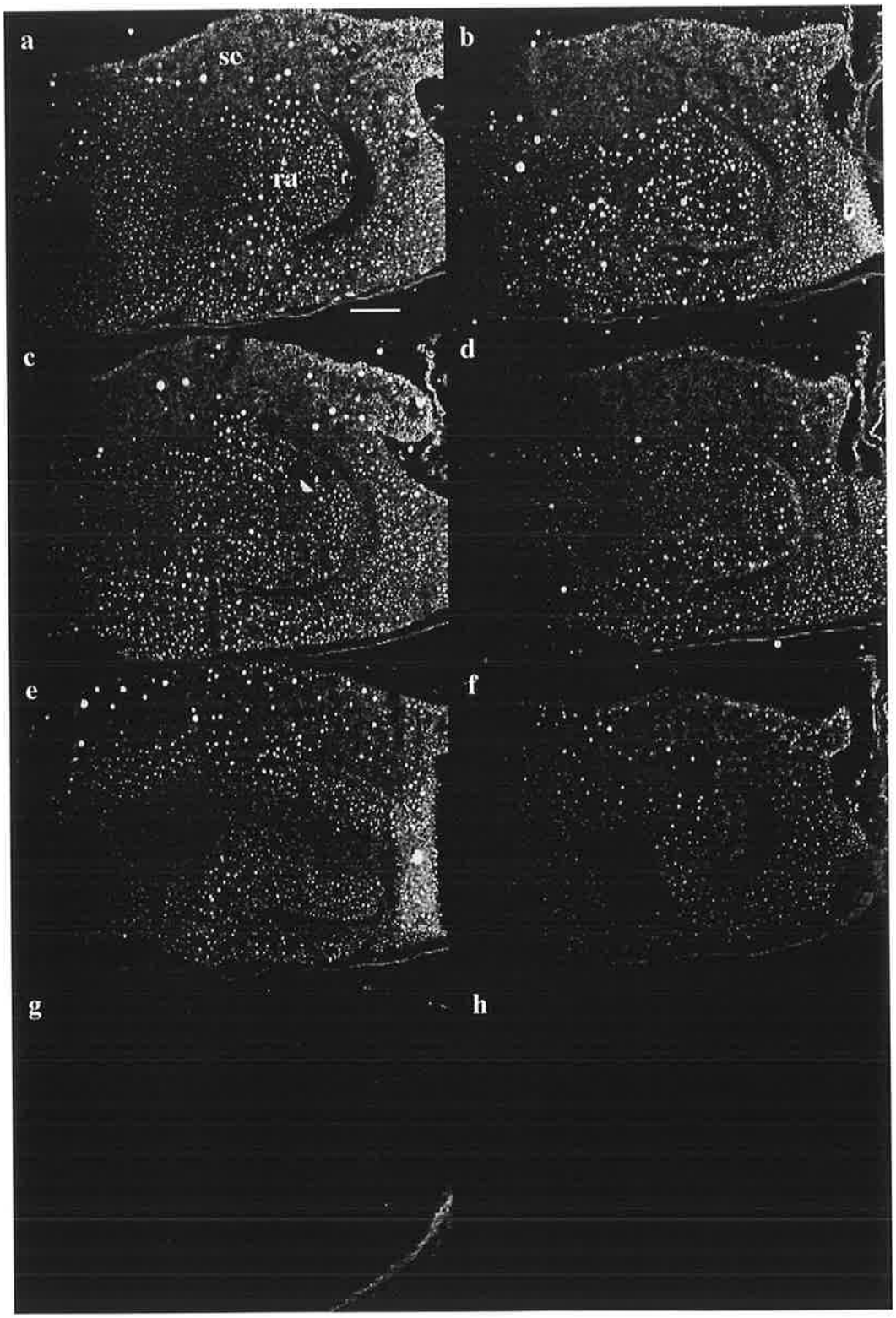
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Fig. 5.6 Lipid droplets in wheat embryos at 15 days after anthesis (DAA) after drying for 5 days. Images for 6 seeds (a-f) plus two controls, a delipidated sample (g) and an autofluorescence test (h). *ra*, radicle; *sc*, scutellum; bar = 250 μm. Each image represents radicle side of longitudinal section of embryo.

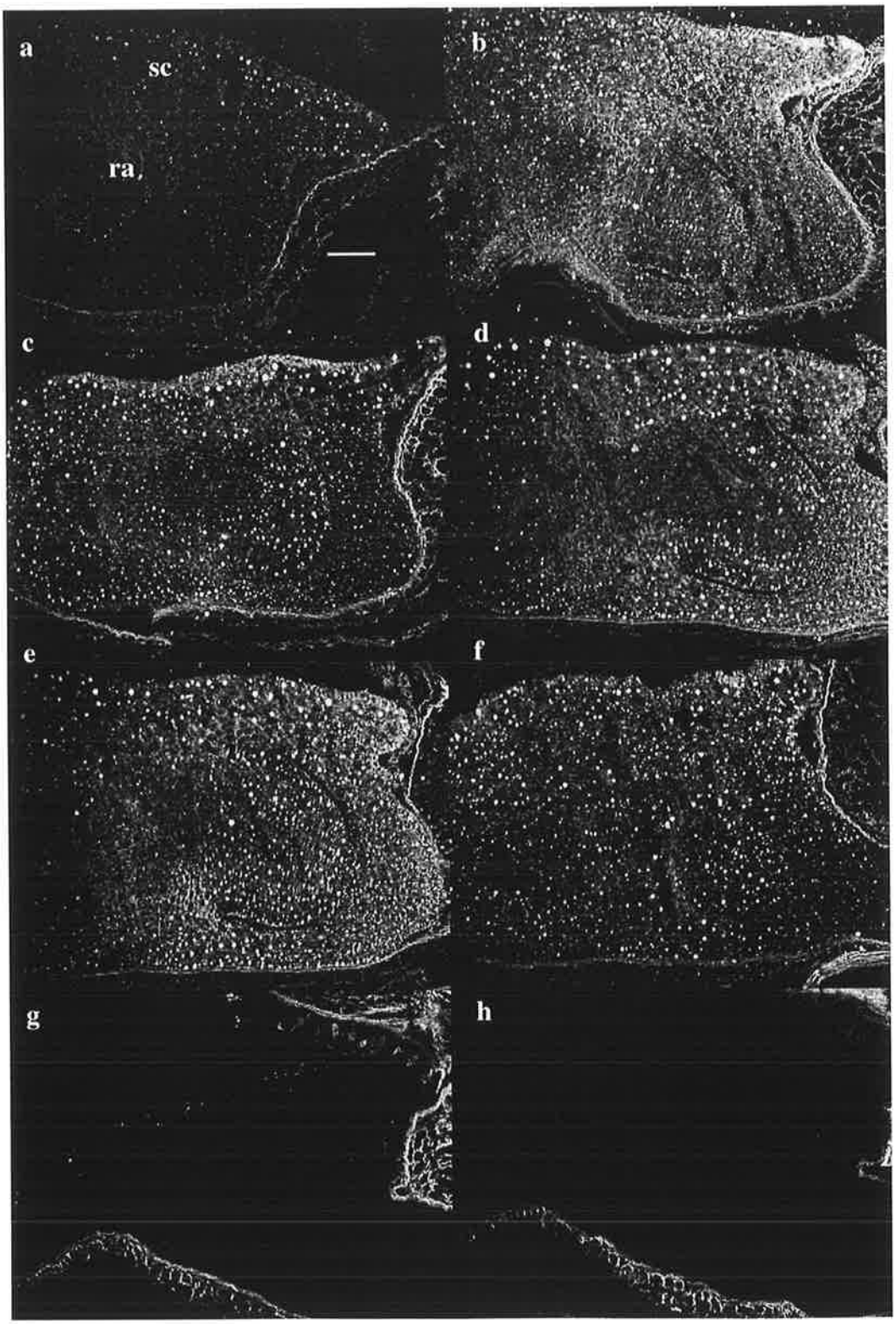
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Fig. 5.7 Lipid droplets in fresh wheat embryos at 25 days after anthesis (DAA). Images for 6 seeds (a-f) plus two controls, a delipidated sample (g) and an autofluorescence test (h). *sh,* shoot; *sc*, scutellum; bar = 250 μm. Each image represents shoot side of longitudinal section of embryo.

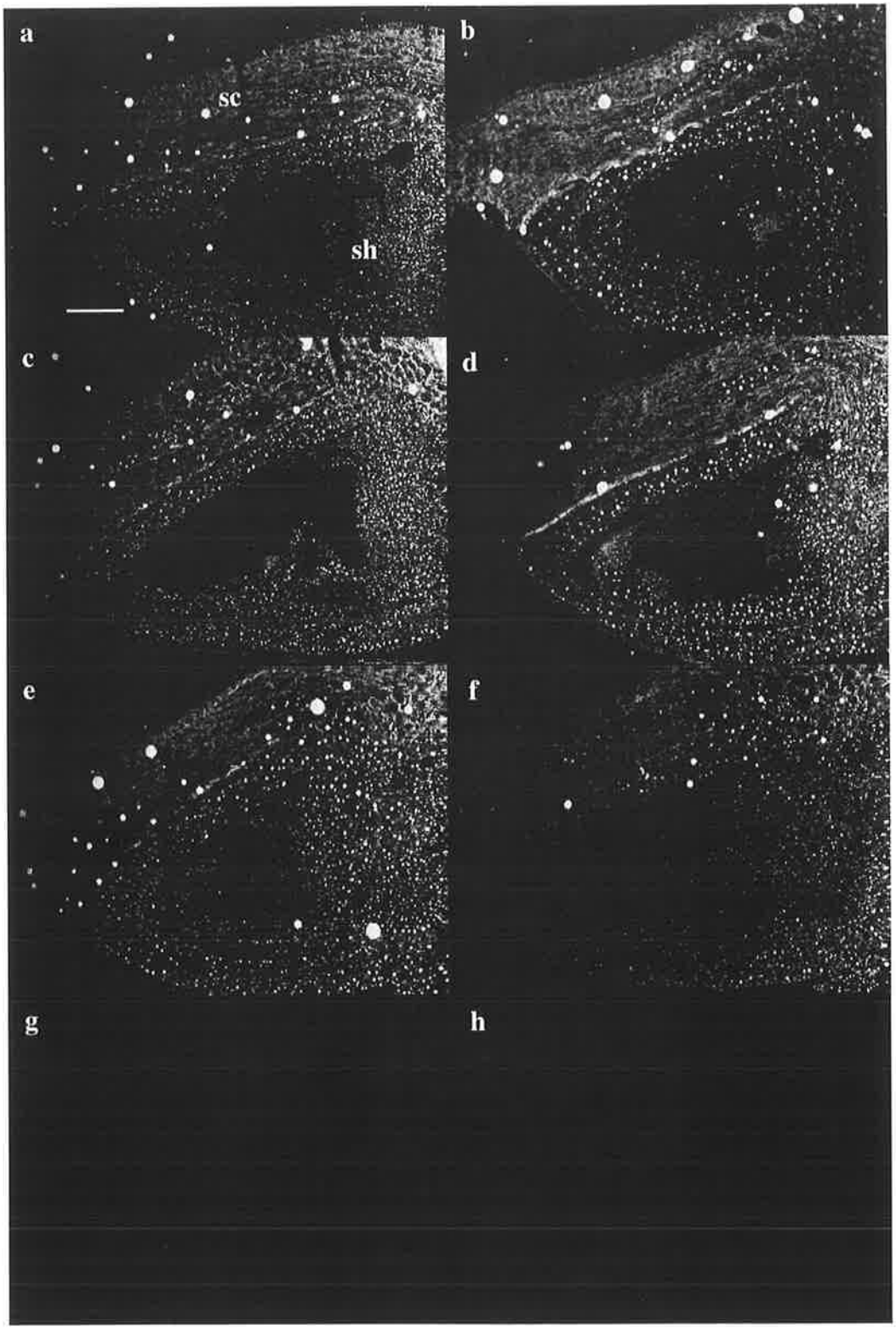
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Fig. 5.8 Lipid droplets in wheat embryos at 20 days after anthesis (DAA) after drying for 5 days. Images for 6 seeds (a-f) plus two controls, a delipidated sample (g) and an autofluorescence test (h). *sh*, shoot; *sc*, scutellum; bar = 250 μm. Each image represents shoot side of longitudinal section of embryo.

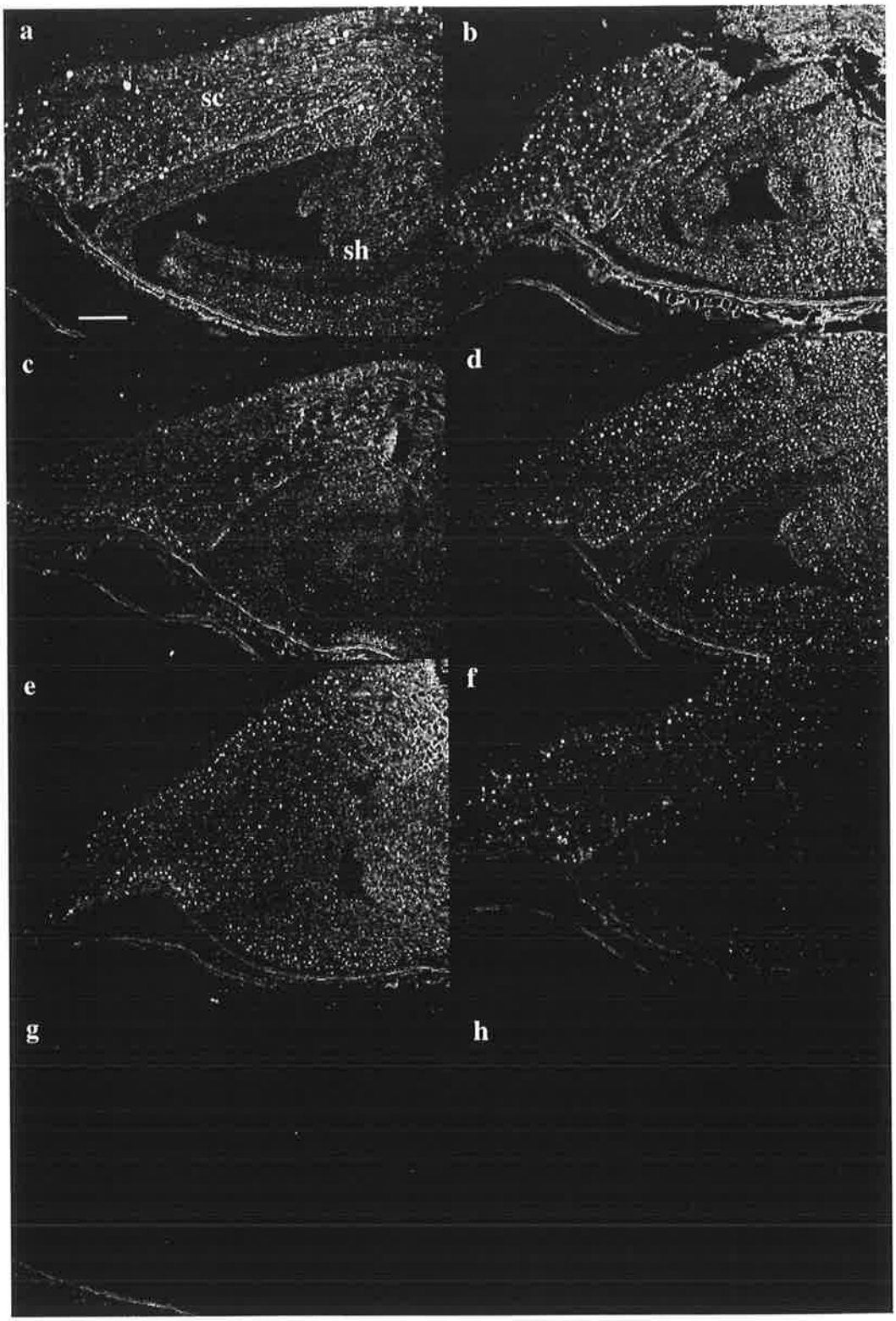
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Fig. 5.9 Lipid droplets in fresh wheat embryos at 25 days after anthesis (DAA). Images for 6 seeds (a-f) plus two controls, a delipidated sample (g) and an autofluorescence test (h). *ra*, radicle; *sc*, scutellum; bar = 250 µm. Each image represents radicle side of longitudinal section of embryo.

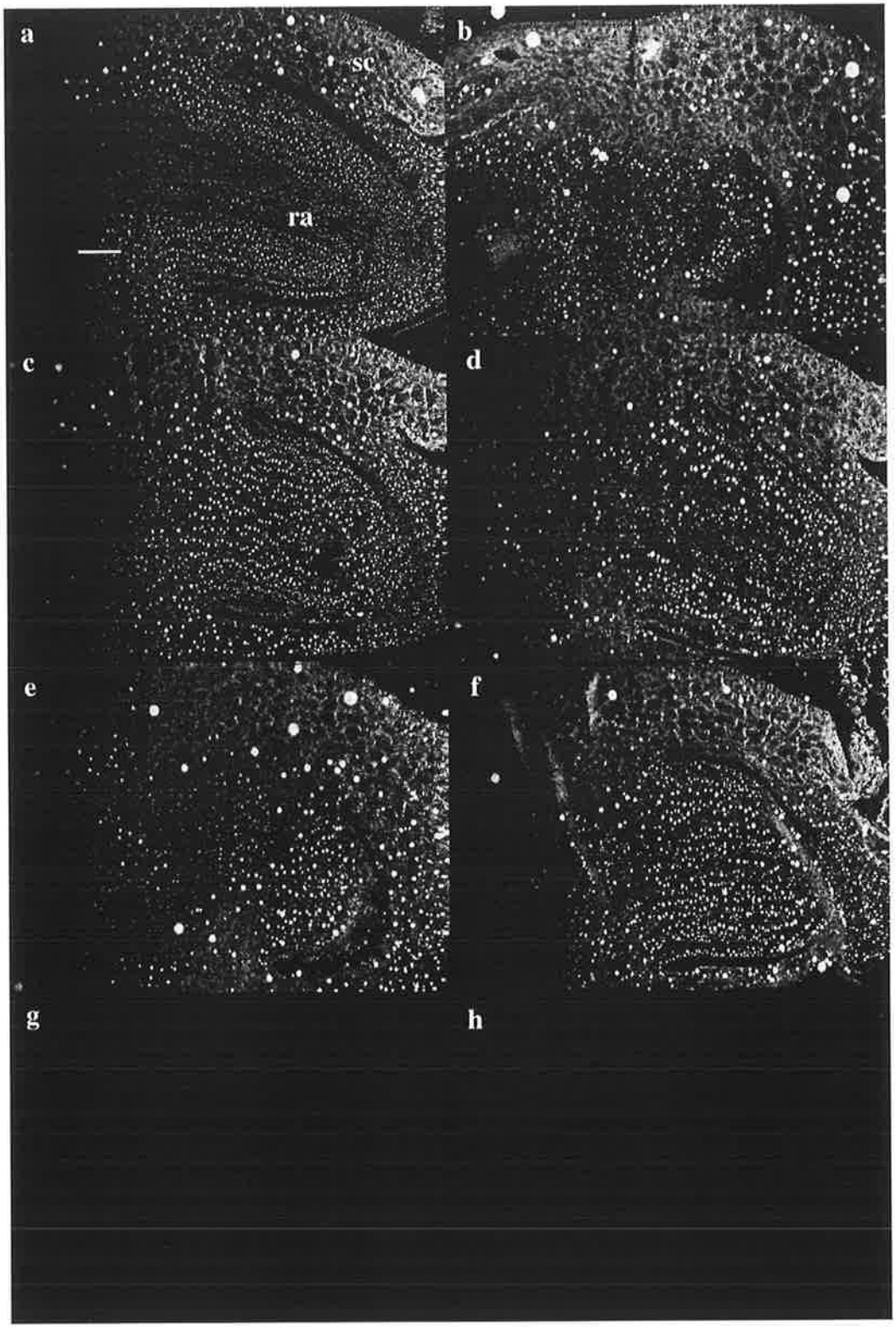
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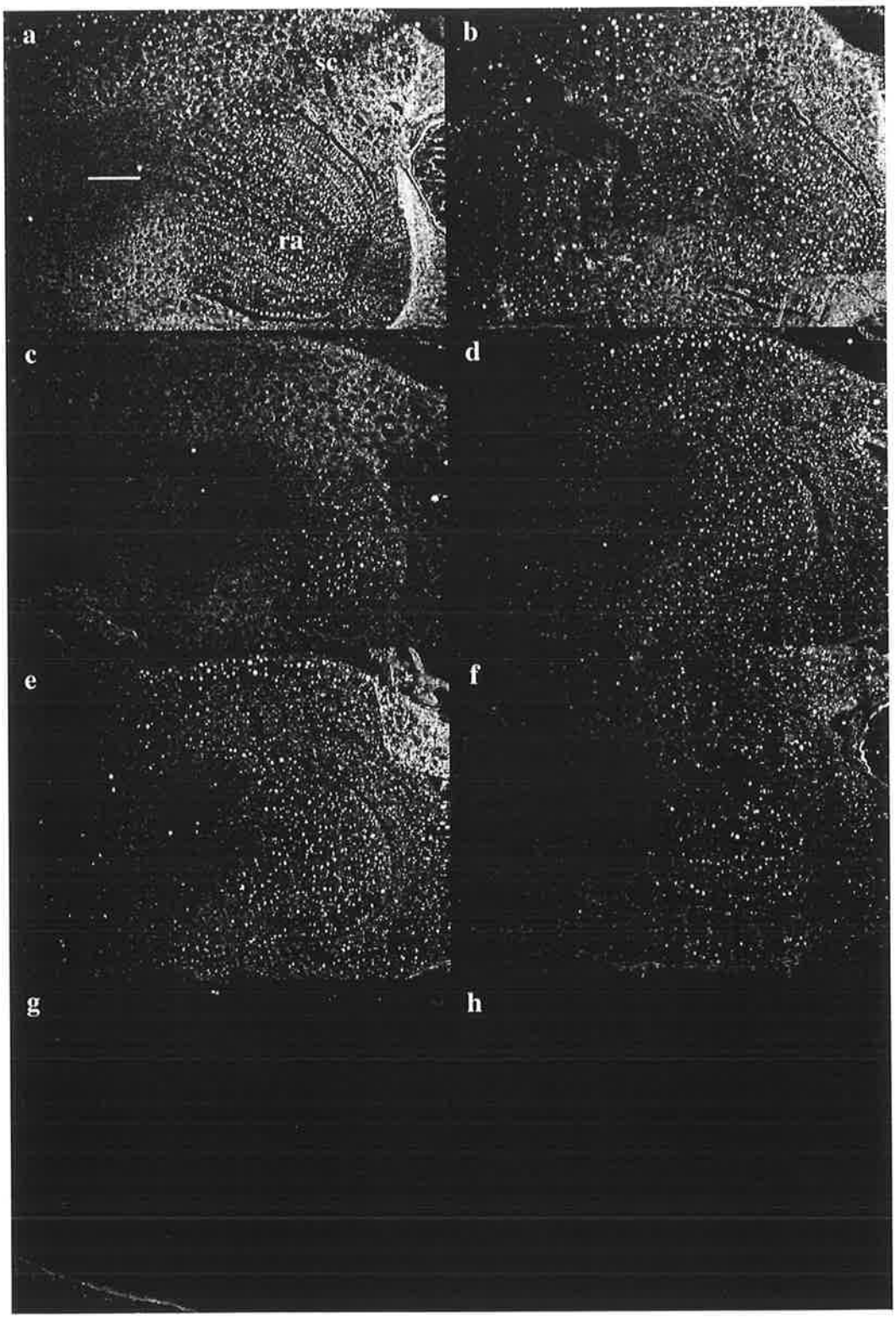
Fig. 5.10 Lipid droplets in wheat embryos at 20 days after anthesis (DAA) after drying for 5 days. Images for 6 seeds (a-f) plus two controls, a delipidated sample (g) and an autofluorescence test (h). *ra*, radicle; *sc*, scutellum. bar = 250 μm. Each image represents radicle side of longitudinal section of embryo.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5.3.2 Area (%) comparisons of lipid droplet fluorescence in fresh embryos and desiccated embryos

Lipid droplets in 15 DAA fresh embryos are small and fluoresce weakly. The fluorescent areas represent only 0.2% of the sampling area in the shoot and 0.3% in the radicle (Table 5.1). In the scutellum, which appears to be the first place of lipid deposition, the fluorescent area is 0.6%. The fluorescent area increases when the embryos are at 20 and 25 DAA. There is a statistically significant difference between TAG lipid areas in the shoot and radicle of embryos at 15 DAA (the desiccation-sensitive stage) and 20, 25 DAA (the desiccation-tolerant stage). At the desiccation-tolerant stage, the lipid-rich tissues which fluoresce most intensely are the shoot and radicle, but in the scutellum there is no significant increase in the area of lipid fluorescence. The differences in the amounts of lipid droplets in these various tissues are shown as the area of lipid droplet fluorescence (%) in Table 5.1. The area of lipid fluorescence in the shoot and radicle of 20 DAA fresh embryos are 4.3 and 4.9 % respectively compared to only 1.1% in the scutellum. Similarly, at 25 DAA, the corresponding areas are 3.3 % in the shoot and 4.0 % in the radicle but only 1.0 % in the scutellum.

The number of small lipid droplets rapidly increase in the scutellum of 15 DAA and 20 DAA seeds after drying (Figs. 5.4, 5.6, 5.8 and 5.10). The fluorescence area of lipid droplets in the scutellum of dried seeds is shown in Table 5.1. The area in 15 DAA embryos after drying for 5 days is 5.6 % which is higher than the 1.1 % in fresh 20 DAA embryos. Also, the area in 20 DAA embryos after drying for 5 days is 3.7 % which is higher than 1.0 % in fresh 25 DAA embryos.

There was no significant difference in area of lipid droplet fluorescence between the shoot and radicle in embryos of 15 and 20 DAA seeds, after drying for 5 days (Table 5.1). However, and possibly of importance, some individual sections of 15 DAA embryo, which are desiccation sensitive (Figs. 5.4a and 5.6a) exhibited

substantial loss of fluorescent lipid droplets from the shoot, scutellum and radicle after drying for 5 days. The complete loss of lipid droplets in the shoot and radicle suggests that lipid degradation takes place in these tissue prior to that of scutellum (Figs. 5.4a and 5.6a). One seed (Fig. 5.8f) of a 20 DAA embryo after drying (in the desiccation tolerant stage), showed that loss of lipid fluorescence had occured in the shoot and scutellum, but not in the radicle (Fig. 5.10).

Table 5.1 Areas of lipid fluorescence (%) in wheat embryos in fresh developing seeds (fresh) and in seeds after drying at 25°C over silica gel (Si) for 5 days (Si 5 D). Areas of 4451 μm^2 were counted for % fluorescence, at a setting of 50% brightness for all sections. Data for each treatment represents the mean from 18 observations. Data followed by the same letters for all treatments in the same column are not significant at $p \leq 0.01$. DAA is days after anthesis.

Treatment	Lipid droplet area (%)		
	Shoot	Radicle	Scutellum
15 DAA fresh	0.2*b*	0.3*b*	0.6*c*
15 DAA Si5D	2.8*a*	4.1*a*	5.6*a*
20 DAA fresh	4.3*a*	4.9*a*	1.1*bc*
20 DAA Si5D	2.2*a*	4.6*a*	3.7*ab*
25 DAA fresh	3.3*a*	4.0*a*	1.0*c*

5.3.3 Neutral lipid fluorescence using Nile red as an indicator

When applied to sections of wheat embryos, Nile red exhibits an intense fluorescence (522 nm) with neutral lipids, i.e. TAG (Figs. 5.3-5.10). Lipid droplets in wheat embryos show a spherical shape and they are very intense in the embryonic axis. Substantially lower fluorescence is observable in the immature

fresh embryos at 15 DAA (Fig. 5.2). Evidence supporting the assignment specificity of this dye to lipid droplets is supported by the complete loss of droplet fluorescence in all delipidated sections (Figs. 5.2g-5.10g). In fresh tissue, Nile red is a sensitive indicator for the presence of triacylglycerols. However after drying, triacylglycerols are no longer detectable by TLC or IR (Chapter 4), nevertheless, intense neutral lipid fluorescence is apparent (Figs. 5.4, 5.6, 5.8 and 5.10). This suggests that Nile red reacts with other neutral lipids in the sections of the seeds after drying.

5.4 Discussion

Lipid has been reported as the primary energy reserve in the immature embryo of wheat (Evers and Bechtel 1988). The droplets are deposited in the embryos during early development. They accumulate rapidly from the start of the grain filling period until embryo maturation (Evers and Bechtel 1988). In this experiment, lipid droplets start to accumulate in 15 DAA (the desiccation-sensitive stage) embryos but to a low detectable amount, as shown by the low % area of lipid droplets (Table 5.1). Numerous lipid droplets are observed in the embryonic axis tissue of 20 DAA and 25 DAA (the desiccation-tolerant stage) embryos and in the vascular tissue.

A substantial difference in the size of lipid droplets present in the scutellum compared with the embryonic axis can be seen in Figs. 5.3, 5.5, 5.7 and 5.9. Trelease (1969) has also reported that lipid droplets in the scutellum of maize are larger than those in the embryonic axis. It has been suggested (Murphy *et al.* 1993; Ross *et al.* 1993) that the size of lipid droplets depends upon their function in the plant tissue. For example, lipid droplets in seed tissue such as endosperm and embryo are smaller than those in fresh fruits. It has also been suggested that for rapid utilisation lipid droplets in seeds are small because they must withstand desiccation and provide a large surface for hydrolysis following germination,

whereas large lipid droplets in fruits are not used for such rapid metabolism (reviewed by Murphy *et al.* 1993). If this is so, then the data presented here indicates that in the developing embryo, the embryonic axis has a higher metabolic activity than does the scutellum. The TAG in small droplets may be used to provide a rapidly utilisable source of energy in contrast to the large droplets of the scutellum.

The dispersion of lipid droplets in wheat seeds after drying as observed in this study is consistent with prior reports of the commencement of lipid degradation in maize (Nir *et al.* 1970*a*, *b*), rye (Hallam *et al.* 1972) and wheat (Swift and O'Brien 1972*b*) following germination. These authors found that lipid droplets in cells migrate from the wall of the epithelial cell and lie scattered within the cytoplasm. There is no clear explanation why lipid droplets disperse from the epithelial cell wall at the beginning of lipid degradation, however, it is possible that the migration of these droplets from the wall facilitates contact with lipase prior to lipolysis (Huang 1992).

The apparent loss of lipid droplets during desiccation in the embryonic axis prior to that in the scutellum was observed in one seed (Figs. 5.4a and 5.6a). Similar findings have been reported for germinated wheat seeds (Tavener and Laidman 1968). Lipids in the embryonic axis are broken down within 24 hours of imbibition while the breakdown in the scutellum begins at around 48 hours of imbibition. The distribution of the smaller droplets in the embryonic axis may provide a larger surface for binding sites with lipase enzymes (Wang and Huang 1987; Huang 1992) and thus, degradation takes place more rapidly than in the scutellum.

The loss of these lipid droplets from the shoot and radicle of 15 DAA seed after drying (Figs. 5.4a and 5.6a) may result in an inadequate energy supply for the seeds to complete their growth following reimbibition. A loss of lipid droplets

was observed in the shoot of one seed at a 20 DAA seed after drying (Fig. 5.8f). No seeds at 20 DAA were found to have completely lost lipid droplets from the radicle upon drying (Fig. 5.10). Because these samples are at the desiccation-tolerant stage, these data suggest that lipids in the radicle have a more important role for embryo survival after desiccation than those in the shoot, but this tentative conclusion requires further study.

In the previous experiment (Chapter 4), the ester carbonyl peak (1743 cm^{-1}) of TAG was observed to decrease in embryos after drying, indicating the breakdown of these neutral lipids during desiccation. However, the results obtained with the confocal microscope show no difference in the lipid fluorescence area in the embryonic axis and an actual increase of the lipid fluorescence area in the scutellum in the seeds after drying. This apparent paradox can be explained by the fact that triacylglycerols are degraded by lipase activity (Huang 1992) to yield glycerol and fatty acids (Clarke *et al.* 1983; Huang 1992) which no longer retain the ester linkage. An increase of fatty acids is supported by 1) a strong peak of the carboxyl (1710 cm^{-1}) and the strong C-H stretching peaks (2922 cm^{-1} and 2853 cm^{-1}) in the hexane extraction from the embryos of dried seeds. This carboxyl peak (1710 cm^{-1}) is not apparent in the hexane extraction from the embryos of fresh seeds (see Chapter 4); 2) the numerous lipid droplets in the embryos of the seeds observed after desiccation are completely removed in the delipidated sections (the sections were washed with chloroform followed by ethanol prior to staining with Nile red). This fluorescence increase is consistent with the characteristics of the un-ionised fatty acids as neutral lipids which are soluble in non-polar solvents. Thus, these uncharged free fatty acids, being strongly hydrophobic form droplets and stain strongly with Nile red.

The solvent delipidated and non stained autofluorescent controls (sections g and h) for each treatment (Figs 5.2-5.10) showed a virtually complete absence of significant autofluorescence of lipid droplets. Hence, the strong bright field after

desiccation cannot be ascribed to polar phenolic compounds which commonly autofluoresce in the fluorescent microscope (Smart and O'Brien 1979). Thus, the strong fluorescence observed after desiccation coupled with the absence or decrease of the ester carbonyl at 1743 cm^{-1} and the appearance of the carboxyl carbonyl at 1710 cm^{-1} is consistent with a rapid increase in free fatty acids during desiccation.

5.5 Conclusions

1) In fresh developing embryos at 15 DAA (the desiccation-sensitive stage), few lipid droplets are evident. These droplets are tiny with a weak fluorescence. At 20 DAA and 25 DAA (the desiccation-tolerant stage), numerous lipid droplets accumulate in the meristematic tissue, i.e. the embryonic axis (shoot and radicle) and vascular tissue. At this stage, TAG lipids induce an intense fluorescence with Nile red. No substantial increase in lipid droplets is observed in the scutellum of the developing seed between 15-25 DAA.

2) After desiccation, lipid droplets are dispersed throughout the scutellum and the embryonic axis. There is no significant change in lipid droplet fluorescence in the shoot and radicle but the % areas of lipid droplet fluorescence increase in the scutellum. The evidence (confocal, IR, TLC) taken together is consistent with an increase in lipid fluorescence area due to an increase of free fatty acids after triacylglycerol hydrolysis.

3) In some seeds at the desiccation sensitive stage of 15 DAA (when dried for 5 days) sections were found wherein lipid fluorescence areas were completely absent from both the shoot and radicle, and only a few droplets were visible in the scutellum. At the desiccation tolerant stage, in one of 20 DAA seeds (dried for 5 days), the lipids were occasionally absent from the shoot, never from the radicle. This correlation between the lack of radicle lipid reserves and desiccation sensitivity implies that the radicle is more sensitive to desiccation than the shoot.

4) To summarise, it is reasonable to suggest that during desiccation of developing seeds which are at the desiccation sensitive stage, substantial cleavage and catabolism of the limited lipid reserves occurs, which results in almost total degradation of TAG lipids. Such seeds may not survive desiccation, because of significant depletion of the lipid reserves required for the germination process (Clarke *et al.* 1983). A logical extension of this conclusion is that embryo TAG may play an important role in the drought tolerance of wheat seeds at the early stages of seed development.

CHAPTER 6

General discussion

Desiccation tolerance in seeds has been studied for decades and the possibility that sugars play a major role in conferring desiccation tolerance has been reported for many plants. However, there are many inconsistencies in the literature which may be due to differences in the methods used to measure sugars or the criteria used for desiccation tolerance. For example, Koster and Leopold (1988) reported that the high contents of sugars that accumulate in maize, pea and soybean embryo tissue (prior to the desiccation tolerance test) coincided with the ability to withstand desiccation, and that loss of sugars from embryos resulted in the loss of desiccation tolerance. Chen and Burris (1990) and Blackman *et al.* (1992) have found that initial sugar contents in the maize embryos were relatively low but that slow drying induced sugars to increase in amount. Maize embryos exhibiting these higher sugar contents were found to tolerate desiccation whereas the embryos with low sugar contents did not. In contrast, Bochicchio *et al.* (1994) have reported that the initial sugar contents in maize embryos were zero (before the desiccation tolerance test), but these embryos were still able tolerate desiccation.

Part of the reason for continued doubt about the role of sugar in desiccation tolerance resides in the lack of unequivocal experiment data in the literature. For instance, Bochicchio *et al.* (1994) and Koster and Leopold (1988) did not indicate whether or not sugar accumulated during the drying period. As another example, the relationship between sugar contents and the loss of desiccation tolerance has been studied in germinating seeds at the time of the protrusion of the radicle (Senaratna and McKersie 1983; Senaratna *et al.* 1983; Koster and Leopold 1988) In this example there were no clear data to show whether or not the loss of

desiccation tolerance was due to insufficient sugars or whether it was due to the sensitivity of the rapidly expanding cells to drying.

In this thesis, measurement of sugar contents in the embryos after desiccation shows clearly that sugars contents can be enhanced by a variety of conditions which include slow drying, rapid drying and even moist conditions under which no significant desiccation occurs. The response of increased embryo raffinose content under all these conditions is always more pronounced than that of increased sucrose contents. As expected, the %mole ratio raffinose/sucrose also exhibits the same behaviour. The results obtained are in accord with the observations of Bochicchio *et al.* (1994). However, the results in this thesis show that low contents of raffinose initially present in embryos can be enhanced during the desiccation tolerance test.

Although many results in this thesis have shown an increase of sugars during desiccation, it is not always so. For examples, in wheat seeds where raffinose contents are low and these contents do not change after rapid drying, the seeds will still tolerate desiccation (Fig. 3.8b). In sweet corn seeds, the %mole ratio raffinose/sucrose after slow drying is very low (2.4%) but again the seeds will tolerate desiccation (Tables 3.2 and 3.3). Even at the desiccation tolerant stage, Table 3.4 shows there can be rapid changes in the %mole ratio raffinose/sucrose from 26.5% to 4.7% in developing wheat seeds. Furthermore, embryo raffinose is also enhanced in ~~wheat~~ corn seeds stored under HRH conditions where there is no stress from moisture loss from the seeds (Table 3.2).

This evidence suggests that increased raffinose synthesis occurs in sweet corn seeds and wheat seeds which are exposed to a stress condition such as detachment rather than as a specific stress response to moisture loss. These results are in marked contrast to the report of Blackman *et al.* (1992) for soybeans which did not accumulate oligosaccharides under HRH conditions at 25°C. It seems that seeds of

different plant species may exhibit different saccharide responses to stress conditions and hence there is no universality to the saccharide response to stress.

These discordant results cast considerable doubt upon the hypothesis that sugars alone are responsible for all the aspects of desiccation tolerance. The inevitable conclusion has been reached that sugars are not a reliable indicator for desiccation tolerance. At this stage, it is not at all clear whether an increase of embryo raffinose provides any particular benefits for wheat embryos undergoing stress. Jones *et al.* (1980) have reported that there are various mechanisms whereby soluble sugars could accumulate under water stress conditions. This is because increased sugar concentrations may result either from increased starch hydrolysis, synthesis by other pathways or decreased rates of conversion to other products.

Apart from the apparently inconsistent role of oligosaccharides in desiccation tolerance, other authors have reported increased contents of other solutes in plant tissue under water stress (Wang *et al.* 1995; Rascio *et al.* 1994; Handa *et al.* 1983; Jones *et al.* 1980; Spollen and Nelson 1994). These solutes include reducing sugars, Cl^-, K^+ ion and proline (Rascio *et al.* 1994; Handa *et al.* 1983). It has been suggested that these increased solute contents under water stress provide a mechanism for an osmotic adjustment (Neumann 1995). Where oligosaccharides are synthesised during desiccation, it is conceivable that osmotic adjustment also have a role to play. However, it appears osmotic adjustment cannot be invoked to explain an increase of embryo sugars in seeds stored under HRH conditions (Fig. 3.9f).

Although ^{13}C NMR signals for the carbons of carbohydrates can readily be detected in wheat embryos, the interpretation of this data requires care. Large changes in chemical shifts are detectable in the difference spectra after desiccation (Figs. 4.20a, b) but are not prominent in the original spectra (Figs. 4.18, 4.19). The results support the interpretation in Chapter 3 that starch breakdown is probably not a

dominant mechanism during desiccation. The reasoning here, is that if the degradation of starch takes place, the spectrum characteristic of the major α, β pyranosyl forms of glucose would be dominant in the difference spectra (Breitmaier and Voelter 1990). What is observed in the difference spectra is most consistent with a mainly fructofuranosyl glycoside spectrum in the wheat embryos at the desiccation-tolerant stage (Fig. 4.20b). Therefore, in the limited case of wheat embryos, the data are consistent with the presence of a fructofuranosyl species such as a fructan at the desiccation-tolerant stage. Independent confirmation of this hypothesis is now required.

An alternative interpretation of the ^{13}C NMR data (Doyle *et al.* 1986; Jarvis and Apperley 1990) is that there are alterations to the chemical shift of C_4 of a glucan, once it changes from the amorphous to the crystalline form with increased cell wall strength which is then a feature of desiccation tolerant embryos (Figs. 4.20a, b).

The results from diffuse reflectance using infrared dispersion spectra (DRUIDS) have provided new insight into the rapid changes in lipid composition during desiccation. It is obvious that at the desiccation sensitive stage (15 DAA), the ester carbonyl peak of triacylglycerols (TAG) is very small and consequently the peak to trough absorbance ratio is close to 1.0 (Figs. 4.7, 4.11). At the desiccation tolerant stage (20 DAA), the ester carbonyl peak is dominant and the absorbance ratio is high ~1.1 (Figs. 4.10, 4.11). Based on the hypothesis that TAG may act as a storage depot of unoxidised free fatty acids for repairing membrane damage due to free radicals induced during desiccation, a decrease in the absorbance ratio during desiccation would correlate with poorer membrane repair mechanism.

There are two alternative mechanisms in the literature for the formation of free fatty acids during desiccation. The simplest mechanism would be that of lipolysis mediated by a lipase enzyme involved in TAG degradation as reported by Huang (1985). The second possible mechanism is based upon the observations of Niehaus

(1978) and Senaratna *et al.* (1985*b*). These workers noted that in addition to the well documented free radical attack on unsaturated groups in lipids, there was also a rapid superoxide anion mediated de-esterification of even saturated fatty acyl esters in phospholipids. To satisfactorily elucidate the mechanism (s) of TAG lipid de-esterification during desiccation, it will be necessary to consider both the free radical and lipase mediated ester cleavage.

The confocal microscopy results show that at the desiccation-sensitive stage (15 DAA), there are few lipid droplets and these exhibit only a weak fluorescence in fresh wheat embryos (Figs. 5.1a-f). However, at the desiccation-tolerant stage (Figs. 5.2a-f and 5.4a-f), numerous lipid droplets with a strong fluorescence are evident which agree well with the accumulation of TAG during development (Fig. 4.11). There was no significant change in the mean areas of lipid fluorescence between desiccated and fresh embryos at the same corresponding ages in the shoot or radicle (Table 5.1). In view of the virtual complete cleavage of TAG ester bonds, this residual fluorescence after desiccation must be due to the presence of free fatty acid residues (Figs. 4.16, 5.7 and 5.9). However, by examining individual sections, one seed was found to show a decrease in lipid fluorescence area in both shoot and radicle after desiccation at the desiccation-sensitive stage (Figs. 5.3*a*, 5.5*a*). At the desiccation-tolerant stage, no loss of lipid fluorescence was apparent in the radicle of wheat embryos (Figs. 5.9a-f). These data are consistent with some degree of catabolic activity occurring during desiccation. The results suggest that not only is TAG a potential reserve source of free fatty acids for membrane repair systems, it is also a potentially important energy source for germinating embryos. Thus, the depletion of both TAG and free fatty acids in immature wheat embryos especially at the desiccation-sensitive stage could result in insufficient energy reserves for germination following imbibition which is in accord with the conclusion of Clarke *et al.* (1983) and Evers and Bechtel (1988).

By the use of a variety of independent methods, this thesis has demonstrated the shortcomings in the current literature concerning the widely accepted role of low molecular weight carbohydrates in the development of desiccation tolerance in embryos and has focussed attention on two hitherto unrecognised features of the desiccation-tolerant stage. First, the presence of strong infrared signals for triacylglycerols in desiccation-tolerant embryos and secondly the ^{13}C NMR data which suggest the presence of unique carbohydrate carbon signals which are absent from the difference spectra of embryos at the desiccation sensitive stage. These ^{13}C NMR data show strong anomeric signals and may well be coming from hitherto unrecognised polysaccharide (s) which may play an important role in the desiccation tolerance of wheat embryos.

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