AN EVALUATION OF ANTIOXIDANT AND HYDRATION TREATMENTS FOR THE IMPROVEMENT OF THE STORABILITY OF SOYBEAN *[Glycine max (L.) Merr.]* SEEDS.

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Dedication

To my father, who inspired those to work hard.

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ABSTRACT

Antioxidant and hydration treatments were evaluated for their potential to improve the storability of soybean *cvs*. Amsoy and Davis under different ageing conditions (accelerated ageing at 40°C, 36-100% RH; controlled deterioration at 40°C, 20% seed moisture; or slow ageing at 35°C, 9 or 12% seed moisture).

Despite previous reports in the literature, no protective effects of treatment were found using 1% α-tocopherol, 0.1% butylated hyroxytoluene (BHT) in acetone solution for 16 h, β-mercaptoethanol 0.52-52 *ppm* in aqueous solution or 0.1-1% iodine in calcium carbonate. High concentrations of BHT (2.2%) or iodine (1%) caused toxic effects to germination performance (as determined by normal germination, viability, fresh and dry weights of normal seedling axes). Acetone, used as a carrier for antioxidant treatments, was toxic to soybean seeds. Initial seed moisture contents, levels of mechanical damage, treatment duration and seed lot variation were key factors affecting susceptibility to acetone toxicity. Tetrazolium staining showed that acetone did not cause damage to a specific tissue but rather increased the area of dead tissue which had been mechanically damaged.

Hydration-dehydration pre-storage treatments of soybean also showed damaging effects. Soaking treatments caused injury to low vigour lots immediately and reduced ageing resistance in high vigour material. Moisture equilibration (ME) for 24-48 h had no effects on seed performance, but an extended moisture equilibration period up to 72 h increased rates of germination loss during subsequent ageing. However, post-storage hydration treatments showed some capacity to repair damaged seeds.

Increased conductivity of seed leachate was always significantly correlated with loss of germination performance, suggesting that membrane damage was related to seed deterioration. Changes in lipid and membranes were assessed in seeds aged at 40°C, ~100%

RH or 35°C, 9% constant seed moisture. No changes in total lipid content due to ageing or treatments suggested that changes in storage lipid were not related to germination performance. Significant losses of phospholipid (PL) from cotyledons occurred 4 days after accelerated ageing. These losses were correlated with loss of seed performance, but no changes in PL contents from axes were detected throughout the ageing period. By itself, this result suggests that cotyledon damage may be an important contributing factor to seed deterioration, but the transmission electron microscopy (TEM) study indicated that damage occurring in axes due to accelerated ageing was more severe than in cotyledons.

Acetone or water soaking pretreatments increased rates of loss of germination performance which were associated with PL losses or ultrastructural abnormalities in both axes and cotyledons. In addition, ME treatment (72 h) applied to slowly aged seeds accelerated PL loss in axes but, this was unrelated to seed performance. These data therefore indicate that PL losses *per se* are not a fundamental cause of seed deterioration and may not be the first event in membrane damage. Also germinating seeds seem to be able to repair some damage of this type. No changes in proportions of polyunsaturated fatty acids were observed in either total lipid from seed tissues or microsome fractions, indicating that lipid peroxidation was not involved in seed deterioration due to ageing or treatments. This, of course, explains the ineffectiveness of the antioxidant treatments in this study and suggests that hydrolytic damage may be involved in PL loss and seed deterioration.

Possible reasons for the differences between these results and the small amount of other work published on seed treatments in soybean are discussed, but it was clear that chemical or hydration treatments of soybean are unlikely to be a commercially viable option for maintenance of soybean quality where poor storage conditions are inevitable. Nevertheless, this study indicates some interesting areas for future research into the critical events involved in soybean deterioration.

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ABBREVIATIONS

AA	= Accelerated ageing
AOSA	= Association of Official Seed Analysts
BHA	= Butylated hydroxyanisole
BHT	= Butylated hydroxytoluene
CD	= Controlled deterioration
d	= day
ER	= Endoplasmic reticulum
GL	= Glycolipid
GLC	= Gas liquid chromatography
h	= hour
ISTA	= International Seed Testing Association
kD	= kilo-Dalton
ME	= Moisture equilibration
µS	= micro-Siemens
mth	= month
NL	= Neutral lipid
PA	= Phosphatidic acid
PC	= Phosphatidyl choline
PE	= Phosphatidyl ethanolamine
PEG	= Polyethylene glycol
PG	= Phosphatidyl glycerol
PL	= Phospholipid
PUFA	= Polyunsaturated fatty acid
RCR	= Respiratory control ratio
RH	= Relative humidity
RQ	= Respiratory quotient
SA	= Slow ageing
SMC	= Seed moisture content
TEM	= Transmission Electron Microscopy
TL	= Total lipid
wk	= week
WSB	= Water saturated butanol

CHAPTER 1

INTRODUCTION

1.1 RATIONALE FOR THIS STUDY

The need to store seeds arises from the fact that seeds are seldom planted immediately after harvesting. Normally, most bulk seeds are stored for relatively short periods to await planting or sale, but longer storage periods may be necessary for insurance against crop failure, or for seeds which are expensive or difficult to produce. Germplasm banks, however, require seed storage for as long as possible for future breeding programmes (Roos, 1980). Seeds with high germination and vigour are required for all the above purposes. Unfortunately, seeds deteriorate continuously after reaching maximum germinability and vigour, usually attained at physiological maturity (Delouche, 1982). The rate of deterioration depends on genotype, pre-storage history and, ultimately, the ambient conditions of seed storage (Roberts and Ellis, 1978).

Soybean seed has a high oil content and is inherently short-lived (Delouche *et al.*, 1973); it can be stored successfully under low relative humidity, low temperature conditions, but otherwise it deteriorates rapidly, especially under hot and humid storage conditions of tropical regions. For example, in Thailand, one experimental study conducted at Kasetsart University showed that recommended soybean cultivars (*i.e.* SJ.2 and SJ.4) lost >35% germinability after 6 months in store at ambient conditions during January-July in Bangkok (Ratanaubol, 1988). The difficulty in maintaining vigour or geminability of soybean seed from harvest to the next season is one of the major impediments to extension of soybean production in South East Asia because of the problems of shortages of high quality seed (Gregg, 1982). Preserving the vigour of soybean seed is a challenging problem, especially as ideal storage conditions are often difficult to achieve in these countries. Although there is an enormous body of literature on soybean storage, the key factors at the biochemical

level, which may limit seed performance and its preservation are still poorly understood. An understanding of the metabolism of seed deterioration may lead to better ideas and practice for preserving or improving seed quality.

Seed deterioration is a consequence of various deteriorative biochemical events (see Priestley, 1986 for review). Peroxidation of membrane lipids has been suggested to be a primary event in seed ageing (*e.g.* Harrington, 1973; Parrish and Leopold, 1978; Wilson and McDonald, 1986). Damage to membranes relates to conductivity of seed leachate, loss of phospholipid (PL) content, or changes in ultrastructure of cell organelles. In soybean, determination of membrane damage by examination of ultrastructural changes has not been reported, and reports on PL changes have been contradictory (*e.g.* Priestley and Leopold, 1979; Nakayama *et al.*, 1981; Chapman and Robertson, 1978). There have also been some conflicts of opinion about the role of oxidation damage in soybean seed deterioration under different conditions (Buchvarov and Gantcheff, 1984; Priestley *et al.*, 1985; see also Section 2.2.1).

Generally, the lower the temperature and moisture content at which seeds are stored, the longer their storage life (Roberts, 1972). As indicated above, ideal storage conditions may be expensive to maintain, and techniques of antioxidant or hydration-dehydration seed treatments have received considerable attention as alternative means of improving the storability of some crop seeds. Some methods have shown good potential for a range of species even under adverse conditions (*e.g.* Basu, 1976; Woodstock *et al.*, 1983; Dey and Mukherjee, 1984; Francis, 1985; Gorecki and Harman, 1987; Saha and Basu, 1984). Lipid antioxidants have the potential to protect dry seed by neutralizing destructive free radicals and free radical derived compounds (*e.g.* Woodstock *et al.*, 1983; Gorecki and Harman, 1987), while hydration treatments may have the potential both to terminate free radical chain reactions by quenching and to activate enzymes for repair (*e.g.* Saha and Basu, 1984).

Seed treatments are not without their problems however: for instance, soybean is well known to suffer from soaking injury, and this may be an obstacle to a successful hydration treatment. Several techniques have been attempted to minimize soaking damage by slowing down rates of water uptake (*e.g.* Woodstock and Taylorson, 1981; Tilden and West, 1985). Moisture equilibration pretreatment (*i.e.* holding the seeds for

specified periods at high humidity) is one method which shows promising effects both to avoid soaking injury and to protect and/or repair soybean seeds (Saha and Basu, 1984). As an alternative option, acetone has often been recommended as a suitable solvent for non-aqueous seed treatments when it is desirable to introduce chemicals into dry seed (*e.g.* Milborrow, 1963; Lewis *et al.*, 1979). However, for soybean, there is a dearth of information about effects and mechanisms of acetone based treatments, particularly for storage purposes.

1.2 OBJECTIVES OF THIS STUDY

Specifically the objectives of this research were as follows:

1) To evaluate the effectiveness of different types of antioxidants for prolonging storability of soybean seeds subjected to both rapid and slow ageing regimes,

2) To characterise any effects of acetone *per se* on the performance of soybean seed lots of different quality,

3) To assess the utility of hydration-dehydration treatments, which have been shown to be effective for this or other species in the literature, for protection and/or repair of soybean seeds stored under accelerated or slow ageing conditions.

4) To investigate how changes in seed performance due to ageing and seed treatment relate to the status of total and membrane lipids and the ultrastructure of cell organelles and membranes.

5) To characterise the role of lipid peroxidation as a possible cause of seed deterioration in soybean.

There has been a lot of conflict in the literature on the effectiveness of seed treatments for storage improvement. It was clear that a careful study designed to evaluate some of the different effects which have been reported may lead to a better understanding of

the limitations of the techniques used and allow suggestion of appropriate modifications. Therefore, an emphasis on the comparison of effects in different ageing regimes, careful evaluation of different parameters of germination, and characterisation of seed lot variability were underlying features of the experiments carried out in this research.

As mention above, differences of opinion on possible causes of seed deterioration have arisen. This may be due to variations in methodology, choice of tissue and/or storage conditions. In this study investigations on lipid and membrane damage were conducted on axes and cotyledons separately and on different lipid fractions under two different ageing conditions. All data were carefully related to changes in germination performance due to ageing and/or seed treatments.

1.3 ORGANISATION OF THE THESIS

This thesis comprises 8 chapters. Following this introduction is a review of the literature on the physiology of seed deterioration with a critical discussion on seed treatments for storage protection or repair and their problems (Chapter 2). Chapter 3 describes the general materials and methods used in this research. The results from the four main series of experiments are presented in Chapters 4-7. Each of these chapters contains a brief introduction, results, discussion and conclusions. The effects of antioxidants and solvent treatments are evaluated in Chapter 4, emphasis being placed on the toxic effects of acetone when used as a solvent for pre-storage treatments of soybean and how to overcome these problems to make antioxidant treatments more effective. In Chapter 5 the effectiveness of hydration-dehydration treatments for both protection and repair are assessed, with particular attention being given to soaking injury problems. The effects of accelerated or slow ageing with and without treatments on total and membrane lipids are characterised, and relationships between germination performance and some aspects of membrane damage are examined in Chapter 6. Some changes in the ultrastructure of cell membranes and organelles as a result of accelerated ageing, soaking or acetone pretreatments are identified in Chapter 7. In the last chapter (8), the achievements and key conclusions of this work are discussed, and suggestions for future work are given.

CHAPTER 2

REVIEW OF LITERATURE

2.1 INTRODUCTION

In order to develop successful seed treatments for the improvement of seed storability, it is necessary to develop an understanding of mechanisms of seed deterioration. This review first discusses the physiology of seed deterioration, and evidence to suggest possible crucial events during this process is highlighted. Techniques of seed treatment for protecting seed in storage and/or improving seed performance after deterioration are then discussed, including the reported problems of using such methods.

2.2 THE PHYSIOLOGY OF SEED DETERIORATION

Deterioration is a common phenomenon occurring in all organisms and ultimately leads to loss of viability. Deterioration of seeds has been defined as "irreversible degenerative changes in the quality of a seed after it has reached its maximum quality level" (Abdul-Baki and Anderson, 1972). Although seed deterioration is ultimately inevitable, it is now clear that, in the early stages of deterioration at least, some damage is repairable (*e.g.* Coolbear *et al.*, 1984; Saha and Basu, 1984). Deteriorative processes may begin during seed development itself (Harrington, 1973); and the subsequent rate of seed deterioration is influenced by genotype, prestorage history conditions (*e.g.* mechanical damage, maturity) and storage conditions, particularly relative humidity and temperature (Roberts and Ellis, 1978). The consequence of seed deterioration is the production of aged seeds with symptoms of reduced vigour, possibly loss in germinability (the ability to produce a normal seedling) and ultimately losses in viability (Bewley and Black, 1982). Normally, individual seeds within a seed lot deteriorate at different rates. Consequently, a partially deteriorated seed lot may include seeds of high and low vigour as well as non-germinable seeds (Priestley, 1986).

From the physiological point of view, if a single mechanism is a cause of seed deterioration under all storage conditions, the concept of primary cause or secondary effect may be less important. In fact, different conditions of ageing appear to initiate different ageing mechanisms (see following discussion). Each may lead directly to the death of seeds or may affect changes in other mechanisms associated with deterioration. Thus, it is important to be careful to distinguish between first causes of seed deterioration and secondary effects in studying seed ageing. There is a difficulty in this type of research: although there is much available literature on seed deterioration, our understanding of this process is still incomplete. Further, it must be remembered that microorganisms and external agents are also important causes of seed deterioration (Halloin, 1986). This discussion is, however, confined to a consideration of important physiological aspects, especially factors affecting the integrity of cell membranes.

2.2.1 **Membrane deterioration**

Membranes have been suggested as a primary site of age-induced lesions in seeds (Abdul-Baki and Baker, 1973; Parrish and Leopold, 1978). General symptoms of membrane damage include both compositional and structural changes. A possible cause of the damage may be free radicals. Both symptoms and causes are discussed in detail below.

2.2.1.1 *Symptoms of membrane damage*

Loss of lipid from membrane

Membranes comprise lipids and proteins. Phospholipids (PL) are the major component of membrane lipids (Bewley, 1986); and, thus, losses of PL can evaluated to determine membrane damage. Two suggestion have been offered to explain decreased PL levels in aged seed: either the lipids have been subjected to peroxidation or they may have been degraded by lipolytic enzymes, particularly phospholipases (both of these possibilities will be discussed in detail later in Sections 2.2.1.2 and 2.2.2).

There is much evidence showing loss of PL is associated with seed deterioration during ageing. The results reported in the literature summarised in Table 2.1 show that moisture status during ageing, seed species, cultivars used, or extent of seed deterioration may cause differences in PL changes. Care must be taken in these studies to equate changes in viability or vigour resulting from seed ageing by different methods, otherwise some comparisons may be misleading. In Priestley and Leopold's studies (Table 2.1), for example, naturally aged soybean seeds lost only 12% viability with no PL loss (Priestley and Leopold, 1983), while under their high humidity artificial ageing regime, seeds lost 95% viability and approximately 50% PC (although little total PL) (Priestley and Leopold, 1979).

In case changes in PL extracted from membrane *per se* may be different from PL extracted from total seed tissues, a few workers have attempted to determine changes of PL from specific organelle membranes. In soybean, Senaratna *et al.* (1988) reported dramatic decreases in PL from the microsomal fraction taken from axes of naturally aged soybean as seed lost more than 80% viability (Table 2.1). However, even in this case, the evidence is purely correlative: no causal relationship has been demonstrated between membrane damage and loss of germinability, and possible trivial explanations for results cannot be excluded. For example, in the microsomal study there may be differences in extraction efficiency of membranes from aged and unaged tissues.

There have even been a few reports showing increased PL content in seed during ageing *e.g.* in cucumber (Koostra and Harrington, 1969) or corn (Lin and Pearce, 1990), but the increases in these species are only marginal. Surprisingly, Chapman and Robertson (1977) have reported a marked increase in PL from whole seed of soybean after ageing at high RH. They have explained that the increased moisture content during storage may account for increases in PL levels. Since no germination data were provided in this study, how increased PL relate to germination performance is not known.

Although an association between PL loss and viability does not confirm cause and effect, and there are other uncertainties, as discussed above, the bulk of evidence presented in Table 2.1 suggests that this is one primary cause of seed damage, especially when losses of PL are detected early in deterioration, *e.g.* in corn (under AA), peas, sunflower, wheat and tomato (depending on cultivar).

Table 2.1 Effects of ageing conditions on viability/germinability and percentage of phospholipid loss in different crop seeds.

Species/ cultivars	Ageing conditions	Ageing period	Effects on viability or germinability (%) Initial	Final	Tissue assayed	%PL loss	References
Cucumber	38°C, ~100% RH	4 wks	99	2	Whole seed	67	Koostra and Harrington (1969)
	24°C, 40% RH	10 yrs	100	70		14↑	
Corn	45°C, ~100% RH	4 days	90[a]	60	Whole seed	33	Lin and Pearce (1990)
	40°C, 80% RH	28 days	90[a]	0		9	
	25°C, 79% RH	92 days	90[a]	0		12↑	
French bean	45°C, ~100% RH	4 days	90[a]	0	Whole seed	46	Lin and Pearce (1990)
	40°C, 80% RH	28 days	90[a]	0		44	
	25°C, 79% RH	92 days	90[a]	0		31	
Pea	25°C, 94% RH	9 wks	97[b]	44	Cotyledons	48	Powell and Matthews (1981)
Pea	25°C, 94% RH	6 wks	98[a]	88	Cotyledons	32	Powell and Harman(1985)
	30°C, 94% RH	8 wks	98[a]	83		8	
	45°C, 20% SMC	18 hrs	98[a]	100		17	
Sunflower *cv.* EC-68414	28°C, 95% RH	50 days	90	0	Whole seed	22	Halder *et al.* (1983)
	28°C, 15% SMC	40 days	90	0		22	
Sunflower *cv.* Rodeo	42°C, ~100% RH	8 days	95	70	Whole seed	40	Gidrol *et al.* (1989)

Table 2.1 (continued)

Soybean *cv.* Chippewa	40°C, ~100% RH	5 days	98	5	Whole seed	10 (~50% PC)	Priestley and Leopold (1979)
Soybean *cv.* Wayne	4°C, 8-10% SMC	44 mths	98	86	Whole seed	0	Priestley and Leopold (1983)
Soybean	35°C, 85% RH	23 days	NI	NI	Whole seed	65↑	Chapman and Robertson (1977)
Soybean	35°C, 13% SMC	6 mths	NI	NI	Whole seed	41	Nakayama *et al.* (1981)
Soybean *cv.* Maple Arrow	23°C, NI	5 yrs	93	16	Axis microsomes	50	Senaratna *et al.* (1988)
Wheat	20°C, 50-70% RH	24 mths	83	57	Axes	14	Petruzzelli and Taranto (1984)
	30°C, 12.5% SMC	40 days	83	60		27	
	30°C, 14.5% SMC	35 days	83	57		31	
	30°C, 16.5% SMC	35 days	83	55		34	
Tomato *cv.* Moneymaker	45°C, 70% SMC	168 hrs	100	0	Whole seed	34	Francis and Coolbear (1984)
Tomato *cv.* Kingley cross	45°C, 70% SMC	48 hrs	86	29	Whole seed	6	Francis and Coolbear (1987)
	Natural ageing	7 yrs	95	5		71	

a = *% normal seedlings, otherwise radicle emergence, except b = viable seeds from Tetrazolium staining test.*
NI = *not indicated; ↑ indicates increase compared to control.*

Ultrastructural changes

Changes in the ultrastructure of cell organelles are another indicative phenomenon of membrane damage due to ageing (*e.g.* Hallam *et al.*, 1973; Vishnyakova *et al.*, 1976). One of the most common features observed from unimbibed, fixed aged seeds is coalescence of lipid bodies (*e.g.* Anderson *et al.*, 1970 in wheat; Harman and Granett, 1972 in peas; Villiers, 1972 in lettuce). Evidence of fusion of lipid bodies was also detected in both unimbibed and imbibed rye embryos (Hallam *et al.*, 1973), or imbibed tomato seeds (Francis, 1985) after ageing. Vishnyakova *et al.* (1976), on the other hand, found that lipid bodies in dry embryos of viable and non-viable rice were in contact with each other but did not fuse; whereas the onset of fusion was observed after 24 h soaking, and the process progressed after 48 h. This suggests that detection at different states of imbibition may give different results. Although to date, there is not enough information to explain the mechanisms responsible for this kind of damage, the suggested possibility is membrane rupture (Anderson *et al.*, 1970; Francis, 1985).

Withdrawal of plasmalemma from cell walls is also often observed in aged seeds. Events of this kind have been reported in both dry (Anderson *et al*, 1970 in wheat; Öpik, 1972 in rice; Harman and Granett, 1972 in pea) and imbibed embryos (Hallam *et al.*, 1973 in rye; Villiers, 1972 in lettuce). There have been some queries as to whether these are fixation artifacts when dry tissues were fixed in aqueous solution, because some plasmalemma disruption was also observed from unaged seeds (Chabot and Leopold, 1982 in soybean; Öpik, 1972 in rice). However, Hallam *et al.* (1973) reported the occurrence of disruption in both unimbibed and imbibed non-viable tissues of rye embryos; and, although the lesions became more frequent and more obvious after imbibition in these tissues, this kind of event was not apparent in either unimbibed or imbibed tissues of viable embryos.

There are different types of mitochondrial abnormality found in aged seeds. These include distorted mitochondrial profiles or abnormal inner membranes (Berjak, 1978 in maize; Harman and Granett, 1972 in peas; Hallam *et al.*, 1973 in rye; Vishnyakova *et al.*, 1976 in rice). Damage to mitochondria appears to be an early event during ageing. Berjak and Villiers (1972a) showed that maize embryos held for only 6 days at 14% SMC, 40°C and still possessing a high viability (>95%) exhibited degenerative changes in mitochondrial structure which could be repaired within 48 h of imbibition.

Evidence of dense patches of chromatin in cells of aged seed (Villiers, 1972 in lettuce; Francis, 1985 in tomato; Hallam *et al.*, 1973 in rye), shrinkage of nuclei and a decrease in distinctness of the nuclear membrane (Vishnyakova *et al.*, 1976 in rice), and an increase in the number of lobed nuclei (Berjak and Villiers, 1972a in maize; Öpik, 1972 in rice), indicates deterioration of nuclei. However, most of these characteristics have been observed in non-viable seeds. Villiers (1972) reported the sequence of nucleus alteration in lettuce as follows: in unaged embryos the nuclei stained homogeneously except for a fine network of darker material of heterochromatin, but, as ageing proceeded; deeply stained heterochromatin occurred first, followed by dense patches of chromatin in a low viability lot, with the nuclear membrane eventually becoming difficult to distinguish as a continuous membrane system as viability was lost completely.

Changes in nuclear structure may result in (or be the result of) damage to genetic material. Chromosome aberrations have been shown to be a good index of genetic damage in many species including soybean (*e.g.* Roberts *et al.*, 1967; Chauhan and Swaminathan, 1984; Murata and Vig, 1985). Many chromosomal aberrations may be lethal to the cells, and loss of a critical number of cells can compromise viability (Roberts *et al.*, 1967). Although total levels of DNA do not change much as seed age, it has been shown that certain qualitative changes may take place, *e.g.* the amount of spoolable DNA from rye embryos of reduced viability was decreased (Osborne *et al.*, 1980). This is indicative of DNA fragmentation and was confirmed by separation of total nuclear DNA on alkaline sucrose density gradients, which revealed that randomly sized fragments of low molecular weight DNA were accumulated in stored aged seeds. Recently, Guy *et al.* (1991) have used a Restriction Fragment Length Polymorphism (RFLP) analysis technique to determine DNA changes during accelerated ageing. They found non-random changes in the restriction pattern of the embryo DNA probed with wheat ribosomal DNA. Such changes were first detectable after only 12 h ageing, while vigour loss was detected after 4 days ageing. Further, in grains which have only lost vigour, the restriction pattern of the extracted DNA almost completely repaired during imbibition. The finding of non-random fragmentation of DNA is quite surprising because, in general, DNA is expected to be randomly fragmented. Normally repair enzymes (*e.g.* ligases) may repair the breaks in DNA, providing fragments are still held in position by histone protein; for example, DNA synthesis is more intense in partially deteriorated seed (Osborne, 1983).

Reports on changes in other organelles appear to be limited to a few species. For example, loosely associated golgi stacks (Berjak, 1978), distension of ER cisternae, or distortions of plastid membranes were found in aged maize (Berjak and Villiers, 1972a, b, c). A distorted ribosomal matrix or ruptured plastids were observed in peas as a result of ageing and/or fungal infection (Harman and Granett, 1972).

Loss of selective permeability

Loss of membrane permeability results in metabolites leaking into the imbibition medium. Leakage from seeds often increases with ageing (*e.g.* Ching and Schoolcraft, 1968 in crimson clover; Powell and Matthews, 1977 in pea; Parrish and Leopold, 1978 in soybean). Accordingly, conductivity has become a routine test for determining the integrity of cell membranes and thus seed vigour (AOSA, 1983; Matthews and Powell, 1987a). However, the relationship between imbibitional leakage and ageing cannot be assumed for all species or all types of ageing. In seeds with a large inert endosperm (*i.e.* sorghum: Perl *et al.*, 1978; wheat: Ram and Wiesner, 1988; tomato: Coolbear *et al.*, 1984; ryegrass: Ching and Schoolcraft, 1968) the conductivity of seed leachates does not always correlate with viability. For example, Coolbear *et al.* (1984) reported a considerable increase in leakage of reducing sugars and amino acids in tomato being associated with ageing, which suggests hydrolytic damage had occurred during ageing, but there was no associated increase in conductivity of leachate.

There are other factors which may affect solute leakage. Mechanical damage (Abdul-Baki and Anderson, 1970) or cellular rupture due to rapid water uptake (Schoettle and Leopold, 1984; Spaeth, 1987) reflects complete breakage of cells and tissues, rather than membranes *per se*, and thus strictly is not comparable with that resulting from ageing. The permeability of the seed coat and amount of solutes available to leak out may also mask the effect of ageing.

However, most reports simply present correlative evidence associating leakage of solutes with ageing with no evidence suggesting primary causes or effects. Moreover, much evidence may be merely indicative of an increase in the proportion of dead seeds in a population or an increase in the area of dead tissue in individual viable seeds (Priestley, 1986). Firm evidence to suggest membrane damage increases as living cells in seeds become damaged by ageing was given by Powell and Matthews (1977). They

showed that storage of pea seeds in both humid and excessively dry conditions resulted in increased leakage before any significant loss of viability (indicated by TZ staining studies) had occurred. This finding does indeed suggest that membrane damage is a primary event in seed deterioration.

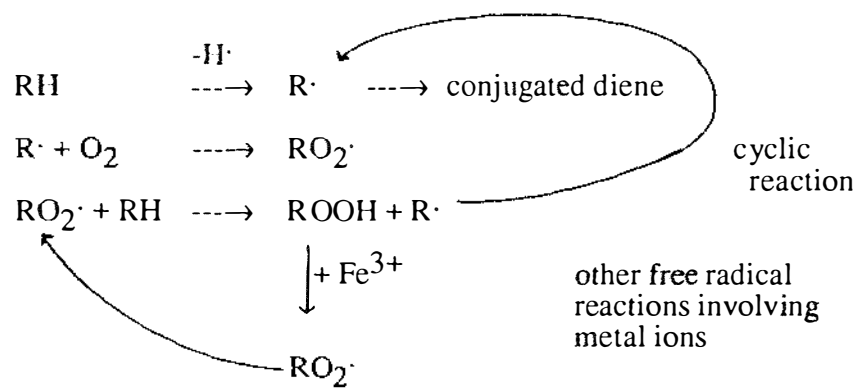
To interpret evidence based on leakage, it is important to understand how membrane leakage occurs. Cellular membranes are based on a lipid bilayer structure composed of polar heads of phospholipids and apolar tails of fatty acid chains. According to Simon's (1974) ideas, an inverted hexagonal (H_{II}) phase is formed in dry seeds. When rehydration occurs the bilayer is formed. Although, these ideas are such an attractive explanation for leakage through the membranes of imbibing seeds, no evidence has yet been found to show that this membrane structure is found in living dry tissue. The reason for this is that the membrane phospholipid, phosphatidyl ethanolamine (PE) is the only membrane component likely to go into the H_{II} phase under physiological conditions, but the fact that this compound is mixed with other membrane phospholipids probably inhibits this phase change: moreover, the presence of sugars such as sucrose is likely to prevent membrane inversion occurring in the way that Simon suggests (Crowe *et al.*, 1989). These observations led Crowe *et al.* to suggest that the reason for leakage might lie in a different type of phase change affected by dehydration, that of the transition from the gel to the liquid crystalline state. The status of a particular type of phospholipid bilayer depends on temperature, ambient moisture and/or sugar concentration. Increase in one or a combination of these factors may result in membrane phospholipids passing from the gel to the liquid crystalline state. Although both gel and liquid crystalline states are impermeable, leakage occurs, during the transition phase, especially when different areas of the membrane may have different transition temperatures due to different phospholipid composition or contact with different sugar concentrations. This may be the state in which the cell is most prone to damage if, during hydration it cannot do any necessary repair and reorganisation in pace with the physical expansion and increased enzymatic activity of the cell.

2.2.1.2 *Free radicals: a cause of membrane damage*

Free radical damage to cell membranes is a main candidate for a primary cause of seed deterioration. Free radicals are molecular species which contain an unpaired electron

and are thus highly reactive. There are many species of free radicals, with the superoxide ($O_2^{\cdot -}$) being the most damaging (Benson, 1990). The superoxide radical is reactive in hydrophobic environments such as the interior of the membrane bilayer (Thompson *et al.*, 1987). It has been proposed that during ageing free radicals are formed and accumulate in dry seeds (Harrington, 1973). The formation and significance of free radicals has been reviewed by a number of workers (*e.g.* Harrington, 1973; Priestley, 1986; Thompson *et al.*, 1987; Benson, 1990). Although several research studies have dealt with free radical actions on seed ageing, the evidence available is still in disagreement. This part of the review will include only reports on changes in proportions of fatty acid composition, a relevant approach to detect lipid peroxidation in seeds.

Free radical damage to membranes is mediated through the process of lipid peroxidation. Since membrane lipids contain high levels of fatty acids with two or more double bonds (polyunsaturated fatty acids, PUFA's), they are particularly electron rich and ideal targets for free radical attack. The process of lipid peroxidation starts when a hydrogen atom is abstracted from a PUFA resulting in fatty acid free radicals. These free radicals can be stabilised and form a conjugated diene independently of oxygen by molecular rearrangement of double bond system, or in the presence of oxygen, react further with oxygen producing new free radical species such as peroxides, which can then react with another unsaturated fatty acid resulting in formation of lipid radical and hydroperoxides, thus initiating a chain reaction ultimately leading to a whole range of toxic breakdown products. The series of process can be summarised as follows:



Where RH = PUFA, R· = fatty acid or lipid radical, $RO_2^{\cdot}$ = peroxide radical, ROOH = hydroperoxide (Benson, 1990).

As this process may occur spontaneously, the term 'autoxidation' is often used, but the reaction can also be enzyme driven at greatly accelerated rates by lipoxygenases which are found in many different seeds, especially soybeans (Wilson and McDonald, 1986; Benson, 1990). Lipid peroxidation may result in losses of PL's due to disruption of the membrane bilayer (Priestley, 1986) and accumulation of free fatty acids (Dey and Mukherjee, 1986). Free radicals may also attack membrane proteins or nucleic acids (Benson, 1990). Free radical chain reactions can be terminated by reaction with another free radical, natural scavengers, quenching with water, or superoxide dismutase activity (*e.g.* Porter and Wagner, 1986; Priestley *et al.*, 1985; Puntarulo and Boveris, 1990).

From the above discussion, it can be seen that, if lipid peroxidation occurs, polyunsaturated acids would be expected to decrease as the seed deteriorates, while other common fatty acids such as 16:0, 18:0 and 18:1 carbon acids should remain relatively constant. If lipids were being damaged by other enzyme activity (*e.g.* β-oxidation or hydrolytic damage), all fatty acids might be expected to decrease at equal rates. The technique of fatty acid analysis has widely been used by many workers for various seed species, and a summary of results reported in literature are presented in Table 2.2. Clearly, species, seed tissues, or ageing conditions influence changes in proportions of fatty acids. For example, Lin and Pearce (1990) showed the responses of different species (French bean and corn) under various conditions.

The conditions of seed ageing are a crucial factor affecting the mechanisms of seed deterioration. Although several reports indicate that lipid peroxidation occurs in dry storage conditions (*e.g.* Priestley and Leopold, 1983; Ferguson *et al.*,1990b in soybean; Flood and Sinclair, 1981 in clover), this is not always the case because lipid peroxidation has also been detected under high RH conditions (*e.g.* Priestley and Leopold, 1979 in soybean; Harman and Mattick, 1976 in pea).

In soybean, Ferguson *et al.* (1990b) showed the presence of lipid peroxidation in one out of two cultivars detected, which confirms the idea of cultivar differences (Table 2.2). Further, generally soybean axes contain a higher proportion of PUFA's than cotyledon tissue (Priestley and Leopold, 1979 and 1983; Ferguson *et al.*, 1990b) and might thus be expected to be more susceptible to free radical attack. In addition, changes occurring in the polar lipid fraction would more convincingly suggest membrane damage rather than measurements of total lipid.

Table 2.2 Changes in the proportions of fatty acids in deteriorating seeds.

Species/ cultivars	Ageing conditions	% Viability change	Lipid analysed	Tissue assayed	Change in proportion of fatty acids	References
Corn	45°C, ~100% RH	90 → 60%	TL	Whole seed	No change	Lin and Pearce (1990)
	40°C, 80% RH	90 → 0%	TL		No change	
	25°C, 79% RH	90 → 0%	TL		No change	
French bean	45°C, ~100% RH	90 → 0%	TL	Whole seed	↓ in 18:3	Lin and Pearce (1990)
	40°C, 80% RH	90 → 0%	TL		↓ in 18:3	
	25°C, 79% RH	90 → 0%	TL		↑ in 18:3	
Pea	30°C, 92% RH	100 → 5%	TL	Whole seed and Axes	Preferential ↓ in PUFA's	Harman and Mattick (1976)
Peanut	'Natural'	94 → 39%	NL, GL, PL	Whole seed	No change	Pearce and Abdel Samad (1980)
	38°C, 90% RH	95 → 15%	NL, GL, PL		No change	
Soybean *cv.* Chippewa	40°C, ~100% RH	100 → 5%	TL	Whole seed	No change	Priestley and Leopold (1979)
Soybean *cv.* Chippewa	'Natural'	98 → 28%	TL	Whole seed	30% ↓ in 18:3, 8% ↓ in 18:2, 37% ↑ in 18:1, no change in others	Priestley and Leopold (1983)
	'Natural'	98 → 28%	Polar	Whole seed	20% ↓ in 18:3, no change in others	Priestley and Leopold (1983)

Table 2.2 (continued)

Soybean *cv.* Chippewa	'Natural'	98 → 28%	TL	Axes	40% ↓ in 18:3, 15% ↓ in 18:2, no change in others	Priestley and Leopold (1983)
Soybean *cv.* Wayne	'Natural'	98 → 86%	TL	Whole seed	32% ↓ in 18:3, 7% ↓ in 18:2, no change in others	Priestley and Leopold (1983)
	40°C, ~100% RH	98 → 38%	TL	Whole seed	No change	Priestley and Leopold (1983)
Soybean *cv.*Pride x005	45°C, ~100% RH	60 → 0%	Polar	Axes	Preferential ↓ in PUFA's	Stewart and Bewley (1980)
	45°C, low RH	60 → 60%	Polar	Axes	No change	
Soybean *cv.* Maple arrow	'Natural'	93 → 16%	TL	Axis microsomes	No change	Senaratna *et al.* (1988)
Soybean *cv.* Union	'Natural'	No germination change, decrease vigour	TL	Whole seed	No change	Ferguson *et al.* (1990b)
			TL	Axes	No change	Ferguson *et al.* (1990b)
			Polar	Axes	No change	
			TL	Axis mitrochondria	10% ↑ in 16:0	
Soybean *cv.* Desoto	'Natural'	No germination change, decrease vigour	TL	Cotyledons	No change	Ferguson *et al.* (1990b)

Table 2.2 (continued)

Soybean *cv.* Desoto	'Natural'	No germination change, decrease vigour	TL Polar TL	Axes Axes Axis mitrochondria	No change No change 16% ↑ in 16:0, 18% ↑ in 18:0, 15% ↓ in 18:3, no change in others	Ferguson *et al.* (1990b)
Soybean *cv.* William	105°C, 9% SMC	Non viable	TL Polar TL Polar	Cotyledons Cotyledons Axes Axes	No change No change No change Preferential ↓ in PUFA's	Priestley *et al.* (1985)
Soybean *cv.* Amsoy	25°C, 20% SMC	89 → 8%	Polar	Whole seed	No change	Ohlrogge and Kernan (1982)
Subterranean Clover	'Natural'	? → 10%		Whole seed	Preferential ↓ in PUFA's	Flood and Sinclair (1981)
Sunflower	42°C, ~100% RH	98 → 75%	TL Diacyl-glyceral Polar	Whole seed (dehulled)	No change Preferential ↓ in PUFA's No change	Gidrol *et al.* (1989)
Tomato	45°C, 70% SMC		TL	Whole seed	↓16:0, ↑18:0, ↓18:2	Francis and Coolbear (1988)

TL = Total lipid, NL = Neutral lipid, GL = Glycolipid, PL = Phospholipid, ↑ or ↓ indicates increase or decrease, repectively, as compared to control.

Lipid peroxidation is expected to cause damage to membranes as mentioned earlier, and mitochondria or microsomes have been reported to contain high proportions of PUFA's (cf. Demopoulos, 1973). Studies on these organelle membranes may result in some interesting information. Very few reports in this area are available so far. Also the results obtained are contradictory. Senaratna *et al.* (1988) reported no changes in proportions of fatty acids in the microsome fraction from soybean axes in 5 years stored seeds despite significant decline in normal germination, while Ferguson *et al.* (1990b) detected reduction of 18:3 fatty acid in the mitochondrial fraction from axes even before seed lost vigour.

According to Wilson and McDonald (1986), besides loss of PUFA's, there are several other practical approaches to detect the action of free radicals in seed tissue. However, as stated earlier, clear cut evidence seems to be difficult to achieve. Examples of these methods and their associated problems are discussed briefly below:

1) Direct detection of free radical levels by electron resonance techniques at any time during storage may not always suggest its action, because free radicals are highly unstable and may be trapped by drying or quenched by high moisture content. The reports in the literature are also conflicting, *e.g.* while Priesley *et al.* (1985) found little change in free radical levels in soybean axes under 'natural' ageing but a ~100% increase under an artificial regime, Buchvarov and Gantcheff (1984) contended that marked increases occurred in both types of ageing conditions.

2) The often used method of detection of primary products of lipid peroxidation, which involves oxidation of iodide ions to iodine by hydroperoxides, is not entirely specific; because other compounds may reduce the hydroperoxides naturally. Sharma (1977) reported a 100% increase in hydroperoxides by this method in sesame stored for 18 months, and a 30% increase in cotton and castor bean. On the other hand, Pearce and Abdel Samad (1980) showed no evidence of hydroperoxides in aged peanut; while Powell and Harman (1986) found only a trace in aged peas.

3) Secondary product analysis centres around the determination of malondialdehyde. This is a product of peroxidation of 18:3 fatty acids and thus dependent on their concentration. Stewart and Bewley (1980) showed increased malondialdehyde levels in deteriorated soybean, which support the free radical hypothesis; but this work has been criticised, because the ageing conditions were too severe. In this case, both viable and non-viable embryos produced malondialdehyde, but the increased levels were evident only on non-viable embryos.

4) Data from work using protective antioxidants may provide some of the most convincing, if indirect evidence, in favour of the free radical hypothesis. This involves determining the levels of endogenous compounds which may play a role as free radical scavengers such as the superoxide dismutase enzymes (Puntarulo and Boveris, 1990), α-tocopherol (Priestley *et al.*, 1980), or the effects of applied antioxidants *e.g.* cathodic protection used by Pammenter *et al.* (1974). This topic is discussed in detail in Section 2.3.2.

2.2.2 Changes in enzyme activity as a cause of seed deterioration

Enzyme activities are often considerably reduced in aged seed (*e.g.* Anderson, 1970a), although according to Perl *et al.* (1978), some hydrolytic enzymes *e.g.* amylase, glutamic-pyruvic-transaminase, ribonuclease, glutamic acid decarboxylase increased in the early stage of accelerated ageing of sorghum seeds, while protease increased throughout the ageing process. The increase in hydrolytic enzymes could be ignored on the basis that under high moisture conditions in the initial stages of the ageing process, seeds begin processes leading to germination. The increase of protease activity may result in destruction of other enzymes in the seed. So, determination of gross changes in enzyme activity may be misleading. A loss of compartmentalisation is likely to be crucial. Conflicting evidence may also arise when different parts of tissue are analysed. For example, Kunert (*cf.* Priestley, 1986) reported that lipase in castor bean decreased more rapidly in the peripheral layers of the endosperm than deeper within the tissue.

Kole and Gupta (1982) have shown that the rate of decline in activity of amylase and lipase was the most sensitive to ageing in safflower and may be a cause of seed deterioration. Losses of phospholipids which are related to accumulation of phosphatidic acid or lysophospholipid during ageing (Priestley and Leopold, 1979; Nakayama *et al.*, 1981) may suggest the activity of phospholipases. Priestley (1986) assumed that phospholipase-D cleaves the polar head group from PL's to leave phosphatidic acid while phospholipase-A may remove single fatty acids from PL's to form lysophospholipids, but there is little evidence from plant systems at present.

Loss of activity of detoxification or repair enzymes can also be an important component of seed ageing. Puntarulo and Boveris (1990) reported that superoxide dismutase (SOD) activity measured after 2 h imbibition showed a marked decrease in

activity that correlated with losses of vigour during ageing, and after 30 h of imbibition, the SOD activity in aged axes was only 1/5 of that in fresh axes. These observations were similar in both naturally and artificially aged seeds. Similarly, Stewart and Bewley (1980) found that non-viable soybean seeds failed to show any SOD activity during the early stages of germination. However, working with non-viable seeds does not identify a crucial role for this enzyme.

2.2.3 Changes in respiration

Seed deterioration is often associated with changes in one or more aspects of respiratory metabolism such as a decrease in the rate of O_2 uptake (*e.g.* Abdul-Baki, 1980; Parrish and Leopold, 1978), an increase in respiratory quotient (RQ) (Woodstock *et al.*, 1984) or changes in ATP levels (Lunn and Madson, 1981). However, this correlation is not universal; and, once again, correlative relationships do not necessarily imply cause and effect. Several studies show that changes in respiration do not always correlate with vigour loss (*e.g.* Abdul-Baki, 1969 in barley and wheat; Anderson, 1970b in wheat). For example, Byrd and Delouche (1971) demonstrated that there was little difference in rate of oxygen consumption among ageing treatments in soybean seeds despite significant losses of vigour in some samples. This suggests that oxygen uptake *per se* may not always be a reliable index of seed deterioration. Furthermore, care must be taken when comparisons are made between different tissues, because embryonic tissues seem to be more sensitive to ageing than cotyledonary or endospermic tissues (Buchvarov and Alekhina, 1984; Anderson and Abdul-Baki, 1971).

Working with soybean, Woodstock and coworkers (Woodstock and Taylorson, 1981a, b; Woodstock *et al.*, 1984) reported a dramatic decrease in rates of O_2 uptake but an increased RQ in both whole seeds and axes with vigour loss. They suggested that imbalances in the components of respiratory metabolism, *e.g.* the uncoupling of oxidative phosphorylation or a loss of coordination between the activity of glycolysis and the Krebs cycle, may be an early indication of ageing. Excess glycolysis may result in anaerobic respiration and the production of ethanol and acetaldehyde, both of which are toxic (Woodstock and Taylorson, 1981b; Gorecki *et al.*, 1985; Amable Obendorf, 1986 and Reedy and Knapp, 1988). Similarly, Abu-Shakra and Ching (1967) confirmed that there were only small differences between new and old soybean

seeds (4 years storage) in the rates of O_2 consumption in mitochondria isolated from 4 day- old seedlings, although the ADP:O ratios of mitochondria from aged material were about half of those unaged ones which suggests that direct measurements of damage to mitochondria may be sensitive indicators of deterioration. Crawford (1977) suggested that high levels of ethanol may cause destruction of mitochondrial membranes, but he provided no evidence to support. Recently, Ferguson *et al.* (1990a) presented experimental evidence showing that the early occurrence of respiratory changes was associated with mitochondrial damage in soybean axes, which strongly suggests that this may be an important cause of seed deterioration.

During ageing, respiratory pathways may shift from normal respiration to the Pentose Phosphate Pathway (PPP) (*e.g.* Takayanagi, 1977 in rape seed; Kharlukhi and Agrawal, 1984 in chickpea, wheat and green gram; Leopold and Musgrave, 1980 in soybean). The occurrence of alternative respiration may have little significance *per se*, except that it is less sensitive to ageing damage than normal respiration and it can thus compete for oxygen more effectively when conventional respiration loses efficiency.

2.2.4 Impaired RNA and protein synthesis

Protein synthesis, which is usually evident at very early stages of imbibition, is reduced in aged seeds (*e.g.* Osborne *et al.*, 1974; Anderson, 1977). The declining rate of protein synthesis in deteriorated seeds may be due to damage to conserved mRNA, impaired synthesis of new mRNA, damage to ribosomes or losses of activating components in protein synthesis. Several or all of these factors may serve to reduce the overall ability of aged seed to synthesize protein. This deficiency, in turn, has severe consequences for the effectiveness of cellular repair mechanisms that operate during the early stages of imbibition. Bray and Smith (1985) suggested that lesions affecting the pattern of decay of stored mRNA could be an early indication of deterioration in wheat. Recently, Gidrol *et al.* (1988) demonstrated that accelerated ageing greatly decreased protein synthesis during the first hour of imbibition in both pea and soybean seeds, but effects were greater in soybean. They attributed this to degradation of pre-existing 'long-lived' mRNA's during ageing in both pea and soybean, and that soybean aged seeds were also not able to synthesize new mRNA nor to translate messages during the first hour of imbibition because of lack of ATP, which is in agreement with Anderson (1977). However, lesions appearing at the level of

ribosomal activity as well as tRNA synthetase activity have been demonstrated to be a cause of vigour loss in wheat seeds (Blowers *et al.*, 1985). Usually protein synthesis is organised on a membrane surface. Therefore, membrane damage may be an important factor contributing to loss of activity, and loss of activity may in turn impede opportunities for detoxification and/or repair.

2.3 SEED TREATMENTS FOR THE PROTECTION OR REPAIR OF STORED SEEDS

Seed treatment is a broad term and there are a vast number of different treatment options available (Scott, 1989). Some are already commercially viable, some are still at the experimental or developmental stage. This section discusses methods and mechanisms of seed treatments designed to improve and/or repair the physiological condition of stored seeds with particular references on hydration-dehydration and antioxidant treatments.

2.3.1 **Hydration-dehydration treatments**

2.3.1.1 *Background and principles*

The fact that aged seeds can germinate and develop into apparently normal seedlings suggests that seeds are capable of recovery by rehydration. This hypothesis was supported by Berjak and Villiers (1972a), who demonstrated that in maize which had previously been subjected to accelerated ageing, ultrastructural abnormalities present upon rehydration gradually disappeared 24 to 48 h after the start of imbibition. Villiers (1974) showed that lettuce seeds which were stored fully imbibed but unable to germinate had very low levels of chromosome aberrations compared to seeds stored at moisture contents below 18%. Alternatively, subjecting lettuce seeds to occasional brief interludes of wetting and drying during conventional low moisture content storage largely eliminated the usual aged-induced accumulation of chromosome damage (Villiers and Edgcumbe, 1975). These data all provided evidence for the existence of a continuous repair system in imbibed seeds; and, consequently, interest

has developed in hydration-dehydration treatments for storage improvement. Such treatments may be aimed at either improving the vigour and germination performance of aged seed or protecting seed from deterioration under less than ideal conditions.

2.3.1.2 *Methods of hydration-dehydration treatments*

The principle of hydration-dehydration treatment involves allowing seeds to take up a controlled amount of water followed by drying back to the required moisture level. Seeds may be allowed to take up water by different methods: soaking or dipping in water (*e.g.* Basu, 1976; Kundu and Basu, 1981; Dey and Mukherjee, 1986), equilibration with water vapour from a high humidity atmosphere (*e.g.* Pan and Basu, 1985; Rudrapal and Basu, 1982; Saha and Basu, 1984), imbibition from a wet substrate (Tilden and West, 1985; Pandey, 1988), priming in a solution of polyethylene glycol (PEG) (Knypl and Khan, 1981; Burgass and Powell, 1984)) or salt (Haigh *et al.*, 1986; Argerich and Bradford, 1989), or low temperature presowing treatment (LTPST) (Coolbear *et al.*, 1984). As only soaking and moisture equilibration treatments are relevant to this study, detailed discussion is restricted to these methods.

Soaking has been found to be effective in several species. For example, Savino *et al.* (1979) showed that soaking pea, carrot or tomato seeds for 18 or 24 h at 20°C and then drying back prior to accelerated ageing maintained their vigour and viability for a longer period of time than untreated controls. Soaking for only 2 h and drying also successfully extended viability of wheat seeds stored under a range of conditions (Rudrapal and Basu, 1982). Similarly, Dey and Mukherjee (1986) found a 6 h soaking-drying treatment showed a remarkable effect in prolonging the storability of maize or mustard held in different ageing conditions. Soaking techniques show a good potential for protection in many other species, *e.g.* carrot (Kundu and Basu, 1981), sugar beet (Basu and Dhar, 1979), jute, sunflower, rice, pulses and vegetables (Basu, 1976).

Beneficial effects often seem to be more pronounced in relatively low vigour rather than high vigour or badly deteriorated seeds (*e.g.* Basu, 1976; Brocklehurst and Dearman, 1983; Dey and Mukherjee, 1986). Working with brussels sprouts, Burgass

and Powell (1984) found that a 3 h soaking-drying treatment showed both protective and repair effects. The repair effects of short soaking treatments have also been claimed to work well in wheat for many seed lots (Goldsworthy *et al.*, 1982), but as the results in this study lacked complete statistical analysis, these data are not fully convincing. In soybean, variations in response have been reported. For example, Saha and Basu (1984) found that pretreatment of soybean by soaking for 2 h at 28°C followed by drying, reduced germinability in medium and low vigour seeds even before ageing, and reduced ageing resistance in high vigour seeds, although the treatment provided protection of remaining germinable seeds of medium vigour against both accelerated and natural ageing. In contrast, Dey and Mukherjee (1986) also reported (without presenting the data) that soaking-drying treatments caused detrimental effects on soybean seeds after subsequent storage under three different conditions.

While it is generally accepted that cells must approach a state nearing full imbibition for metabolic repair to be active, Kundu and Basu (1981) showed that soaking for either 2 h or 2 min followed by drying in the same manner were equally effective in ageing protection for carrot seeds. Similar evidence has been reported in several other species (*e.g.* wheat, sunflower, rice or pulses: Basu, 1976; mustard: Rudrapal and Basu, 1982; maize: Dey and Mukherjee, 1986). It was suggested that the maximum benefits of hydration treatments for wheat seeds could be obtained from 5 or 30 minutes, provided that a slow rate of drying was used for the former case so as to allow the seeds to remain wet for longer after soaking, thus increasing the time available for repair (Goldsworthy *et al.*, 1982). It seems possible that in many species a brief soaking period may be enough for embryo axis tissue to become imbibed well before the rest of seed (*e.g.* Grosh and Milner, 1959 in wheat; McDonald *et al.*, 1988 in soybean). Self repair thus might possibly be facilitated in these seeds. Clearly the effectiveness of the soaking period may vary depending on the seed itself and the speed of subsequent drying.

Although soaking treatments show a tendency to give positive results in a variety of seeds, in some species, when seeds or embryonic axes take up water rapidly, soaking injury may occur, especially in low vigour lots (Powell and Matthews, 1978 in pea;

Woodstock and Taylorson, 1981b, Woodstock and Tao, 1981, Saha and Basu, 1984, Tilden and West, 1985 all in soybeans; Pandey, 1988 in French bean). These problems may be overcome by slowing down the rate of water uptake, *e.g.* by moisture equilibration (ME) techniques. Such treatments, followed by drying, have shown to be effective in prolonging storability in various species, *e.g.* carrot (Kundu and Basu, 1981), mustard and wheat (Rudrapal and Basu, 1982), maize (Dey and Mukherjee, 1986) and lettuce (Pan and Basu, 1985). For soybean, Saha and Basu (1984) have evaluated the effectiveness of ME-drying treatment on seeds of different vigour. They found that 24 h ME where SMC's were raised from ~9% to ~20% before drying back, protected high vigour seeds from ageing and repaired medium and low vigour seeds; but Dey and Mukherjee (1986) found only little benefit from this type of treatment. However, as neither germination nor SMC data were provided by the latter, it is difficult to evaluate why these results are different: possibly it was due to variation between the seed lots and/or cultivars used or the moisture contents attained during ME. Kundu and Basu (1981) have shown that ME treatment for 24 h increased the SMC of carrot from 7 to 18.6%. These changes were paralleled by increased germinability of 5 d aged seeds from 17% in the untreated aged sample to 67%. Prolonged ME treatment for up to 48 h resulted in a significant decrease in germination, although the SMC reached 23%. This may be due to accelerated seed deterioration (Rudrapal and Basu, 1982) or fungal attack (Rao *et al.*, 1987).

2.3.1.3 *Mechanisms related to hydration-dehydration treatments*

Although hydration-dehydration treatments have been shown to have beneficial effects on storability in many species, our understanding of the physiological mechanisms to explain how such treatments affect vigour and germination performance of seeds is very limited. The key basis for effective treatments of seeds seems to be to allow self-repair and detoxification, and benefits of the treatments are retained after drying back.

As has already been indicated (Section 2.2), it has been often suggested that free radical chain reactions in aged seeds are terminated by the treatments through quenching and the induction of scavenging mechanisms (*e.g.* Basu, 1976; Mitra and Basu, 1979). Evidence to support this is provided by Dey and coworkers. By using the thiobarbituric acid (TBA) method to determine changes in levels of malondialdehyde, Dey and Mukherjee (1986) found that hydration-dehydration

treatments (soaking, dipping or ME followed by drying) reduced lipid peroxidation and free fatty acids in maize and mustard seeds during different subsequent storage conditions. Similar observations were reported previously in sunflower (Dey and Basu, 1982). Their claims were made without statistical analysis, and no other reports to support such observations have been published, thus it is difficult to draw any clear conclusion. Reduced free radical activity may also be a function of the enzyme superoxide dismutase which has been found to appear in hydrated seeds (Stewart and Bewley, 1980; Puntarulo and Boveris, 1990).

Additionally, protective effects of the treatments may relate to prevention of changes in activities of some enzymes. Dey and Mukherjee (1986) have reported that maize or mustard seeds treated with soaking, dipping or ME followed by drying maintained high germinability accompanied by greater dehydrogenase and peroxidase activities, but lesser lipase activities than untreated seeds. Once again, because the data were presented without statistical analysis, the validity of such findings is questionable.

It has been established that hydrated seeds have a high metabolic activity. For example, respiration is evident (Powell *et al.*, 1983), membrane lipids and proteins are subject to turnover (Cuming and Osborne, 1978a, b), active polysomes are present (Fountain and Bewley, 1973), and chromosome aberrations are reduced (Villiers, 1974; Villiers and Edgcumbe, 1975). Tilden and West (1985) assumed that the existence of such active metabolic functions may allow repair of damage within soybean seeds as it accumulates, but they presented no direct evidence.

As repair is a metabolic process, conditions to allow the process to operate effectively are important. The moisture content of the seed is a key factor. As discussed in a previous section (2.3.1.2), a full imbibition state is not necessarily important for metabolic repair mechanisms to begin to become functional. This idea has been supported by experimental evidence. For example, Saha and Basu (1984) showed that SMC ~20% (from ME treatment) or~ 50% (from ME + soaking treatment) gave similar effects for repair on aged soybean (Saha and Basu, 1984). These effects are not surprising, because the findings of Vertucci and Roos (1990) confirm that the onset of respiration occurred at a SMC in equilibrium with 91% RH (~27% SMC for soybean seeds.

There are a few reports showing an importance of temperature and/or oxygen pressure for effective repair processes. Goldsworthy *et al.* (1982) demonstrated that after soaking treatment at 35°C wheat seeds germinated faster and produced longer coleoptiles than after treatment at lower temperatures. In soybean, Tilden and West (1985) reported that imbibing seeds at 25°C followed by drying at 25°C was the most effective repair treatment (in terms of reduction of electrolyte leakage) relative to combinations of other lower imbibing and/or drying temperatures.

Goldsworthy *et al.* (1982) showed that soaking at 35°C for 2 h in water bubbling with air offered no advantage over bubbling with nitrogen which led them concluded that oxygen might not be required for the initial repair mechanisms of imbibed seeds. There is, however, no other evidence in this or other species to support this finding. Most reports suggest that repair processes during storage are strictly dependent on the availability of oxygen. Ibrahim and Roberts (1983) showed that longevity of lettuce seeds was promoted when their SMC was increased above 15-20% providing oxygen was being supplied (Ibrahim *et al.*, 1983).

2.3.2 **Antioxidant treatments**

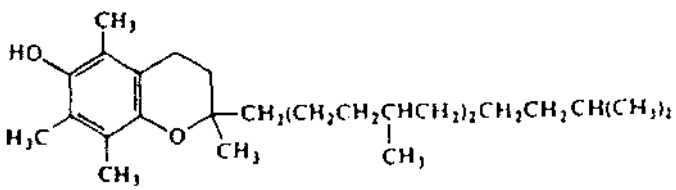
Pammenter *et al.*'s (1974) demonstration that cathodic protection can protect maize seed in high temperature storage conditions (13.6% SMC, 40°C RH) supports the hypothesis that free radicals and/or lipid peroxidation could be a major cause of seed deterioration. It seems likely, therefore, that the use of chemical antioxidants may have the potential to be a cost-effective mechanism for prolonging storage life (Wilson and McDonald, 1986). Although attempts to use such treatments have become an increasing focus of interest for many workers, they have met with variable success. Different responses to antioxidants reported in the literature seem to depend on three main factors: selection of antioxidants, techniques of application, and storage conditions. These factors are discussed in detail below.

2.3.2.1 *Selection of antioxidants*

There are several types of natural or synthetic antioxidants available. Alpha-tocopherol, vitamin E (a mixture of α,β,γ-tocopherol), and ascorbic acid are examples of naturally occurring antioxidants, while butylated hydroxytoluene (BHT), butylated

hydroxyanisole (BHA), Propyl gallate (PG), Monotertiary butylated hydroquinone (TBHQ), β-mercaptoethanol and sodium thiosulphate are synthetic. Iodine is an antioxidant element (Dey and Mukherjee, 1984). These chemicals are commercially available and have been widely evaluated by many workers (*e.g.* Woodstock *et al.*, 1983; Gorecki and Harman, 1987; Okundaye, 1977; Parrish and Bahler, 1983; Francis, 1985; Basu, 1976; Dey and Mukherjee, 1984). Structure and/or properties of some antioxidants which are relevant to this research are given as follows:

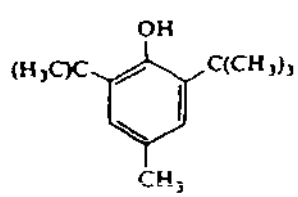
a) α-tocopherol



2,5,7,8-tetramethyl-2-(4,8,12-trimethyl tridecyl)-6-chromanol.

Chromanols derived from plastoquinones are ubiquitous in plants and are known as natural antioxidants. Total tocopherol contents in whole seed soybeans range from 1.2-1.8 mg per g lipid with only 7% being α-tocopherol (Priestley *et al.*, 1980), but α-tocopherol in axes may be as high as 50 $mg.g^{-1}$ lipid (Senaratna *et al.*, 1985). Alpha-tocopherol is light and moisture sensitive, nearly insoluble in water but soluble in acetone (BDH, 1986).

b) BHT (Butylated hydroxytoluene)



2,6-di tert-butyl-4 methylphenol.

BHT has extremely low solubility in water but is highly soluble in fats (BDH, 1986) and acetone (Woodstock *et al.*, 1983)

c) β-mercaptoethanol

$HS\text{-}CH_2\text{-}CH_2OH$

Beta-mercaptoethanol is a volatile liquid and completely soluble in water or most organic solvents (BDH, 1986).

d) Iodine (I_2) has a crystalline structure and easily sublimes.

To deal with antioxidant seed treatments, it is important to understand how antioxidants play a role in seeds. If lipid peroxidation is involved in ageing, antioxidants would, at least in theory, be effective. In plant tissue: for example, tocopherol (α,β or γ isomer) is a naturally occurring lipid peroxidation inhibitor (Porter and Wagner, 1986) found in association with lipid bodies. *In vitro*, one molecule of α-tocopherol can prevent 2,000 molecules of unsaturated fatty acids from oxidation (Gruger and Tappel, 1971), while γ-tocopherol has been found to be 1.4 times as effective as the α-isomer (Wu *et al.*, 1979). The protective role of α-tocopherol is due to its ability to quench both the superoxide and lipid peroxy free radicals (Leibovitz and Siegel, 1980). In the process of quenching, α-tocopherol is first oxidised to the tocopherol radical and subsequently to tocopheryl quinone (Gorecki and Harman, 1987). Since tocopherol is consumed in the process of quenching free radicals, lipid peroxidation accelerates only after tocopherol levels have declined. Applied antioxidants may thus substitute for any shortages of tocopherol, resulting in ageing protection. However, reports on levels of natural tocopherols in seeds during ageing are inconsistent. Sharma (1977) found a decrease of tocopherol in sesame, castor bean or cotton embryos which paralleled loss of seed viability. Similarly Ramarathnam *et al.* (1989) reported that α-tocopherol decreased markedly when rice seeds were artificially aged by γ-irradiation. In contrast, Fielding and Goldsworthy (1980) and Priestley *et al.* (1980) showed no changes of α-tocopherol levels in whole seed of wheat and soybean, respectively during rapid ageing. It is possible that other natural antioxidants may provide more resistance to free radical damage *e.g.* oryzanol in rice (Ramarathnam *et al.*, 1989); procyanidins in *Vigna angularis* (Ariga *et al.*, 1988); phytic acid in legumes or cereals (Graf *et al.*, 1987) have stronger activity than α-tocopherol.

Apart from free radical quenching, Basu and Rudrapal (1980) have proposed that antioxidants may play a role on lipid stabilization by reacting with carbon-carbon double bonds of polyunsaturated fatty acids. This idea might explain why antioxidants are effective even in newly harvested seeds, although free radical levels may be high in senescing material (Benson, 1990). However, there is no hard evidence for this idea.

At present, there are only a few reports providing evidence of mechanisms involving antioxidant treatments. Using iodine, Dey and Mukherjee (1984) found that treatments which slowed rates of loss in dehydrogenase or amylolytic activity, reduced lipid peroxidation, and decreased seed leachate conductivity in sunflower and soybean. Similar effects of iodine or *p*-hydroxybenzoic acid were found in maize or mustard, respectively (Basu and Rudrapal, 1980; Dey and Mukherjee, 1988). Antioxidants may react synergistically with phospholipids to increase lipid oxidative stability (Hildebrand, 1984). In addition to the amine groups on phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the reducing sugar on phosphatidylinositol (PI) can facilitate hydrogen or electron donation to partially oxidized tocopherol. By delaying the oxidation of tocopherol to an irreversible stage, PL's can extend the effectiveness of tocopherol. Working on soybean, Aho (1990) found that improved germination as a result of TBHQ treatment was associated with loss of phospholipids from embryos. He concluded that TBHQ may act in conjuction with PL's to quench free radicals within the seed.

Although a wide range of antioxidants have been tested on various seed species and evaluated under different storage conditions, there are no generalisations about which antioxidants are more effective. An ideal chemical would be expected to be effective on a wide range of seed species and storage conditions. In fact, often seeds of different species respond differently to the same antioxidant, or different antioxidants have different effects on the same species. Woodstock *et al.* (1983) have evaluated the effectiveness of butylated hydroxytoluene (BHT) and vitamin E on the storage life of onion, parsley and pepper seeds held in desiccators over zeolite at ambient temperature, and found that parsley seeds soaked in acetone solutions of both BHT (0.1 M or 2.2%) and vitamin E (20 units.ml^{-1} or 2%) had improved storability, while these antioxidants accelerated the rate of deterioration of pepper seeds. BHT has the

capacity to protect onions in store, whereas vitamin E accelerated their deterioration under identical storage conditions. Similarly, Gorecki and Harman (1987) applied 8 different antioxidants to pea seeds and found that only 0.1% (*w/w*) BHT and α-tocopherol (1.0%, *w/v*) impregnated via acetone for 16 h gave some measure of protection under high humidity conditions (92% RH, 30°C). Protective effects of α-tocopherol were also found in mustard, maize (Dey and Mukherjee, 1988) and soybean (Okundaye, 1977).

Sodium thiosulphate in aqueous solution has been claimed to have protective effects during both natural and accelerated ageing of a variety of species including wheat, rice, pulses, sunflower (Basu 1976), but as the effectiveness of these treatments was compared without statistical analysis in this report, it is difficult to ensure that the claims are reliable. Other workers have used various water soluble antioxidants, including mercaptoethanol and sodium thiosulphate, and showed that they were effective in improving the storability of carrot (Kundu and Basu, 1981) and tomato (Francis, 1985). However, little or no effect was observed over hydration treatment alone on sugar beet (Basu and Dhar, 1979), maize (Barnes and Berjak, 1978), pea (Gorecki and Harman, 1987) or wheat (Sharma, 1988).

2.3.2.2 *Techniques of application*

Generally, the aim of these techniques is to allow the required concentration of antioxidant to be available to the embryos of seeds without causing severe damage. There are three main factors interacting here: solvent used, antioxidant concentration and treatment duration. Antioxidants can be applied in aqueous solution, via organic solvents or as a solid. Choice of solvent is limited if the selected antioxidant is sparingly soluble in water, but soluble in organic solvents. Treatment durations vary with seed types and carrier solvents (Tao and Khan, 1974). It seems that choice of concentrations of antioxidants and treatment durations are quite arbitrary in this type of study. Basu (1976) suggested that 2-6 h in aqueous solution is enough for a wide range of species, while a period of 2-4 h in organic solvents (*e.g.* acetone, benzene, dichloromethane, xylene), with 4 h in acetone being the most favoured, has been reported to be effective for various crop seeds (*e.g.* maize and mustard: Dey and

Mukherjee, 1988; parsley and onion: Woodstock *et al.*, 1983; soybean: Okundaye, 1977 and Parrish and Bahler, 1983). Gorecki and Harman (1987) successfully used a 16 h acetone soak for pea seeds. Recently, Aho (1990) applied 50 mM BHT, BHA, PG or TBHQ to soybean seeds by soaking in dimethyl sulphoxide (DMSO) for 15 min, and reported that BHT, BHA or PG did not show any effect, although the toxicity of TBHQ was detected. It appears that no validation of treatment times were undertaken in this study. Thus, it is unclear whether these antioxidants could reach the embryo with this method of treatment.

To evaluate effectiveness of antioxidants, care must be taken when solvents are used as a vehicle to carry antioxidants. In aqueous solutions, water itself can quench or scavenge free radicals or, alternatively, cause soaking injury to seed which results in antioxidants being less effective (*e.g.* Basu and Dhar, 1979 in sugar beet; Kundu and Basu, 1981 in carrot; Gorecki and Harman, 1987 in pea; see also Section 2.4.1 for a more complete discussion). On the other hand, organic solvents (*e.g.* 2,4-dinitrophenol, acetone) may be toxic to seeds or induce seed dormancy (Dey and Mukherjee, 1988; Woodstock *et al., 1983*; see also Section 2.4.2). An alternative to the use of solutions is to treat seeds with chemicals in the vapour phase or via a solid medium. Basu and Rudrapal (1980) described a procedure for iodine application to mustard seeds by iodine vapour. The technique involves allowing solid iodine to volatilize in a closed container before exposing seeds in that atmosphere for a required period. This technique has been modified to be more convenient by using a mixture of solid iodine and an inert carrier powder (such as $CaCO_3$). A proportion of one part of 0.1% iodine/$CaCO_3$ mixture to four parts seeds (*w/w*) has successfully improved the seedling growth of soybean and sunflower compared to untreated controls after both rapid and slow ageing conditions (Dey and Mukherjee, 1984).

2.3.2.3 *Storage conditions*

As dicussed in Section 2.2, the mechanisms of seed deterioration under different storage conditions may be different. This may be one of the key reasons why reported effects of antioxidant treatments are variable. Okundaye (1977) showed evidence of interactions between antioxidants and subsequent storage conditions in soybean and

corn in that α-tocopherol, BHA and $FeCl_3$ were all effective in retarding the rate of viability loss under high temperature conditions (40°C, 30% RH) with the most effective being BHA, but these antioxidants did not give any beneficial response under low temperature and low RH (30°C, 35% RH) conditions. At low temperature and medium RH (30°C, 78% RH) α-tocopherol was more effective than BHA. There was no explanation given why different low RH conditions (30 and 35%) were used to compare effects of temperatures in this study. Parrish and Bahler (1983) also reported that in spite of initially harmful effects, PG and TBHQ proved to have some protective value on soybean during storage (at 40°C, 8.3% SMC), while PG was toxic when seeds were stored at 13% SMC and ambient temperature. However, Dey and Mukherjee (1988) reported that α-tocopherol, *p*-hydroxybenzoic acid and aminobenzoic acid were effective in controlling deterioration of maize and mustard under both accelerated and natural ageing conditions. Similar evidence has been provided by the same group for iodine treatments (Dey and Mukherjee, 1984 and Rudrapal and Basu, 1980) and water soluble antioxidants (Basu, 1976).

2.4 POTENTIAL PROBLEMS WITH SEED TREATMENTS

2.4.1 **Soaking injury in hydration-dehydration treatments**

Soaking injury is a damaging phenomenon occurring in any tissue as a result of a rapid inrush of water. In seed, this type of damage may be reflected by loss of vigour and germinability. There is much evidence showing that soaking causes damage to dry seed particularly to large-seeded legumes (*e.g.* pea: Powell and Matthews, 1978; soybean: Woodstock and Tao, 1981; French bean: Pandey, 1988). Factors which may affect the extent of soaking damage include seed species/cultivars, mechanical damage, initial moisture contents, vigour of seeds and ambient temperatures. These factors relate to each other (*e.g.* Hobbs and Obendorf, 1972; Wolk *et al.*, 1989; Saha and Basu 1984). Among these, seed moisture is crucial: for instance, soaking at very low initial SMC's, at low temperatures, and to low vigour seeds is likely to cause much more severe damage than the sum effects of these factors individually.

According to Vertucci and Leopold (1984), damage to soybean seeds due to imbibing water is greatest when initial SMC's are below 7.5% (fresh weight basis), while

damage is lessened when SMC's are increased to 7.5-19.5% and is absent at SMC's greater than 19.5%. Saha and Basu (1984) have provided evidence that no damage occurred when low vigour soybeans were soaked at an initial SMC of 20% for 2 h at 28°C, while at 9% these seeds lost more than 50% of their germinability. High vigour seeds were resistant to soaking injury. Different responses associated with variations in seed vigour in soybean have also been reported by other workers (*e.g.* Woodstock and Tao, 1981; Woodstock and Taylorson, 1981; Tilden and West, 1985).

In general, intact seed coats can protect seeds against imbibitional injury by slowing rate of water uptake. Without seed coats, seeds may be prone to soaking damage (*e.g.* Rowland and Gusta, 1977; Powell and Matthews, 1978; Ashworth and Obendorf, 1980; Duke and Kakefuda, 1981). Considering temperature effects, Wolk *et al.* (1989) showed that levels of SMC's that mark the onset of imbibitional injury (breakpoint) in *Phaseolus vulgaris* varied with temperature and cultivar. At 20°C, the breakpoints were 13% for *cv.* Tendercrop and 10% for *cv.* Kinghorn Wax, while at 5°C the breakpoints were 16% and 14% for Tendercrop and Kinghorn Wax, respectively. In soybean, Hobbs and Obendorf (1972) reported that 13% SMC was safe for soaking at both 5 and 25°C; at 5% SMC, soaking injury occurred at both temperatures, but was greater at 5°C.

In an attempt to avoid problems of soaking injury, several techniques have been evaluated by many workers on different species. The concept of the techniques is to slow down the rate of water uptake. In addition to moisture equilibration (discussed in Section 2.2.1), other approaches to avoid soaking injury have been evaluated. Woodstock and Tao (1981) showed that when excised soybean axes from accelerated aged seeds were imbibed on blotters containing 30% polyethylene glycol (PEG) the injury during an 8 h imbibition period was greatly reduced (determined by an improved axis length and decreased conductivity of axis leachates) in both high and low vigour lots. Likewise, Tilden and West (1985) assessed the technique of imbibition on different layers of absorbent paper soaked with the same amount of water. They reported that viability of 50 h accelerated aged seeds was increased from 10 to 90% on 1 to 5 layers. Pandey (1988) also reported the effectiveness of this method to improve vigour of French bean after long-term storage.

Damaging effects of soaking on seed performance relate to both physical and physiological changes. Meristematic regions of the radicle and plumule, and possibly

some of the surrounding tissue seem to be the first areas to be damaged as a result of soaking effects (Harrison, 1973; Ashworth and Obendorf, 1980). Initially the damaging effects of soaking are physical rather than chemical (Powell and Matthews, 1978; Vertucci and Leopold, 1983); the former group showed that when seeds were imbibed without seed coats, cell death in pea cotyledons occurred within 2 minutes of imbibition. They proposed that cell death occurred as a consequence of the complete disorganisation of cell structure resulting in physical disruption of membrane. Spaeth (1987) used light and scanning electron microscopy to examine quantities, forms and composition of materials released from bean (*Phaseolus vulgaris* L.) and pea cotyledons without seed coats during imbibition. He found that there were both large and small sizes of extrusion streams being released. Large streams contained starch grains and protein bodies, and small streams contained protein bodies. The size of the streams and the presence of starch grains led him conclude that cellular rupture occurred during imbibition. Similarly, Duke and Kakefuda (1981) found leakage of macromolecules such as mitochondria marker enzymes from bean or soybean embryos when seeds were imbibed without testae, but not from embryos of seeds with testae.

There are a few reports showing that soaking may cause damage to metabolic processes. Powell and Matthews (1978) showed that, after 24 h imbibition, the respiration of imbibitionally damaged pea embryos was less than half of that of intact ones. Woodstock and Taylorson (1981b) reported increased RQ values and levels of acetaldehyde or ethanol in soybean, which also suggested respiratory damage. Most recently, Pretorius and Small (1991) found that soaking reduced the capacity for protein synthesis as well as altered the complement of proteins produced. Working with lima bean, Roos and Pollock (1971) reported a reduction of RNase activity in submerged axes parallelled with decreased axis growth, although the reduction of the enzyme was more dramatic than that of the growth of seedling.

2.4.2 **Organic solvent toxicity**

Recently there has been renewed interest in the use of organic solvents for introducing chemicals into seeds (*e.g.* Shortt and Sinclair, 1980; Woodstock *et al.*, 1983; Petruzzelli and Taranto, 1985). There are two main reasons of using such solvents.

Firstly, some chemicals are insoluble or only sparingly soluble in water. Secondly, the susceptibility of seeds to soaking injury in some species may mask the effects of beneficial seed treatment. Thus, it may be prudent to avoid aqueous solutions for application of chemicals.

There have been many reports showing that organic solvents have been successfully used in a wide range of seed species as carriers for applying fungicides (*e.g.* Shortt and Sinclair, 1980), pesticides (Eckenrode *et al.*, 1974), growth regulators (Persson, 1988; Petruzzelli and Taranto, 1985) and anti-ageing chemicals (*e.g.* Gorecki and Harman, 1987; Dey and Mukherjee, 1988).

Acetone has been recommended as one of the most effective solvents, as it has the properties of being a good carrier (Eckenrode *et al.*, 1974; Persson, 1988) and causing little damage (Milborrow, 1963; Dadlani and Agrawal, 1985; Persson, 1988). Nevertheless, there are occasional, if inconsistent, reports in the literature of toxic effects of acetone on a range of species. The effect of acetone may differ with seed species/cultivars, quality of seeds to be treated, duration of treatment, purity of the acetone and the drying method after treatment. Table 2.3 summarises the reports in the literature of effects of acetone *per se* on various seed crop species when seeds were treated for different durations and dried by different methods. For example, the report of Milborrow (1963) showed that nine out of ten species had no germination losses after 3 months soaking in acetone. Lewis *et al.* (1979) found no damaging effects of acetone on different cultivars of soybeans and peas soaked for 24 h, although in contrast, soaking for 24 h decreased germinability of some cultivars of snapbean. The susceptibility of snapbean to acetone had been also reported by Muchovej and Dhingra (1980), who found that these seeds lost viability if soaked in acetone for longer than 1.5 h.

Table 2.3 Effects of acetone on different crop seeds.

Crop	Duration	Drying	Effects	References
Barley	3 mths	12h, 35°C	54% reduction of germination	Milborrow (1963)
Bean (*Phaseolus vulgaris*)	6 h	overnight, air	reduced germination of 3 out of 4 cultivars	Muchovej and and Dhingra (1980)
Carrot	24 h	24h, 25-26°C	no effect on normal germination in unaged seeds, 38% reduction in aged seeds	Dadlani and Agrawal (1985)
Cotton	72 h	24h, air	no effect on sound seed but killed excised embryos	Halloin (1977)
	72 h	24h, vacuum	70% necrotic seedlings	Halloin (1977)
	24 h	overnight, air	75% reduction of viability	Lewis *et al.* (1979)
Cucumber	4 h	not indicated	no effect on viability	Tao and Khan (1974)
	16 h	24h, air	200% increase in viability after 21 d sowing	Nelson and Sharples (1980)
Lentil	24 h	24h, 25-26°C	no effect on normal germination in unaged seeds, 43% reduction in aged seeds	Dadlani and Agrawal (1985)
Lettuce	44 h	vacuum	no effect on viability	Meyer and Mayer (1971)
	24 h	30 min, vacuum	no effect on viability after 20 months storage	Khan *et al.* (1973)
	1 h	1h, vacuum	no effect on viability	Tao *et al.* (1974)
Lima bean	1 h	air	increased susceptibility to maggot damage	Eckenrode *et al.* (1974)

Table 2.3 (continued)

Crop	Duration	Drying	Effects	References
Linseed	3 mths	12h, 35°C	no effect on germination	Milborrow (1963)
Maize	10 h	3h, 35°C	no effect on germination	Dey and Mukherjee (1988)
Mungbean	3 mths	12h, 35°C	small increase in germination	Milborrow (1963)
Muskmelon	16 h	24h, air	70% reduction of viability after 21 d sowing	Nelson and Sharples (1980)
Mustard	3 mths	12h, 35°C	small increase in germination	Milborrow (1963)
	10 h	3h, 35°C	no effect on germination	Dey and Mukherjee (1988)
Oat	3 mths	12h, 35°C	no effect on germination	Milborrow (1963)
Parseley	4 h	overnight, air	stimulated germination during early stage of ageing (2-3 months), decreased germination of low germinable old seeds (6 years)	Woodstock *et al.* (1983)
Pea	3 mths	12h, 35°C	no effect on germination	Milborrow (1963)
	44 h	vacuum	small reduction in viability	Meyer and Mayer (1971)
	24 h	overnight, air	no effect on viability	Lewis *et al.* (1979)
	16 h	24h, 43°C	no effect on viability in unaged seeds, 20% reduction after 8 weeks ageing at 92% RH and 30°C	Gorecki and Harman (1987)

Table 2.3 (continued)

Crop	Duration	Drying	Effects	References
Pepper	4 h	overnight, air	no effect on normal germination in unaged seeds, 43% reduction after 9 mths stored dry at ambient	Woodstock *et al.* (1983)
Ryegrass	3 mths	12h, 35°C	small increase in germination	Milborrow (1963)
Snapbean	24 h	overnight, air	no effect on viability in 2 out of 4 cultivars tested	Lewis *et al.* (1979)
Soybean	24 h	overnight, air	no effect on viability	Lewis *et al.* (1979)
	24 h	air	no effect on viability, increased damage to exposed cotyledons, reduced fungal infection to seedcoat	Shortt and Sinclair (1980)
	4 h	air	no effect on normal germination in unaged seeds 13% reduction after 90d ageing at 8% SMC, 40°C	Parrish and Bahler (1983)
Sugar beet	3 mths	12h, 35°C	22% improvement of germination	Milborrow (1963)
Tomato	3 mths	12h, 35°C	94% improvement of germination	Milborrow (1963)
Wheat	3 mths	12h, 35°C	80% improvement of germination	Milborrow (1963)
	24 h	24h, 25-26°C	germination reduced 11% for unaged seeds and 30% for aged seeds, reduced seedling growth on aged seeds	Dadlani and Agrawal (1985)
Wheat	30 min	vacuum	no effect on viability in unaged seeds, 45% reduction after 35d ageing at 14.5% SMC, 30°C	Petruzzelli and Taranto (1985)

It is possible that the penetration of the solvent into seeds may be an important variable as Lewis *et al.* (1979) found that no further solvent uptake occurred after the first hour of a 24 h soak in pea and snapbean, but soybean still continued taking up the solvent during this period. On the other hand, Dadlani and Agrawal (1985), using methyl red dye, argued that no penetration of acetone occurred beyond the seed coat of soybean even after 24 h soaking: it is not clear whether these differences represent variation between seed lots/cultivars or methodological problems. Determination of weight increase in Lewis *et al.*'s (1979) study can be misleading if solvent accumulates between the seed coat and the cotyledons. It has been shown in cotton (Halloin, 1977) and soybean (Shortt and Sinclair, 1980) that if acetone penetrates beyond the barrier of the seed coat into living tissues, damage may occur. On the other hand, penetration of a dye into seeds may not directly reflect penetration of the carrier solvent.

There are a few reports showing that acetone may cause more severe damage in combination with other reagents. Lewis *et al.* (1979) presented evidence that while soybean seeds were not sensitive to 1 h immersion in acetone, they were to an acetone solution of 50% PEG (Polyethylene glycol), 10% TEA (Triethanolamine) or 20% water. After treatment, seeds are normally dried to remove the solvent from the seeds. Again, drying may affect seed performance. Halloin (1977) found that cotton seeds treated with acetone and then vacuum-dried produced more abnormal seedlings when they were germinated in a covered container. They suggested that this was due to residual acetone because similar types of abnormal seedlings were found when non-treated seeds were germinated in a container in the presence of acetone.

As acetone appears to cause no problems in a wide range of seed species, information on its mechanism of action in seeds is lacking. Halloin (1977) found increased leachate from acetone treated tissue of cotton embryos, and proposed, without data support, that acetone might cause disruption of lipid systems. Acetone may decrease the activity of invertase (Eldan and Mayer, 1976) or depress oxygen uptake (Meyer and Mayer, 1971) in lettuce seeds, but in each case germinability was unaffected.

CHAPTER 3

MATERIALS AND METHODS

This chapter deals with general methodology. Details of experimental design and any special approaches will be indicated alongside the appropriate data in the subsequent chapters.

3.1 SEED MATERIALS

Nine seed lots of *cv.* Amsoy and one large lot of *cv.* Davis were used during the course of this study. These seed lots were harvested in different years and obtained from different sources (Table 3.1). Seed lot A-7 was further sorted visually into damaged and undamaged seeds.

Table 3.1 Soybean seed lots used in experiments.

Cultivar	Source[1]	Year of harvest	Initial germination	Initial SMC	Code
Amsoy	Wrightson	1987	86.5%	10.8%	A-0
	Wrightson	1987	81%	11.6%	A-1
	Wrightson	1987	86%	10.9%[3]	A-2
	Corson	1988	83%	11.8%	A-3
	STC	1988	82%	10.1%	A-4
	Corson	1988	12%	10.6%	A-5
	STC	1986[2]	83%	8.2%	A-6
	Corson	1988	79%	12.0%	A-7
	Corson	1988	99%	12.1%	A-8
Davis	Wright-Stephenson	1988	81%	9.1%	Davis

1 Addresses: *Wrightson Ltd (now Challenge Seeds Ltd), Palmerston North, New Zealand*
Corson Grain Ltd, PO Box 1046, Gisborne, New Zealand
STC = Seed Technology Centre, Massey University, Palmerston North, New Zealand
Wright-Stephenson and Co (Australia) Pty Ltd, PO Box 357, Seven Hills, NSW, Australia

2 Hand threshed

3 SMC later increased in this lot due to moisture accumulation in 5°C store.

3.2 SEED TREATMENTS

3.2.1 **Antioxidant treatments**

Antioxidant treatments were conducted using different carriers:

3.2.1.1 *β-mercaptoethanol in water*

Four concentrations: 0, 1, 10, or 100 *ppm* (*v/v*) of β-mercaptoethanol in water were added to seed samples of 10.8% moisture content in moisture proof packages to raise the moisture content of seed (SMC) up to 20% followed by ageing treatments. The active concentrations of β-mercaptoethanol per total seed moisture were thus 0, 0.52, 5.2 or 52 *ppm*.

3.2.1.2 *α-tocopherol or BHT in organic solvents*

In order to avoid the effects of soaking injury when water was used as a carrier (*e.g.* Woodstock and Taylorson, 1981b) and because α-tocopherol and BHT are sparsely soluble in water, solutions of 1% α-tocopherol or either 0.1 M (2.2%) or 0.1% BHT were prepared in acetone or hexane.

Analar grade acetone and Hipersolv hexane (BDH chemical NZ Ltd) were used as supplied throughout. Previous work by the group of Coolbear *et al.* (1991) had shown that redistilling the acetone provided no advantage. Soaking was carried out by completely immersing each replicate of seeds in acetone or hexane for up to 16 h at a constant 20°C. Following soaking, samples were spread out on one layer of absorbent paper and left to dry at 20°C for 24 h unless otherwise stated. Controls were acetone or hexane soaking without antioxidants. Subsequently, untreated seeds will be referred to seeds without chemical or hydration treatment, though they may still have been subjected to ageing.

3.2.1.3 *Iodine with calcium carbonate*

Iodine treatments were carried out by mixing crystallised iodine with calcium carbonate after the method described by Dey and Mukherjee (1984). Each replicate sample of seeds was placed into a conical flask containing powdered calcium carbonate with 0, 0.1, 1 % iodine in the proportions of 4:1 (*w/w*) seeds : $CaCO_3$ + iodine. These flasks were tightly covered with parafilm and kept at 25°C in the dark for up to 24 h with frequent shaking. After treatment, excess calcium carbonate was removed using a fine sieve. Seeds were then wiped with a muslin cloth before drying at 35°C or at ambient in fume hood for 24 or 48 h depending on experiments.

3.2.2 Hydration treatments

Three methods of hydration treatment were carried out in this study.

3.2.2.1 *Moisture equilibration* (ME)

Seed samples were held in closed jars above water at 25°C for up to 72 h. Since seeds were bagged up rather than in a monolayer, variations in moisture content within the seeds may be a major source of variation. There were also variations in SMC's obtained during moisture equilibration in different experiments. Possible variables include seed lots, initial seed moisture contents and/or the quantity of seeds within the container. Relationships between initial and final SMC and quantities of seed for the experiments with *cv.* Davis are shown in Table 3.2. The SMC's before ME differed between experiments due to drying methods after ageing as indicated alongside the table.

Table 3.2 Measured seed moisture contents (SMC) after 72 h moisture equilibration of different quantities of seeds within a container of *cv.* Davis. Data presented are means of 3 replicates (±S.E.).

Experiment	Seed quantity	Seed sample	SMC* before ME	SMC after ME
A	1 x 120 g	SA 0 wk	9.2±0.07	18.3±0.17
		SA 8 wk	8.7±0.03	18.8±0.27
		SA 15 wk	8.9±0.00	18.6±0.22
B	4 x 80 g	AA 0 d	13.6±0.03	17.4±0.15
		AA 1 d	14.8±0.06	17.3±0.17
		AA 2 d	14.3±0.07	17.9±0.25
		AA 3 d	14.3±0.03	17.4±0.14
C	4 x 90 g	AA 0 d	10.2±0.53	14.9±0.53
		AA 2 d	10.3±0.33	15.5±0.10
		AA 4 d	10.2±0.07	14.9±0.59
		AA 6 d	10.3±0.03	15.2±0.13

Note: *SA* = *Slow ageing at 30°C, 9% SMC*
AA = *Accelerated ageing at 40°C, ~100% RH*
* = *without drying after slow ageing (Expt. A)*
= *drying at 20°C for 2 d after AA (Expt. B)*
= *drying at 20°C for 2 d and then at ambient for 2 d after AA (Expt. C)*

3.2.2.2 *Imbibition*

Absorbent papers wetted with tap water as used for normal germination testing (ISTA, 1985) were used for seed imbibition. Seeds were placed onto two layers and covered with another layer of paper before being rolled as a tube and kept in a plastic bag at 25°C until the SMC reached required levels. Imbibed seeds were blotted dry before determining SMC or drying back in a laminar flow cabinet at ambient temperature for 4 days.

3.2.2.3 *Soaking*

Soaking treatments were conducted by immersing each replicate of seeds either directly into deionized water for 30 min (Expts. in Section 5.2.1.2 and in Chapter 6), or for 2 h immediately after 24 h moisture equilibration (Section 5.2.1.2). After seeds were hydrated, they were either germinated immediately or dried-back under various conditions. Appropriate details are noted with each experiment.

3.2.3 Surface sterilization treatment

Seeds of each replicate were placed in a nylon bag and completely submerged in 1% sodium hypochlorite (NaOCl) for 15 min, and then transferred to 0.01 N HCl for 10 min in order to completely remove residual NaOCl from seed surface, as suggested by Abdul-Baki (1974). These seeds were then washed 3 times in distilled water for 5 min followed by drying back in a laminar flow cabinet at ambient temperature for 4 days (until original weights were obtained).

3.3 GENERAL METHODS FOR SEED QUALITY ASSESSMENT

3.3.1 Seed moisture contents

Moisture contents were determined by the oven method according to the International Seed Testing Association (ISTA) Rules (1985). Seeds were ground and then dried at 103°C for 17 h. The SMC's were expressed as % of wet weight basis. In case of wet

seeds (>10% SMC), which were difficult to grind properly, a two-stage drying was used. Firstly, seeds were predried at 103°C for 5-10 min, and then dried in a warm condition (for at least 2 h) until the SMC's were low enough to be ground (ISTA, 1985). Secondly, seeds were processed as to a general method described above.

Where seed moisture needed to be adjusted downwards, drying was achieved by storing the seeds over silica gel in a desiccator at room temperature until the required calculated weight was reached. Alternatively, moisture contents were raised by adding the calculated amount of distilled water to a weighed sample of known initial moisture content and then equilibrating overnight at 10°C in heat-sealed polyester-aluminium foil-polyethylene laminated packages. These packages are moisture proof (*i.e.* water vapour transmission rate is zero). After these adjustments the actual moisture contents were checked for each replicate.

3.3.2 Ageing treatments

Accelerated ageing treatments were carried out by holding seed samples in 100%, 90% or 36% RH for up to 6, 10 or 30 days at 40°C. A nominal 100% RH was achieved by equilibrating seeds over water after the method described by Baskin (1987). However, Tomes *et al.* (1988) have shown that the actual RH under such conditions during 24 to 72 h is in the range of 90-95%. Thus, the RH obtained from these conditions will be referred as an approximate (~) 100% RH. Proportions of 32:68 or 84:16 (*v/v*) glycerine and water were used as per Hill (1987) to modify RH to 90 or 36% respectively. SMC's were determined before and after subjecting seeds to these conditions. After ageing (both AA and CD) seeds were germinated directly without drying back. Except that AA or SA seeds, which were used for post-storage ME treatments were dried back before treatment. After various treatments the SMC's of dried-back seeds could vary by several percentage points from that of the controls. Details will be given with each experiment as appropriate.

Controlled deterioration treatments were a modification of the technique of Matthews and Powell (1987b). To obtain the required moisture contents, the calculated amount of water was added and the seeds allowed to equilibrate overnight at 10°C for 24 h as described above. The conditions used were seed moisture contents of 20, 12, or 9% and a temperature of 40 or 35°C. The durations of ageing treatment varied from 1 day to 15 weeks depending on the experiment.

3.3.3 Normal seed germination and seedling growth testing

Germination trials were normally conducted on 50 seeds per replicate kept at 25°C in the dark. The between paper method (ISTA, 1985) was used, recording percentage normal seedlings 8 days after sowing. These normal seedlings are seedlings which show the potential for continued development into satisfactory plants when grown under favourable conditions (ISTA, 1985). Seeds were dusted with thiram prior setting up germination trials. Percentage seed viability (normal and abnormal seedlings plus fresh ungerminated seeds) was also recorded, as were the fresh and dry weights of embryonic axes (cotyledons removed) of normal seedlings 8 days from sowing (Association of Official Seed Analysts or AOSA, 1983). Fresh ungerminated seeds are ungerminated seeds which are still firm and therefore regarded as still viable. Dry weights were measured after drying for 4 days at 65°C.

3.3.4 Germination rate trials

In addition to the standard germination test, germination rate trials were initially carried out to compare the effect of treatments. Seeds were germinated on top of two layers of Whatman No.1 filter paper wetted with 25 ml distilled water in 12.5 cm petri-dishes. Seeds showing radicle protrusion were counted and removed at least three times daily during peak periods of germination activity until no further germination occurred. The median germination time (T_{50}) was calculated according to Coolbear *et al.* (1984). As the results seem to be unreliable (discussed in Chapter 4), this technique was not used in further experiments.

3.3.5 Conductivity testing

Conductivity measurements were carried out on 50 seeds per replicate as described by Matthews and Powell (1987a). Seeds were soaked in a 250-ml conical flask with 100 ml deionized water at 20°C. Conductivity of seed leachates was measured after seeds were soaked for up to 24 h using a direct reading conductivity meter. The results were expressed as $\mu S.cm^{-1}.seed^{-1}$ rather than per gram of seeds as in the original method so that results can be compared when seeds of different SMC's were used.

3.3.6 Staining procedures

3.3.6.1 *Tetrazolium test*

Tetrazolium (TZ) testing was carried out as per AOSA (1970). Seeds were placed between moist paper towels overnight for imbibition before soaking in a 1% aqueous solution of 2,3,5-triphenyl tetrazolium chloride and kept in 35°C for 3 h. After staining, the seeds were washed in running water. If not evaluated immediately, seeds were held in water and stored at 5°C until evaluations were made. The seeds were individually examined for the staining pattern of embryos and cotyledons. The position and size of unstained areas were examined.

3.3.6.2 *Ferric chloride ($FeCl_3$) test*

A ferric chloride test was used to visualise mechanical damage on seeds. A 20% (*w/w*) solution of ferric chloride was prepared in distilled water (Lankford, personal communication). Seed were submerged in the solution for up to 15 min. Separation of damaged and undamaged seeds was done within 5-15 min after soaking. Seeds with black stain were considered as mechanically damaged (Plate 3.1). Seeds of each group were dried back to required SMC's above silica gel in a desiccator at 20°C. However, preliminary trials suggest that seeds separated by ferric chloride cannot be used for further study because the ferric chloride itself had toxic effects on soybean as revealed by the TZ test (Plate 3.2) and confirmed by germination results (data not shown). As the two staining procedures were obviously incompatible, damaged seeds were then separated visually. The toxic effects of ferric chloride do not appear to have been reported in literature before.

3.3.6.3 *Methyl red penetration*

A saturated solution of methyl red in acetone was used in an attempt to examine acetone penetration of the seed. Seeds were submerged in the solution for 16 h at 20°C. Solutions were decanted and seeds were then dried for 24 h to evaporate the acetone. Staining patterns were evaluated individually on cut through or halved seeds.

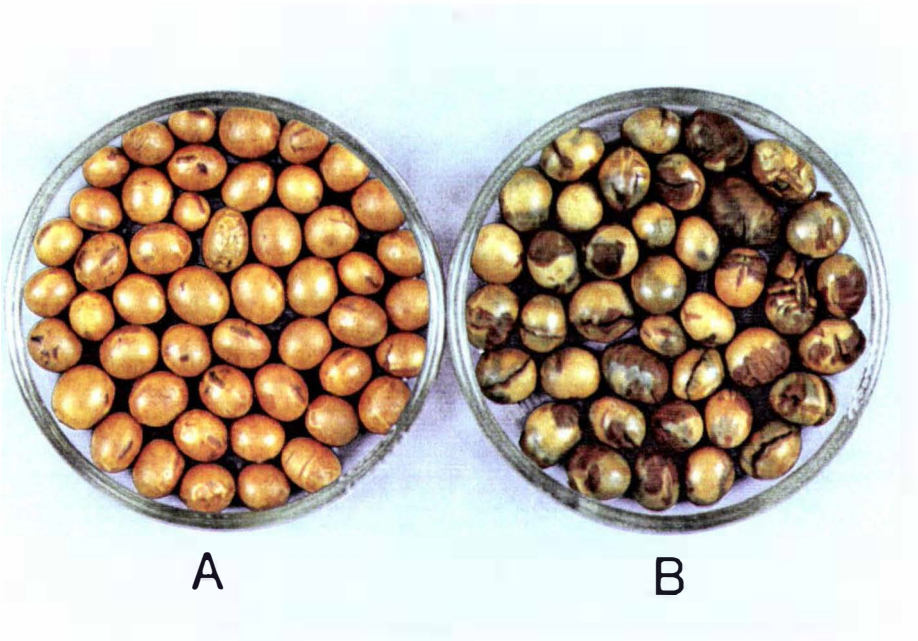


Plate 3.1 Responses of undamaged (A) or mechanically damaged seeds (B) of soybean lot A-7, *cv.* Amsoy to 20% ferric chloride soaked for 5-15 min. The black staining on the testa indicates sites of mechanical damage penetrated by the salt which is subsequently oxidised.

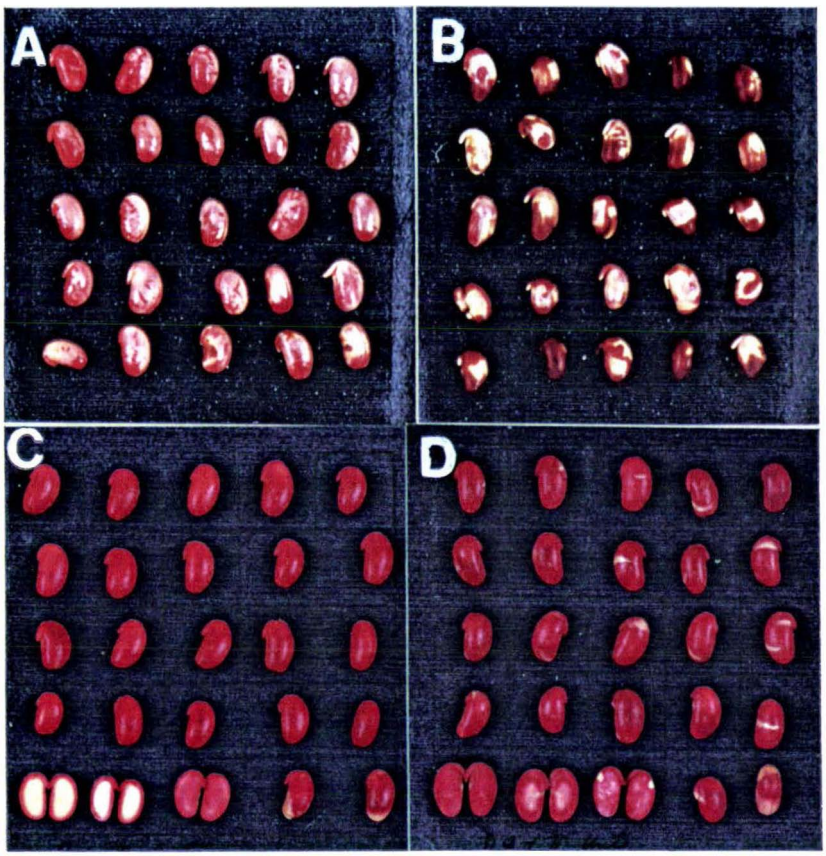


Plate 3.2 Responses to tetrazolium chloride of undamaged (A, C) and mechanically damaged seeds (B, D) sorted by 20% ferric chloride staining (A, B) or visual separation (C, D) of soybean seed lot A-7, *cv* Amsoy. $FeCl_3$ clearly affects the tetrazolium staining response and makes evaluation difficult and inaccurate.

3.4 SOIL EMERGENCE TRIALS

A soil emergence trial was conducted to compare the effects of ageing and moisture equilibration treatments on emergence and growth of seedlings. Smith's potting mix was placed in Plix bedding root trainers and watered until the potting mix was well moistened and compact. A single seed was then placed in each trainer and covered with potting mix to a depth of 2 cm. Watering was supplied daily. Three replicates of 50 seeds were sown per treatment. Emergence (appearance of a visible hypocotyl hook) and establishment of photosynthetic seedlings (cotyledons emerged above the soil surface) was scored daily until no more emergence occurred. The experiment was conducted in a ventilated glasshouse where the temperature was regulated to approximately 20°C. After 17 days from 50% establishment (T_{50}) in each separate plot, all seedlings were removed and percentages of normal and abnormal shoots were recorded. Fresh and dry weights of shoots and roots of normal seedlings were then determined as described in Section 3.3.3.

3.5 LIPID EXTRACTION FROM SEED TISSUES AND MEMBRANES

3.5.1 **Lipid extraction from seed tissues**

The total lipid from seed tissues was extracted from excised axes and cotyledons using a modification of the method described by Francis and Coolbear (1988). Twenty axes or an equivalent weight of cotyledons were boiled for 2 min in 1 ml water saturated butanol (WSB) containing 100 $\mu g.ml^{-1}$ of BHT and then hand ground in a mortar and pestle to a slurry and washed into a centrifuge tube with a further 1 ml WSB. The suspension was centrifuged for 10 min at 1300 g and the supernatant decanted. The precipitate was then re-extracted with an additional 1 ml of WSB and recentrifuged. Supernatants were pooled and dried under nitrogen. The dry weight of total lipid extracted was measured.

3.5.2 **Membrane lipid extraction**

The microsomal membrane fractions from axes and cotyledons were prepared as described by Senaratna *et al.* (1984). For each treatment, 20 axes or an equivalent weight of cotyledons were homogenized in 2.0-2.5 ml of 0.3 M sucrose, 50 mM $NaHCO_3$, pH 7.0 using a glass homogenizer. The homogenate was filtered through 2

layers of silk cloth and centrifuged at 10,000 g for 20 min. The resulting supernatant was made 15 mM with CsCl before layering on 750 µl of 1.3 M sucrose and centrifuged at 165,000 g for 2 h. Smooth microsomal membranes were collected at the interface by Pasteur pipette. The membranes were diluted with 3 volumes of 50 mM $NaHCO_3$, and pelleted by centrifugation at 165,000 g for 1.5 h.

Total membrane lipid was extracted from isolated microsomal membrane fractions. Pelleted membrane fractions were dissolved in 2 ml of WSB and then boiled for 2 min. The suspension was centrifuged, re-extracted and dried as per the procedures for seed tissues described in a previous Section (3.5.1).

3.6 LIPID ANALYSIS

3.6.1 **Phospholipid extraction**

Phospholipid in the total lipid extract was quantified by measuring the amount of phosphorus (P) according to the method of Bartlett (1959). A 200 µl aliquot of supernatant (lipid in butanol) extracted from seed tissue before drying or the total amount of dried microsomal membrane lipid was used. To each sample was added 0.5 ml of 10 N H_2SO_4 before heating on a 150-160°C digestion block for at least 3 h. Two drops of 30% H_2O_2 were then added, and the digestion was continued for at least 1.5 h in order to ensure completion of reaction and decomposition of all the peroxide. After adding 4.4 ml distilled water, 0.2 ml of 5% ammonium molybdate and 0.2 ml of Fiske-SubbaRow reagent (total volume of the solution was 5 ml), the solutions were thoroughly mixed and heated for 7 min in boiling water. The optical density at 830 mµ was recorded and compared to a standard curve which was prepared using inorganic orthophosphate. Solutions containing 0-5 µg phosphorus were prepared in 4.1 ml with water to which was added 0.5 ml 10 N H_2SO_4, 0.2 ml of 5% ammonium molybdate and 0.2 ml of Fiske-SubbaRow reagent before processing in the same way as the sample solutions.

In an attempt to assess the reproducibility of the method used for phospholipid extraction, three subsamples of 200 µl aliquots were taken from the same sample of seed lipid (taken from water soaked seed samples aged at different durations) and digested as described previously. Results showed that variation occurring between these internal replicates was very low (maximum range was only 8.7 or 4.8% of the

means of PL from axis or cotyledon, respectively) which indicated that there was no need to use internal replications. Thus, except for this set of experiments the results presented in Chapter 6 are always externally replicated.

For membrane phospholipid analysis, an additional standard curve made from H_2SO_4-digested inorganic orthophosphate was also included so that if there are any losses of phosphorus occurring during digestion, the amounts of P detected were still comparable. The results were calculated from this 'digested standard curve' and expressed as a proportion of phosphorus present in total membrane lipid (P:L ratios).

3.6.2 Fatty acid analysis

In preliminary trials, fatty acid methylation for GLC analysis was carried out using boron fluoride (BF_3) in methanol (described by Francis and Coolbear, 1988). 500 µl of 10% H_2SO_4 in methanol, and 50 µl of 100 $\mu g.ml^{-1}$ heptadecanoic acid (as an internal standard) were added to lipid dried from a 200 µl aliquot) and incubated for 3 h at 70°C before adding 1 ml of 14% BF_3 in methanol and incubating at 80°C for 1 h. The samples were washed into separation funnels with hexane (15 ml) and 2 ml of distilled water were added. After thorough mixing, the funnels were left to stand for 30 min. The lower layer was discarded, the upper part was filtered through Watson No.1 filter paper and dried under nitrogen. These methylated samples were redissolved in 100 µl hexane before injection into the GLC (Perkin-Elmer 8500). A J&W DB-wax megabore 30 m column was used with a 6 $ml.min^{-1}$ flow rate of helium and an oven temperature of 160-230°C, while both detector and injector ports were maintained at 300°C. Detection was by a flame ionisation detector. Peak areas of the samples were estimated from the GLC signal by integration controlled by a data acquisition and control system (Software: Delta Chromatography System, Digital Solutions Pty Ltd). Identification of fatty acid methyl esters was based on the presence of heptadecanoic acid (C17:0) and by comparison of peak retention times to authentic fatty acids methyl ester standard. Results were expressed as relative proportions of fatty acid from lipid extracts. For this trial, an oxygen trap was not connected into the gas line. After an extensive series of trials, there was a gradual loss of resolution particularly between 18:0 and 18:1 fatty acids, which indicated column deterioration. Details of problems with fatty acid analysis and their possible causes are discussed in Appendix 3.1.

Accordingly, in the studies described in Chapter 6, a modified analysis procedure was used from the method of Ferguson *et al.* (1990b). Briefly, dried lipid (from a dried 1-3

ml aliquot) was dissolved in 3 ml hexane, and fatty acids were esterified by the addition of 3 ml 1% H_2SO_4 in methanol including 3 ml of 100 $\mu g.ml^{-1}$ heptadecanoic acid. For membrane lipid, 1 ml of each reagent was used for the whole sample of dried lipid. The hexane layer was evaporated at 60°C for 3 h and methylation completed at 85°C for a further 1 h. The fatty acid methyl esters were partitioned and analysed by GLC as described above, except that an oven temperature of 160-190°C was used, and the carrier gas (helium) first flowed through an oxygen trap so that oxygen contaminants were removed.

For membrane analysis, variations occurred both between the two replicated extractions and between injections of subsamples from the same extraction (Appendix 3.2). In general internal replications were used when there were anomalies between external replicates or there were problems of interpretation of individual peak areas on the trace obtained. For the latter case, injections were repeated with varied amount of sample. Where the second injection did not agree with the first, a third replicate injection of the same sample was assayed. Data for membrane fatty acid proportions presented in Chapter 6 were analysed from means of all injections from two replicated extractions.

3.7 ELECTROPHORESIS OF MEMBRANE PROTEINS

Gels (7.5% resolving and 2.5% stacking) were prepared between glass plates (as described by Hames and Rickwood, 1981) using the "PROTEAN II SLAB CELL" vertical slab electrophoresis system. The protein precipitate remaining after lipid extraction of membrane fractions was solubilised in 1 ml of extracting solution (2% SDS, 5% 2-mercaptoethanol, 10% glycerol in 62.5 mM Tris-HCl) and stored at -20°C until required. After thawing, urea was added to a concentration of 6 M. Five µl of bromophenol (tracking dye) were added to 195 µl of sample, which was then denatured by heating at 103°C for 5 min. A 30-60 µl of denatured sample solution was loaded per sample well. Gels were run at a temperature of 5°C and initially at a constant current of 25 mA until the dye front entered the resolving gel, when the current was increased to 35 mA. The power was disconnected when the dye front was about 1 cm from the end of the resolving gel. Gels were then stained with Coomassie Blue overnight, and destained with 600 ml distilled water : 300 ml methanol : 100 ml acetic acid until the gel background was clear and then over stained with silver (Merril *et al.*, 1981).

3.8 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Axes and cotyledons of untreated, 16 h acetone soaked and 30 min water soaked seeds of unaged and 6 day-accelerated aged samples were prepared. Two seeds from each treatment were imbibed between moistened absorbent paper for 24 h. The seed coats were removed and the axes excised. Radicles or cotyledons were transversely sliced into several pieces of approximately 0.5-1 mm thick. These sliced tissues (excluding a 1 mm section from a root tip (Figure 3.1)) were characterised under the light microscope. Sections cut through epidermal, procortical and provascular tissues were chosen for TEM study (examples of tissue types are shown in Plate 3.3). These tissues were then processed as described in Appendix 3.3 by Mr. D. Hopcroft of Fruits and Trees Division, DSIR, Pamerston North. Electron micrographs were taken on a Philips 201C TEM at Fruit and Trees Division, DSIR, Palmerston North.

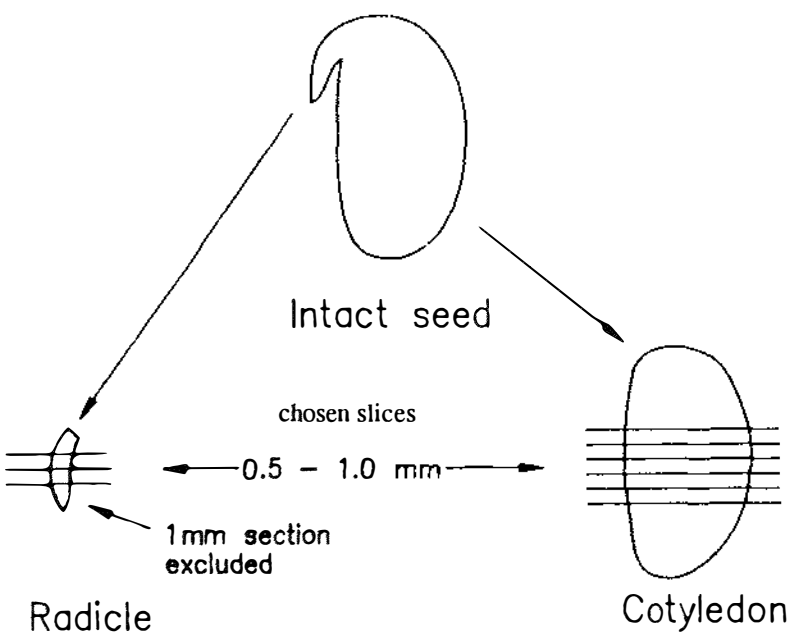


Figure 3.1 Side view diagram of radicle and cotyledon of soybean *cv.* Davis. Tissue slices were taken for TEM study as indicated in the diagram.

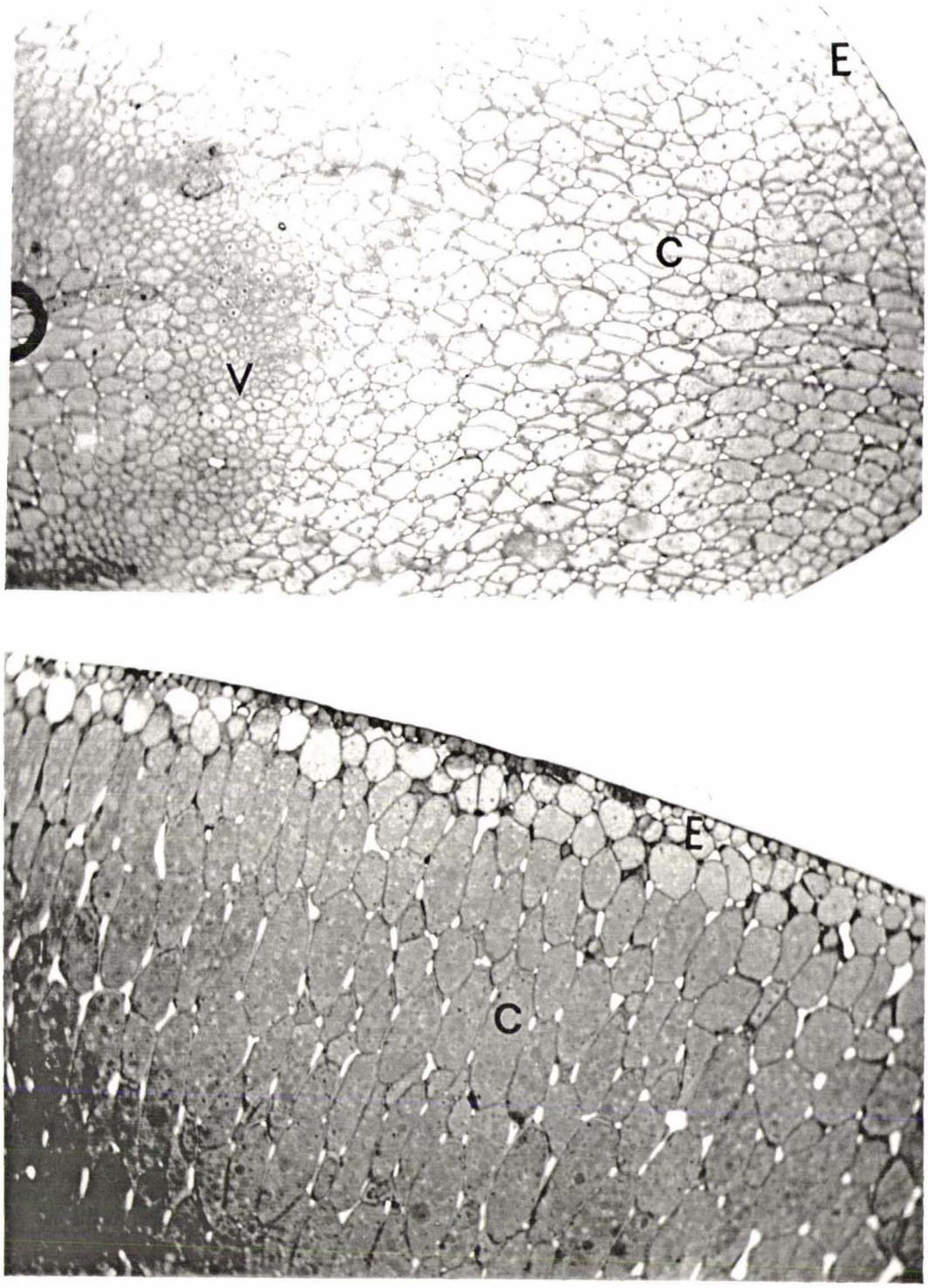


Plate 3.3 Light micrographs of transverse sections of the radicle (A) or cotyledon (B) showing the relationship between epidermal cells (E), adjacent procortical (C) and provascular (V) cells in soybean seeds *cv.* Davis after 24 h imbibition. Magnification 640X.

3.9 DATA ANALYSIS

Data were normally collected from four replications of all treatments for the different lots of *cvs.* Amsoy and Davis, except that (due to limited stocks of seed) for *cv.* Davis three replicates were gathered from experiments on post-storage ME treatments, slow ageing and lipid analysis, and two replicates on membrane lipid analysis. All data were replicated externally. For phospholipid from seed tissues (Section 3.6.1) and membrane fatty acid analyses (Section 3.6.2, Appendix 3.3) internal replicates were also additionally done as to evaluate reproducibility of the techniques.

Either split plot or randomized complete block (RCB) designs were used depending on the experiment. In the split plot designs, seed treatments were regarded as main plots and ageing durations as subplots unless otherwise stated. The least significant difference (LSD) values for comparisons across main plots were calculated according to Mead and Curnow (1983), using the appropriate t-value with the main plot degrees of freedom. Germination and viability data were transformed to arcsin $\sqrt{}$percentage for analysis, unless otherwise stated.

CHAPTER 4

ANTIOXIDANT AND SOLVENT TREATMENTS

4.1 INTRODUCTION

Several reports in the literature suggest that antioxidants have the potential to improve storability of seeds, and that acetone is a safe solvent to use for such seed treatments on various species including soybean (reviewed in Sections 2.3.2 and 2.4.2). The effects of antioxidants and acetone were thus characterised on the performance of several seed lots of soybean *cvs.* Amsoy and Davis under both accelerated and slow ageing conditions. The results presented are grouped into three main sections. Firstly, the effects of antioxidant treatments administered via organic solvents are presented, followed by the effects of acetone treatment on seeds *per se*. The experiments carried out for these two sections are summarised in Table 4.1. The last section describes the results of investigations of treatments using applications of either aqueous solutions of β-mercaptoethanol or iodine in inert powder. Variations in responses of seeds to these treatments are then discussed.

4.2 RESULTS

4.2.1 Effects of α-tocopherol and BHT treatments in acetone and/or hexane

The results reported here are collated from 6 separate experiments on two cultivars, where the effect of the treatment has been variously assessed on seeds subjected to controlled deterioration, accelerated ageing or slow ageing techniques (Table 4.1, experiments I-VI). The data presented are accordingly grouped by methods of ageing applied to the seed after treatment.

Table 4.1 Summary of experiments of acetone series.

Experiment	Seed lot[a]	Chemical treatment[b]	Ageing conditions	Maximum ageing durations
I	A-1	UC, Ac, Ac + 1% α-toc	40°C, 20% SMC	5 d
II	A-1	UC, Ac, Ac + 0.1 M BHT	40°C, ~100% RH 40°C, 20% SMC	5 d
III	Davis	UC, Ac, Ac + 1% α-toc	40°C, ~100% RH	6 d
IV	Davis	UC, Ac, Ac + 0.1 M BHT	40°C, ~100% RH	6 d
V	Davis	UC, Ac, Ac + 1% α-toc, Ac + 0.1% BHT	35°C, 12% SMC	12 wk
VI	Davis	UC, Hex, Hex + 1% α-toc	40°C, ~100% RH	6 d
VII	A-2	UC, Ac	40°C, 20% SMC	3 d
VIII	A-2	UC, Ac	40°C, ~100% RH	3 d
IX	A-2	UC, Ac	40°C, 20% SMC	3 d
X	A-2,3,4,5	UC, Ac	40°C, 20% SMC	3 d
XI	A-3	UC, Ac	40°C, 20% SMC	3 d
XII	A-8	UC, Ac	40°C, 20% SMC	3 d
XIII	A-7	UC, Ac	40°C, 20% SMC	3 d

[a] *For a full description of cv. Amsoy seed lots, A-1 through A-8, refer to Table 3.1.*

[b] *UC = untreated controls*
Ac = acetone treatment
α-toc = α-tocopherol
BHT = butylated hydroxytoluene
Hex = hexane

4.2.1.1 *Treatment prior to controlled deterioration (CD)*

Analysis of normal germination and viability data for untreated, acetone and α-tocopherol in acetone treated seeds of lot A-1 (Expt. I) showed no interactions between treatments and durations of ageing. Accordingly, data are plotted in Figure 4.1 showing the overall mean effects of ageing or treatment. A significant decline ($P<0.001$) in germination and viability was observed during ageing. Seeds began to lose germinability after 3 d, and after 5 d the proportion of normal germinants was less than 20%. There was no significant effect of treatments on germinability, but acetone caused a small, but significant ($P<0.05$), decrease in overall viability.

BHT treatment (Expt. II) resulted in many abnormal seedlings with retarded roots from unaged seeds (Plate 4.1). As a result, normal germination of unaged seeds was significantly ($P<0.01$) decreased by BHT, but in contrast to this, viability was slightly improved compared to seeds treated with acetone alone ($P<0.05$) (Figure 4.2). Subsequent damage during CD was a function of acetone rather than BHT, but there was no evidence of any significant protective effects of the antioxidant.

Data on times to 50% radicle emergence (Table 4.2) indicated that a significant ($P<0.05$) inhibitory effect of α-tocopherol is evident after 3 days CD, while BHT marginally increased the T_{50} of only unaged seeds. It was also noted, however, that the effects of acetone treatment differed slightly between experiments, as no significant toxic effect of acetone occurred in experiment I, but in experiment II it was evident after 1 d CD. In both experiments, the general pattern of changes in seedling growth (Appendix 4.1) followed that of the normal germination percentage data.

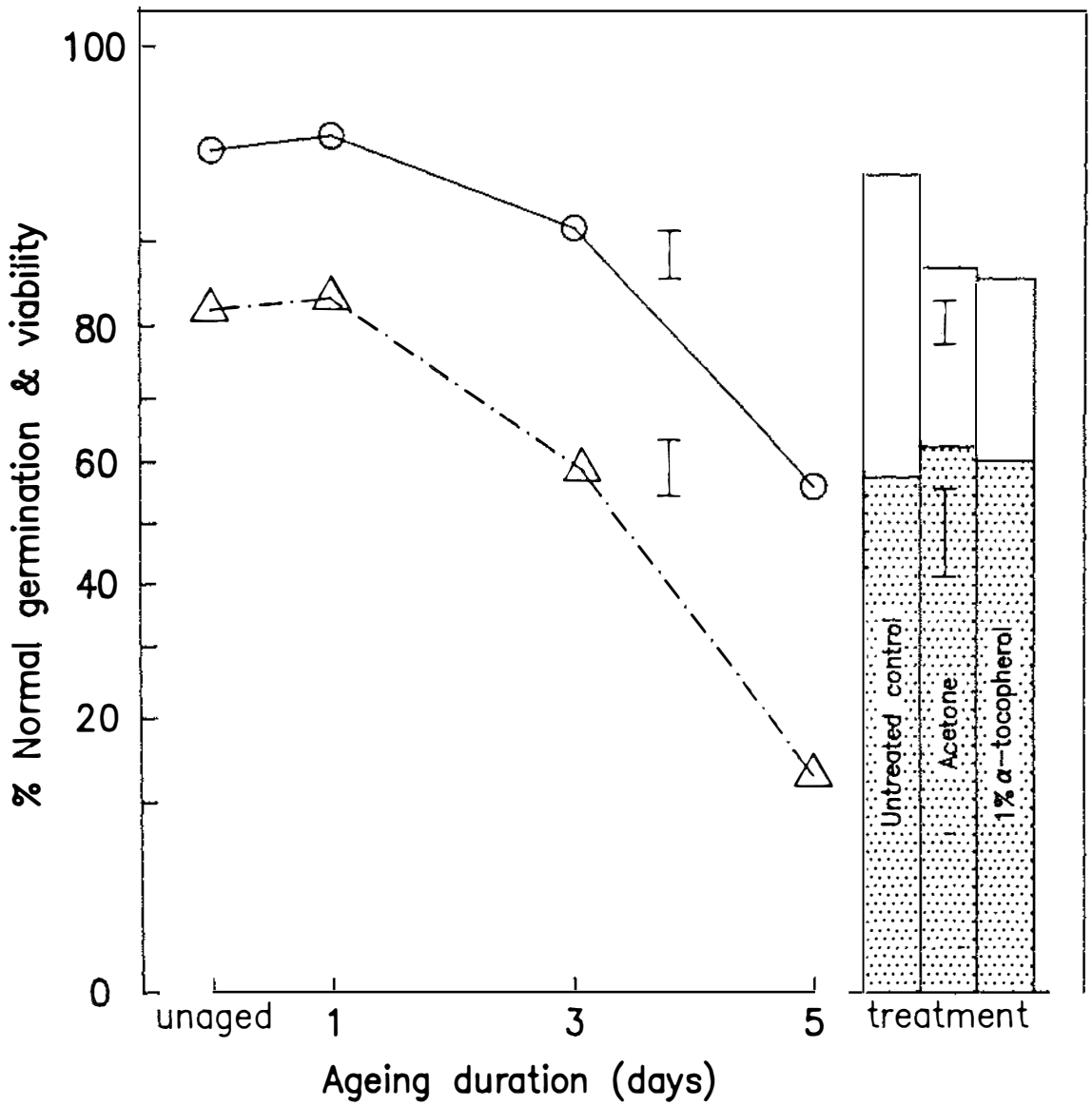


Figure 4.1 The effects of subsequent controlled deterioration (40°C, 20% SMC) (averaged over treatment) or chemical treatments (averaged over duration of CD) on viability (○ , open histogram) and normal germination (△ , closed histogram) of soybean seed lot A-1, *cv.* Amsoy. Data presented are arcsin √% transformed means of 4 replicates. Bars represent least significant differences (P=0.05).

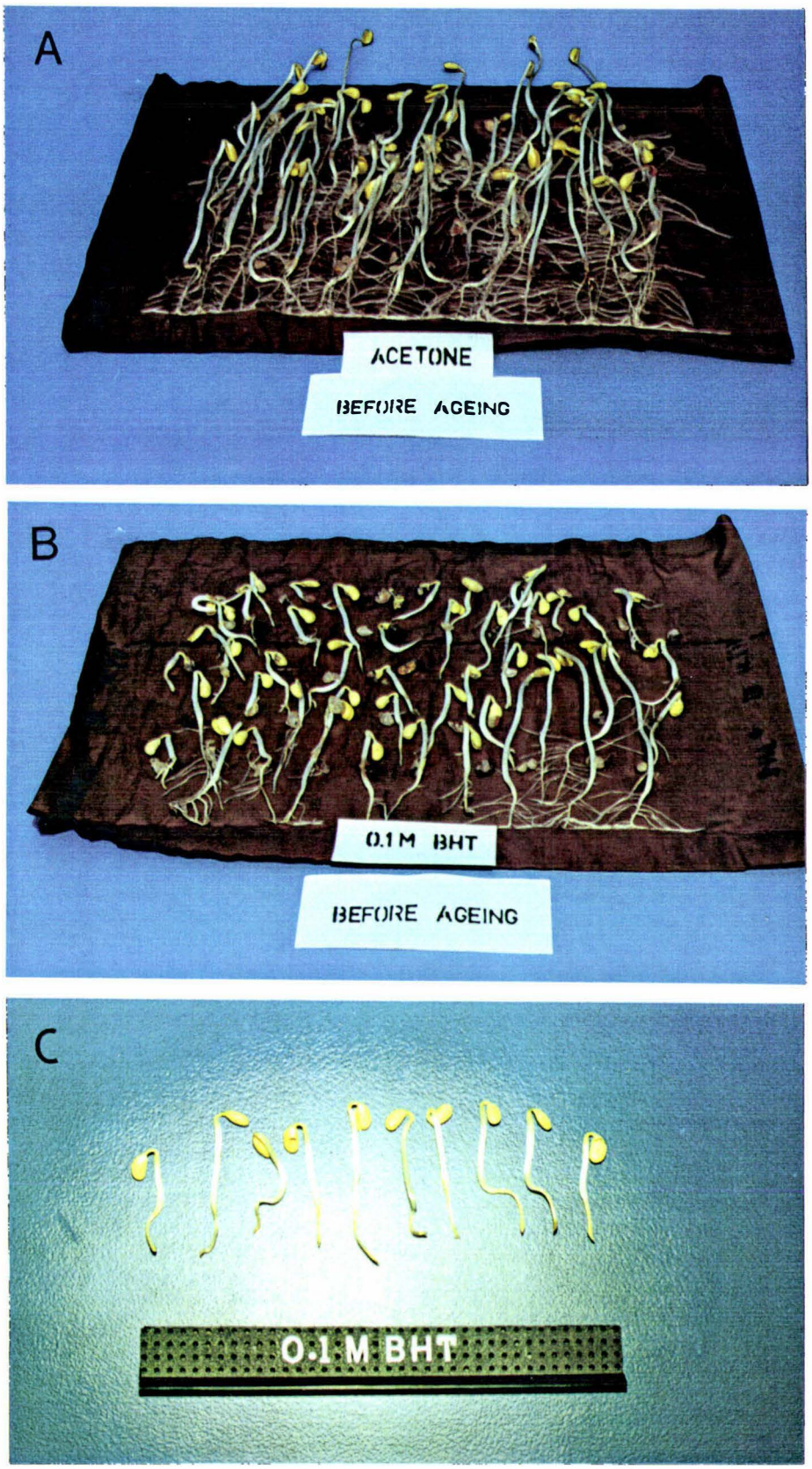


Plate 4.1 The effects of acetone and BHT on germinability and seedling growth of unaged soybean seed lot A-1 *cv.* Amsoy.

A: Acetone alone;
B: 0.1 M BHT in acetone;
C: Stunted root abnormalities from BHT treatment.

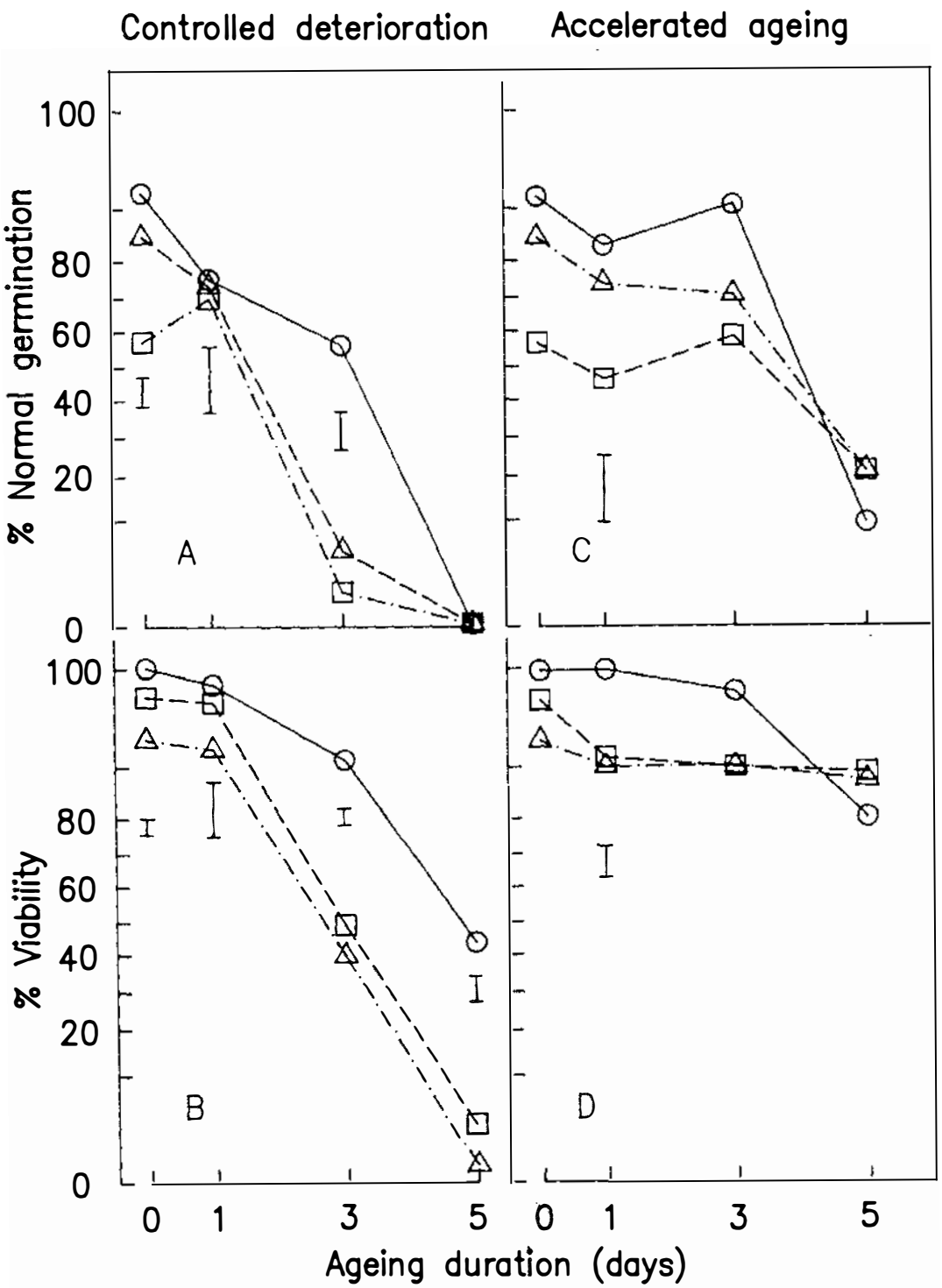


Figure 4.2 The effects of chemical treatments and subsequent controlled deterioration (40°C, 20% SMC) or accelerated ageing (40°C,~100% RH) on normal germination (A and C) and viability (B and D) of soybean seed lot A-1, *cv.* Amsoy. Data presented are arcsin √% transformed means of 4 replicates. ○—○: Untreated controls; △···△: Acetone alone; □– –□: 0.1 M BHT in acetone. Bars represent least significant differences (P=0.05).

Table 4.2 The variations in time to 50% radicle emergence between experiments after chemical treatment and controlled deterioration (40°C, 20% SMC) of soybean seed lot A-1, *cv.* Amsoy.

	Time to 50% radicle emergence (T_{50}) (h)					
	Experiment I			Experiment II		
Chemical treatment	Unaged	1d CD	3d CD	Unaged	1d CD	3d CD
Untreated controls	14.0	15.8	20.3	16.5	13.8	17.5
Acetone alone	12.5	13.8	21.8	16.5	15.8	21.3
1% α-tocopherol in acetone	12.5	15.8	31.8	-	-	-
0.1 M BHT in acetone	-	-	-	18.5	14.0	19.8
$LSD_{0.05}$	1.69	2.40	5.39	1.77	1.25	2.53

(Note that % radicle emergence after 5 d CD was too low for accurate estimation of T_{50}).

4.2.1.2 *Treatment prior to accelerated ageing (AA)*

Effects on cv. Amsoy (Expt. II)

In lot A-1 the effects of acetone and BHT treatments were similar to those under CD conditions, but AA caused less damage to seeds than did CD for equivalent periods of time (Figure 4.2). Acetone treatment had some inconsistent effects on T_{50} during accelerated ageing (compare 1 and 5 days AA, Appendix 4.2). Data for seedling growth reveal that the effects of treatments on this parameter were similar to the germination data, although 1 d AA reduced the normal seedling weight while germination itself was only reduced at 5 d (compare Appendix 4.3 and Figure 4.2C).

Effects on cv. Davis (Expts. III and IV)

When α-tocopherol and BHT were applied to *cv.* Davis, the pattern of response was broadly similar to that found for *cv.* Amsoy. In order to avoid the toxic effects of acetone at high seed moisture contents, the moisture contents of seeds were adjusted prior to soaking (for a detailed experimental evaluation see Section 4.2.2.4). The effects of α-tocopherol and acetone treatments on normal germination and viability at each level of initial moisture content are shown in Figure 4.3. AA caused significant reductions in germination and viability percentages. At 6% and 9% SMC, a dramatic deterioration in normal germination occurred after 4 d ageing, while at 12% SMC seeds deteriorated immediately. There was no significant effect of α-tocopherol on normal germination and viability at any level of seed moisture when compared to acetone treatment (Figure 4.3). Similar results were obtained from BHT treatment (Appendix 4.4) except that BHT significantly reduced normal germination of unaged seeds at all levels of seed moisture (data summarised in Table 4.3). At 6% seed moisture, BHT also inhibited normal seedling growth in unaged seeds (Figure 4.4), but note that accelerated ageing for 2 days significantly improved seedling weight.

Table 4.3 The effects of BHT treatment on normal germination percentage of unaged soybean seeds, *cv.* Davis at different seed moisture contents (% FWT) before soaking. Data presented are means of 4 replicates (Arcsin transformed data ± SE in brackets).

	% normal germination (arcsin √% transforms)		
Chemical treatment	6% SMC	9% SMC	12% SMC
Acetone alone	89 (1.23 ± 0.02)	85 (1.18 ± 0.04)	75 (1.04 ± 0.03)
O.1 M BHT in acetone	62 (0.91 ± 0.04)	66 (0.95 ± 0.04)	61 (0.89 ± 0.04)

For untreated control data and effects of accelerated ageing, refer to Appendix 4.4.

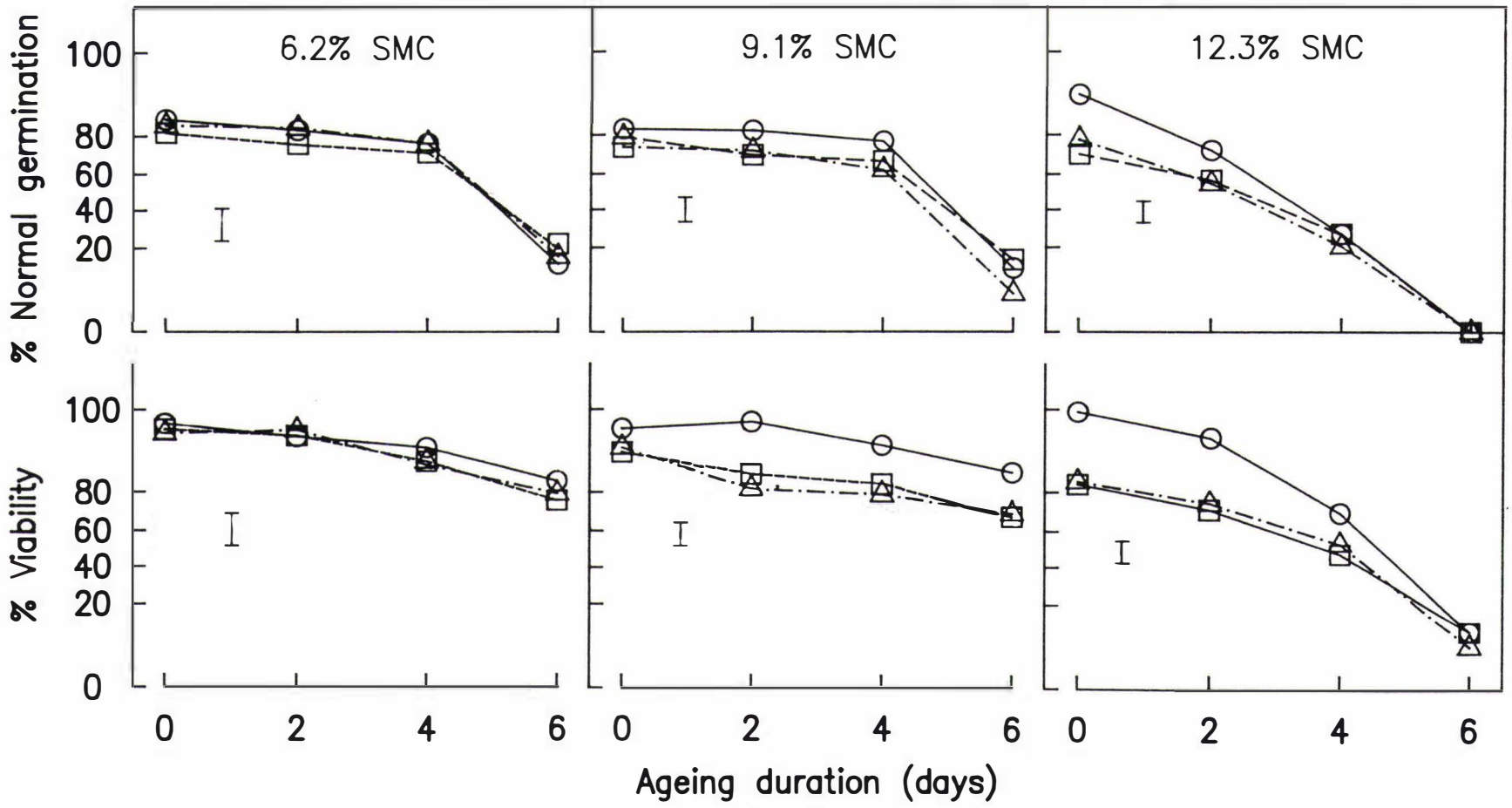


Figure 4.3 The effects of chemical treatments, soaking seeds at different initial moisture contents and subsequent accelerated ageing (40°C,~100% RH) on normal germination and viability of soybean seeds,*cv.* Davis. Data presented are arcsin √% transformed means of 4 replicates. ○—○: Untreated controls;△-△: Acetone alone;□-□: 1% α-tocopherol in acetone. Bars represent least significant differences (P=0.05).

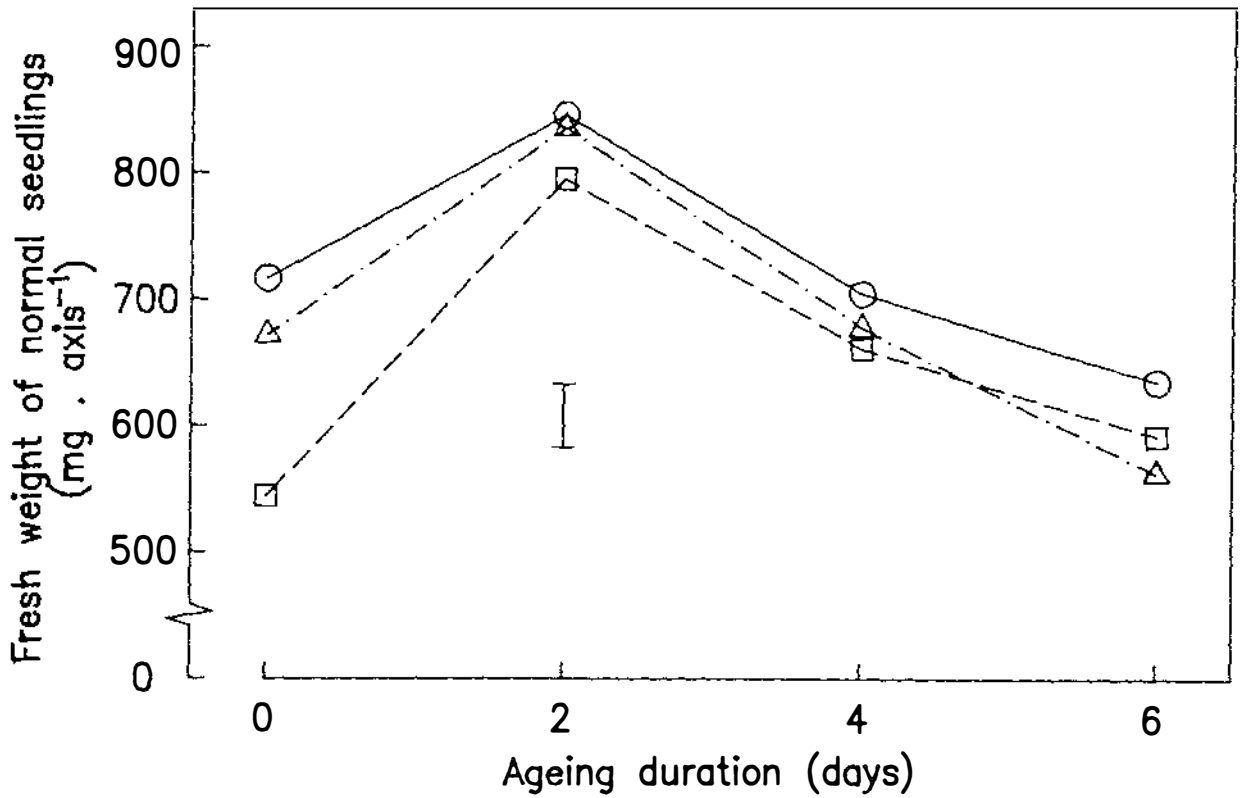


Figure 4.4 The effects of chemical treatments, soaking seeds at 6% SMC and subsequent accelerated ageing (40°C, ~100% RH) on axis fresh weights of normal soybean, *cv.* Davis seedlings 8 d after sowing. ○—○: Untreated controls; △–·–△: Acetone alone; □– –□: 0.1 M BHT in acetone. Bar represents least significant difference (P=0.05).

Hexane used as an alternative solvent (Expt. VI)

When hexane was used as an alternative solvent to introduce α-tocopherol to *cv.* Davis seeds before ageing under AA conditions, no toxic effects of hexane were observed on seeds with either 6% or 9% moisture contents prior to soaking for 16 h. However, once again α-tocopherol showed no significant effect on seed storability. Germination data for seeds treated at 9% SMC are presented in Figure 4.5. There were also some indications of a small toxic effect of α-tocopherol on seedling fresh weight at 6% SMC (Table 4.4), although this effect was not observed in any other experiment and requires confirmation.

Table 4.4 The effects of chemical treatments (averaged over durations of AA) on fresh weights of normal seedlings of soybean *cv.* Davis, after 8 d from sowing seeds soaked at 6% seed moisture content in hexane as a solvent for α-tocopherol.

Chemical treatment	Seedling fresh weight (mg.embryonic axis^{-1})
Untreated controls	832.00
Hexane alone	840.67
1% α-tocopherol in hexane	799.11
$LSD_{0.05}$	17.91

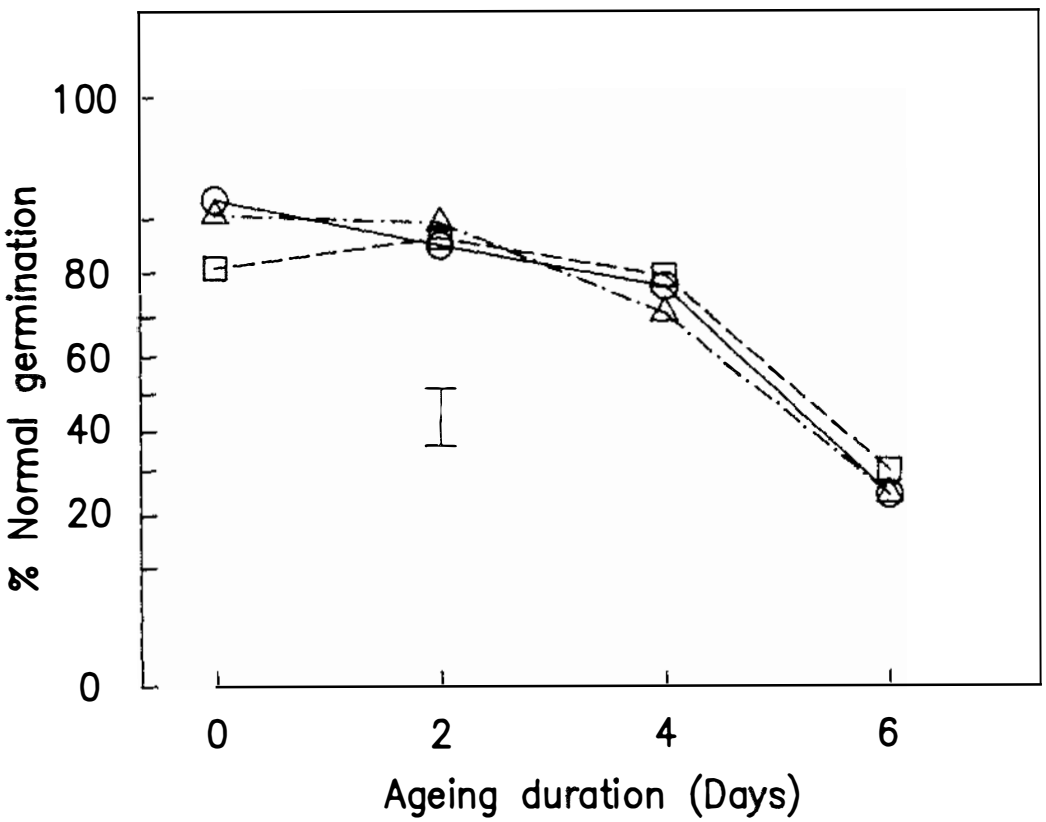


Figure 4.5 The effects of chemical treatments, soaking seeds at 9% SMC and subsequent accelerated ageing (40°C,-100% RH) on normal germination of soybean seeds, *cv.* Davis. Data presented are arcsin √% transformed means of 4 replicates. ○—○: Untreated controls; △-△: Hexane alone; □-□: 1% α-tocopherol in hexane. Bar represents least significant difference (P=0.05).

4.2.1.3 *Slow ageing*

In case the conditions of seed ageing affected the mechanism(s) of seed deterioration (see Chapter 2) and thus the efficiency of the applied antioxidants, a further experiment (Expt. V) was conducted using a slow ageing regime, storing seeds for up to 12 weeks at 35°C, 12% SMC. Due to the apparent toxicity of 0.1 M (2.2%) BHT, a lower concentration of 0.1% BHT (effective in prolonging storage life in pea, Gorecki and Harman, 1987) was used.

The effects of acetone, 1% α-tocopherol and 0.1% BHT on normal germination when seeds of *cv.* Davis were soaked at 6% SMC and then aged at 35°C, 12% SMC for different periods are given in Figure 4.6. Again, no protective effects of antioxidants were found, and there was clear evidence of acetone toxicity. Viability changes were similar to germination, and the data are not shown. The treatments did not affect the growth of normal seedlings in relation to untreated controls at any ageing times (Appendix 4.5).

4.2.1.4 *Conductivity measurements*

In all cases, there were significant ($P<0.01$) negative correlations between normal germination percentage and conductivity of seed leachate during ageing. Combined and individual treatment correlation coefficients (r) between germination and conductivity leachates from seeds of *cv.* Davis are presented in Table 4.5. Note that the correlations for slow aged seeds are significantly better than those subjected to accelerated ageing. The relationship between the two parameters was not changed by antioxidant treatments nor acetone. As an example, the correlations of data from each treatment for slow ageing are shown in Figure 4.7.

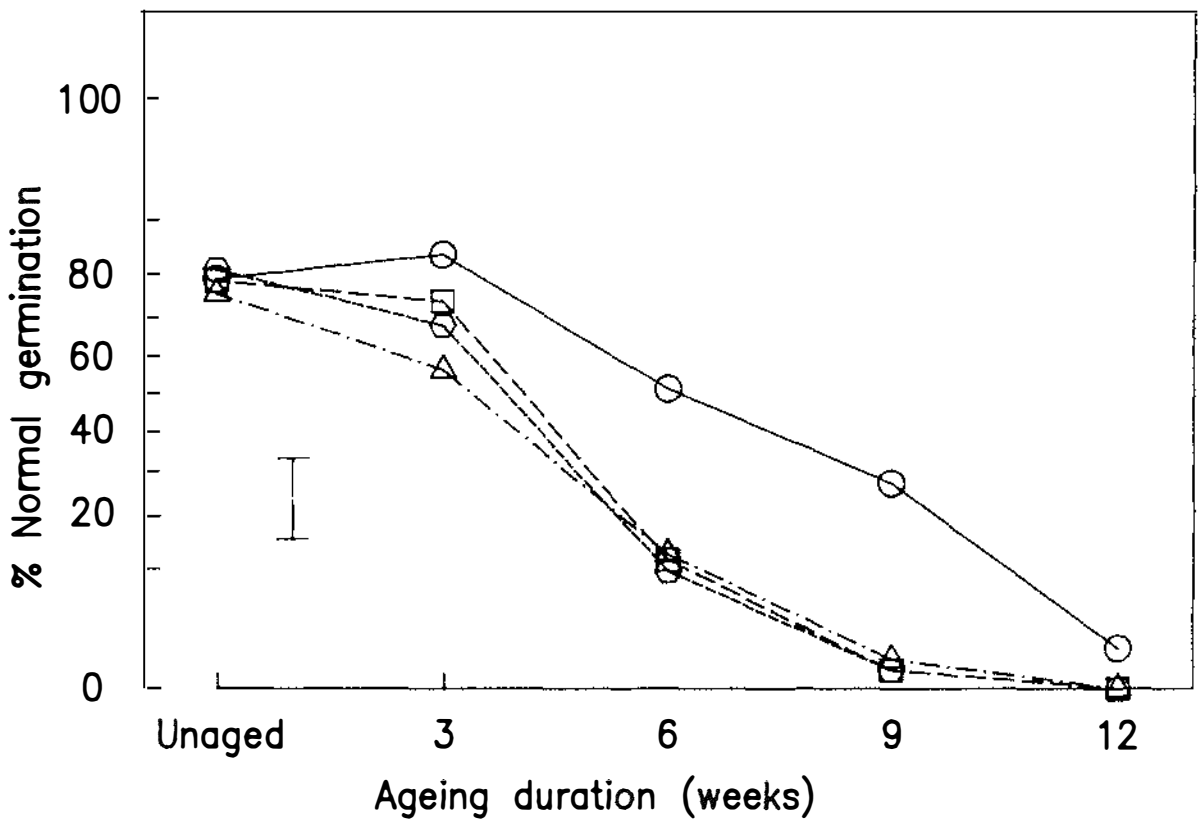


Figure 4.6 The effects of chemical treatments, soaking seeds at 6% SMC and subsequent slow ageing (35°C, 12% RH) on normal germination of soybean seeds, *cv.* Davis. Data presented are arcsin √% transformed means of 3 replicates. ○—○ : Untreated controls; △—·—△: Acetone alone; □—□ : 1% α-tocopherol in acetone; ⬡----⬡ : 0.1% BHT in acetone. Bar represents least significant difference (P=0.05).

Table 4.5 The effects of chemical treatments and ageing conditions on the correlations between conductivity and normal germination of soybean, *cv.* Davis, when seeds were soaked at 6% seed moisture content.

Chemical treatment	Correlation (r)[1]	
	AA (40°C, ~100% RH)	Slow ageing (35°C, 12% SMC)
All treatments	-0.73	-0.92
Untreated controls	-0.80	-0.89
Acetone alone	-0.63	-0.96
α-tocopherol in acetone	-0.83[2]	-0.92
BHT in acetone	-0.86	-0.91

[1] *All significant at P = 0.01.*
[2] *From separate experiment.*

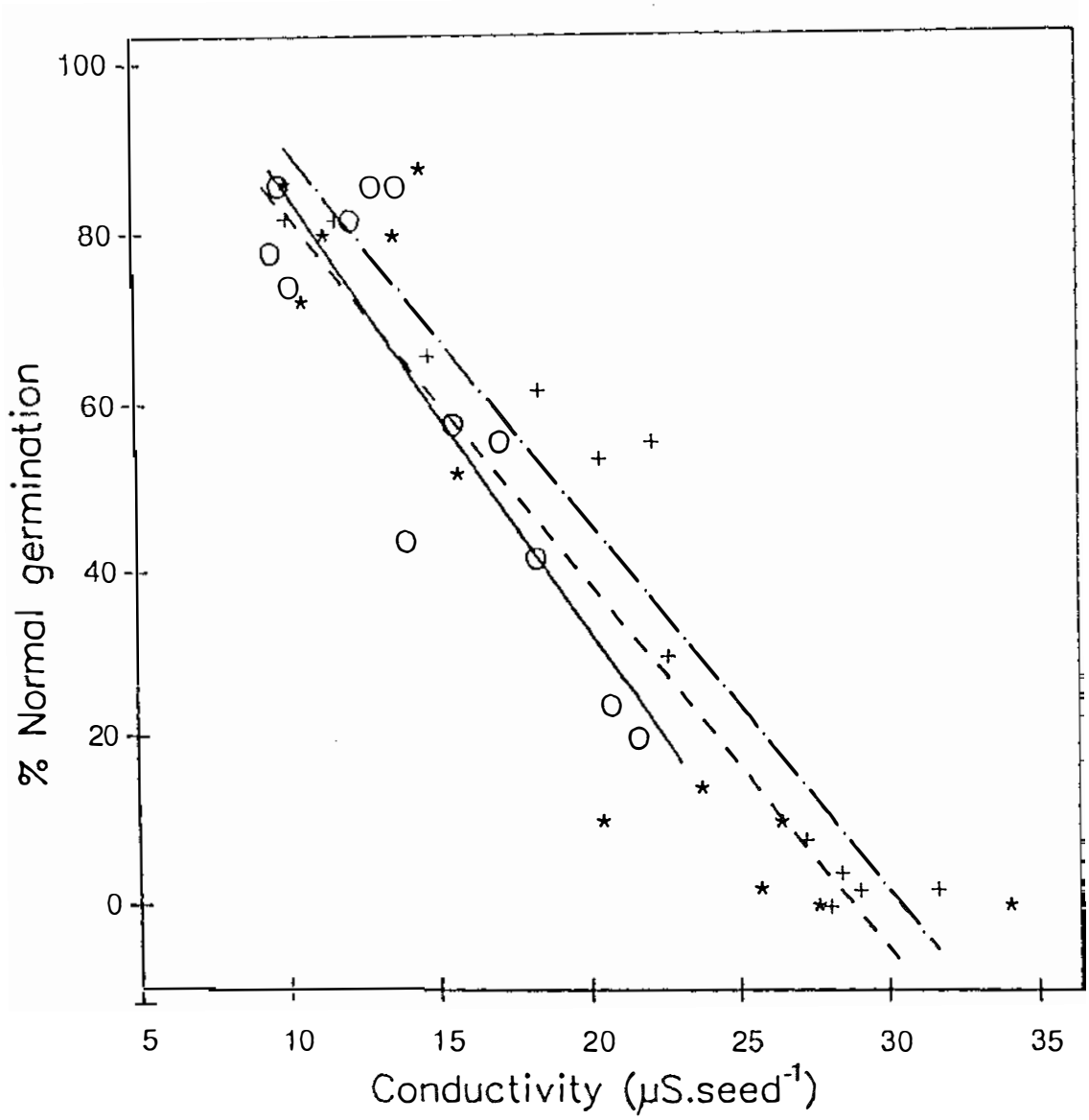


Figure 4.7 The correlations between normal germination and conductivity during slow ageing (35°C, 12% SMC) of untreated controls [o , —]; acetone alone [+, - · -]; and 1% α-tocopherol in acetone [*, – –] for soybean seeds, *cv.* Davis (data from experiment V). Correlations are significant for all treatments (see Table 4.5, 10 degrees of freedom). Slopes are not significantly different.

4.2.2 Evaluation of acetone as a solvent medium for delivering antioxidants to seeds

It is evident from section 4.2.1 that a 16 h acetone soak caused damage to soybean seeds, particularly when they were subsequently aged. Experiments VII-XIII were designed to characterise the effects of acetone using different techniques.

4.2.2.1 *Effects of duration of soaking in acetone*

The effects of duration of acetone treatment were first assessed in seed lot A-2 under both CD and AA conditions. Data for the effects on germination percentage under CD conditions (Exp. VII) are shown in Figure 4.8. There was a small, but significant ($P<0.05$) increase in normal germination as a result of raising SMC to 20% (0 d CD) compared to untreated unaged controls (Figure 4.8). A significant ($P<0.05$) toxic effect of acetone treatment was evident on unaged seeds (0 d CD) after just 2 h soaking, percentage germination decreasing from 95 to below 90. However, there was no evidence of further effects due to longer soaking times. There was no evidence of acetone toxicity after 1 d CD, but clear progressive toxic effects were evident after 3 d CD. Seeds soaked for 2 h lost 25% of their germinability, while the numbers of normal germinants of seeds soaked for 16 h dropped sharply to less than 10%. Data for viability followed the pattern of germination and are not shown. There was no change in either fresh or dry weights of remaining normal seedlings as a result of soaking duration during CD (Appendix 4.6).

Under AA conditions (Expt. VIII) there were no interactions between durations of soaking and ageing. Accordingly, data are tabulated in Table 4.6 and show overall mean effects of soaking duration. Increased times of soaking in acetone significantly ($P<0.01$) decreased germinability and viability. Seeds began to lose germination significantly after soaking for 8 h, while only 78% of seeds soaked for 16 h were germinable. A small, but significant reduction in viability was evident after only 2 h soaking. Although seedling dry weights were not affected (data not shown), 4 h acetone soaking significantly ($P<0.05$) reduced fresh weights of normal seedlings of unaged seeds, and interestingly, the seedling fresh weights of acetone soaked materials were significantly ($P<0.001$) improved after 1 d AA and, with less pronounced effects, after 3 d AA (Figure 4.9).

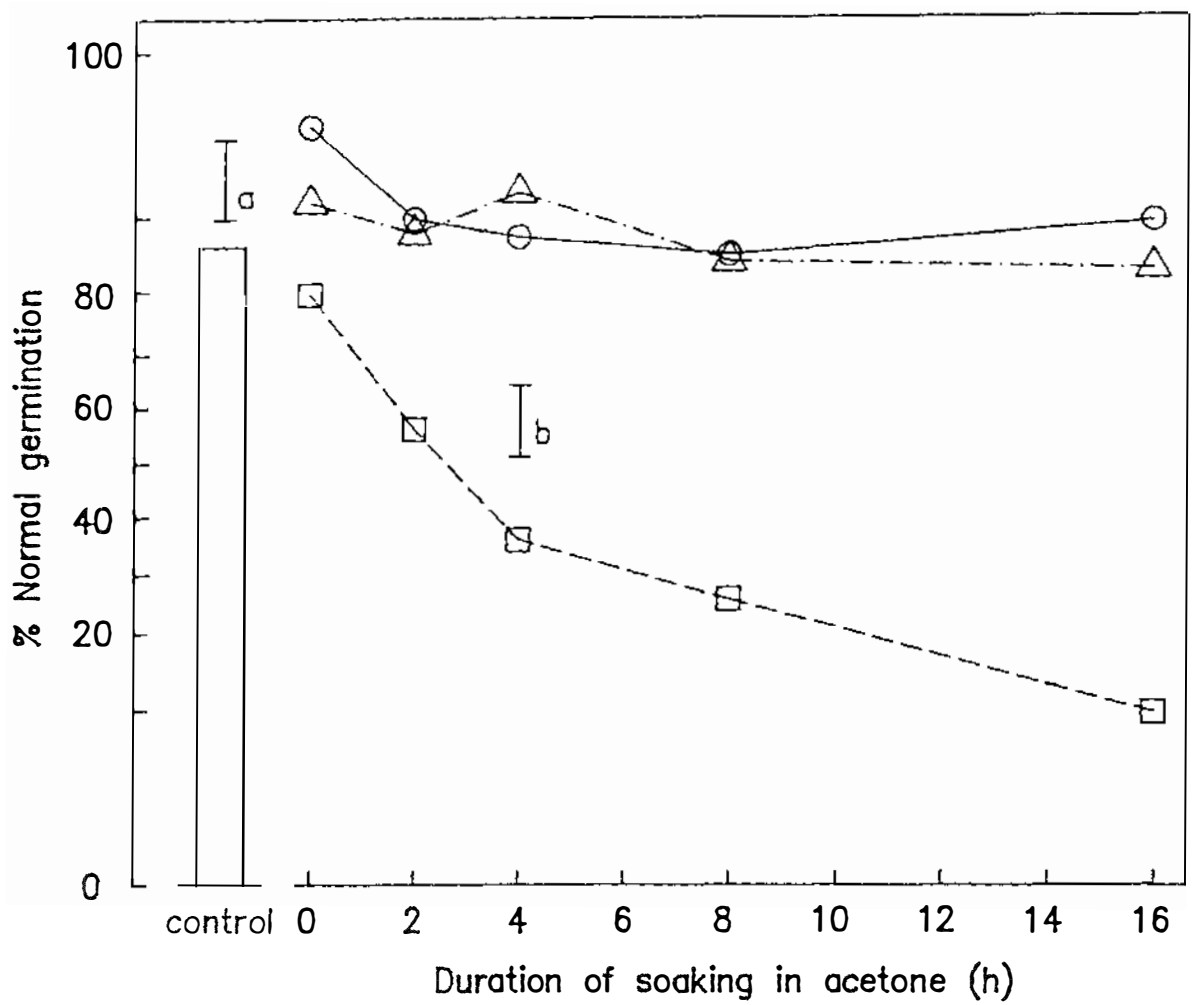


Figure 4.8 The effects of different durations of soaking in acetone and subsequent controlled deterioration (40°C, 20% SMC) on the normal germination of soybean seed lot A-2, *cv.* Amsoy. Data presented are arcsin √% transformed means of 4 replicates. ○–○: 0 d CD; △–△: 1 d CD; □–□: 3 d CD; histogram: Untreated unaged controls. Bars represent least significant differences (P=0.05) between untreated unaged controls and unsoaked, 0 d CD seeds (a), and between means of CD aged seeds (b).

Table 4.6 The effects of durations of soaking in acetone on germinability and viability of soybean seed lot A-2, *cv.* Amsoy, after accelerated ageing (40°C, ~100% RH). Data presented are overall means for 0, 1 and 3 d AA (see text). (Arcsin √% transformed data in brackets).

Soaking period, h	% Germination	% Viability
0	91.0 (1.27)	100 (1.56)
2	88.0 (1.22)	98.5 (1.44)
4	88.0 (1.22)	96.0 (1.37)
8	84.5 (1.17)	93.5 (1.31)
16	78.0 (1.08)	89.5 (1.24)
$LSD_{0.05}$	(0.063)	(0.065)
CV (%)	6.44	5.75

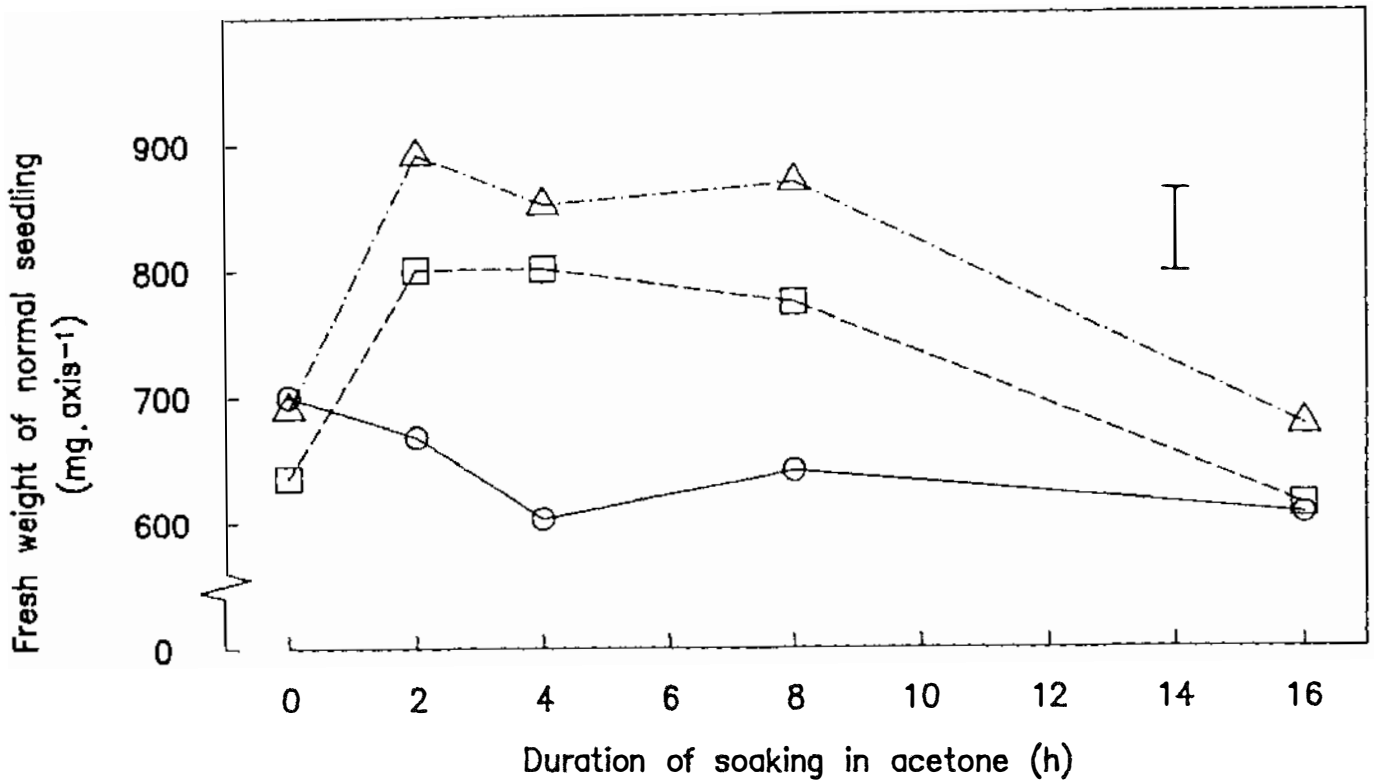


Figure 4.9 The effects of different durations of soaking in acetone and subsequent accelerated ageing (40°C, ~100% RH) on axis fresh weights of normal soybean seedlings 8 d after sowing of lot A-2, *cv.* Amsoy. ○—○: 0 d AA; △···△: 1 d AA; □ – □: 3 d AA. Data are means of 4 replicates. Bar represents least significant difference (P=0.05) between any two means.

4.2.2.2 *Drying methods* (Expt. IX)

In order to confirm that all the acetone in the seed was completely removed after soaking in acetone for 16 h, seeds were dried at 20°C or 35°C for 24 h or 48 h. The effects on moisture contents and germinability after drying are presented in Table 4.7. There were no significant ($P<0.05$) differences between drying regimes on normal germination, although the SMC's of seeds dried at 35°C were 3-4% lower than those of seeds dried at 20°C. In all cases, the damaging effects of acetone were evident ($P<0.05$). The toxicity of acetone was particularly evident in 3 d CD seeds. Neither drying nor acetone treatment affected growth of remaining normal seedlings (data not shown).

Table 4.7 The effects of different drying conditions after 16 h soaking in acetone on seed moisture content (% FWT) and normal germination of soybean seed lot A-2, *cv.* Amsoy. Data in brackets are arcsin √% transformed.

Acetone treatment and drying	% SMC after drying	% Germination	
		unaged	3 d CD
Untreated controls	12.4	98.0 (1.43)	85.5 (1.18)
16 h Ac[1] - 20°/24 h	9.9	84.0 (1.16)	0
16 h Ac - 35°C/24 h	7.0	80.0 (1.11)	0
16 h Ac - 20°C/48 h	9.4	84.5 (1.17)	0
16 h Ac - 35°C/48 h	5.6	90.5 (1.26)	2 (0.04)
$LSD_{0.05}$	0.16	(0.17)	(0.10)
CV (%)	1.28	9.14	27.8

1 Ac = Acetone.

4.2.2.3 *Seed lot variation* (Expt. X)

A soaking duration of 16 h followed by ageing under CD conditions for 3 days was chosen to investigate the effects of seed lot variation on susceptibility to acetone toxicity. There was high variation of seed moisture and germination quality among seed lots before acetone treatment (Figure 4.10).

A considerable variation in susceptibility of seed lots to both ageing and acetone toxicity was noted. The toxicity of acetone was not observed in unaged seed of high vigour, lots A-2 and A-3, but after 3 d ageing acetone soaked seeds of lot A-2 were completely killed and lot A-3 lost 80% germination. While acetone caused a highly significant reduction in germination percentage of lot A-4 on both unaged and aged seeds, these effects were undetectable in the remaining germinable seeds of the very low quality seed lot A-5.

4.2.2.4 *Influence of seed moisture before soaking in acetone*

From the results of the previous experiment, the possibility arose that differences in initial seed moisture before treatment might affect susceptibility to acetone. This was investigated in three further studies (Expts. XI-XIII) as follows. When the moisture contents of seeds of lots A-3 and A-8 were adjusted prior to soaking in acetone for 16 h, the seeds showed a similar pattern of response (Figure 4.11). At a moisture content below 7.5% the toxic effects of acetone were not evident in unaged seeds (although they still were in aged seeds, *e.g.* Appendix 4.7 for lot A-8). Increasing SMC from 7.5-10.5% increased the seeds susceptibility to damage, as viability of unaged seeds of lot A-3 dropped from 95 to below 60%, and lot A-8 from more than 99 to 90%. No further damage occurred beyond 10.5%. Germination data (not shown) followed a similar pattern. Seeds of lot A-3 were more prone to ageing damage than those of A-8 (data not shown), and untreated seeds at low SMC were more susceptible to CD effects than seeds at 9% SMC or higher (*e.g.* Appendix 4.7 for data for lot A-8).

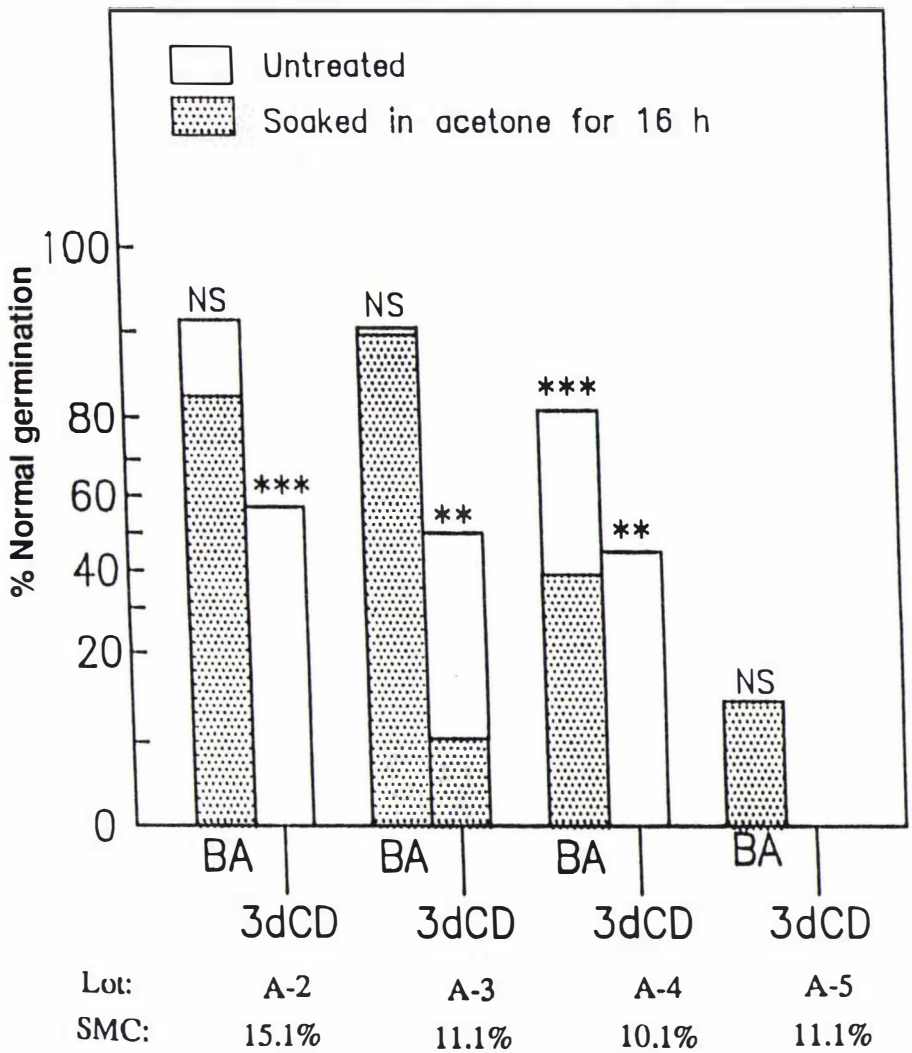


Figure 4.10 The germination responses of different seed lots of soybean *cv.* Amsoy to 16 h soaking in acetone and subsequent ageing under 40°C, 20% SMC (CD). Data are arcsin √% transformed means of 4 replicates. The effects of acetone treatment were compared to untreated controls within seed lots at each ageing period using a T-test. BA: before ageing; 3 d CD: after 3 days controlled deterioration; NS: Not significant (P>0.05); **: significant at P=0.01; ***: significant at P=0.001.

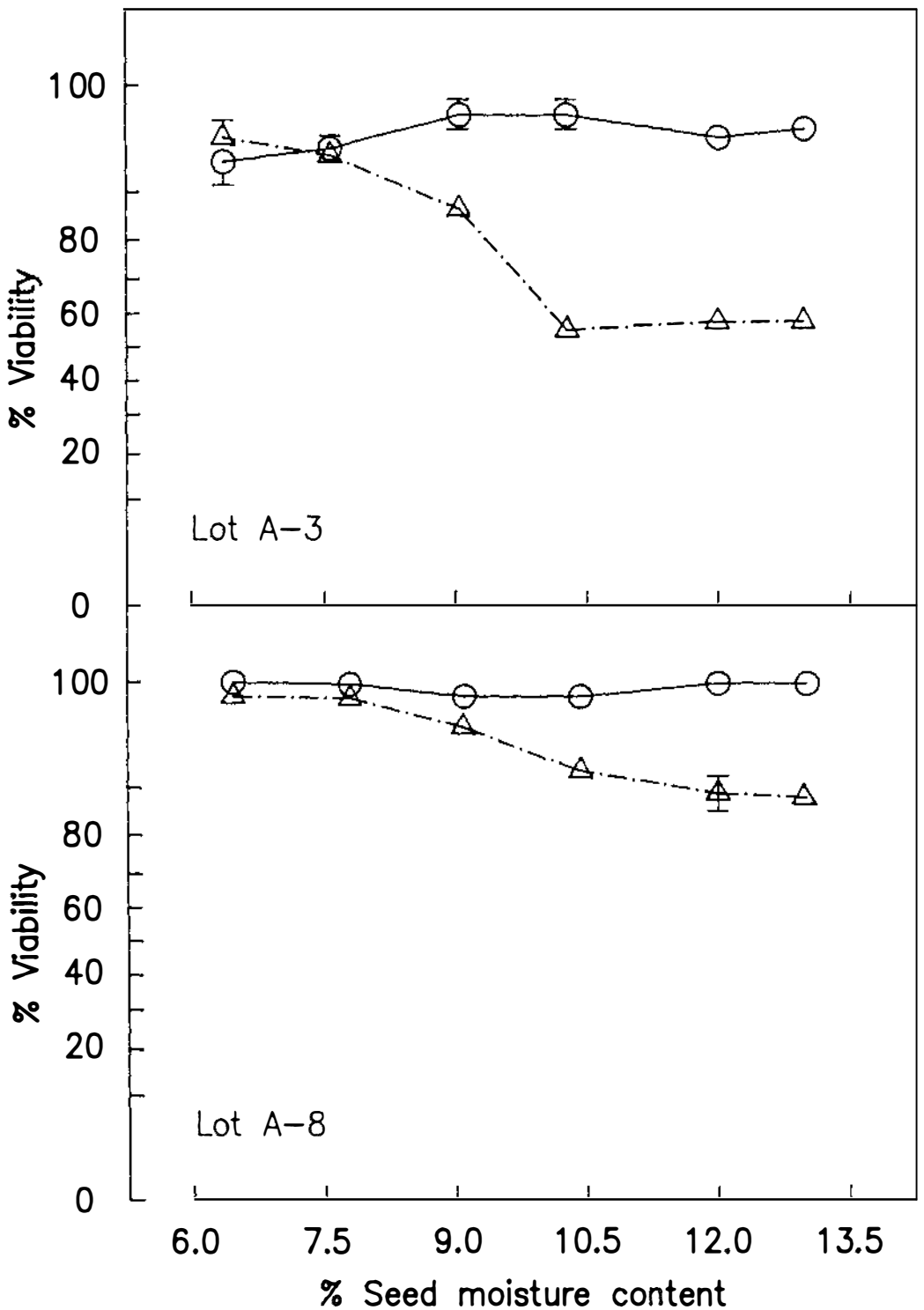


Figure 4.11 The effects of initial SMC on viability of unaged soybean seed lots A-3 and A-8, *cv.* Amsoy, after 16 h soaking in acetone. Data presented are arcsin √% transformed means. ○—○: Untreated controls; △-·-△: Acetone. Vertical bars represent SE's calculated for individual means from 4 replicates and are shown when larger than the symbols used.

For *cv.* Davis, data were collated from experiments III and IV which have already been mentioned (Figure 4.3 and Appendix 4.4). While at 6% SMC neither normal germination nor viability were affected by previous acetone soaking either before or after AA, at 9% SMC the toxic effects of acetone were evident on the viability of aged seeds. However, at 12% SMC losses of both normal germination and viability were evident immediately after soaking. Under slow ageing at 35°C and 12% SMC, even though seeds were soaked in acetone at 6% SMC, the toxicity of acetone became obvious during subsequent ageing, for example, acetone treated seeds lost more than 80% of their remaining germinability after 6 weeks of ageing (Figure 4.6).

4.2.2.5 *Effects of seed damage*

When seeds from a single lot (A-7) were sorted into damaged and undamaged groups, and their moisture adjusted before soaking in acetone, the solvent had a much greater toxic effect on damaged samples compared to undamaged seeds (Figure 4.12, Plate 4.2). The safe levels of seed moisture content which protect against acetone toxicity are also lower for damaged seeds than undamaged ones. While no appearance of acetone toxicity occurred in undamaged seeds at moisture contents below 7.5%, damaged seeds required around 6% of moisture for safe treatment. At 9 to 10.5% seed moisture, the rate of viability loss increased considerably, dropping from 96 to 83% and from 46 to 14% in undamaged and damaged seeds, respectively. A similar pattern of response was obtained from germination data (not shown).

4.2.2.6 *Penetration and site of acetone damage*

In an attempt to follow solvent penetration in soybean seed, the addition of methyl red to acetone showed that in intact seed there was little or no movement of dye beyond the seed coat, compared to extensive penetration in mechanically damaged material (Plate 4.3). Seeds either sorted by $FeCl_3$ staining (Plate 4.3A) or by visible damage (Plate 4.3B) gave similar results. The results from tetrazolium staining (Plates 4.4 and 4.5) also reveal that cracked or high SMC seeds were much more sensitive to acetone damage.

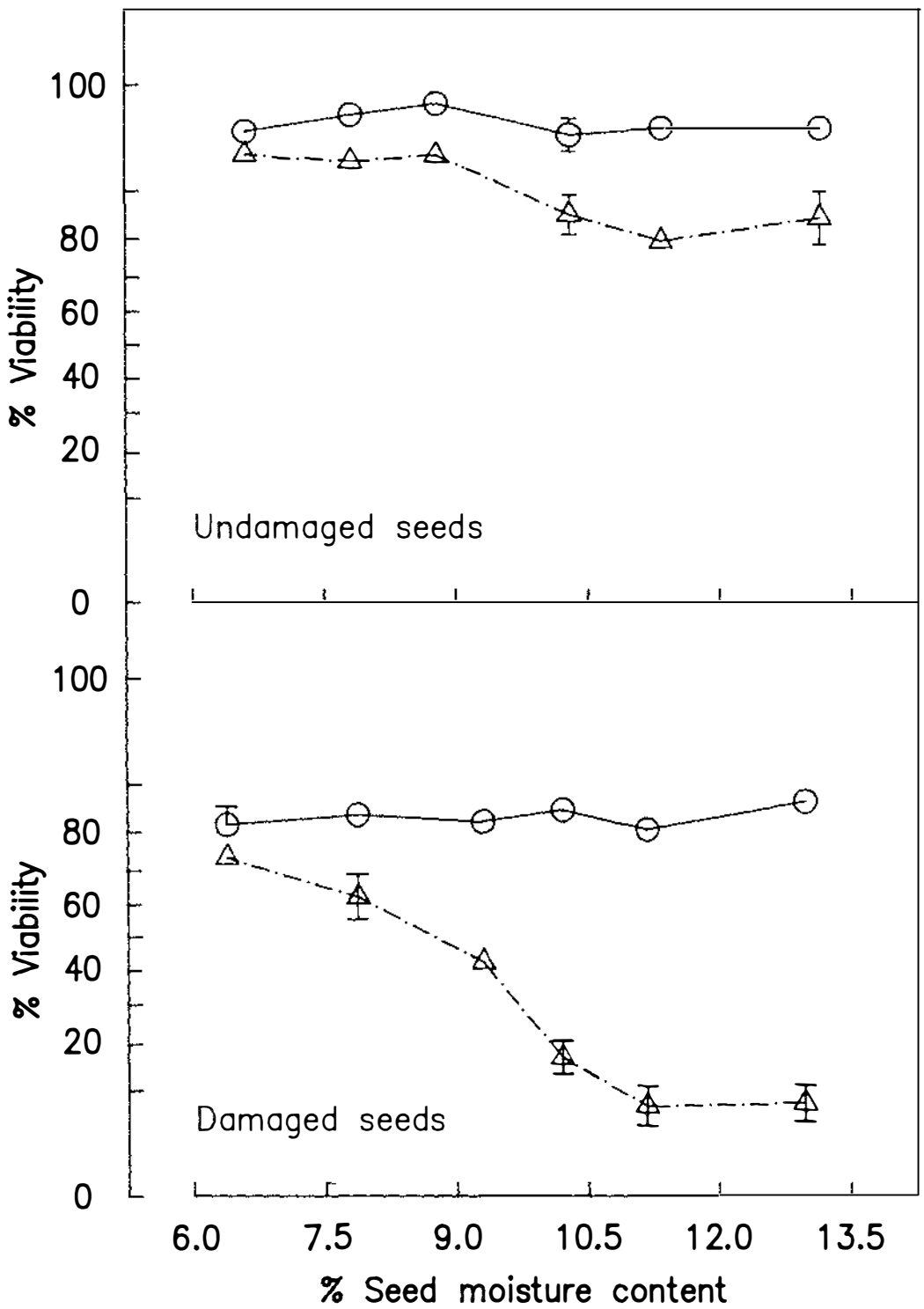


Figure 4.12 The effects of seed damage and SMC on viability of unaged soybean seed lot A-7, *cv.* Amsoy, after 16 h soaking in acetone. Data presented are arcsin √% transformed means. ○—○ : Untreated controls; △···△: Acetone. Vertical bars represent SE's calculated for individual means of 4 replicates and are shown when larger than the symbols used.

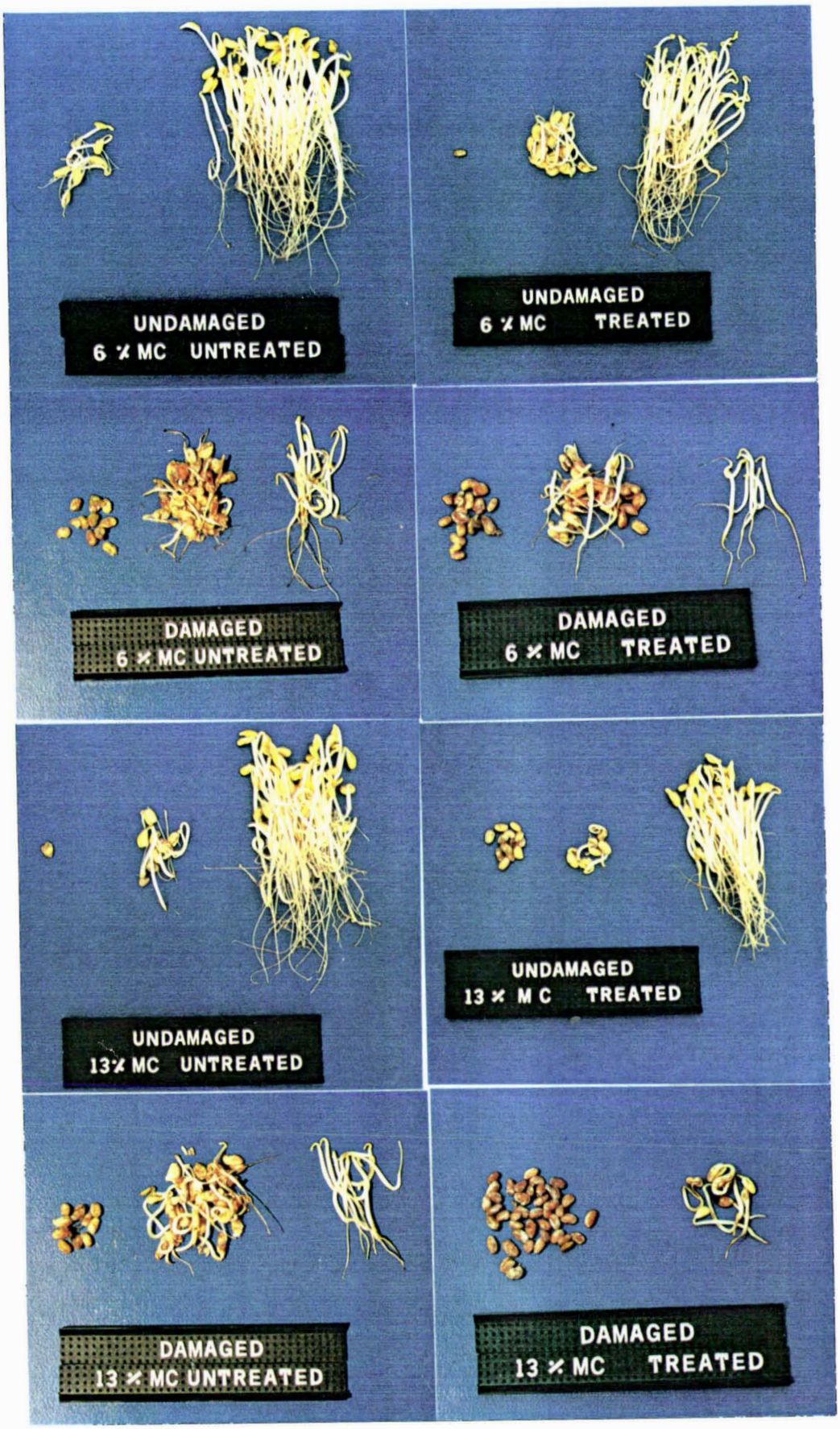


Plate 4.2 Germination responses as a result of initial seed moisture content or mechanical damage and its interactions with 16 h acetone treatment of unaged soybean seed lot A-7, *cv.* Amsoy.

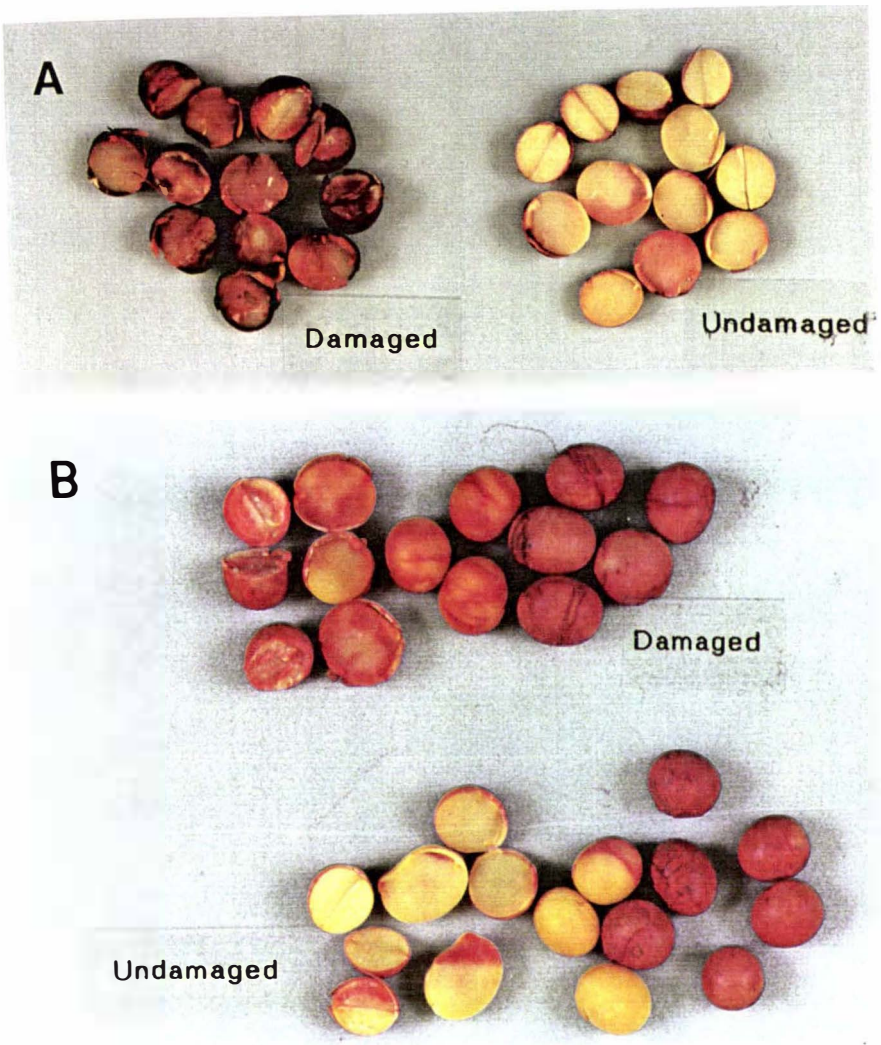


Plate 4.3 The penetration pattern of methyl red in acetone in mechanically damaged and undamaged seeds of soybean lot A-7 sorted by (A) 20% $FeCl_3$ staining or (B) evaluation by eye. The black staining on the testa indicates sites of mechanical damage penetrated by the salt which is subsequently oxidised.

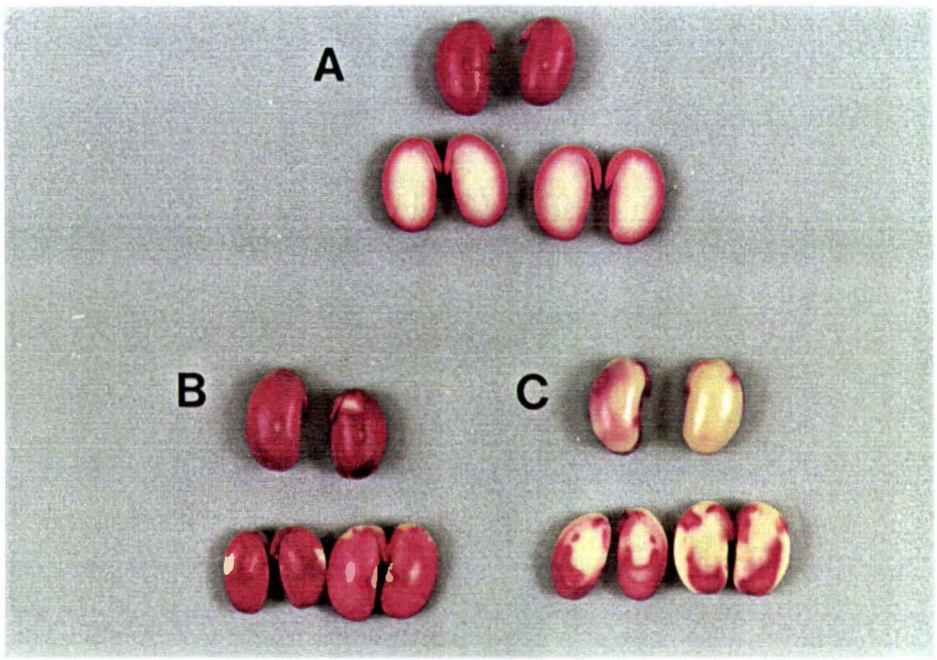


Plate 4.4 Tetrazolium staining patterns of undamaged untreated controls (A), damaged untreated seeds (B), or damaged seeds after 16 h soaking in acetone at 9% SMC (C) of soybean seed lot A-7, *cv.* Amsoy.

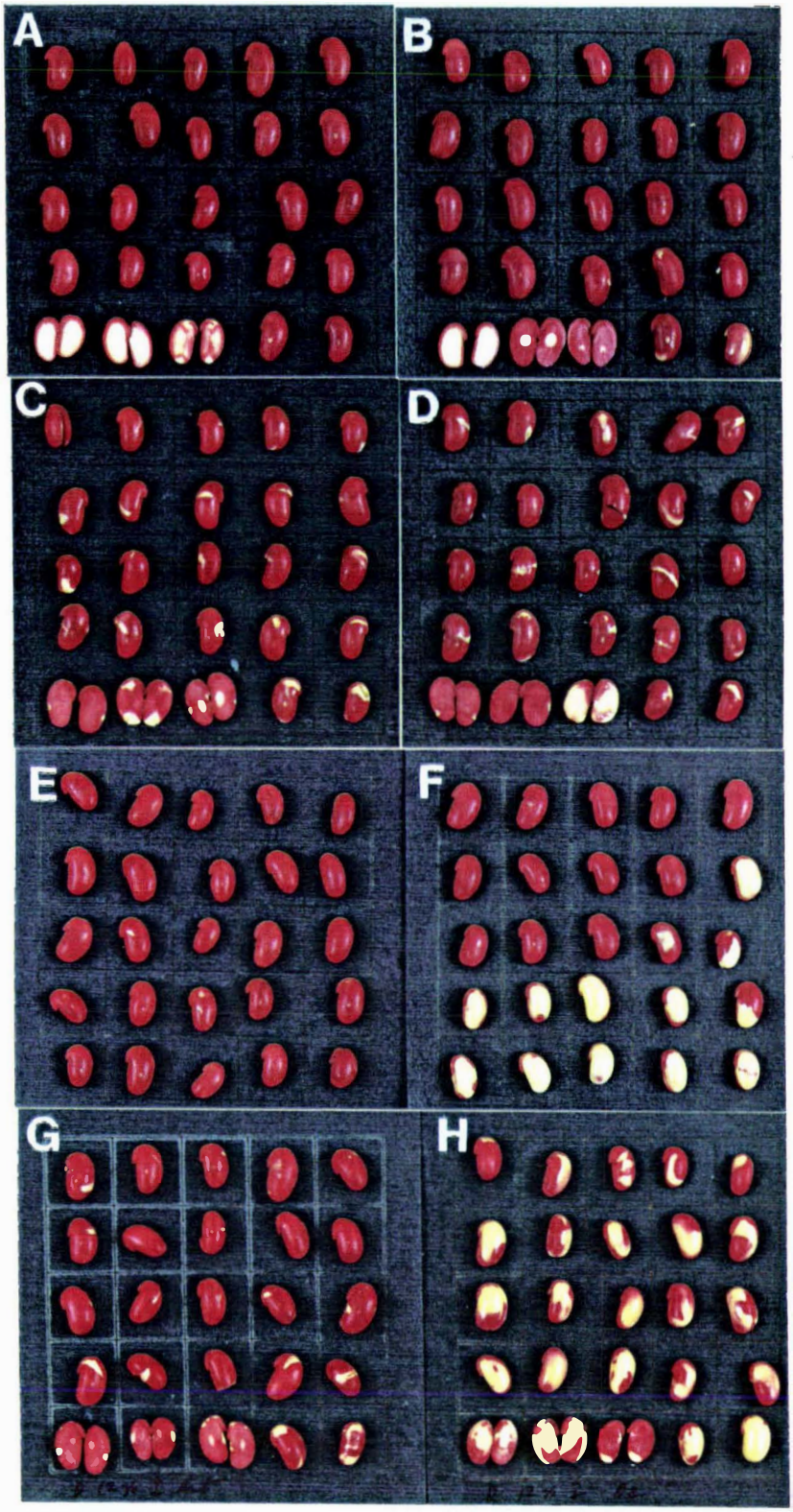


Plate 4.5 Tetrazolium staining patterns as a result of the interactions between initial moisture content (SMC) or mechanical damage and 16 h acetone treatment on unaged soybean seed lot A-7, *cv.* Amsoy.

A: 6% SMC, undamaged, untreated control;
B: 6% SMC, undamaged, acetone soaked seeds;
C: 6% SMC, damaged, untreated control;
D: 6% SMC, damaged, acetone soaked seeds;
E: 12% SMC, undamaged, untreated control;
F: 12% SMC, undamaged, acetone soaked seeds;
G: 12% SMC, damaged, untreated control;
H: 12% SMC, damaged, acetone soaked seeds.

4.2.3 Other antioxidants delivered as aqueous solutions or as solids

4.2.3.1 β-*Mercaptoethanol*

β-Mercaptoethanol in aqueous solution was applied to *cv.* Amsoy seed lot A-0, and seeds were aged under controlled deterioration conditions (40°C, 20% SMC). There was no significant effect of any β-mercaptoethanol treatment (0-100 *ppm*) on normal germination, viability and seedling growth during ageing, although significant ($P<0.01$) losses of these attributes were detected due to the effects of ageing (Appendix 4.8).

4.2.3.2 *Iodine treatments*

Effects of storage regime

In an attempt to confirm whether 0.1% iodine applied for 2 h in the $CaCO_3$ mixture had the potential of prolonging the storage life of soybean seeds (as reported by Dey and Mukherjee, 1984), this was applied to seed lot A-2 which was subsequently aged under three different conditions. Analysis of each ageing condition indicated that there were no significant interactions between iodine treatment and ageing duration, thus overall means of germination responses to ageing under different storage regimes are shown in Figure 4.13. After ageing for 3 days under 40°C, ~100% RH, seeds lost their germinability significantly ($P<0.05$), and no more normal germination was detected after 5 days. Under the less stressful conditions of 40°C, 90% RH, deterioration was a little slower. Under slow ageing of 40°C, 36% RH, seeds lost only 5% normal germination ($P<0.001$) over the duration of the experiment. Only under 5 d ageing at 40°C and ~100% RH was the viability of iodine treated seeds significantly ($P<0.001$) improved and was 4 fold that of untreated seeds (Figure 4.14). Note that under 40°C and ~100% RH seeds were not dried to drive off excess iodine after treatment, while they were for the other two storage conditions (see details in Chapter 3). No other evidence of improved germination performance due to iodine treatment (either in germinability or seedling growth) was detected under any storage conditions. In an attempt to confirm these results, seeds of *cv.* Davis with or without iodine treatment were aged at 35°C, 12% SMC for up to 12 weeks. There was no significant effect of iodine treatment on any aspect of seed performance (data not shown).

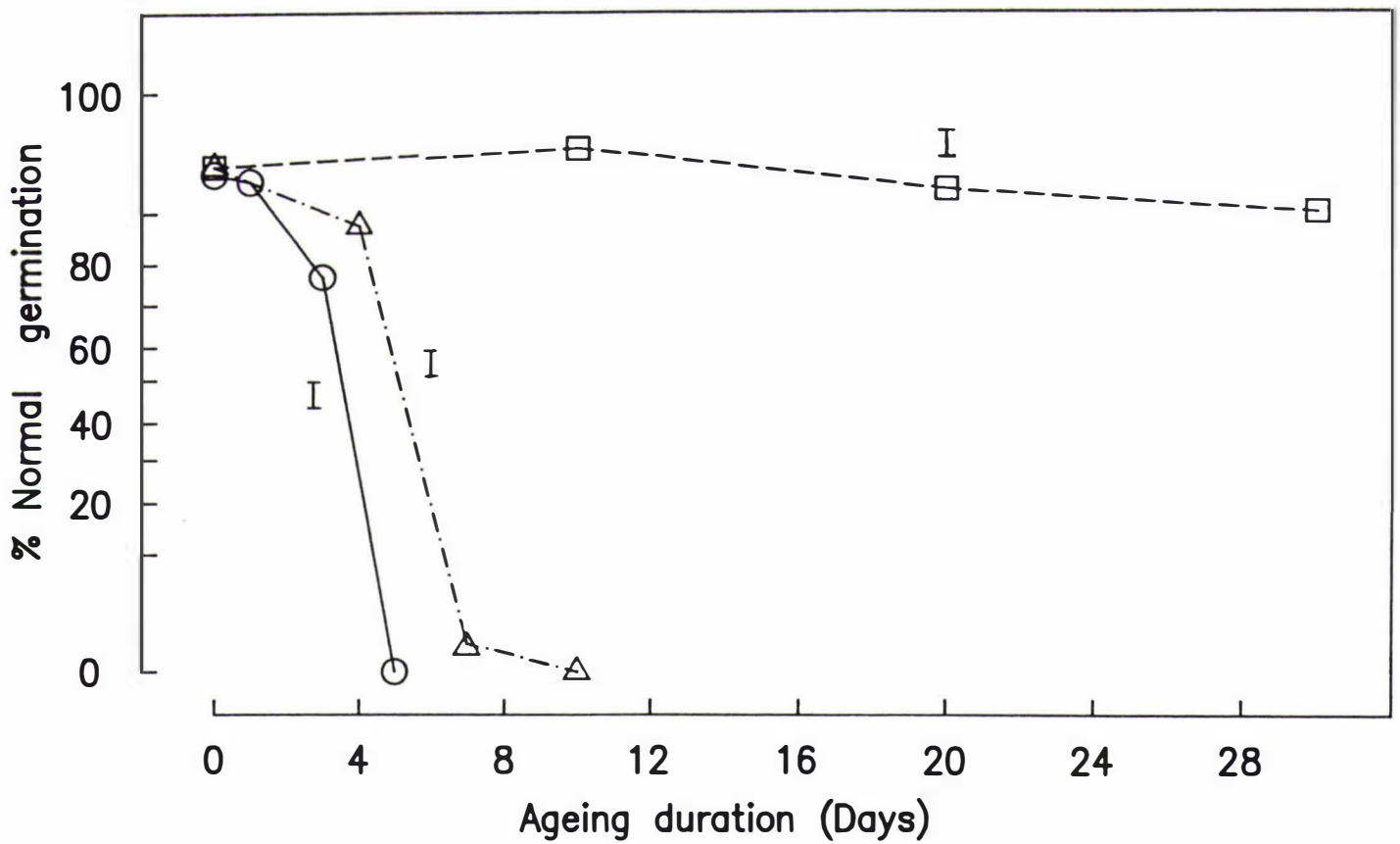


Figure 4.13 Germinability changes of soybean seed lot A-2 *cv.* Amsoy under three different storage regimes. ○—○: 40°C, ~100% RH; △···△: 40°C, 90% RH; ⊟ – ⊟: 40°C, 36% RH. Data plotted are arcsin √% transformed means (averaged over untreated controls and 0.1% iodine treatment). Bars represent least significant differences (P=0.05) between ageing durations under each set of conditions.

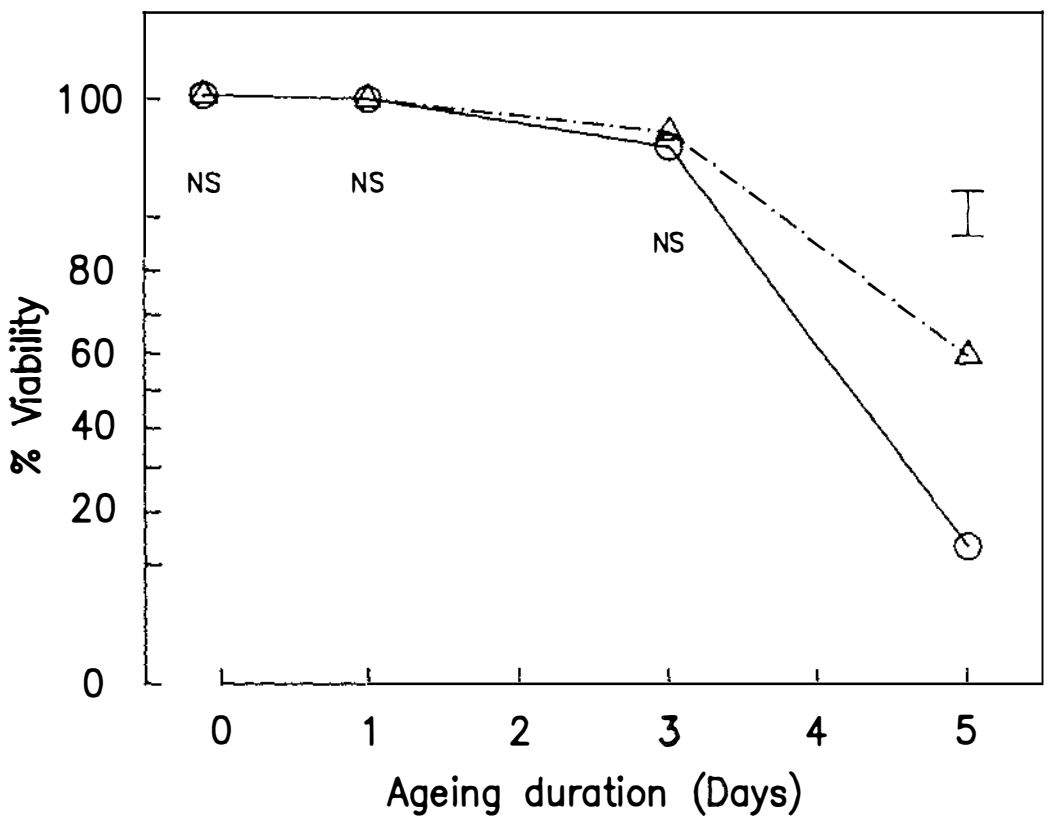


Figure 4.14 The effect on viability of accelerated ageing (40°C, ~100% RH) and its interactions with previous iodine treatment (0.1%) of soybean seed lot A-2, *cv.* Amsoy. Data plotted are arcsin √% transformed means of 4 replicates. ○—○ : Untreated controls; △···△ : 0.1% iodine. Bar represents the least significant difference (P=0.05) between treatments at 5 d AA. NS: No significant effects at other ageing times.

Effects of treatment duration and iodine concentration

In a further attempt to reproduce the one positive result with iodine, seeds of lot A-3 were treated for up to 24 h with 0.1% iodine prior to accelerated ageing but no beneficial effects were observed (data not shown). In a final study using 0.1 and 1% iodine, there was a small, but significant ($P<0.05$) reduction of seed viability caused by 1% iodine treatment, but normal germination was not affected (Figure 4.15). One percent iodine treatment also showed toxic effects on seedling fresh weight (Appendix 4.9).

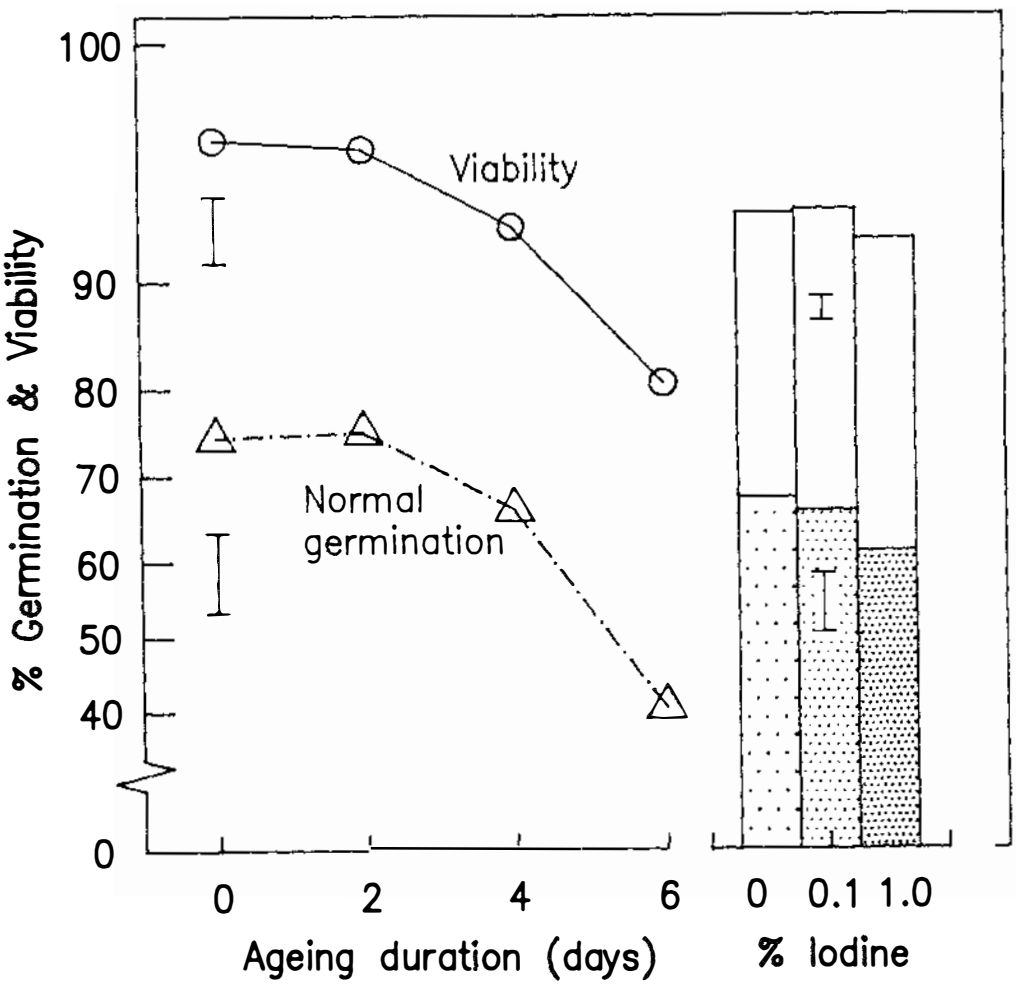


Figure 4.15 The effects of subsequent accelerated ageing (40°C,~100% RH) (averaged over treatments) or iodine treatment for 24 h (different concentrations averaged over duration of AA) on viability (○—○, or open histogram) and normal germination (△·△, or closed histogram) of soybean seed lot A-3, *cv.* Amsoy. Data presented are arcsin √% transformed means of 4 replicates. Bars represent least significant differences (P=0.05). There were no significant interactions between ageing and iodine treatments.

4.3 DISCUSSION

4.3.1 **Effects of antioxidant treatments**

4.3.1.1 *Antioxidants in acetone or hexane*

The effectiveness of α-tocopherol and BHT dissolved in acetone or hexane as storage protectants were assessed in six experiments on two cultivars of soybean using different storage regimes. Unexpectedly, these antioxidants did not give any benefit (Figures 4.1 to 4.6 and Appendix, 4.4). In fact these two antioxidants appeared to have minor toxic effects on some aspects of seed performance. Contrasting results have been reported by Gorecki and Harman (1987) who found that 1% α-tocopherol or 0.1% BHT maintained germinability and vigour in pea stored at 30°C, 92% RH. Beneficial effects of α-tocopherol were also found for maize and mustard stored under both 40°C, 40% RH, and 27-32°C, 65-75% RH with the treatment being more effective under the former conditions than the latter (Dey and Mukherjee, 1988). Okundaye (1977) claimed that seeds treated for 4 h with 1% α-tocopherol in acetone solution caused a reduced rate of viability loss in both maize and soybean. However, effects on soybean were very marginal: after 90 d storage at 30°C, 78% RH, α-tocopherol treated seeds showed just 2% germination compared to 0% in untreated controls, and there was only a 6% improvement of germination in seeds stored at 40°C, 30% RH. Clearly the viability of this evidence is questionable.

In this study, the higher concentration (2.2% or 0.1 M) of BHT was toxic particularly to unaged seeds (Figure 4.2, Appendix 4.4). The toxic effect of BHT found in this study did not correspond with a recent report on soybean, where Aho (1990) found that 1% BHT treatment had no effect on germination performance of soybean seeds. Possibly the 15 min soaking in DMSO solution in Aho's study may be not enough to allow the chemicals to reach the embryo. According to Woodstock *et al.* (1983), application of vitamin E (a mixture of α,β,γ-tocopherol) or BHT to different species of small seeded vegetables, produces variable results. They found that, in some cases, germinability after 6 years storage was markedly improved by both antioxidants. 2.2% BHT treatment had protective potential on onion, but it caused damage to pepper. A high concentration (2%) of vitamin E was toxic to both onion and pepper. In all

species, low concentrations (0.8% Vitamin E or 0.2% BHT) showed no or little effect. Although soybean is an oily seed like onion, it responds to the treatments applied in a similar fashion to pepper seeds; however, the toxic effects observed in this study are less marked than those in Woodstock's report.

In the present study, BHT (2.2%) reduced normal germination and seedling weights of unaged material (Figures 4.2 and 4.4, Table 4.3, Appendix 4.3), and 1% α-tocopherol occasionally increased the time to 50% radicle emergence (Table 4.1) and decreased fresh weights of normal seedlings (Table 4.3). It could be that α-tocopherol may reduce rate of water absorption of seeds, due to its lipid properties, while BHT seems to specifically inhibit root growth, resulting in retarded roots (Plates 4.1B and C). This latter observation corresponds with Bekendam and Grob (1979), who described that chemical toxicity is a possible cause of retarded seedling root growth; however, BHT is not mentioned specifically. Similarly, Parrish and Bahler (1983) also found harmful effects of PG (propyl gallate) and TBHQ (monotertiary butylated hydroquinone) on soybean seedling growth, but these antioxidants proved to have some protective value on germination during storage at 40°C, 8% SMC. In contrast, treatment of soybean with 100 mM PG prior to 6 months storage at ambient temperatures, 13% SMC decreased germinability (Parrish and Bahler, 1983). The toxic effects of BHT disappeared during ageing under both CD and AA conditions (Figure 4.2, Appendix 4.3). This is in agreement with the report on the related compound BHA by Okundaye (1977), who found that treatment with this chemical reduced seedling weight of unaged seeds, but that the toxic effect was not detected after 90 days storage at 40°C, 30% RH.

Explanations for these variable results may include the following:

i) damage caused by BHT can be repaired when SMC is increased, as occurs during CD or 3-5 d AA where SMC's were around 20% or more (Figure 4.2, Appendix 4.3). This was also supported by data presented in Figures 4.4, 4.8 and 4.9 showing that high SMC seeds (1-3 d AA or 0 d CD) are capable of some repair or invigoration despite elevated temperatures. High SMC may increase the rate of growth in normal seedlings (Figures 4.4 and 4.9) or amend some defect of abnormal seedlings resulting in improved normal germination (Figure 4.8). This type of finding is explored further in Chapter 5.

ii) It may also be the case that loss or improvement of vigour or germination is the outcome of interactions between both damaging and protective effects of these antioxidants.

Because antioxidants are expected to prevent membrane damage (*e.g.* Basu and Rudrapal, 1980), one possibility was that antioxidants might have prevented membrane damage even though there was no protection of viability. Data on conductivity of seed leachates suggested that this was not the case, since there was no evidence of changes in these measurements being induced by antioxidants (*e.g.* Figure 4.7).

4.3.1.2 β-*Mercaptoethanol and iodine*

Although β-mercaptoethanol (1-20 *ppm*) has been reported to prevent the loss of viability of tomato seeds (Francis, 1985), beneficial effects of β-mercaptoethanol on soybean were not found in this study. Similar negative results were reported in maize by Barnes and Berjak (1978) while Sharma (1988) and Coolbear (personal communication) were unable to find any protective effects on wheat and tomatoes, respectively. Differences in responses may depend on the species and/or seed lots used and be related to factors like endogenous levels of antioxidants and type of damage sustained during deterioration.

Iodine is another type of chemical antioxidant which has been used to avoid solvent toxicity and/or water soaking injury. In the study here, application of 0.1% iodine to a single seed lot under different ageing regimes (Figure 4.13) and also to different seed lots under the same storage regime (Figures 4.13-4.15) had no effect on any aspects of seed performance with the exception of when lot A-2 was held at 40°C, ~100% RH, where iodine showed promising effects of protection of viability loss on 5 d aged seeds. However, a higher concentration of iodine (1%) used for a longer period (24 h) caused loss of viability (Figure 4.15) and vigour (Appendix 4.9). These results are not in agreement with the report of Dey and Mukherjee (1984) who found that 0.1% iodine treated for 2 h in $CaCO_3$ powder protected soybean and sunflower seeds from vigour and germination losses during ageing at 40°C and 90% or 36% RH. They also reported that the high concentrations of iodine (up to 2%) did not cause harmful effects to soybean, although the advantage gained was less than with 0.1%. Successful use of

iodine treatment for prolonging the storage life of soybean was associated with protection of losses in membrane integrity and/or dehydrogenase activity (Dey and Mukherjee, 1984). Similarly, Basu and Rudrapal (1980) also reported these types of changes in mustard as a result of iodination. Because these antioxidants used were not effective in the lots of soybean used here, attempts to investigate any related changes at a molecular level were not included in this study.

4.3.1.3 *Actions of applied antioxidants*

It is unclear why all antioxidants used in this study were not effective. Possibilities for their ineffectiveness may be:

i) antioxidants may not reach the site of action,
ii) antioxidants may not be completely soluble in the solvents used,
iii) endogenous antioxidants may be already sufficient, or
iv) the mechanisms of deterioration in those seeds used in these studies may not involve peroxidation.

The idea that applied antioxidants cannot reach the embryo is supported by the evidence of little penetration of acetone into undamaged seeds (Plate 4.3: as shown by the movement of solute dye (methyl red) in acetone; Shortt and Sinclair, 1980; Halloin, 1977). However, whether penetration of chemicals and transport to the active site in the required concentration has occurred is difficult to evaluate, as discussed in Section 4.3.2.3.

Besides this factor, effectiveness of applied antioxidants may depend on levels of endogenous antioxidants in seeds. According to Porter and Wagner (1986), endogenous or natural antioxidants are consumed in the process of quenching free radicals, and levels would be expected to decline considerably under conditions where lipid peroxidation continues. As a result, additional applied antioxidants would be expected to be effective. The literature, however, is inconsistent on the role of endogenous antioxidants, as determinations of levels of endogenous antioxidants in seeds during ageing have given variable results (reviewed in Chapter 2). Declines in α-tocopherol associated with viability losses have been found in maize, mustard (Dey

and Mukherjee, 1988), cotton, castor bean or sesame (Sharma, 1977), but not in wheat (Fielding and Goldsworthy, 1980). For soybean, Priestley *et al.* (1980) found no changes in levels of total tocopherol nor the proportions of their principal homologues (α,γ,δ) during either slow or rapid ageing conditions. Further, they also reported no changes in organic radical levels throughout the course of storage. These findings support both the ideas that seeds have enough endogenous antioxidants so that when extra antioxidants are added they may be in surplus resulting in inhibitory effects, and that mechanisms of seed deterioration may not always involve lipid peroxidation. Although it was not the objective here to determine levels of natural antioxidants, the results on lipid changes during ageing reported in Chapter 6 may provide a reason why antioxidants were not effective.

4.3.2 **Effects of solvents**

4.3.2.1 *Effects of acetone used as a solvent for delivering antioxidant to seeds*

Under all ageing conditions acetone, either alone or as a solvent for antioxidant, caused significant toxic effects on seed performance in both cultivars Amsoy and Davis. Effects were more evident on aged, high SMC or cracked seeds (*e.g.* Figures 4.2, 4.3 and 4.12). A few similar observations have been reported by other workers in different species (*e.g.* soybean: Parrish and Bahler, 1983; pepper: Woodstock *et al.*, 1983; pea: Gorecki and Harman, 1987; wheat, lentil and carrot: Dadlani and Agrawal, 1985), but, in contrast, most reports in the literature show no toxic effect of acetone on germination or viability even for long periods of treatment (*e.g.* various species: Milborrow, 1963; pea: Meyer and Mayer, 1971; soybean: Lewis *et al.*, 1979 and Shortt and Sinclair, 1980) or in connection with ageing (lettuce: Khan *et al.*, 1973; maize and mustard: Dey and Mukherjee, 1988).

4.3.2.2 *Effects of acetone on other aspects of seed performance*

Different aspects of seed performance indicated the heterogeneous responses of seeds in that acetone tended to cause damage to seed viability before normal germination (*e.g.* Figures 4.1 and 4.2, Table 4.5), while weights of residual normal seedlings remained unchanged (*e.g.* Figure 4.4). This contrasts with usual ideas suggesting that

losses in vigour occur earlier than losses of germinability and then viability during ageing: here abnormals and/or low vigour seeds are severely damaged before higher vigour ones are affected at all. Furthermore, changes in fresh but not dry weights of normal seedlings for acetone soaked seeds after AA (Figure 4.9) imply that acetone treatment may also affect water relations during germination.

Interestingly, T_{50} data, often used as a sensitive vigour index (*e.g.* Coolbear *et al.*, 1984) showed a lack of reproducibility between experiments in this present study (Table 4.1, Appendix 4.2) and are thus too unreliable to be used for evaluating the effects of acetone treatments. This may be due to the following confounding factors:

i) treatments causing variation in moisture contents of unaged seeds (Appendix 4.10). In earlier experiments (Expts. II-IV) involving accelerated ageing, seeds were not re-equilibrated after pretreatment with acetone alone or antioxidants in acetone. Data for actual seed moisture contents after treatment are shown in Appendix 4.10. It was thought possible that this may have affected the rate of change of seed performance during ageing, since at high initial SMC seeds lost their germinability due to ageing faster than seeds of low SMC (Figure 4.3 and Appendix 4.4, compared between different SMC's of untreated controls). However, results in experiments VI (where SMC's were equilibrated to similar levels before ageing) show that interactions between treatments and seed moisture are in fact related to SMC's before treatment and not the SMC's attained afterwards.

ii) a variation in sensitivity of soybean seeds to imbibition injury during the germination test which may mask the effects of treatments in some cases.

iii) variations in ability of seeds to undertake repair at early stages of ageing (Figure 4.4).

4.3.2.3 *Acetone soaking durations and drying*

The time course of acetone treatment (Figure 4.8 and Table 4.5) clearly indicated that toxic effects of acetone are related to durations of soaking and ageing. The longer the

soaking duration, the more pronounced were toxic effects, particularly on aged seeds. According to Milborrow (1963), seeds of various species were safe from acetone toxicity even though seeds were soaked for up to 3 months, provided that solvent was removed before sowing. The present results (Table 4.6) show that drying did not alter the toxic effects of acetone, and adverse effects of acetone were apparent even when seeds were soaked for only 2 h (Figure 4.8). Lewis *et al.* (1979) provided contrasting evidence, as they did not find toxic effects of acetone in 4 cultivars of soybean even when seeds were soaked for 24 h.

Brewer and Wilson (1975) reported in lettuce that the absence of toxic effects of acetone was due to the failure of solvent penetration to embryo tissues. It is questionable whether acetone penetrated into embryos of those seeds used by Lewis *et al.* (1979) or not. Although, by determining changes of seed weights, Lewis *et al.* (1979) claimed that seeds took up considerable amount of acetone when soaking durations were prolonged, it does, of course, not guarantee that acetone actually penetrated into the seeds. The results in Figure 4.12 and Plate 4.2 also demonstrate that seeds with less mechanical damage can resist detrimental effects of acetone soaking even at the higher moisture levels. In other words, mechanically damaged seeds were prone to acetone damage because acetone could penetrate deeper into damaged seeds (Plate 4.3) and kill those tissues it reached. The results are in agreement with Shortt and Sinclair (1980), who found that acetone killed cotyledonary tissues in soybean seeds with cracked seed coats. The findings of Halloin (1977) also showed no toxic effects of acetone on germination of sound seeds of cotton, but excised embryos or damaged seeds were injured by acetone treatment. He also demonstrated that acetone caused injury only when it reached the embryos. On the contrary, Tao and Khan (1974) reported that, although acetone reached the embryos of cucumber or squash seeds, it did not cause any damaging effect on seed germination.

The evidence in this study based on methyl red penetration (Plate 4.3) indicated that acetone accumulated only around the seed coat of intact seed without or with little penetration. The results found in intact seeds here do not conform to O'Neill *et al.* (1979), who reported that the quantity of ethazol (dye) infused into soybean seed after 1 h immersion was directly proportional to the ethazol concentration in acetone treatment. When treated for longer times, greater quantities of ethazol were not

infused into the seed, but deeper penetration of ethazol occurred. They did not, however, mention the quality of the seeds used. Relationships between permeation time and the depth of chemical penetration were also reported by Khan *et al.* (1973) and Tao and Khan (1974) for lettuce. However, in the present study there is a query whether methyl red was left behind the acetone front as it moved into the seed, because it was also observed that while both high and low moisture seeds showed a similar pattern of methyl red movement, the high SMC seeds were damaged more than the low SMC material.

4.3.2.4 *Interactions between seed quality and moisture content and susceptibility to acetone*

According to data in Figure 4.10, three out of four seed lots were not significantly affected by a 16 h acetone soak when seeds were germinated immediately, but acetone caused significant damage to aged seeds of all seed lots. Further, seed lot A-3 which was not affected by acetone toxicity (Figure 4.10 before ageing), became susceptible to acetone (Figure 4.11) after another 3 months in store, suggesting rapid loss in vigour of this seed lot. It is possible that critical stage of seed deterioration had begun during that period. This indicates that acetone causes hidden damage to seeds which can be repaired if seeds were germinated immediately but cannot be prevented/repaired after ageing. It is possible that Lewis *et al.* (1979) used only high vigour lots in their study, and thus hidden damage effects remained undetected.

Although the response differences between seed lots to acetone did not relate to seed moisture contents (Figure 4.10), the most interesting observation obtained in this study was the results of soaking seeds of the same lots at different initial moisture contents. The data presented here (Figures 4.11 and 4.12, Plate 4.2) demonstrate that acetone toxicity could be reduced if seed moisture contents were lowered. At around 6%, all seed lots tested become more tolerant to 16 h soaking in acetone. However, it must be remembered that hidden damage still shows up after ageing (Figure 4.6). These results generally agree with our findings in peas (Coolbear *et al.*, 1991), where a study of different pea cultivars showed that the most susceptible cultivar became tolerant to a 16 h acetone soak when seed moisture was reduced to below 8%.

These data are quite different from those of other workers, as the other workers did not use the approaches like these to evaluate the effects of acetone. Only two relevant reports were found in the literature. Both mentioned increased toxicity of solvents when relatively large amounts of water were added to the solvent itself. Lewis *et al.* (1979) found that acetone diluted with 20% water reduced germination of cotton seed in the glasshouse and of soybean in the field while pure acetone did not. Similarly Persson (1988) reported that *Brassica* and *Linum* species showed an increase in the degree of ethanol toxicity when the water content of solvent was increase from 0 to 10%. Note that contaminants in the acetone seem not to be a cause of toxicity in this study, because we had shown that redistilling the normal stock solvent did not ameliorate toxicity effects (Coolbear *et al.*, 1991).

All these results emphasise that the interactions between mechanical damage and moisture contents are of crucial importance in the resistance of seeds to acetone treatment. This was supported by the results from tetrazolium test (Plates 4.4-4.5).

An understanding of how acetone causes damage to seeds may facilitate the success of seed treatment for soybean storage. While Meyer and Mayer (1971) found that acetone depressed oxygen uptake, Eldan and Mayer (1974) reported a decrease in activity of invertase, although germinability was unaffected. It has also been proposed that acetone causes injury to aged or mechanically damaged seeds by disrupting some inherent protection against ageing (Woodstock *et al.*, 1983) or by disrupting the lipid system (Halloin, 1977). This latter idea is supported by the evidence of a close association between loss of normal germination and increased leachate during ageing (*e.g.* Figure 4.7). From the results presented here, it is speculated that penetration of acetone into cell membranes may result in disruption of the crystalline lipid bilayer which exists in dry tissue (Crowe *et al.*, 1989). This disruption could be exacerbated in higher SMC seeds if the solvent can bring water molecules into the apolar region of the membrane. High vigour seeds may have some capacity for repair of this kind of damage, but this may become increasingly difficult for low vigour or ageing seeds. Evidence of lipid and membrane changes caused by acetone and ageing is reported in Chapters 6 and 7.

The use of a solvent with a lower dielectric constant such as hexane avoids toxicity problems (Figure 4.5). Hexane could be safer than acetone because of its ability to

penetrate dry membranes without disrupting the amphoteric structure of the bilayer. This idea was supported by Jain and Shivanna (1988a and b), who demonstrated that although neither acetone nor hexane showed toxic effects on viability of pollen grains, acetone caused more extensive leaching of phospholipids than hexane. To date, there are no other reports of the use hexane as a solvent for seed treatment, but its cost and toxicity to humans may prohibit its routine use.

4.4 PRELIMINARY CONCLUSIONS

From these results and discussion on evaluations of effectiveness of different antioxidants and the toxicity of solvents used as a carrier, several conclusions can be drawn as follows:

1) There were no protective effects of any antioxidant treatments used on stored soybean seeds of either *cv.* Amsoy or Davis.

2) Possible reasons of ineffectiveness of antioxidant treatments may be:

   i) antioxidants cannot reach the active site,
   ii) levels of endogenous oxidation inhibitors may be sufficient for protection, or
   iii) mechanisms of seed deterioration may not involve lipid peroxidation.

3) Levels of acetone toxicity vary with seed lot, levels of mechanical damage and seed moisture contents.

4) Although toxicity of acetone could be reduced by lowering initial moisture contents of seeds before treatment, hidden damage was still observed after subsequent storage.

5) Hexane can be used as an alternative solvent for chemical seed treatments; however, the costs of this solvent and its harmfulness to humans should be considered.

CHAPTER 5

HYDRATION-DEHYDRATION TREATMENTS

5.1 INTRODUCTION

In the literature, there is evidence showing promising effects of hydration-dehydration treatments for either the protection of seeds against ageing or for the repair of aged seeds (reviewed in Section 2.3). In particular, Saha and Basu (1984) noted that moisture equilibration (ME) and/or soaking showed beneficial effects in soybean. In this chapter the effects of hydration-dehydration treatments including moisture equilibration, soaking or imbibition and their interactions with subsequent accelerated ageing were identified for different seed lots of *cvs.* Amsoy and Davis in an attempt to improve their storage life. Attempts were also made to assess the effectiveness of moisture equilibration treatments for repair of damage incurred as a result of previous slow or accelerated ageing.

5.2 RESULTS

Results presented are divided into two main sections, firstly pre-storage and then post-storage treatments. For ease of reference, a summary of effects of hydration treatments is given in Table 5.0, with details of results following.

Table 5.0 A summary of results on hydration treatments

Type of ageing	Treatment	Seed lot	Effect on germination performance	References
Pre-storage treatments				
AA	24 or 48h ME	A-3	no effect	Figure 5.1
		A-5	damage	
		A-6	improvement on unaged seeds	
AA	24h ME, 24h ME+S	A-7	ME+S caused damage	Figure 5.2
		A-8	no effect	
	24, 48 or 72h ME, 24h ME+S	Davis	ME+S and 72h ME– caused damage	
AA	24 or 48h ME, 24h ME+S followed by SS	Davis	SS caused damage 48 h ME reduced SS damage	Figure 5.3
AA	S, SS	Davis	damage	Figure 5.4
AA	1, 3 or 5h IB±SS	Davis	damage	Section 5.2.1.2
Post-storage treatments				
A. Laboratory trial				
Slow ageing	72h ME±drying	Davis	improving effects	Figures 5.6 and 5.7
AA	72h ME±drying	Davis	ME without drying caused improving effects	Figure 5.8
B. Soil trial				
Slow ageing	± 72h ME	Davis	occasionally caused improving effects	Figure 5.9

ME = *moisture equilibration*
S = *soaking*
SS = *surface sterilisation*
IB = *imbibition*
AA = *ageing at 40°C, ~100% RH for up to 6 days*
Slow ageing = *ageing at 9% SMC, 35°C for up to 15 weeks*

5.2.1 Effects of pretreatments

5.2.1.1 *Responses of different seed lots to ME treatments*

The moisture contents of different seed lots before or after hydration treatments without and with drying back are shown in Table 5.1. Seed moisture contents increased as the duration of moisture equilibration increased, but rates of water uptake varied considerably with seed lot. For example, after 24 h ME, lots A-6 and Davis had the highest rates of water uptake (4.9%.day^{-1}), while lots A-3 and A-7 gained water at only just half of this rate. The initial SMC did not seem to affect rate of water uptake during ME (*e.g.* compare A-7 and A-8 or A-3 and Davis). ME for 24 h followed by 2 h soaking, regardless of seed lot differences, raised the moisture content up to around 50% (Table 5.1). After drying, the SMC's attained depended on drying methods rather than seed lots. The SMC's of lots (A-3, A-5 and A-6) dried at 25°C for 4 d ranged 7.5-8.0% which were lower than those of lots (A-7, A-8 and Davis) dried at ambient (8.3-11.6%). Except for lots A-3 and A-6, the SMC's of hydrated-dried seeds were similar to those of untreated controls.

The effects of different ME durations on germinability of seed lots both before drying and during subsequent accelerated ageing after drying back are shown in Figure 5.1. There was no significant ($P<0.05$) effect of ME on normal germination of lot A-3 at any time. However, both 24 and 48 h ME treatments significantly ($P<0.05$) improved germinability of unaged seeds of lot A-6 by 10 and 15%, respectively, and the benefits of the treatments were still retained even after drying. There was repair effect of 2 d ageing noted on all treatments of lot A-6, this effect allowed untreated but aged seeds to perform extremely well resulting in no protective effects of the treatments observed on subsequent ageing. In the poor quality seed lot A-5, there was no significant enhancement of performance as a result of ME and, unsurprisingly, no normal germination after 2 d ageing. Drying showed a small, but significant ($P<0.05$) damaging effect on the surviving seeds of this lot after ME.

Table 5.1 Moisture contents (% fresh weight basis) of six seed lots subjected to different hydration treatments. Data presented are means of 4 replicates (S.E.'s are given in brackets below each mean).

Treatments	Seed lot					
	A-3	A-5	A-6	A-7	A-8	Davis
Untreated control	11.6 (0.08)	8.3 (0.00)	10.6 (0.09)	12.0 (0.05)	12.2 (0.05)	9.3 (0.03)
Untreated + D	- -	- -	- -	8.2 (0.05)	8.9 (0.06)	- -
24 h ME	14.3 (0.08)	12.1 (0.13)	15.5 (0.10)	14.6 (0.09)	16.0 (0.17)	14.2 (0.22)
24 h ME + D	7.5 (0.07)	7.6 (0.06)	7.9 (01.05)	8.3 (0.04)	9.0 (0.06)	8.9 (0.03)
48 h ME	16.2 (0.14)	14.4 (0.26)	18.8 (0.21)	- -	- -	18.0 (0.48)
48 h ME + D	7.5 (0.05)	8.1 (0.03)	8.0 (0.06)	- -	- -	9.2 (0.03)
72 h ME	- -	- -	- -	- -	- -	19.9 (0.49)
72 h ME + D	- -	- -	- -	- -	- -	9.6 (0.06)
24 h ME + Soak	- -	- -	- -	51.3 (0.72)	47.1 (0.23)	47.7 (0.78)
24 h ME + Soak + D	- -	- -	- -	8.5 (0.04)	11.6 (0.24)	8.5 (0.04)

ME = *moisture equilibration at 25°C, ~100% RH.*
Soak = *submerge seeds in water at room temperature for 2 h.*
D = *drying under the conditions given below:*
- *lots A-3, A-5, and A-6 at 25°C for 2-3 days*
- *lots A-7 and A-8 at ambient for 4 days*
- *Davis at ambient in laminar flow cabinet for 3 days (fan on occasionally).*

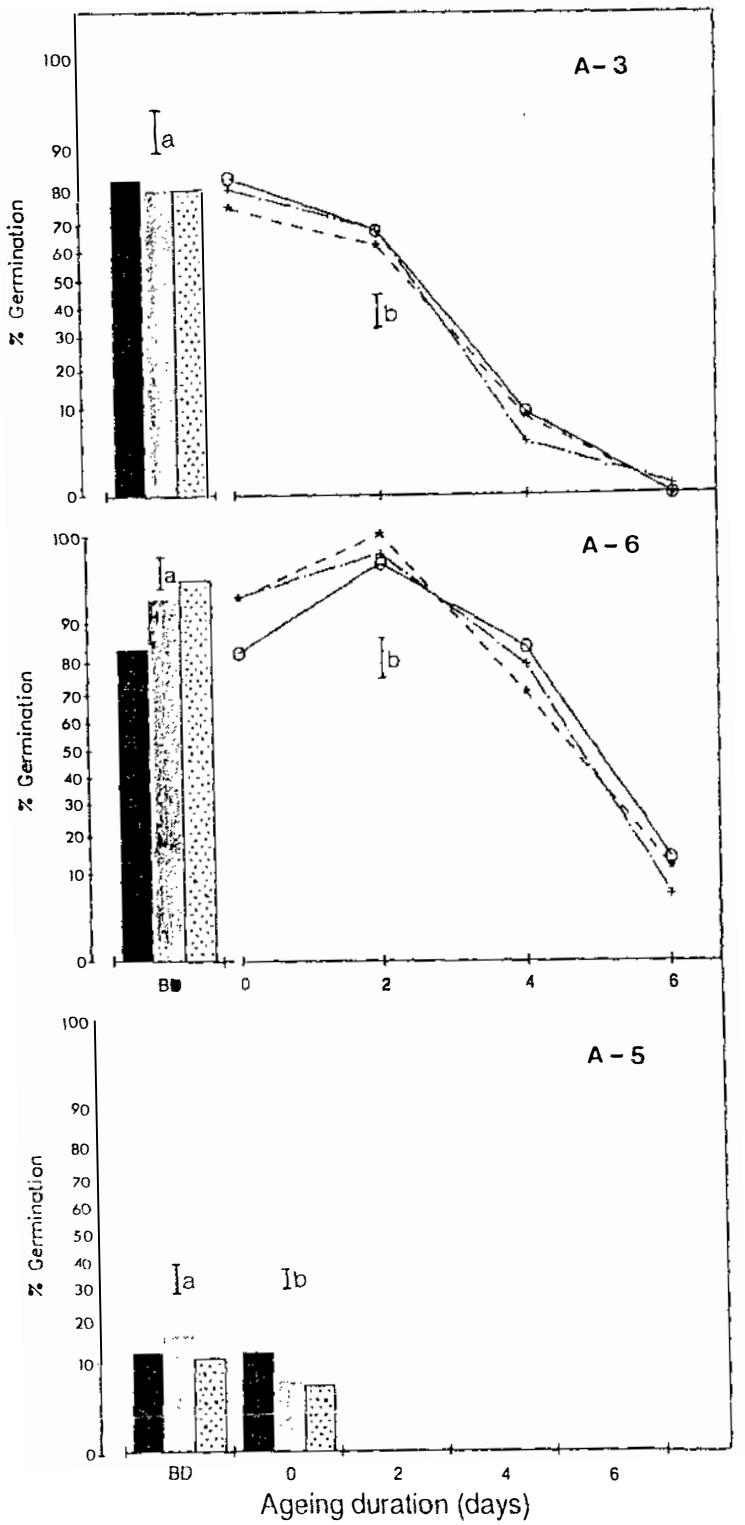


Figure 5.1 The effects of 24 or 48 h moisture equilibration (ME) at 25°C on germinability of three soybean seed lots of *cv.* Amsoy and interactions with subsequent accelerated ageing (40°C, ~100% RH) after drying back. Data presented are arcsin √% transformed means of 4 replicates. ■ and —○—: Untreated controls; ▨ and ·—+—·: 24 h ME; ⬚ and - -*- -: 48 h ME. BD= before drying, bars represent least significant differences (P= 0.05) (a) between treatments before drying (BD), 6 degrees of freedom and (b) between any two single means during ageing (df= 33 for lots A-3 and A-6, and 6 for lot A-5).

Again, there were no significant effects of 24 h ME treatments on seed lots A-7, A-8 (Figure 5.2) nor for Davis after 24 or 48 h ME (data not shown). In all these cases moisture contents were well below the 20% attained in Saha and Basu's (1984) work. Accordingly, the effects of 72 h ME treatment were also assessed on Davis. This duration of treatment allowed 20% SMC to be reached (Table 5.1), but no beneficial effects were observed. Although 72 h treated seeds did not exhibit any differences in germinability from untreated seeds before ageing, they lost germinability more rapidly (Figure 5.2). Another problem was that ME treatment, particularly for 72 h increased the level of fungal infection observed after 6 d ageing.

Except for lot A-6 (viability was unaffected), treatment effects on viability (data not shown) were similar to those of germinability. Growth of normal seedlings was always unaffected by any ME-drying treatments, although it was reduced by ageing (Appendix 5.1).

5.2.1.2 *Effects of soaking treatments*

As an alternative method of raising seed moisture levels during treatment, 2 h soaking following 24 h ME was applied to seed lots A-7, A-8 and Davis; and the resulting germinability data are shown in Figure 5.2. A significant ($P<0.05$) reduction of germinability due to ME-soaking treatment was found on seed lot A-7, while ME-soaking followed by drying caused damage to Davis even on unaged seeds. In all seed lots, viability responded to the treatments similarly to germination, but treatments had no effect on seedling weights (Appendix 5.1).

In an attempt to avoid problems of mould infection during ageing, surface sterilization was applied after ME using the Davis seed lot. Surface sterilization significantly ($P<0.05$) reduced the subsequent ageing tolerance of seeds irrespective of whether moisture equilibration treatments had been applied (Figure 5.3). However, prior ME treatments, especially 48 h, significantly reduced the rate of germination loss due to surface sterilization as compared to untreated sterilized seeds (Figure 5.3). Figure 5.4 shows that surface sterilization and water soaking treatment significantly ($P<0.001$) caused damage to seeds at similar levels, suggesting that surface sterilization caused damage as a result of soaking injury rather than any toxicity of sodium hypochlorite itself.

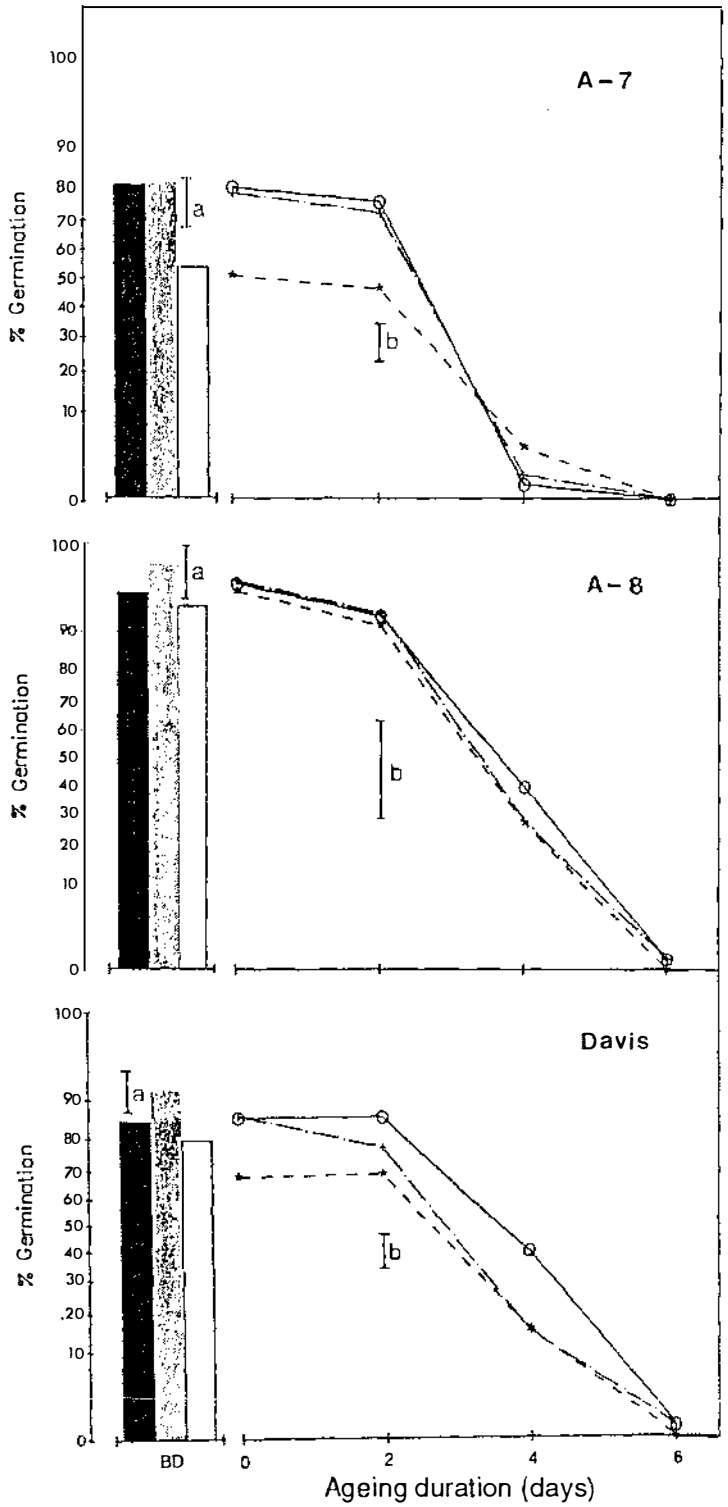


Figure 5.2 The effects of 24 or 72 h moisture equilibration (ME) at 25°C or 24 h ME followed by 2 h soaking in water on germinability of three soybean seed lots of *cvs.* Amsoy or Davis, and interactions with subsequent accelerated ageing (40°C,-100% RH) after drying back. Data presented are arcsin √% transformed means of 4 replicates. ■ and —○— : Untreated controls; ▩ and - +- - : 24 h ME for A-7 and A-8, 72 ME for Davis; □ and - -*- - : 24 h ME followed by 2 h soaking in water. BD= before drying, bars represent least significant differences (P=0.05) (a) between treatments before drying (BD), 6 degrees of freedom and (b) between any two single means during ageing (df= 24 for lot A-7, and 33 for lots A-8 and Davis).

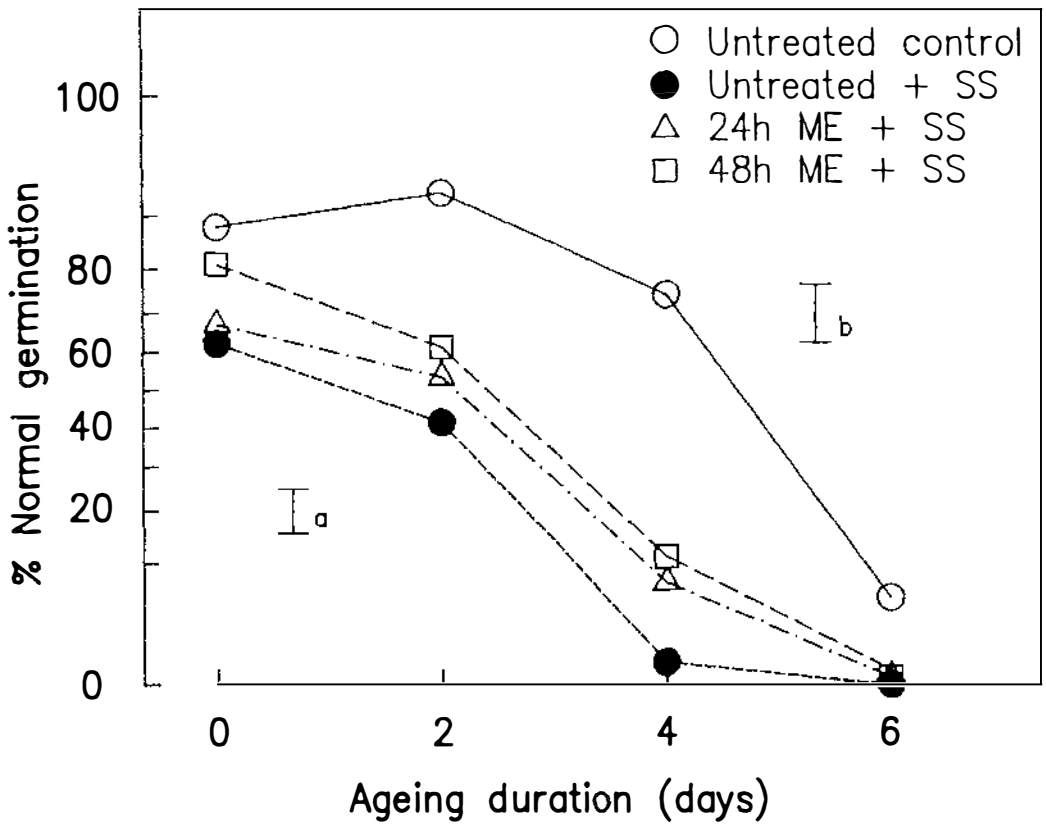


Figure 5.3 The effects of 24 or 48 h moisture equilibration at 25°C followed by surface sterilization (SS) on germinability of soybean seeds, *cv.* Davis, during subsequent accelerated ageing (40°C, 100% RH) after drying back. Data presented are arcsin $\sqrt{\%}$ transformed means of 4 replicates. Bars represent least significant differences between any two single means of (a) surface sterilized seeds, 24 degrees of freedom, and (b) of all seeds, 33 degrees of freedom.

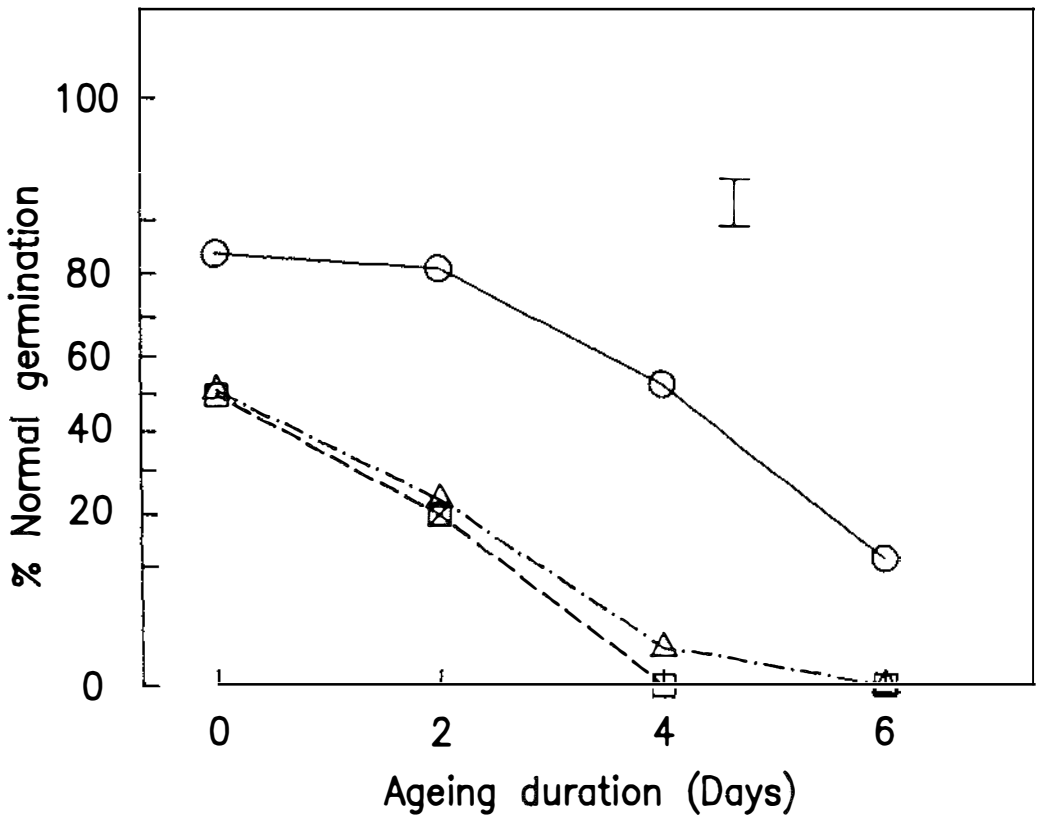


Figure 5.4 The effects of soaking or surface sterilization on germinability of soybean seeds *cv.* Davis. Data presented are arcsin √% transformed means of 3 replicates. ○—○: Untreated controls; △···△: Soaking in water for 30 min; □ - □: Surface sterilization 15 min in NaOCl, 10 min in 0.01N HCl and 3 times in distilled water (5 min) as modified from Abdul-Baki (1974). Bar represents least significant differences (P=0.05) between any two single means (4 df).

In an attempt to speed up the rate of water uptake and also to avoid fungal problems, a preliminary trial using a pre-imbibition treatment of up to 5 h followed by surface sterilization was also conducted on the Davis seed lot. The data from this set of experiments (not shown) suggested that a 5 h preimbibition duration reduced levels of soaking injury due to surface sterilization, however, the storability of these surface sterilised seeds was still poorer than untreated controls. During this study, it was noted that there was a high variation in imbibition rates of individual seeds as shown by the frequency distribution diagrams of Figure 5.5. For example, after 8 h from the start of imbibition, the amounts of water taken up were between 0-200 $mg.seed^{-1}$ with the most common value being 80-100 $mg.seed^{-1}$, while rates of water uptake during 8-11 h imbibition ranged from 0-21 $mg.seed^{-1}.h^{-1}$.

5.2.2 **Effects of treatments on aged seeds**

Both slowly and rapidly aged seeds were used to evaluate repair possible effects of ME treatments, because the deterioration mechanisms incurred under different conditions may be different (reviewed in Section 2.1 Chapter 2). A moisture equilibration treatment for 72 h where the SMC was raised up to around 20% for unaged material (Table 5.1) was chosen as a treatment for repair of aged seeds in *cv.* Davis, since previously Saha and Basu (1984) had reported the effectiveness of ME treatments at this moisture level for repair of aged soybean seeds. However, there was considerable variation in the SMC's obtained after ME treatments between experiments. For example, after 72 h ME the SMC obtained from slowly aged seeds was 18.6 (± 0.3)% higher than that (15.1 ± 0.19%) from accelerated aged seeds. These all were lower than that obtained from pre-storage treatment (Table 5.1).

5.2.2.1 *Slowly aged seeds*

Seeds aged at 35°C and 9% SMC for up to 15 weeks (when normal germination was significantly ($P<0.001$) reduced from 95 to 32%) were treated with ME. Despite no effect on unaged seeds, ME treatments showed significant ($P<0.05$) effects of restoration on 15 weeks aged seeds, where normal germination was improved by more than 60% (Figure 5.6) compared to untreated aged seeds. Neither ageing nor treatments affected viability (Figure 6.2A, data for seeds before drying not shown).

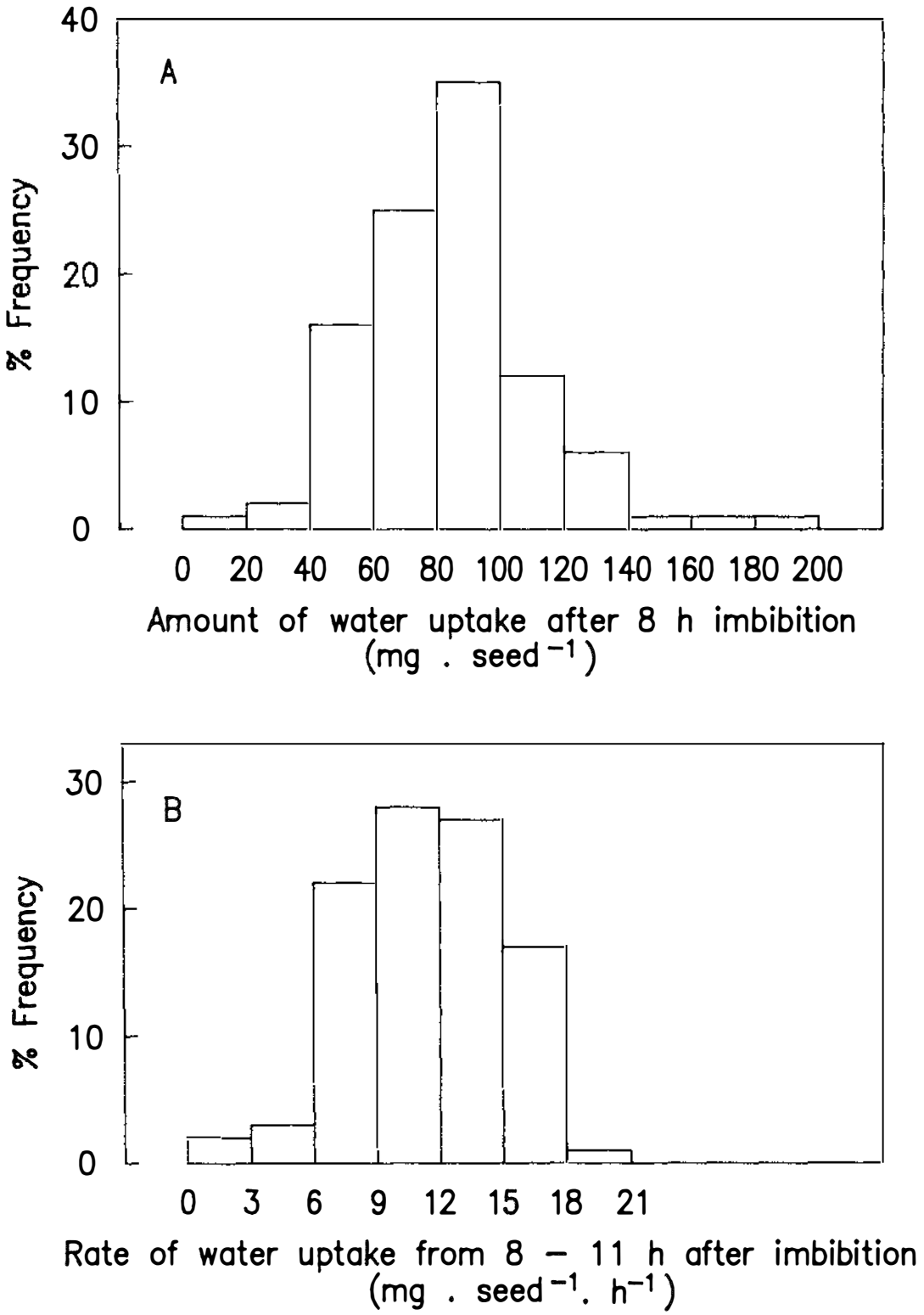


Figure 5.5 Frequency distributions of amount of water taken up by individual seeds at 8 h after the start of imbibition (A), and of rates of water uptake during 8-11 h after the start of imbibition (B).

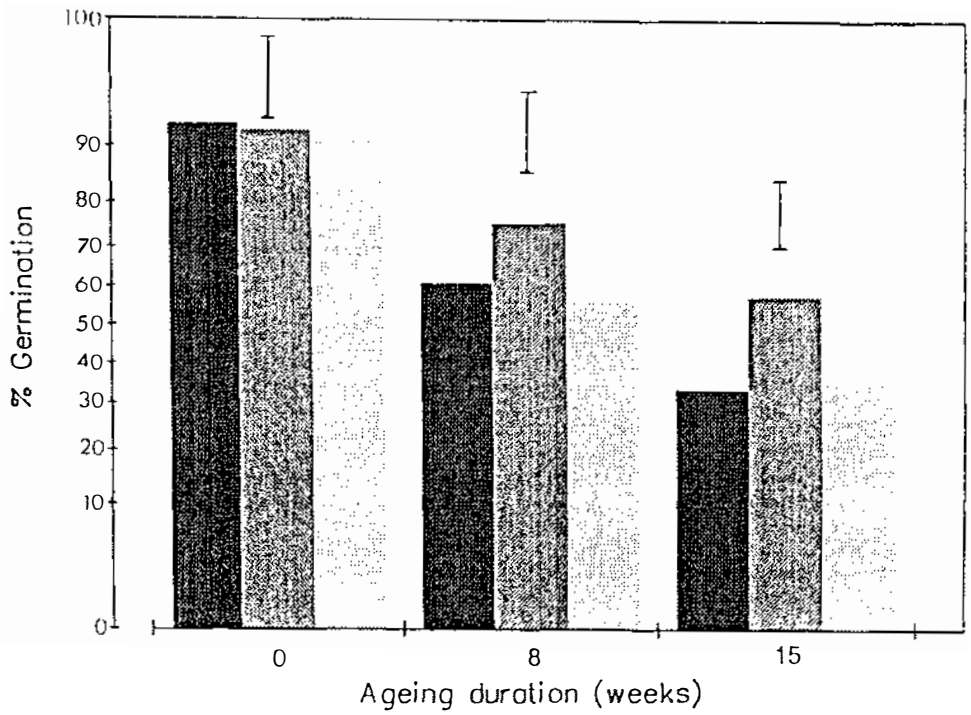


Figure 5.6 The effects of slow ageing (35°C, 9% SMC) alone (■) or followed by 72 h moisture equilibration without (▩) or with drying (□) on germinability of soybean seeds *cv.* Davis. Data presented are arcsin √% transformed means of 3 replicates. Bars represent least significant differences (P=0.05) between treatments at each ageing time (4 df).

Many slowly aged seeds showed primary root abnormalities (Plate 5.1A). It was the frequency of these abnormal seedlings which was reduced by subsequent ME treatment.

There was a significant ($P<0.001$) reduction of both fresh and dry weight of normal seedlings due to slow ageing which were evident after 15 weeks ageing, but ME treatment significantly ($P<0.001$) improved weight of these seedlings in all unaged and aged seeds (Figure 5.7, fresh weight data not shown). Regardless of ageing, seedling dry weights from moisture equilibrated seeds were increased by 20%. The beneficial effects of ME treatments on seedling growth were, however, reduced by drying back (Figure 5.7) as were the effects on germinability (Figure 5.6).

5.2.2.2 *Accelerated aged seeds*

When seeds aged for up to 3 days at 40°C, ~100% RH were used as a material for characterising the effectiveness of ME treatments, there was no significant ($P<0.05$) effects of ageing nor treatments detected (data not shown). As a result, seeds aged for up to 6 days were then used. Seeds lost a significant ($P<0.05$) germinability due to ageing after 4 days, when more than 20% loss was detected, and only 41% normal germinants were noted after 6 d AA. Viability, however, remained unchanged throughout the ageing period (data not shown). On these accelerated aged seeds, neither germinability nor viability was significantly ($P<0.05$) affected by ME treatments (Appendix 5.2).

However, analyses at each ageing period showed that mean fresh weights of normal seedlings were improved >20% and 11-14% on unaged and aged seeds, respectively by 72 h ME treatments (Figure 5.8). Again, as with slowly aged seeds, drying back reversed the advantage of ME treatments. Changes in seedling dry weight showed a similar pattern to fresh weight (data not shown).

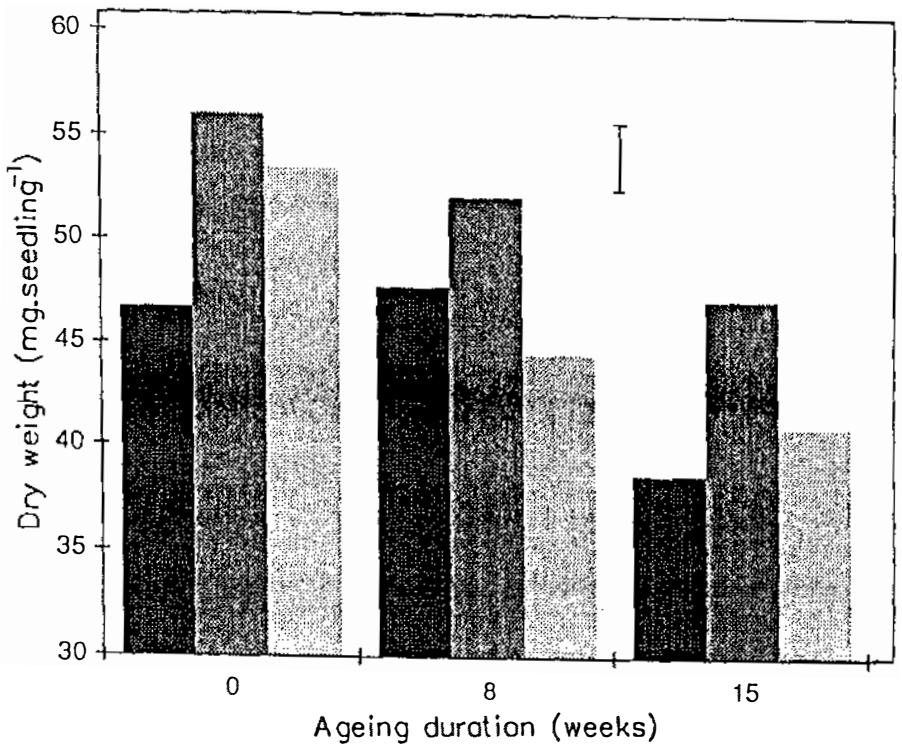


Figure 5.7 The effects of slow ageing (35°C, 9% SMC) alone ( ) or followed by 72 h moisture equilibration without ( ) or with drying ( ) on axis dry weights of normal soybean seedlings, *cv.* Davis. Bar represents least significant differences (P=0.05) between any two single means (22 df).

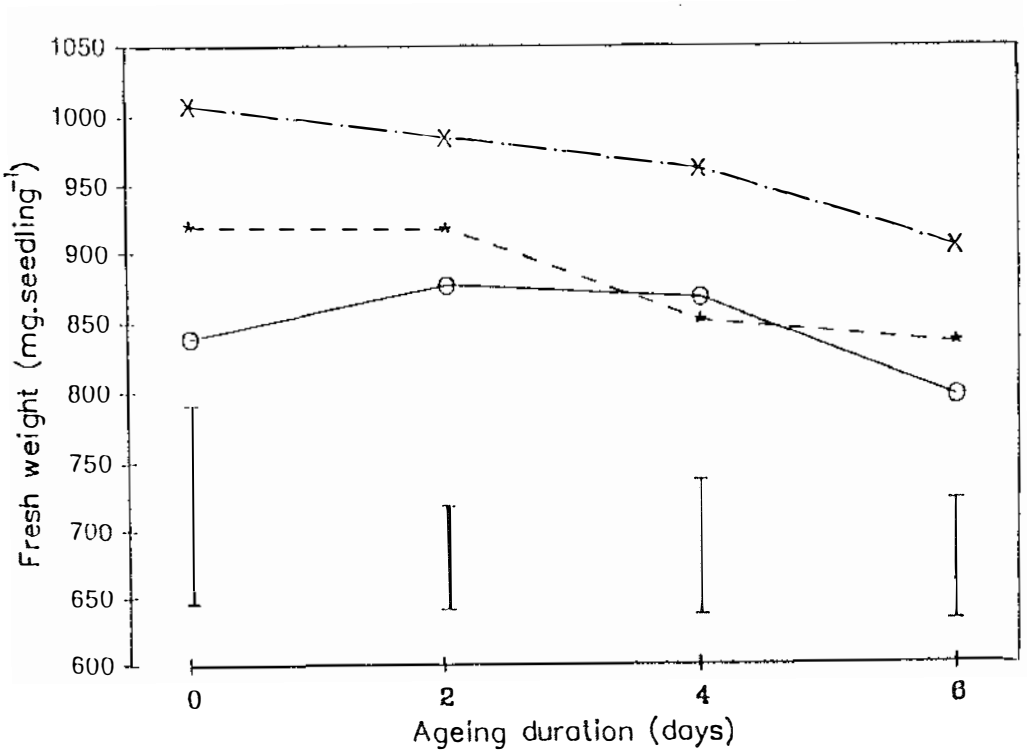


Figure 5.8 The effects of accelerated ageing (40°C,-100% RH) alone or with subsequent moisture equilibration on axis fresh weights of normal soybean seedlings, *cv.* Davis, after 8 days sowing. Bars represent least significant differences (P=0.05) between treatments at each ageing time (6 df). o—o: Untreated control; x- - -x: 72 h ME; *— —*: 72 h ME-drying.

5.2.2.3 *Soil trial*

Since the results from the laboratory test suggested the promising effects of 72 h ME treatment (without drying) for improving normal germination of slowly aged seeds (Figure 5.6), a soil trial was conducted on 15 weeks aged seeds and unaged controls. Although total seedling emergence of 15 weeks aged seeds was significantly ($P<0.05$) lower than that of unaged seeds, both unaged and aged seeds were unaffected by ME treatments compared to untreated controls (Figure 5.9). Neither slow ageing nor 72 h ME treatments had any significant ($P<0.05$) effects on numbers of normal seedlings (based on normal shoot growth as shown in Plate 5.1B) (Figure 5.9) nor weights of seedling axes or roots of those normal seedlings (Appendix 5.3). The rate of establishment of seedlings from soil was not affected by ageing, but ME significantly improved the time to 50% establishment of unaged seeds (Table 5.2).

Table 5.2 The effects of 72 h moisture equilibration (ME) on time to 50% seedling establishment (T_{50}) in soil of unaged or slowly aged (35°C, 9% SMC) soybean seeds, *cv.* Davis. Data presented are means of 3 replicates.

Treatment	T_{50} (d)	
	Unaged	Aged
Control	6.67	7.00
ME	5.67	7.30
$LSD_{0.05}$	0.92	

Note: Least significant difference (LSD) between any two single means at 5%.

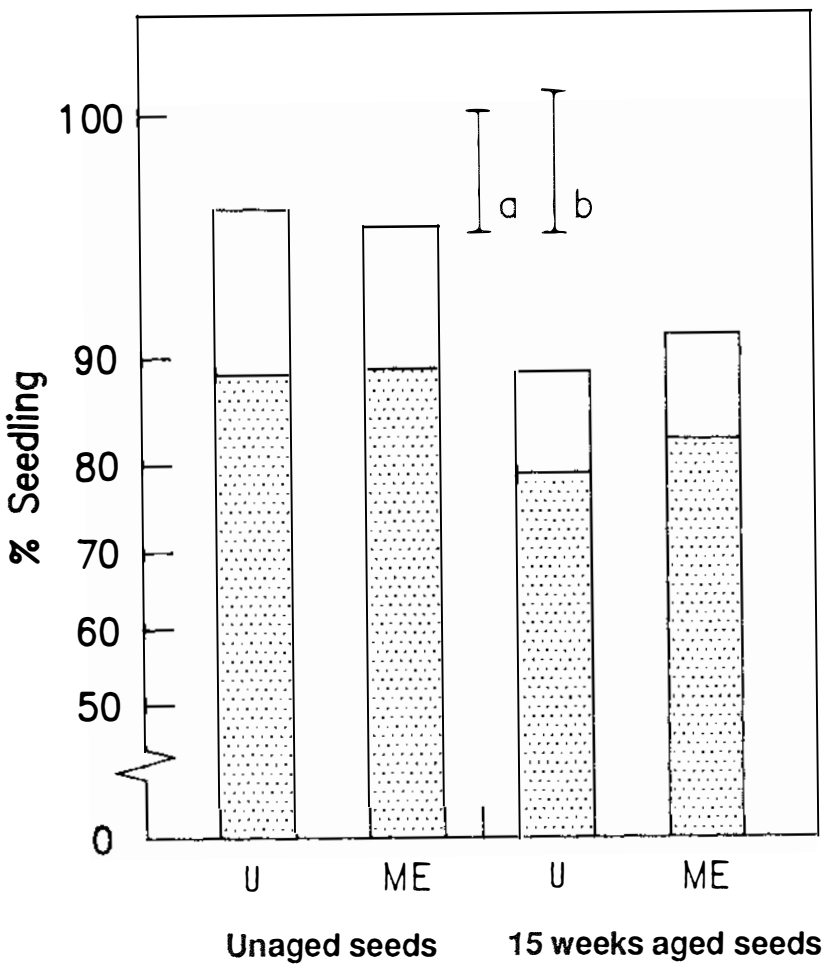


Figure 5.9 The effects of slow ageing (35°C, 9% SMC) alone (U) or with subsequent moisture equilibration (ME) at 25°C for 72 h on the percentages of total seedling emergence (open histogram) and normal seedlings (closed histogram) of soybean, *cv.* Davis, grown in soil and harvested at 17 days after 50% establishment. Data presented are arcsin $\sqrt{\%}$ transformed means of 3 replicates. Bars represent least significant differences (P=0.05) between any two single means, 6 degrees of freedom, of (a) total emergence and (b) normal seedlings.

5.2.2.4 Correlations between conductivity and germination performance

Conductivity measurements were used to determine changes in membrane integrity which may be associated with seed vigour. With one exception, the total correlations between conductivity of leachate (after 24 h soaking) and normal germination or viability were significant ($P<0.01$) in all seed lots tested either with pre- or post-storage treatments (Table 5.3). Interestingly, the exception was *cv.* Davis given treatments before ageing, but without surface sterilisation. Conductivity of leachate from these seeds remained low (6.0-12.3 $\mu S.seed^{-1}$) while seeds lost up to 82% of their germinability.

Table 5.3 Correlation coefficients (r) between conductivity and germinability or viability during ageing for all treatments applied before or after ageing on different soybean seed lots.

Seed lot	Germinability	Viability
Before accelerated ageing treatments		
A-3	-0.86**	-0.90**
A-6	-0.96**	-0.92**
A-8	-0.72**	-0.81**
Davis	-0.27^{NS}	-0.39^{NS}
Davis + SS	-0.73**	-0.75**
After ageing treatments		
Davis after AA	-0.73**	-0.61**
Davis after slow ageing	-0.80**	-0.61**

NS = not significant at P = 0.05
*** = significant at P = 0.01*

Before ageing treatments = 24 and 48 h ME (lots A-3 and A-6) or 24 h ME and 24 h ME + 2 h soaking (lots A-8 and Davis), followed by drying, 34 degrees of freedom.

After ageing treatments = 72 h ME with and without drying after accelerated ageing (34 df) or slow ageing (25 df).

SS = surface sterilization after untreated, 24 and 48 h ME (46 df)

(In all cases untreated controls are included).

5.3 DISCUSSION

5.3.1 **Pretreatment effects and seed lot variations**

The hydration-dehydration pretreatments did not show any protective effects on germinability, viability or seedling growth over untreated controls in all the seed lots tested (Figures 5.1 and 5.2). Moreover, sometimes the treatments, especially those involving soaking, caused damage to seeds. These results do not support the concept that hydration-dehydration treatments have the potential to protect seeds against ageing of various species (*e.g.* Basu, 1976; Savino *et al.*, 1979; Kundu and Basu, 1981; Burgass and Powell, 1984) including soybean (Saha and Basu, 1984). From the literature, the beneficial effects of hydration-dehydration pretreatments are expected to be the detoxification of seeds from damaging free radicals and/or other toxic compounds as well as allowing a gearing up of repair systems to be ready for action so that additional damage incurred during subsequent storage will be prevented. Kundu and Basu (1981) have suggested that ME treatments may primarily counteract free radicals, while soaking may facilitate both removal of free radicals and detoxification. This suggestion was supported by previous evidence in wheat, mustard and maize (Rudrapal and Basu, 1982; Dey and Rudrapal, 1985; Dey and Mukherjee, 1986). All reported evidence that hydration-dehydration treatments reduced physiological deterioration by minimizing lipid peroxidation. However, they only measured changes in malonaldehyde, a secondary product of lipid peroxidation, no direct measurement was made. Additionally, in maize and mustard the treatments were shown to maintain the activities of dehydrogenase and peroxidase enzymes, while minimizing lipase activity and free fatty acid levels (Dey and Mukherjee, 1986). However, the results in the present study are consistent with the findings of Dey and Mukherjee (1986), who claimed, despite no data to support, that only slight improving effects of ME-drying treatment on soybean seeds, and that soaking-drying treatment caused detrimental damage to seeds under three different subsequent storage conditions including 40°C and ~100% RH as used in this study.

Using identical treatments to the present study, Saha and Basu (1984), on the other hand, demonstrated that the effectiveness of the treatments for both protection and repair was vigour dependent. They found that ME-drying had protective effects on

high vigour (1 month-aged) seeds, and had repair effects on medium (6 month-aged) and low vigour (12 month-aged) seeds, while ME-soaking-drying showed both repair and protective effects on those medium and low vigour seeds. Saha and Basu (1984), however, used sub lots of a single soybean seed lot which had been stored for different periods to vary seed quality, the germinabilities of the resulting 'high', 'medium' and 'low vigour' seeds being 76, 76 and 67%, respectively. In this study, the normal germination of 5 seed lots used ranged from 79-99% plus one lot of 12% (Table 3.1).

A possible explanation for the contradiction between the results presented here and those of Saha and Basu (1984) seems to be seed lot and cultivar differences. The inefficacy of hydration-dehydration treatments in this study cannot be simply explained in terms of differences in either initial or obtained SMC's. If the removal of free radicals is the key effect of hydration, SMC's above 12% should be high enough to quench or scavenge free radicals (Priestley *et al.*, 1985). If so, the high initial SMC's of seed lot A-3, A-7 and A-8 (10-12.2%) might be the cause of treatment ineffectiveness. However, the initial SMC of Davis at 9.3% was similar to that of the seeds used by Saha and Basu (1984).

Only the results from lot A-6, which showed improving effects of ME treatments on germinability of seeds prior to ageing (Figure 5.1) are in agreement with the results from medium and low vigour seeds in Saha and Basu's (1984) study. It is possible that damage incurred in lot A-6 during 2 years storage at 5°C may be similar to that in seeds, which Saha and Basu (1984) stored for 6 and 12 months (medium and low vigour) to vary seed vigour; and such damage can be repaired by ME treatments. It is speculated that the seed lot that Saha and Basu (1984) used may have experienced conditions that allowed seeds to accumulate some damaging free radicals, and the benefit derived from hydration treatments was thus caused by their removal.

In seed lot A-6 ME treatment improved only normal germination, but not viability or seedling growth. This suggests that repair mechanisms occurring in this seed lot may be limited to abnormal germinable seeds. Much evidence has suggested that hydration-dehydration treatments can improve only some aspects of seed quality (*e.g.* germination rate in tomato: Coolbear *et al.*, 1984; Argerich *et al.*, 1989 or in soybean: Knypl and Khan, 1981).

Another factor which may contribute to the success of ME treatments is ME duration. If the treatment duration is too short, the required SMC will not be achieved. Saha and Basu (1984) showed that 24 h was long enough to raise the SMC of soybean seeds from ~9% up to around 20%. In this study, on the other hand, ME for 24 or 48 h never allowed seeds to reach 20% SMC (Table 5.1). When ME duration was prolonged up to 72 h to allow 20% SMC to be obtained, pretreated seeds lost their resistance to ageing. It is possible that, for *cv.* Davis at least, the relatively low SMC's (14.2-18%) of 24 or 48 h ME seeds may not be as critical as that at 20% where the moisture content is high enough to allow damage and repair reactions to compete. During the long period of incubation under the conditions of relatively high SMC (near 20%) and 25°C, in this study, seeds may favour deterioration rather than activation and repair (Hegarty, 1978). Anoyher factor may be problems of mould infection induced by extended treatments and subsequent ageing. Further studies to overcome these kinds of problems are reported in Section 5.3.2. The results presented here partly agree with the findings in wheat of Rudrapal and Basu (1982), who found that maximum benefits of ME treatments were obtained at 24-48 h, but these beneficial effects were reduced when the ME duration was continued to 72 h. Surprisingly, they also found that the loss of benefits due to extended ME in their wheat seeds was associated with increases of lipid peroxidation. The possible role of lipid peroxidation and other aspects of membrane integrity in these experiments is considered in subsequent studies (Chapters 6 and 7).

5.3.2 Soaking injury

According to Saha and Basu (1984), ME for 24 h followed by 2 h soaking-drying was effective for both repair and protection of soybean seeds of medium or low vigour from ageing, although it reduced the resistance of high vigour seeds under adverse storage conditions. In contrast, not only was ME-soaking-drying ineffective on all seeds lots used here, but both ME-soaking-drying and soaking-drying treatments also caused damage to lots A-7 and Davis both before and after ageing (Figures 5.2, 5.3 and 5.4). While both Saha and Basu (1984) and Dey and Mukherjee (1986) reported that presoaking-drying treatment reduced ageing resistance of high vigour lots, the seed lots used in this study were damaged prior to exposure to adverse ageing conditions and appear to have suffered soaking injury. Despite the fact that prior ME could reduce soaking damage appreciably, damage from soaking was still evident after

ageing (Figure 5.3). These results conform to various reports in, for example, peas (Ellis *et al.*, 1990) and soybean (Saha and Basu, 1982; Ashworth and Obendorf, 1980). Soaking reduced normal germination (Figure 5.2) and viability but not the growth of remaining normal seedlings (data not shown), suggesting heterogeneity of seeds within a given seed lot, and that while poor seeds do not survive, high vigour seeds were little affected by soaking damage. The evidence of high variation in rate of water uptake between individual seeds (Figure 5.5) supports the idea of heterogeneity within the seed lot which would result in markedly different responses to soaking.

Although many attempts have been made by several workers (*e.g.* Woodstock and Taylorson, 1981b; Woodstock and Tao, 1981, Tilden and West, 1985) to overcome problems of soaking injury, it is still unclear how a seed suffers fom soaking damage. Woodstock and Taylorson (1981b) noted that ageing or soaking had similar effects on soybean seeds as both produced greater ethanol and acetaldehyde concentrations than untreated controls. This supports the idea of Crawford (1977), who suggested that high levels of ethanol may cause destruction of mitochondrial membranes. Changes relating to membranes which may occur as a result of soaking damage were investigated in subsequent studies and are reported in Chapter 6 and 7.

5.3.3 ME treatments on aged seeds

Although ME treatments (either with or without drying) did not alter viability, ME without drying-back considerably improved the normal germination of slowly aged seeds (Figure 5.6), but not of accelerated aged seeds (data not shown). The improving effects of ME on normal germination of *cv.* Davis echoes the action of ME pre-treatment on lot A-6 in this study (Figure 5.1 and Section 5.2.1) and the work of Saha and Basu (1984). These results suggest that the type of damage suffered by seeds during long term storage (2 years at 5°C for lot A-6) or slow ageing (15 weeks at 30°C, 9% SMC for Davis) may be different from that occurring under accelerated ageing.

The ability of ME to improve the growth of normal seedlings, which was found in both slowly and rapidly aged seeds (Figures 5.7 and 5.8), is in agreement with findings of Knypl *et al.* (1980) and Saha and Basu (1984). Knypl *et al.* (1980) suggested that ME treatments improved soybean vigour by repair or re-arrangement of membrane structure and activation of protein turnover, while Woodstock and Tao (1981)

suggested similar effects in their studies with PEG. However, Rao *et al.* (1987) working with lettuce suggested that an alternative key event was the repair of chromosome aberrations. The idea of membrane repair and re-arrangement in soybean seeds is supported by evidence showing considerable reduction of solute leakage from ME treated seeds in this study (Appendix 5.4). Generally, solute leakage measured by the conductivity test is recommended as a useful vigour test for soybean (Matthews and Powell, 1987) and can also be used to determine integrity of cell membranes (Woodstock and Tao, 1981; Tilden and West, 1985). The close relationship between conductivity and germinability or viability usually observed in this study (Table 5.3) supports such recommendations, and emphasises that losses of membrane integrity are a key event in losses of germinability or viability during ageing. The lack of correlation in one set of experiments for *cv.* Davis is quite surprising and remains unexplained. In this case, conductivities were low, suggesting that the deterioration in this lot (shown in Figure 5.2) was due to other causes. However, when soaking injury did occur in this seed lot, correlations between germination and conductivity were evident. This is explored further in Chapter 6.

Interestingly, the soil trial results differed from laboratory germination tests; because, although the emergence percentage was reduced by ageing, there were very few abnormal seedlings when these were evaluated by the condition of shoots. This implies that many seedlings which might show primary root abnormalities in the laboratory test (Plate 5.1A) recover when grown in soil. It is possible that these abnormal primary roots were compensated for by lateral root growth (Plate 5.1C) so that shoot growth was not affected (Plate 5.1B). The optimal conditions of the soil test effectively reproduce the beneficial effects of ME in the laboratory. Both these results emphasise that seedlings of less severely damaged seeds are capable of recovery from ageing damage.

The results from Davis demonstrated that drying after treatments reduced the advantage gained from ME on improving normal germination (Figure 5.6) or seedling growth (Figures 5,7 and 5.8). This does not correspond with the results from lot A-6 (Figure 5.1) where the beneficial effects of ME on germinability were still retained after drying. Similarly, Knypl and Khan (1981) found no loss of benefit due to drying, although their work was conducted on advancement in time of germination and emergence at suboptimal temperature as a result of osmoconditioning.

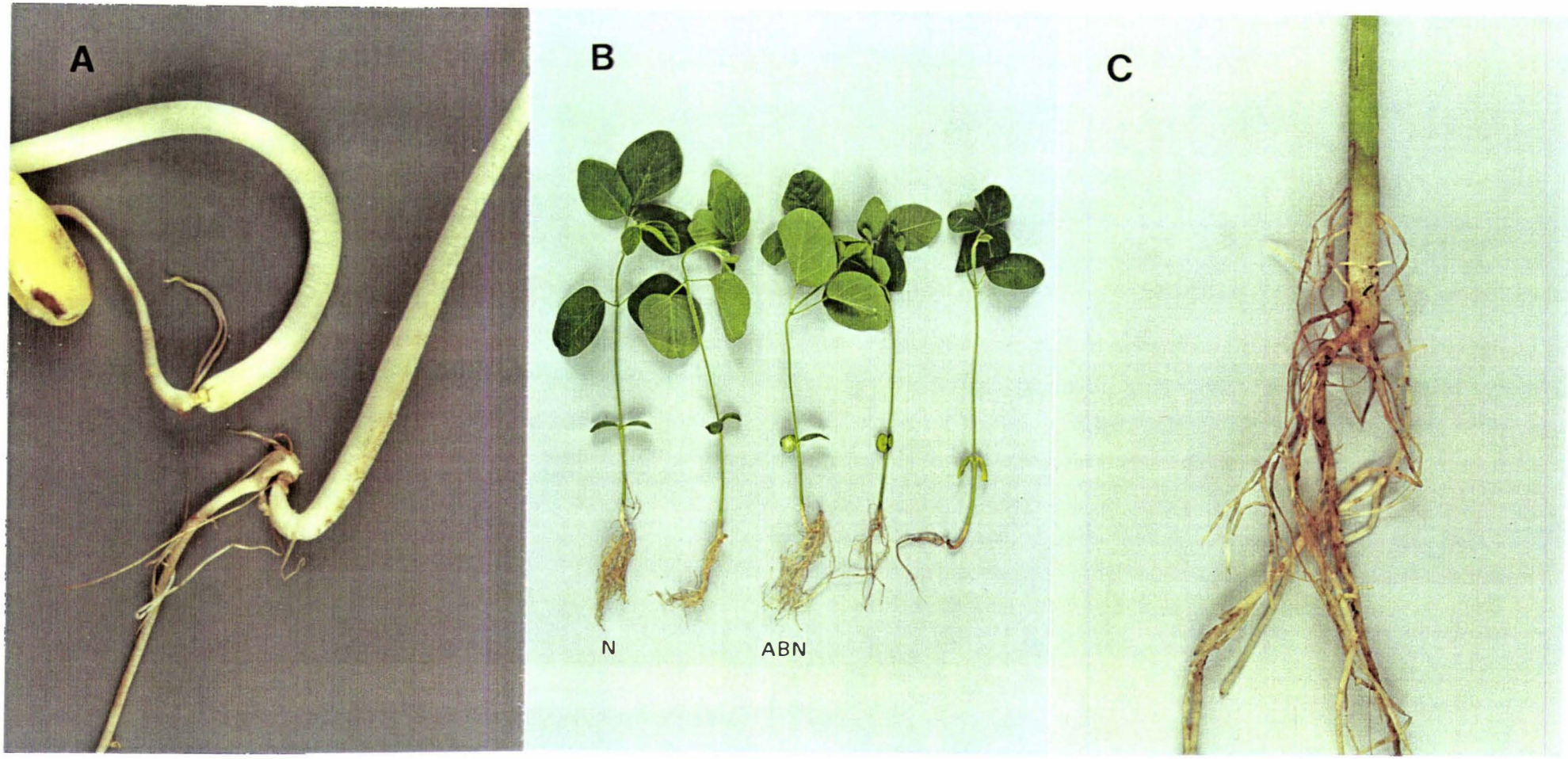


Plate 5.1 Primary root abnormalities in seedlings of slowly aged (35°C, 9% SMC for 15 weeks) soybean, *cv.* Davis. A: from laboratory germination test (8 d after sowing); B: seedlings grown in soil 17 d after 50% establishment: all apparently normal shoots, but all except left hand seedling shown root abnormalities (ABN); C: close up of seedling showing sufficient lateral roots suggesting compensation for some defects of primary root development.

5.4 PRELIMINARY CONCLUSIONS

From the results and discussion presented in this chapter, conclusions can be drawn as follows:

1) Although Saha and Basu (1984) found beneficial effects of hydration-dehydration treatments for both repair and protection for soybean, careful evaluation of several lots found no reproducible protective effects of such treatments in this study, and only occasional beneficial effects of moisture equilibration on seed germinability and vigour providing seeds were not subsequently dried-back.

2) Technical problems were noted with both soaking injury and mould infection. Attempts to avoid the latter by surface sterilisation resulted in increased problems with the former.

Some of these ideas will be discussed further in the context of the whole thesis (Chapter 8).

CHAPTER 6

LIPID AND MEMBRANE STATUS DURING SEED DETERIORATION

6.1 INTRODUCTION

This chapter presents data on the status of both total lipid and an isolated microsomal membrane fraction from seeds of soybean, *cv.* Davis during ageing under the two storage conditions of accelerated ageing and slow ageing. The effects on such status caused by acetone or water soaking treatment applied before AA or by moisture equilibration following slow ageing were also studied. Details of ageing and treatment regimes are shown below:

Table 6.0 Ageing conditions and treatments used for lipid and membrane studies.

Ageing	Treatment
Accelerated ageing (AA) at 40°C, ~100% RH	Untreated seeds Seeds soaked in acetone for 16 h before AA Seeds soaked in water for 30 min before AA
(Note: After treatment seeds were dried to original SMC before AA)	
Slow ageing at 30°C, 9% SMC	Untreated seeds Moisture equilibration above water at 25°C for 3 d after ageing
(Note: After treatment seeds were dried to original SMC before germination testing)	

Samples of all seeds used for lipid and membrane analysis were tested for germination. The data are briefly summarised in the following paragraph. (Results from similar, earlier experiments have already been presented in Chapters 4 and 5)

After 4 days under AA conditions, untreated seeds lost nearly 40% of their germinability (numbers of normal germinants) although viability remained unchanged, whereas acetone and water treated material lost nearly 75 and 98% germinability (37 and 55% viability), respectively (Figures 6.1A, B). After 6 days untreated seeds showed only 12% normal germination; acetone and water soaked seeds none. The viability of seeds from these treatments was 85, 41 and 1%, respectively. In the slow ageing conditions, which are presumed to be less stressful, germinability dropped from 93 to 34% with no significant viability change after 15 weeks. There was no evidence of an improving effect of subsequent moisture equilibration (ME) on either germinability or viability (Figures 6.2A and B). ME increased fresh (data not shown) and dry weights of seedling axes (Appendix 6.1) in unaged seeds relative to untreated controls, but there was no effect in aged seeds. Ageing, acetone and water soaking treatments all increased the rate of electrolyte leakage (Appendices 6.2, 6.3). In all cases, normal germination losses correlated well with conductivity increases (Appendix 6.4), implicating membrane damage as a possible cause of seed deterioration.

Lipid and membrane analysis are valuable tools for the investigation of some of the mechanisms of deterioration occurring under these different sets of conditions. Axis and cotyledon tissues were examined separately in each case. The implications of these results in the context of possible causes of seed deterioration are discussed.

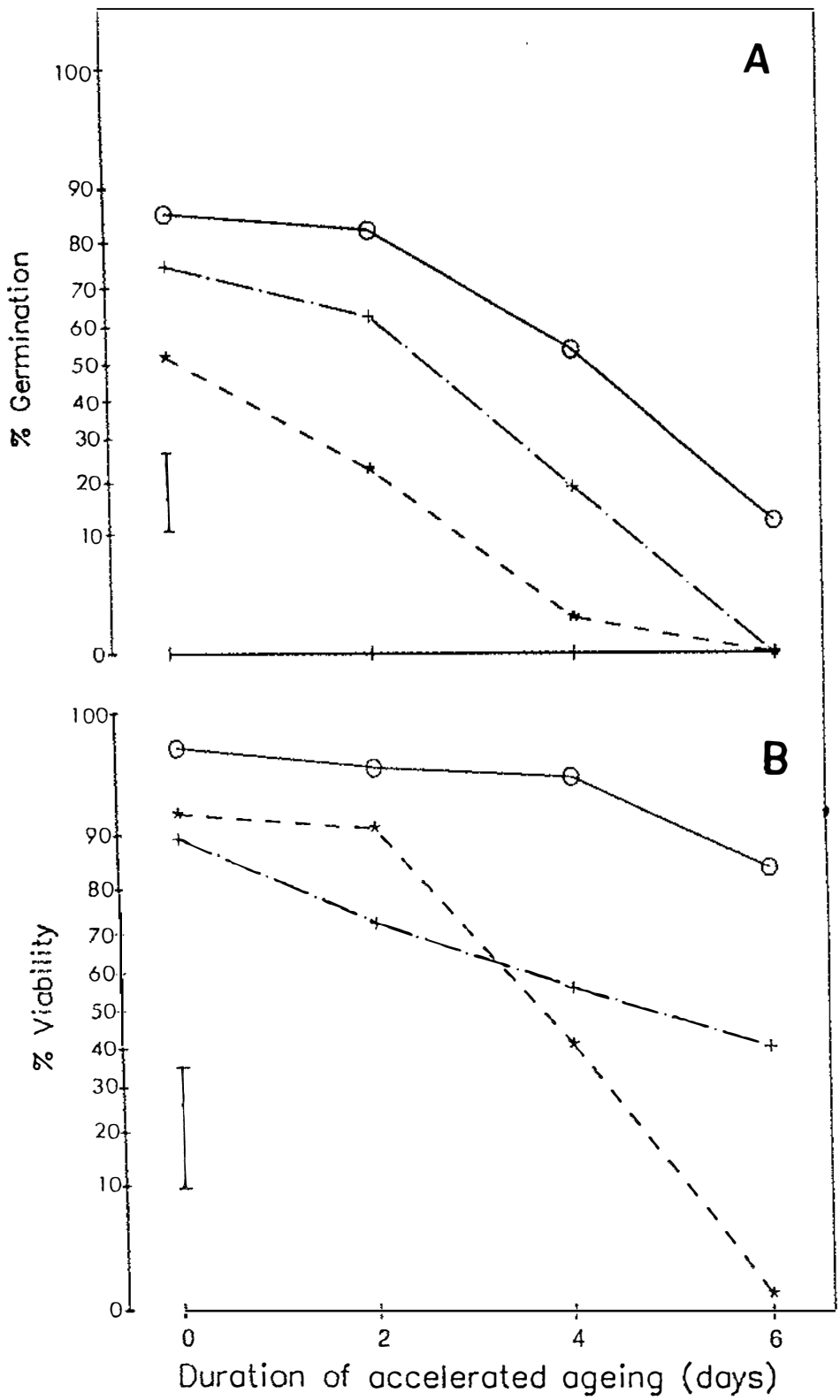


Figure 6.1 Changes in germinability (A) and viability (B) of soybean seeds, *cv.* Davis, as a result of accelerated ageing (40°C,~100% RH) and interactions with preliminary acetone or water soaking. Data presented are arcsin √% transformed means of three replications. ○—○: Untreated controls; +·—·+: Soaked in acetone for 16 h; ★– –★: Soaked in water for 30 min. Bar shows least significant differences (P=0.05) between any single means at df = 6.

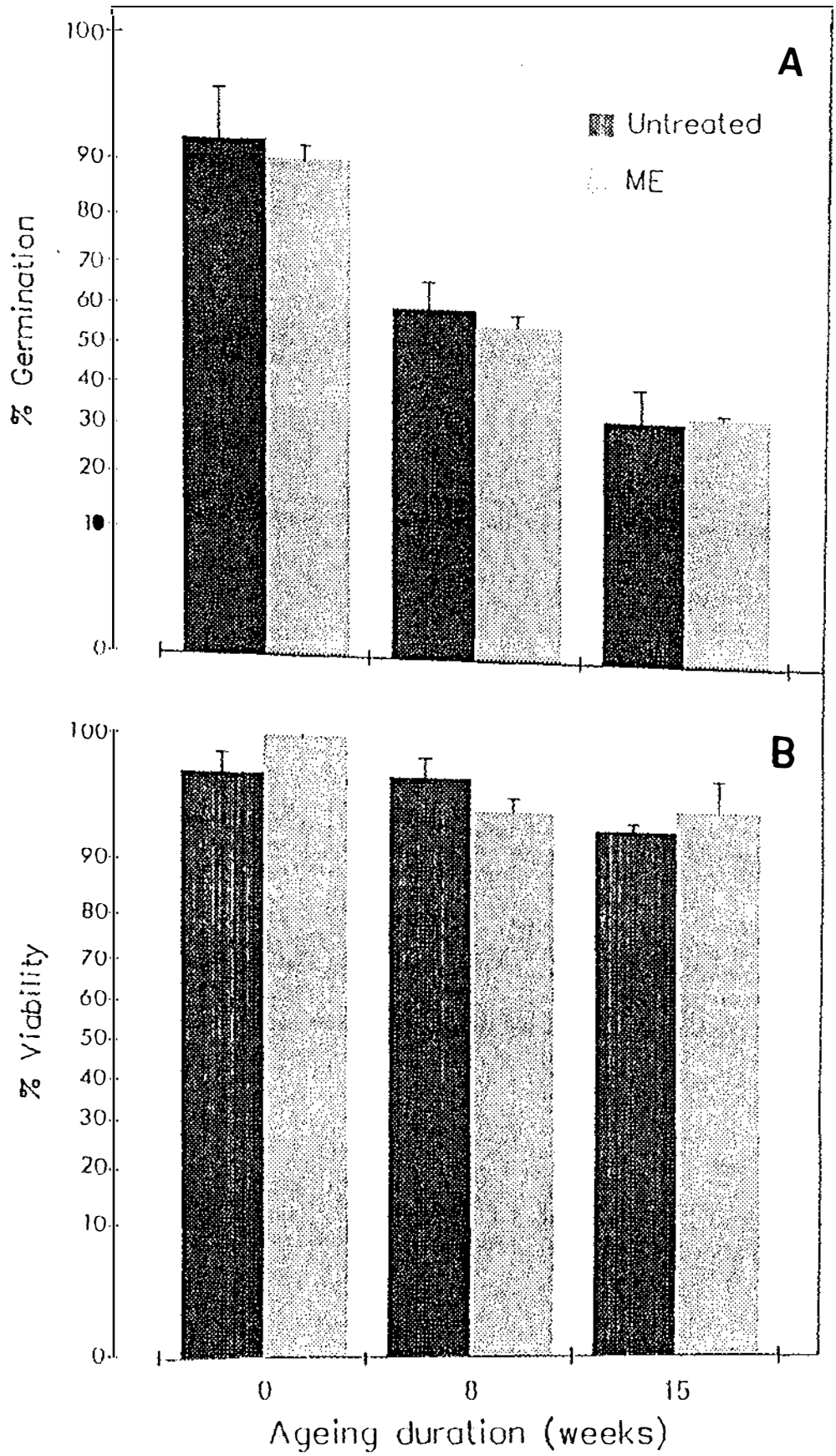


Figure 6.2 Changes in germinability (A) and viability (B) of soybean seeds, *cv.* Davis, as a result of slow ageing (35°C, 9% SMC) alone or with 72 h subsequent moisture equilibration. Data presented are arcsin $\sqrt{\%}$ transformed means of 3 replications. Bars represent SE's calculated for individual means.

6.2 RESULTS

6.2.1 **Total lipid (TL) status**

6.2.1.1 *TL in axes and cotyledons*

The overall mean amount of extracted TL from accelerated aged seeds (regardless of seed treatments) was 1.18 ± 0.02 or 1.08 ± 0.02 mg per axis or equivalent weight of cotyledon. These corresponded well with the values obtained from slow aged seeds in the subsequent study (1.17 ± 0.02 or 1.06 ± 0.01 mg per axis or equivalent weight of cotyledon, respectively). Figure 6.3 shows the effects of accelerated ageing, and interactions between ageing and prior acetone or water soaking on TL extracted from either axes or cotyledons. Analysis of variance of each treatment indicated that there were no significant changes in TL content during AA. The extractable TL from cotyledons was slightly lower than that from axes, on an equivalent weight basis. Acetone or water soaked seeds appeared to yield higher levels of TL than untreated seeds, particularly after 2 days AA, but the differences were not significant. Similarly, the data for changes during slow ageing (Figure 6.4) showed no changes in TL from both axes and cotyledons of either untreated or moisture equilibrated seeds.

6.2.1.2 *Total phospholipid (PL) in the TL extract*

The total phospholipid content of axis and cotyledonary tissue in untreated, unaged seeds (Figure 6.5A, B) was 3.73 ± 0.14 μg P equivalents per axis and 2.73 ± 0.05 μg P equivalents per equivalent weight of cotyledon, respectively. The amount of PL was about 37% higher in axes than in cotyledons on an equivalent weight basis.

An analysis of the PL contents from the TL of untreated, acetone or water soaked seeds showed that prior acetone or water soaking treatment increased the rate of PL depletion from both axes and cotyledons during AA (Figure 6.5). Untreated seeds showed no significant losses of PL from axes and only a small decrease (~20%, $P<0.05$) in cotyledonary PL after 6 days AA. After 4 days AA, losses in cotyledons were significant from acetone or water soaked seeds ($P<0.01$), and there was more than 35% depletion after 6 days. A similar rate of loss was also detected in axis tissue.

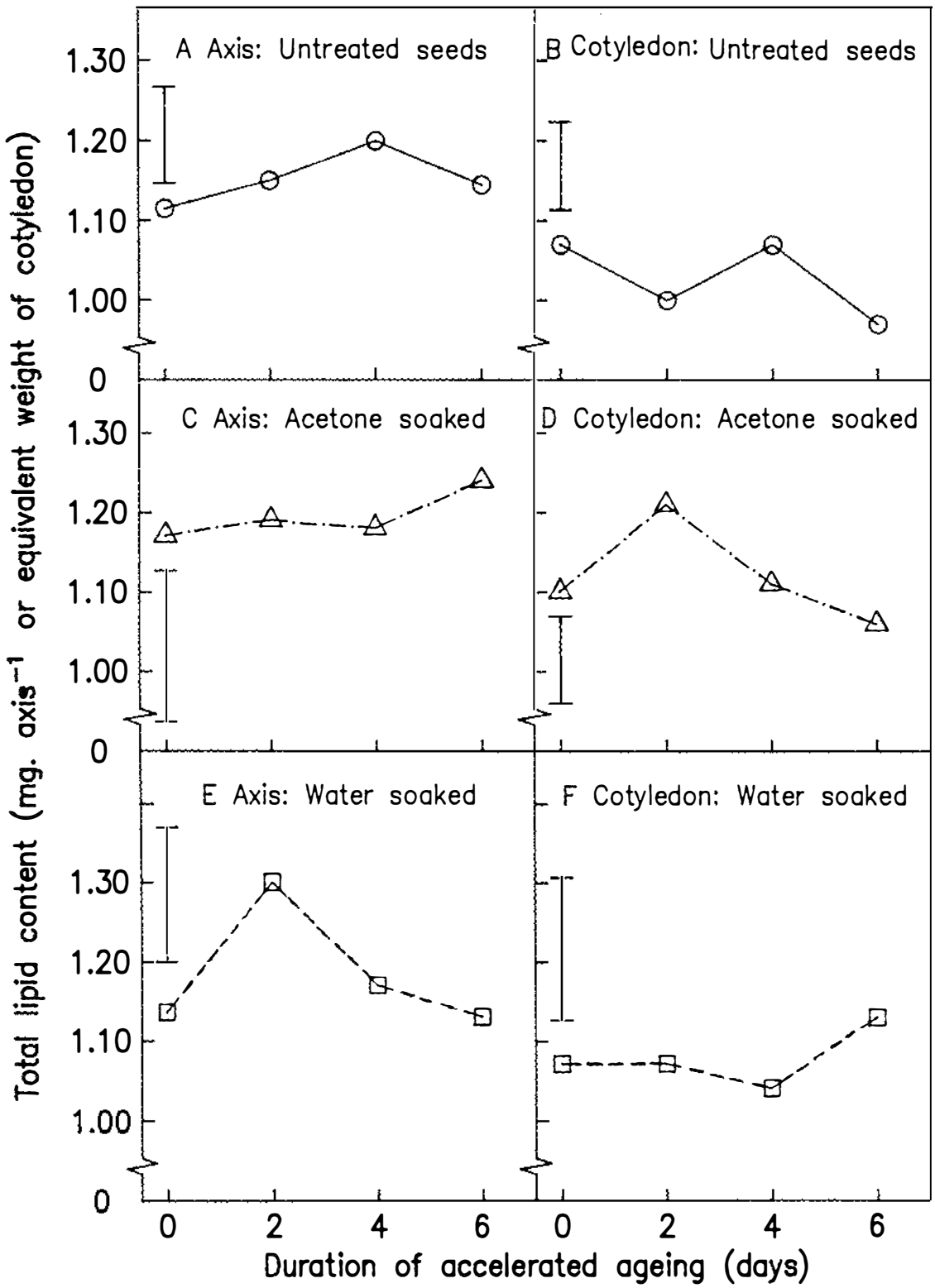


Figure 6.3 The effects of accelerated ageing (40°C,~100% RH) and interactions with preliminary acetone or water soaking on total lipid extracted from axis and cotyledonary tissue of soybean seeds, *cv.* Davis. Data presented are means of 3 replicate extractions. ○—○: Untreated controls; △···△: Soaked in acetone for 16 h; □–□: Soaked in water for 30 min. Bars show least significant differences (P=0.05) between means.

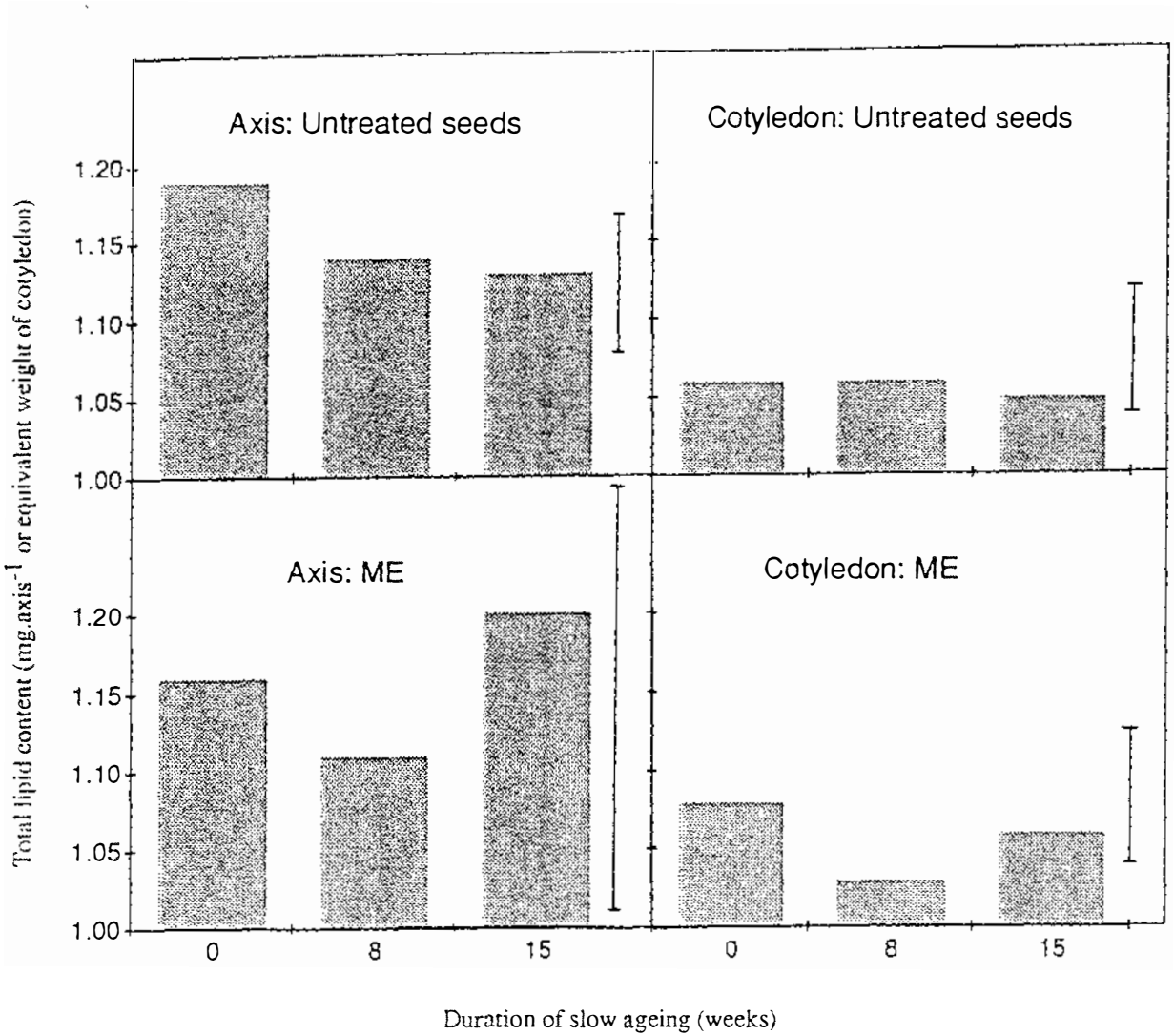


Figure 6.4 The effects of slow ageing (35°C, 9% SMC) alone or with 72 h subsequent moisture equilibration on total lipid extracted from axis and cotyledonary tissue of soybean seeds, *cv.* Davis. Data presented are means of three replicate extractions. Bars show least significant differences (P=0.05) between means.

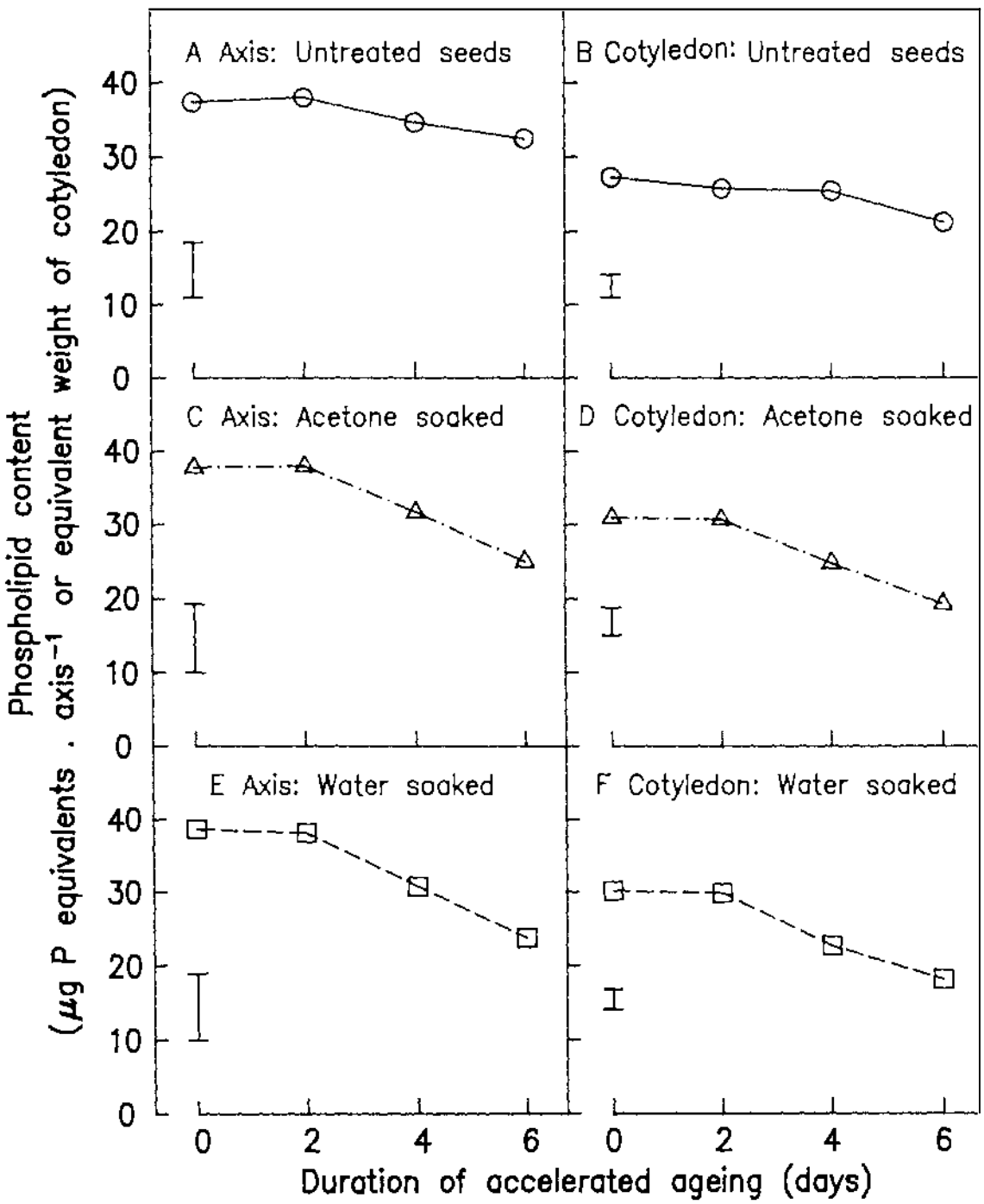


Figure 6.5 The effects of accelerated ageing (40°C,-100% RH) and interactions with preliminary acetone or water soaking on phospholipid content of axis and cotyledonary tissue of soybean seeds *cv.* Davis. Data presented are means of three replicate extractions. ○—○: Untreated control; △-△: Soaked in acetone for 16 h; □-□ : Soaked in water for 30 min. Bars show least significant differences (P=0.05) between means.

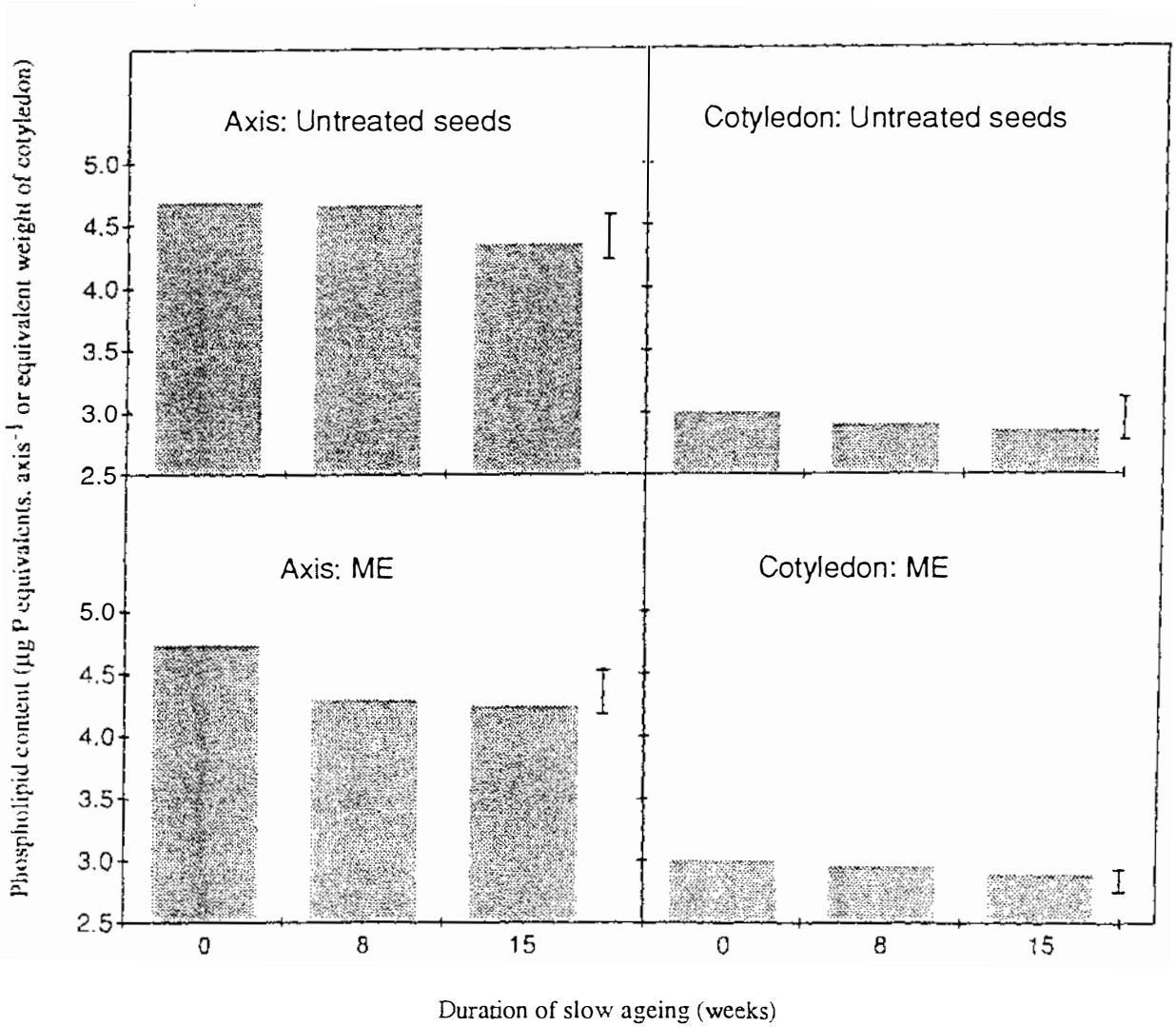


Figure 6.6 The effects of slow ageing (35°C, 9% SMC) alone or 72 h subequent moisture equilibration on phospholipid content of axis and cotyledonary tissue of soybean seeds *cv.* Davis. Data presented are means of three replicate extractions. Bars show least significant differences (P=0.05) between means.

The effects of the less severe slow ageing conditions and subsequent moisture equilibration treatment on PL changes are shown in Figure 6.6. Losses in PL from both axes and cotyledons were not significant in untreated seeds after 15 weeks under these storage conditions, but after subsequent ME treatment significant losses emerged in axes, the PL content declining from 4.74 μg P equivalents.axis^{-1} to 4.31 and 4.25 μg P equivalents.axis^{-1} after ageing for 8 and 15 weeks, respectively ($P<0.05$). The changes were observed despite the fact that, as previously noted (Section 6.1), no significant changes in either germinability or viability were detected as a result of ME treatment.

Relationships between PL losses during AA and other aspects of germination performance

Appendix 6.5 shows details of correlations between PL changes with germinability, viability, seed vigour and conductivity during AA. There were significant relationships between losses of axis PL and germinability for both acetone treated ($P<0.05$) and water soaked ($P<0.01$) seeds during subsequent AA, but not in untreated seeds (Figure 6.7). Only for water soaked seeds was there a significant correlation between losses of PL and viability during AA. There was a significant ($P<0.01$) linear relationship between the levels of PL from cotyledons and decreases in both germinability and viability in all untreated, acetone and water soaked seeds during AA (Appendix 6.5). With respect to germinability, seeds treated with acetone gave the highest positive correlation coefficient ($r=0.83$, $P<0.01$) (Figure 6.8).

Changes in seed vigour of untreated seeds during AA were determined by measuring the fresh and dry weights of seedling axes 8 d after sowing. Only the former was correlated significantly ($P<0.01$) with PL content and then only from cotyledons (Appendix 6.5). Results from acetone and water treatments were not analysed because no normal seedlings were obtained in some or all replications of each treatment after 4 and 6 d AA, respectively.

Neither PL changes in axes nor cotyledons of untreated seeds were significantly correlated with conductivity (Appendix 6.5). For acetone treated seeds, PL changes in axes did not significantly correlate with conductivity, but those in cotyledons did ($P<0.01$, Figure 6.9). There was a highly significant correlation for water soaked seeds from both axes and cotyledons (Figure 6.10 for axes).

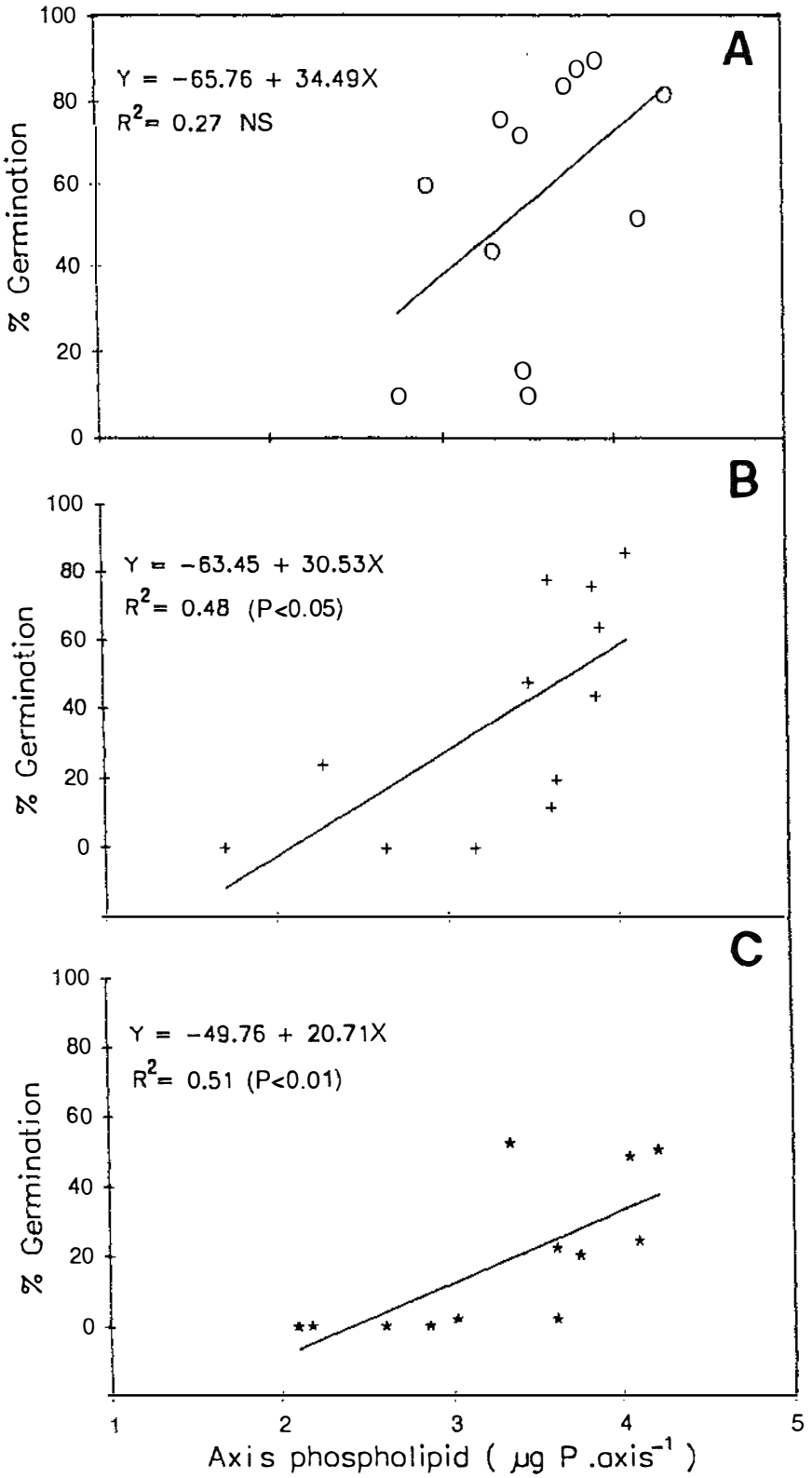


Figure 6.7 Relationships between germinability and phospholipid from axis tissue of untreated (A), acetone (B) and water soaked (C) soybean seeds, *cv.* Davis.

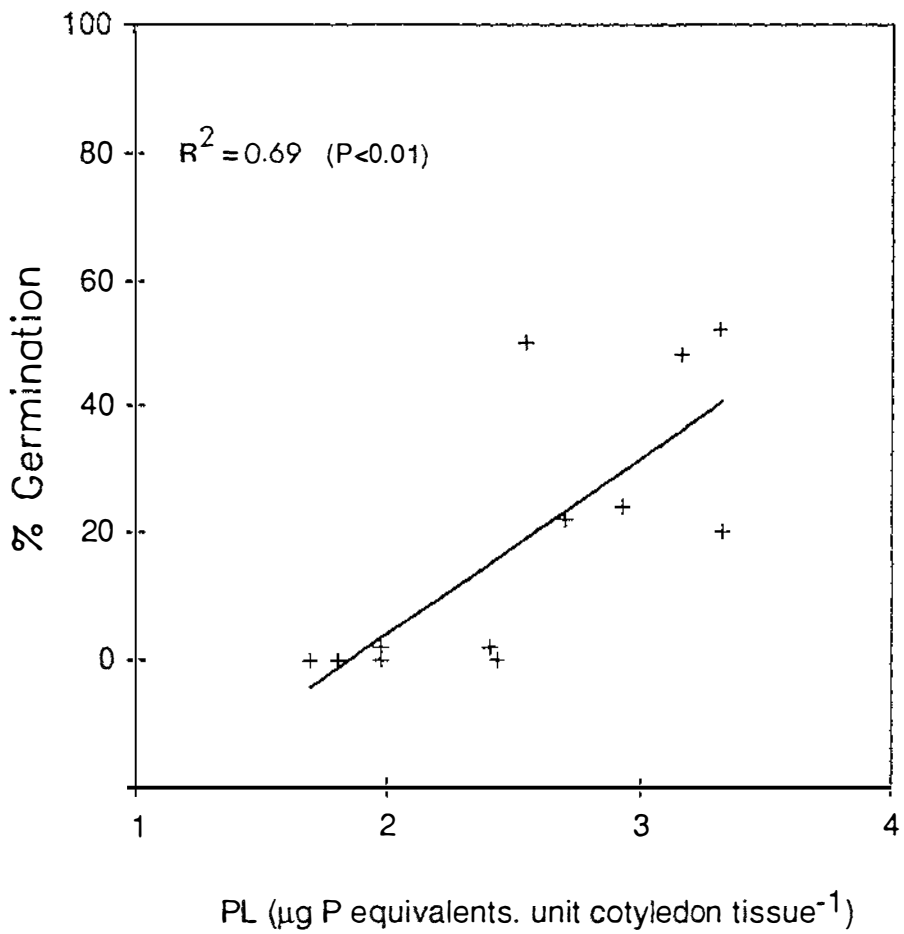


Figure 6.8 Relationship between germinability and phospholipid from cotyledonary tissue of acetone soaked soybean seeds, *cv.* Davis.

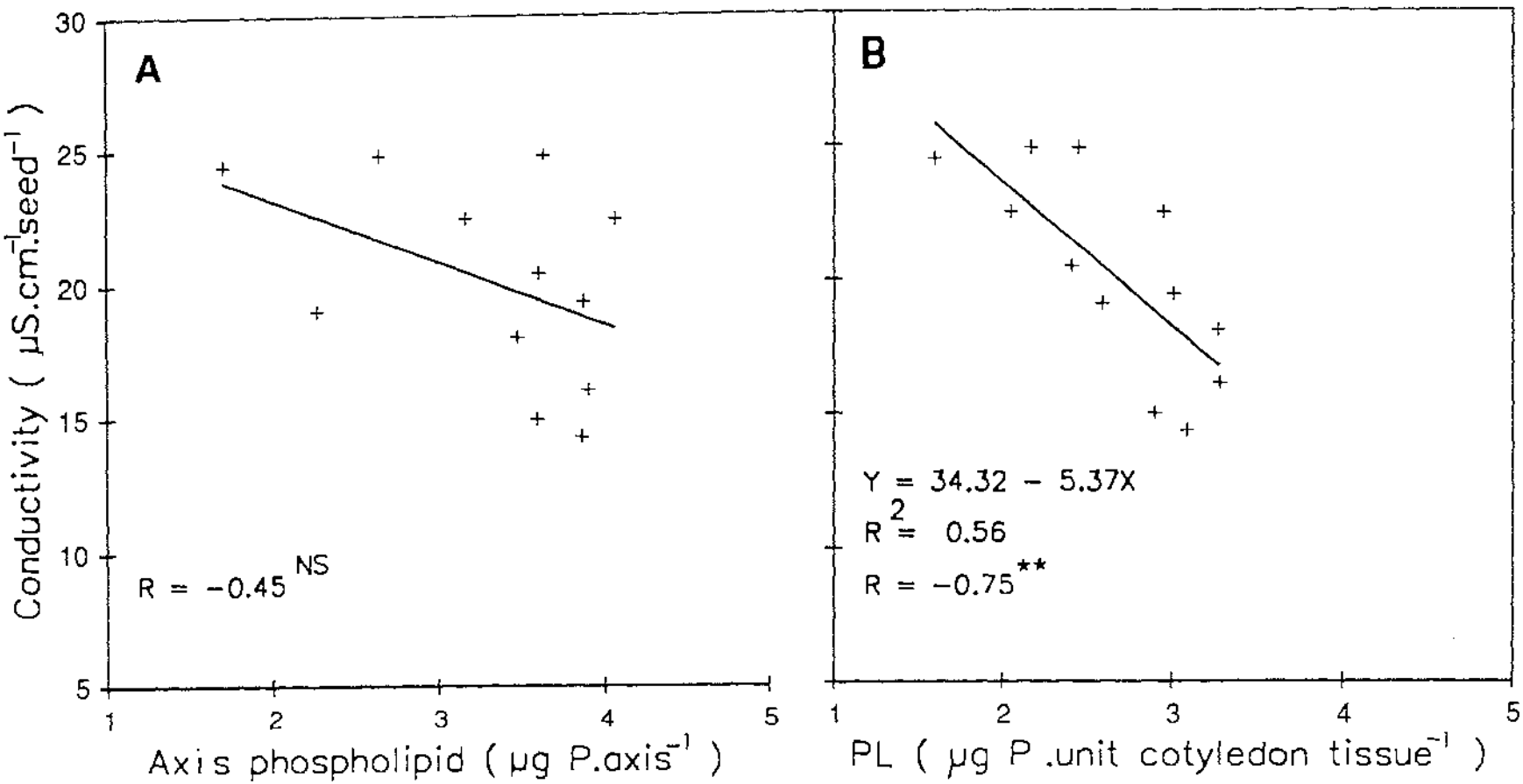


Figure 6.9 Relationship between conductivity and phospholipid from axis (A) and cotyledonary (B) tissue of acetone soaked soybean seeds, *cv.* Davis.

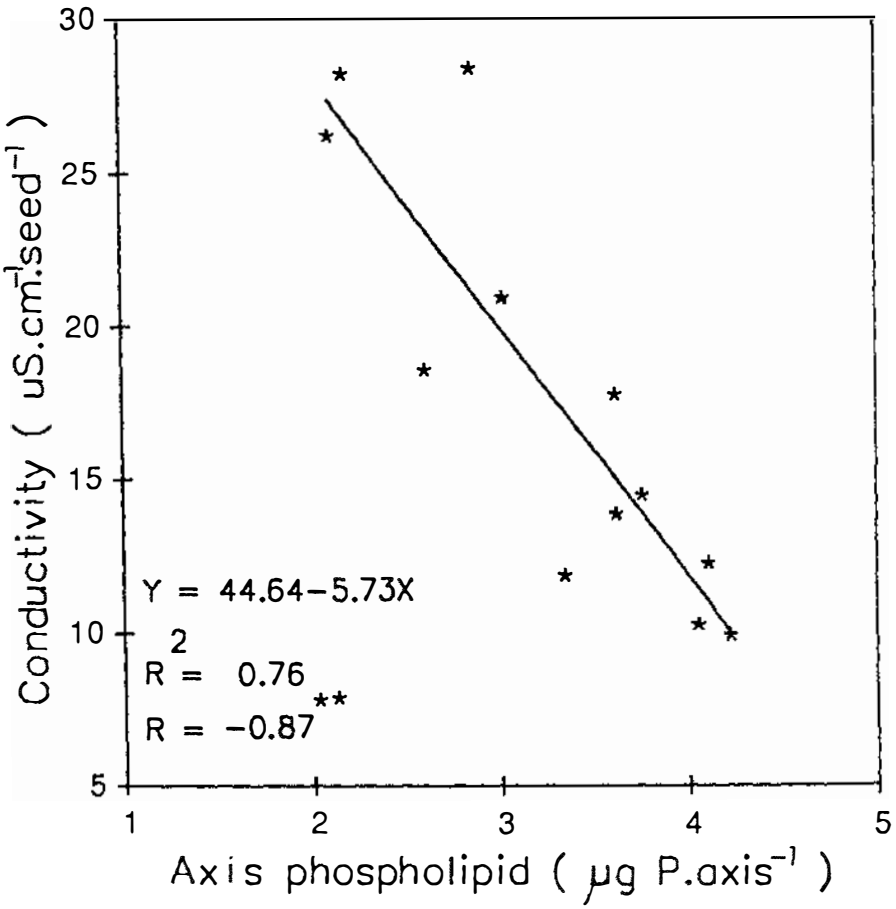


Figure 6.10 Relationship between conductivity and phospholipid from axis tissue of water soaked soybean seeds, *cv.* Davis.

6.2.1.3 *Fatty acid composition in TL*

The relative proportions of fatty acids present in TL extracted from axes and cotyledons are shown in Tables 6.1-6.3. The composition of fatty acids from untreated seeds did not change significantly during either AA (Tables 6.1 and 6.2) or slow ageing (Table 6.3), with no indication of decline in unsaturated forms. Likewise, changes were not detectable as a result of acetone or water soaking prior to AA, or after moisture equilibration following slow ageing. It was however noted that there were small but consistent differences between the AA and slow ageing studies in 'estimates' of proportions of unsaturated fatty acids (compare data for untreated, unaged axes and cotyledons in Tables 6.1-6.3).

The data clearly showed that the proportions of fatty acids from axes differed from cotyledons, the former tending to have a much higher proportion of 16:0 and 18:3 acids. For example, under AA conditions (Table 6.1) the average proportions from all treatments of 16:0 and 18:3 fatty acids from axes were 17.7 and 22.0%, compared to 11.9 and 11.0%, respectively, in cotyledons (Table 6.2). Proportions of 18:1 and 18:2 fatty acids were higher in cotyledons.

6.2.2 **Membrane status**

6.2.2.1 *PL in the membrane fractions*

Microsomal membrane fractions were extracted from unaged and 4 days AA seeds to investigate the effects of ageing and interactions with acetone or water soaking pretreatments. Data obtained are shown in Table 6.4. Analysis of phosphorus (P) from isolated membrane lipids (L) (expressed as P:L ratios) showed that there were no significant effects apparent in any treatment in either preparations from axes or cotyledons. The average mean ratio in axes was 0.46% which was considerably higher than that of 0.25% in cotyledons.

Similarly, Table 6.5 shows that slow ageing with or without subsequent ME had no significant effects on PL of microsomal membranes from both axes and cotyledons. In this study, the average ratio of phosphorus to total lipid (P:L) in the preparations was determined to be 0.30 and 0.20% for axes and cotyledons, respectively.

Table 6.1 The effects of accelerated ageing (40°C, ~100% RH) and interactions with preliminary acetone or water soaking on the fatty acid composition of total lipid from axis tissue of soybean seeds, *cv.* Davis. Data presented are means of three replicate extractions.

Ageing duration (days)	Fatty Acids				
	C16:0	C18:0	C18:1	C18:2	C18:3
	% of Total lipid				
			Untreated controls		
0	17.5	3.0	5.2	51.7	22.5
2	18.3	3.0	5.0	50.8	22.7
4	18.6	2.9	5.3	50.9	22.2
6	18.3	3.1	5.0	51.7	21.5
$LSD_{0.05}$	NS	NS	NS	NS	NS
			Acetone soaked		
0	17.5	3.0	5.1	51.8	22.6
2	17.5	3.0	5.0	51.7	22.7
4	18.3	3.0	5.0	52.0	21.7
6	18.2	3.2	5.3	52.5	21.0
$LSD_{0.05}$	NS	0.14	NS	NS	NS
			Water soaked		
0	17.0	3.0	5.2	52.0	22.7
2	16.9	3.1	5.6	52.3	21.7
4	17.2	3.5	5.4	53.2	21.8
6	17.1	3.3	5.5	53.4	20.7
$LSD_{0.05}$	NS	NS	NS	NS	NS

NS : not significant at P = 0.05

Table 6.2 The effects of accelerated ageing (40°C, ~100% RH) and interactions with preliminary acetone or water soaking on fatty acid composition of total lipid from cotyledonary tissue of soybean seeds, *cv.* Davis. Data presented are means of three replicate extractions.

Ageing duration (days)	Fatty Acids				
	C16:0	C18:0	C18:1	C18:2	C18:3
	% of Total				
			Untreated Controls		
0	11.9	3.1	17.6	57.6	9.8
2	11.8	3.0	15.9	58.1	11.1
4	11.9	3.0	16.1	57.1	11.3
6	11.8	2.9	16.1	57.9	11.3
$LSD_{0.05}$	NS	NS	NS	NS	NS
			Acetone soaked		
0	11.8	2.9	16.4	58.2	10.6
2	11.8	3.0	16.4	57.8	11.0
4	12.5	3.2	16.4	57.2	10.8
6	11.8	3.1	16.1	58.5	10.7
$LSD_{0.05}$	NS	NS	NS	NS	NS
			Water soaked		
0	11.8	3.1	16.0	58.1	10.9
2	11.7	3.0	15.1	58.4	11.8
4	12.1	3.2	15.0	58.2	11.5
6	11.4	3.1	15.6	58.6	11.2
$LSD_{0.05}$	NS	NS	NS	NS	NS

NS : not significant at P = 0.05

Table 6.3 The effects of slow ageing (35°C, 9% SMC) alone or with subsequent moisture equilibration (ME) on fatty acid composition of total lipid from axis and cotyledonary tissue of soybean seeds, *cv.* Davis. Data presented are means of three replicate extractions.

Ageing duration (wks) and treatments	Fatty Acids				
	C16:0	C18:0	C18:1	C18:2	C18:3
	% of Total lipid				
			Axes		
0 -	13.6	2.1	5.4	54.1	24.7
8 -	13.6	2.5	6.0	55.0	22.6
15 -	14.0	2.5	5.7	54.4	23.5
$LSD_{0.05}$	NS	NS	NS	NS	NS
0 + ME	13.1	2.3	5.6	54.4	24.5
8 + ME	12.9	2.3	6.4	54.8	23.6
15 + ME	14.9	2.3	6.1	53.8	22.9
$LSD_{0.05}$	NS	NS	NS	NS	NS
			Cotyledons		
0 -	10.0	2.1	16.9	59.5	11.5
8 -	10.3	2.1	16.4	59.4	11.8
15 -	10.7	2.2	16.7	59.0	11.4
$LSD_{0.05}$	NS	NS	NS	NS	NS
0 + ME	9.8	2.1	16.2	59.4	12.5
8 + ME	10.1	2.2	17.2	58.6	11.9
15 + ME	9.6	2.1	16.3	59.6	12.4
$LSD_{0.05}$	NS	NS	NS	NS	NS

NS : not significant at P = 0.05

Table 6.4 Phosphorus (P):Lipid (L) ratios of microsomal membrane lipid extracted from axes and cotyledons of soybean seeds, *cv.* Davis affected by accelerated ageing (40°C, ~100% RH) and interactions with preliminary acetone or water soaking. Data presented are means of two replicate extractions.

Treatments and ageing	P:L (%)	
	Axes	Cotyledons
Untreated -	0.405	0.230
Untreated + 4 d AA	0.545	0.255
Acetone soaks -	0.490	0.245
Acetone soaks + 4 d AA	0.395	0.230
Water soaks -	0.450	0.320
Water soaks + 4 d AA	0.455	0.235
$LSD_{0.05}$	NS	NS

NS : not significant at P = 0.05

Table 6.5 Phosphorus (P):Lipid (L) ratios of microsomal membrane lipid extracted from axes and cotyledons of soybean seeds, *cv.* Davis affected by slow ageing (35°C, 9% SMC) alone or with subsequent moisture equilibration. Data presented are means of two replicate extractions.

Ageing duration (weeks) and treatments	P:L (%)	
	Axes	Cotyledons
0 -	0.300	0.205
0 + ME	0.325	0.215
15 -	0.280	0.180
15 + ME	0.290	0.205
$LSD_{0.05}$	NS	NS

NS : not significant at P = 0.05

6.2.2.2 *Fatty acid analysis of membrane fractions*

The fatty acid composition of microsomal membrane lipids from both axes and cotyledons is shown in Tables 6.6 and 6.7 for AA and slow ageing, respectively. There was no clear evidence that any treatment or ageing regime caused changes in fatty acid composition of membrane extracts of either axes or cotyledons. Axes had higher proportions of 16:0 and 18:3 fatty acids but lower levels of 18:1 and 18:2 acids than cotyledons. The proportion of 16:0 and 18:0 fatty acids retrieved from membrane lipids (Tables 6.6 and 6.7) was higher than in total lipids (Tables 6.1-6.3) for both axes and cotyledons.

6.2.2.3 *Protein in membrane fractions*

A set of samples of membrane protein from axes and cotyledons of unaged or 4 d AA seeds of untreated, acetone and water soaking treatments was analysed by SDS-PAGE. Plate 6.1 shows banding patterns of these proteins extracted from the microsomal membranes of axis tissue. The analysis showed no evidence of qualitative changes in these proteins resulting from ageing and/or treatments. Six major separate protein components were identified in both axes and cotyledons. The estimated molecular weights of these bands calculated from the relative electrophoretic mobility in comparison to known standards ranged from 72.4 to 20.0 kD.

Table 6.6 Fatty acid composition of microsomal membrane lipid extracted from axes and cotyledons of soybean seeds, *cv.* Davis affected by accelerated ageing (40°C, ~100% RH) and interactions with preliminary acetone or water soaking. Data presented are means of two replicate extractions.

Treatments and ageing	Fatty Acids				
	C16:0	C18:0	C18:1	C18:2	C18:3
	% of Total lipid				
			Axes		
Untreated -	21.3	3.6	7.5	49.3	18.3
Untreated + 4 d AA	22.9	4.2	7.4	45.2	20.3
Acetone soaks -	23.6	4.1	9.2	44.4	18.7
Acetone soaks + 4 d AA	24.9	3.6	7.7	44.3	19.5
Water soaks -	25.0	3.4	8.6	45.5	17.5
Water soaks + 4 d AA	21.9	4.7	7.4	46.9	19.1
$LSD_{0.05}$	NS	NS	NS	NS	NS
			Cotyledons[a]		
Untreated -	14.9	3.0	23.3	49.3	9.4
Untreated + 4 d AA	14.6	2.6	23.2	49.9	9.7
Acetone soaks -	13.8	3.5	22.3	49.1	11.3
Acetone soaks + 4 d AA	13.6	3.7	21.9	51.4	9.4
Water soaks -	14.3	3.5	22.7	49.2	10.3
Water soaks + 4 d AA[b]	10.7	3.0	17.8	56.2	12.3
$LSD_{0.05}$	NS	NS	4.1	NS	NS

a : unequal replication analysis
b : 1 replication
NS : not significant at P = 0.05

Table 6.7 Fatty acid composition of microsomal membrane lipid extracted from axes and cotyledons of soybean seeds, *cv.* Davis affected by slow ageing (35°C, 9% SMC) alone or with subsequent moisture equilibration. Data presented are means of two replicate extractions.

Ageing duration (weeks) and treatments	Fatty Acids				
	C16:0	C18:0	C18:1	C18:2	C18:3
	% of Total lipid				
			Axes		
0 -	18.6	3.8	8.3	52.0	17.3
8 -	21.0	5.3	8.6	50.3	14.8
15 -	17.4	2.7	6.8	53.4	19.7
$LSD_{0.05}$	NS	NS	NS	NS	NS
0 + ME	19.7	3.3	7.9	50.8	18.3
8 + ME	21.8	3.7	8.3	49.5	16.7
15 + ME	18.9	3.3	7.3	51.8	18.7
$LSD_{0.05}$	NS	NS	NS	NS	NS
			Cotyledons		
0 -	13.2	2.8	18.8	53.5	11.7
8 -	12.8	2.8	19.0	53.9	11.5
15 -	10.4	2.5	18.1	56.5	12.5
$LSD_{0.05}$	NS	NS	NS	NS	NS
0 + ME	13.0	2.7	18.6	54.2	11.5
8 + ME	12.8	2.6	18.8	53.6	12.2
15 + ME	13.6	2.5	18.3	54.3	11.3
$LSD_{0.05}$	NS	NS	NS	NS	NS

NS : not significant at p = 0.05

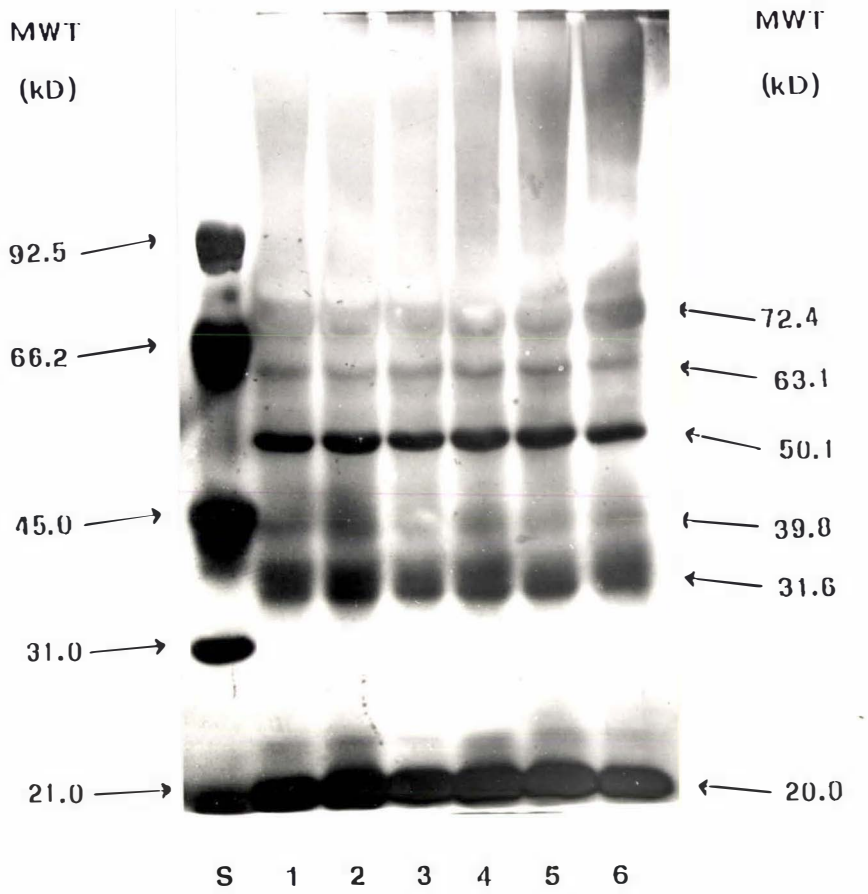


Plate 6.1 SDS-PAGE gel (7.5%) with Coomassie Blue and silver staining showing patterns of membrane proteins from axes of soybean *cv.* Davis.

S: Molecular weight standards
1: Untreated, unaged controls;
2: Untreated, 4 d AA seeds;
3: Acetone treated, unaged seeds;
4: Acetone treated, 4 d AA seeds;
5: Water soaked, unaged seeds;
6: Water soaked, 4 d AA seeds.

6.3 DISCUSSION

Before embarking on this discussion, it is important to clarify the use of the term 'germination' in both this study and the literature so that data are standardized and comparable. On examination of the literature there is ambiguity, because many workers have used 'germination' in the sense of radicle emergence (e.g. Priestley and Leopold, 1979; 1983; Petruzzelli and Taranto, 1984; Koostra and Harrington, 1969). On the other hand, Ferguson *et al.* (1990a) used the term 'germination' in the sense as described by the Association of Official Seed Analysts (AOSA, 1987).

In this study, the term 'germination' is used to refer to normal germination as per the ISTA's rules (1985) (see Chapter 3) and is therefore effectively the same as the criteria used by Ferguson *et al.* (1990a). 'Viability', which includes normal and abnormal seedlings, is taken to have the same meaning as radicle emergence as used in the literature, there being no fresh ungerminated seeds in the seed lot used in this study.

6.3.1 **Lipid changes and their possible association with seed deterioration.**

6.3.1.1 *Lipid content*

Results in this study indicate that, while a decline in germinability during both AA and slow ageing was not related to the amount of extractable TL from axes or cotyledons, losses of PL were sometimes associated with germination and viability losses. This was particularly so for acetone or water soaked seeds. The close relationship between levels of cotyledon PL (but not axis PL) in untreated seeds and germinability is interesting as is the fact that the rate of PL depletion in both axes and cotyledons was increased by the interactions between AA and prior acetone or water soaking treatments.

There are a number of reports dealing with changes in lipids during ageing (*e.g.* Koostra and Harrington, 1969; Priestley and Leopold, 1979 and 1983; Pearce and Abdel Samad, 1980; Stewart and Bewley, 1980; Francis and Coolbear, 1984). Of

these only a few have mentioned changes in amounts of TL, and even these provide contradictory evidence. While Gidrol *et al.* (1989) found that TL content (obtained from summing the amounts reported for individual lipid fractions) in sunflower decreased during AA, Priestley and Leopold (1979) demonstrated a slight increase (from 120 to 155 mg per g seed weight) in TL extracted from whole seed of soybean. Neither of these trends was seen in the data in this study. Figures 6.1 and 6.2 show no evidence of changes in TL content from either axes or cotyledons during accelerated or slow ageing. Extractable TL averaged 275 mg per g seed dry weight (calculated from the TL content of cotyledons at 9.3% SMC), which is about double the amount reported by Priestley and Leopold (1979). In general, the range of lipid contents of soybean seeds has been quoted as 14-26% of seed dry weight (calculated from Trelease and Doman, 1978) or 17-22% (Bewley and Black, 1978). This suggests that the cultivar used in this study belongs to the highest lipid content group, while that used by Priestley and Leopold (1979) is in the lowest. Differences between TL results may be related to species, cultivars or methods of lipid extraction. Gidrol *et al.* (1989) and Priestley and Leopold (1979) both used chloroform / methanol for extraction of sunflower and soybean seeds, respectively. In this study water saturated butanol was used for soybean extractions.

Losses in PL accompanying losses of viability have been reported for various species (*e.g.* cucumber, Koostra and Harrington, 1969; pea, Powell and Matthews; 1981; and tomato, Francis and Coolbear, 1984). Reports on whole soybean seeds however are in disagreement. Nakayama *et al.* (1981) found that 45% of total PL had decomposed after 6 months storage at 35°C, 13% SMC. In contrast, Chapman and Robertson (1977) reported that the amount of PL was doubled after ageing at 35°C, 85% RH for 10 days. However, no germination or viability data were supplied in either case, so, it is difficult to interpret whether a relationship exists between these PL changes and cellular events which might be necessary for germination. Priestley and Leopold (1979) detected a slight decrease of phospholipid in whole seeds during AA when seeds markedly lost their viability from 100 to 5%.

In this study, levels of PL from axes and cotyledons were quantified separately, and data for AA conditions (Figure 6.5) reveal that only the results from cotyledons correspond with findings of Priestley and Leopold (1979) and Nakayama *et al.* (1981).

As the soybean axis represents only about 2.5% of the weight of the whole seed in *cv.* Davis, changes in this part of the seed would be expected to be masked by the huge amount of cotyledon. That the losses of PL from cotyledons, but not axes, are related to germination losses during AA (Appendix 6.5) suggests that a decrease in amount of PL from cotyledon may be an important contribution to seed deterioration under high temperature and relative humidity. However, simple correlations do not imply cause and effect: besides PL losses, ageing may cause damage to seeds via other effects such as changes in respiration or enzymes (see Section 2.2). Further, no information is presented here on changes in different types of PL. It has also been reported that during ageing there are changes in different classes of PL (*e.g.* Nakayama *et al.,* 1981; Simpson and Nakamura, 1989 in soybean, Powell and Matthews, 1981 in pea). These interchanges are, for example, small increases of phosphatidyl glycerol (PG) in pea or phosphatidic acid (PA) in soybean, while phosphatidyl choline (PC) decreases dramatically.

There was no evidence of whole seed phospholipid depletion occurring during slow ageing in the present study, and although the germination capacity of seeds dropped significantly (from 93 to 34%), there were no changes in viability. Slow ageing caused a greater proportion of abnormal seedlings. Data presented in Figure 6.6 are in agreement with Priestley and Leopold (1983) who stored soybean seeds under 'natural ageing' (4°C, low RH), where seeds lost viability from 98 to 86% after 44 months, but suffered no loss in PL. Their results however were with whole seeds. In both cases, it is possible that seed deterioration (indicated by viability losses) has not proceded sufficiently for significant PL depletion to be measured, since the depletion under AA was detected after greater losses of their viability (14% in Figure 6.1B and more than 95% in the report of Priestley and Leopold, 1979). The earliest report on this subject, presented by Koostra and Harrington (1969), showed that there were no PL losses during 'natural ageing' when cucumber seeds lost 30% of their viability, but losses of PL were detected under AA after seeds lost more than 97% viability. This would suggest that PL loss occurs as a consequence of seed deterioration rather than being a cause of it.

Zero or little PL losses during slow ageing have also been reported in wheat embryos, despite the fact that the losses under artificial ageing were significant (Petruzzelli and Taranto, 1984). However, in that study, changes in viability were controlled to similar levels of 83 to 55%. This appears to be the only study where investigators have actually tried to compensate for the extent of viability loss, and suggests that the mechanism(s) which cause damage to these seeds during slow ageing are different from those in rapid ageing. Although, after ME, a small decrease in axis PL was apparent in slowly aged seeds (Figure 6.6), vigour losses were detectable in slowly aged seeds whether or not they had received subsequent ME (Appendix 6.1). All this evidence suggests that PL changes are unlikely to be a primary cause of deterioration.

As correlation may provide a key to possible mechanisms, relationships between losses in germinability or viability during AA and both conductivity of seed leachate and PL changes in axes and cotyledons have been examined extensively in this study. Obviously changes in conductivity of seed leachate are mainly a function of cotyledon integrity, while successful production of a seedling is highly dependent on the level of deterioration in the axes. Once again, the lack of correlation between PL losses and conductivity changes for untreated seeds during AA (Appendix 6.5) and the absence of a clear correlation between axis PL levels and loss of germination (Figure 6.7A) or viability (Appendix 6.5) both argue against PL depletion being the sole primary mechanism of seed deterioration.

The data in Figure 6.5 showed a dramatic decrease in PL of axes and cotyledons due to the interactions of acetone or water soaking treatment with subsequent ageing. In these seeds, there are strong correlations between losses of PL, losses in germinability and increased conductivity of seed leachate (Figures 6.7B and C and Appendix 6.5).

Acetone causes damage to membrane systems. Swanson *et al.* (1973) reported from their electron microscopic work that acetone disrupted membranes in cells of tobacco leaves. This mechanism was originally suggested by Halloin (1977) for acetone toxicity in seeds (Section 2.4.2), although he presented no substantiating evidence apart from measuring an increased conductivity of leachate from acetone soaked embryos of cotton seeds accompanying viability loss. In the present study, although

evidence of membrane disruption was not observed in either axes or cotyledons of unaged, acetone treated seeds (Section 7.2), these seeds were still more susceptible to ageing damage. It could be that acetone may cause damage to seeds via other effects (Meyer and Mayer, 1971; Eldan and Mayer, 1974).

Increased leachate conductivity associated with germination loss has also been reported to be a result of water soaking injury in soybeans (Woodstock and Tao, 1981; Tilden and West, 1985). However, the available evidence for mechanisms involved is mostly concerned with respiratory changes, although the implications are often related to membrane damage. For example, Woodstock and Taylorson (1981b) found increases in subsequent RQ values and acetaldehyde and ethanol levels of water soaked soybean embryos (particularly in aged seeds). Similarly, Powell and Matthews (1978) found a reduction in the respiration rate of pea. Both groups suggested that the alteration in respiratory metabolism resulting in a decline in vigour and viability can be attributed to damage to mitochondrial membranes. Ferguson *et al.* (1990a) showed that mitochondrial membranes (as measured by respiratory control ratio (RCR) and state 3 respiration rates) and tonoplast membranes (as measured by conductivity) deteriorated during storage of soybean. It is possible that damage to these membranes is aggravated by prior water soaking injury and may involve hydrolytic enzyme activity. For example, evidence of soaking injury causing reduced ribonuclease development with an accompanying reduction in axis growth has been reported in lima bean (Roos and Pollock, 1971).

6.3.1.2 *Fatty acid composition in TL*

The data presented in Tables 6.1-6.3 showed no evidence of losses in polyunsaturated fatty acids. The results from the AA and slow ageing studies showed some variation principally due to a small shift between the proportions of 16:0 and 18:2 fatty acids. This is possibly due to slight deterioration of the GLC column during use. In general, however, the proportions of fatty acid obtained from both experiments agree with reports in the literature for soybeans (Table 6.8). From this table it can be seen that there are clear differences between cultivars.

Table 6.8 Fatty acid composition of total lipid from soybean seed tissues as reported in the literature.

Cultivars	Seed materials	Fatty Acids					References
		C16:0	C18:0	C18:1	C18:2	C18:3	
		% of Total lipid					
Chippewa 64	Whole seed	9.8	4.0	17.7	58.5	9.9	Priestley and Leopold (1979)
Wayne	Whole seed	10.1	4.6	25.2	52.8	7.3	Priestley and Leopold (1983)
Union	Whole seed	13.5	4.2	24.1	51.2	6.8	Ferguson *et al.* (1990b)
Chippewa 64	Axes	10.3	2.1	4.1	51.3	32.3	Priestley and Leopold (1979)
Union	Axes	19.0	3.2	7.5	56.5	13.8	Ferguson *et al.* (1990b)
Williams	Axes	13.3	3.0	6.0	60.0	17.7	Priestley *et al.* (1985)
Desoto	Axes	20.0	3.2	6.0	54.6	16.1	Ferguson *et al.* (1990b)
Williams	Cotyledons	10.0	3.7	24.3	54.5	7.5	Priestley *et al.* (1985)
Desoto	Cotyledons	15.2	4.6	25.5	48.4	6.3	Ferguson *et al.* (1990b)

The absence of lipid peroxidation in total lipid which would be indicated by no decline of polyunsaturated fatty acids (18:2, 18:3) during ageing corresponds with the findings of Priestley and Leopold (1979) during AA, Ferguson *et al.* (1990b) during 'natural ageing', and Priestley *et al.* (1985) under high oxygen pressure. In contrast, Priestley and Leopold (1983) reported a decrease in levels of these fatty acids from TL in soybean during 'natural ageing' when viability dropped from 98 to 86%. Possibly, the difference between the present result or that of Ferguson *et al.* (1990b) and Priestley and Leopold's (1983) is a function of greater deterioration under this type of storage regime than in either this or Ferguson's study where no appreciable loss of viability occurred.

Although the acetone or water soaking treatment increased rate of germination losses, there was no evidence of any changes in fatty acid composition being involved. This is novel information, as no other reports have concerned themselves with this area. Work by Francis and Coolbear (1988) on tomato seeds may be relevant, if somewhat contradictory, as they found that a low temperature hydration treatment apparently increased all C 18 fatty acids in the polar lipid fraction of unaged seeds. On ageing, the level of 18:0 and 18:2 fatty acids decreased considerably in treated seeds. These authors also suggested that the initial increases of C 18 fatty acids during treatment or initial ageing of untreated seeds might indicate desaturation reactions of stearic acid (18:0) for membrane repair, and that the losses of fatty acids caused by hydration and ageing might be due to hydrolytic activity. Such hydrolytic enzyme activity seems likely to be involved in the lipid losses in the soybean seeds in this study.

6.3.2 *The role of membrane changes*

Total lipid analysis mainly reflects changes in storage lipid; thus it is possible that any changes in membrane lipids may be hidden. Priestley *et al.* (1985) found a decrease in proportions of 18:2 and 18:3 fatty acids of the polar lipid fraction from soybean axes after exposure of soybean seeds to high oxygen pressure and high temperature but no changes in TL. Stewart and Bewley (1980) analysed polar lipids and reported that the levels of these polyunsaturated fatty acids in soybean decreased due to AA, while none of these changes occurred in seeds stored at high temperature but low RH. Although a number of workers (*e.g.* Pearce and Abdel Samad, 1980 in peanut; Francis and Coolbear, 1988 in tomato; Pukacka and Kuiper, 1988 in maple) have attempted to identify changes in membrane lipids, only three reports prior to this one have been concerned with membranes from specific cell organelles, namely microsomes from soybean axes (Senaratna *et al.*, 1988) and sunflower seeds (Gidrol *et al.,* 1989) or mitochondria from soybean axes (Ferguson *et al.,* 1990b). The present results (Tables 6.4-6.5) indicate that there were no apparent changes in ratios of P:L affected by AA, slow ageing or seed treatments. The average ratios of P:L were only 0.39% from axes or 0.23% from cotyledons. Surprisingly, the ratios from the membrane extracts are little different from those of the TL preparations (Section 6.2.1). An expected ratio (based on a calculation assuming the membrane lipid consisted of dioleoyl phosphatidyl choline) would be 3.3%, ten times the observed value.

Accordingly, we must conclude that, in this study, lipid contaminants have been brought down in the microsomal membrane fraction. TEM examination of the fraction (Plate 6.2) bears out this suggestion, as it is clear that many lipid droplets are apparent. It is also speculated that if there were any damage to the membranes, the extraction technique might bring down only intact membranes which are likely to invest lipid droplets or oil bodies.

There appears to be only one set of reports in the literature where this approach has been used on similar materials: Senaratna *et al.* (1988) used a similar method of microsome extraction for axes from soybean seeds and reported that ~50% PL from microsomal membrane fractions were lost after seeds lost >80% viability. Unfortunately, they reported neither the amount of membrane lipids (L) nor P:L ratios, instead only the amounts of phospholipid extracted from axis tissue and microsome fractions were given. Thus, comparisons with their results may not be validated. In addition, the high level of variation associated with the technique used for this quantitative analysis of membrane lipid may cause difficulties of interpretation. The extremely small amount of lipid retrieved from the membrane fraction was difficult to weigh accurately, while the inherent variation of the seed stock may compound this problem further.

Molecules of membrane lipids are the most susceptible to lipid peroxidation, and many studies demonstrated that lipid peroxidation induces membrane damage (*e.g.* Priestley and Leopold, 1983; Harman and Mattick, 1978). Nevertheless, the results in Tables 6.6 and 6.7 provide no evidence that lipid peroxidation affected lipids from microsomal membranes. The high proportions of more saturated fatty acids (16:0, 18:1) in microsomal membranes (Tables 6.6-6.7) compared to TL (Tables 6.1-6.3) are similar to reports from other workers on polar lipids from soybean, except that in some of these studies, levels of 18:3 fatty acid are also high (Priestley and Leopold, 1983; Priestley *et al.*, 1985; Ferguson *et al.*, 1990b). There was a high variation between replications (both in internal and external replications as discussed in Appendix 3.2), apparent changes in proportions of fatty acids in either axes or cotyledons were thus not significant. Nor was there any clear evidence of changes in protein from the membrane preparation (Plate 6.1) at least from a qualitative point of view. Total protein of the microsome fractions was not determined.

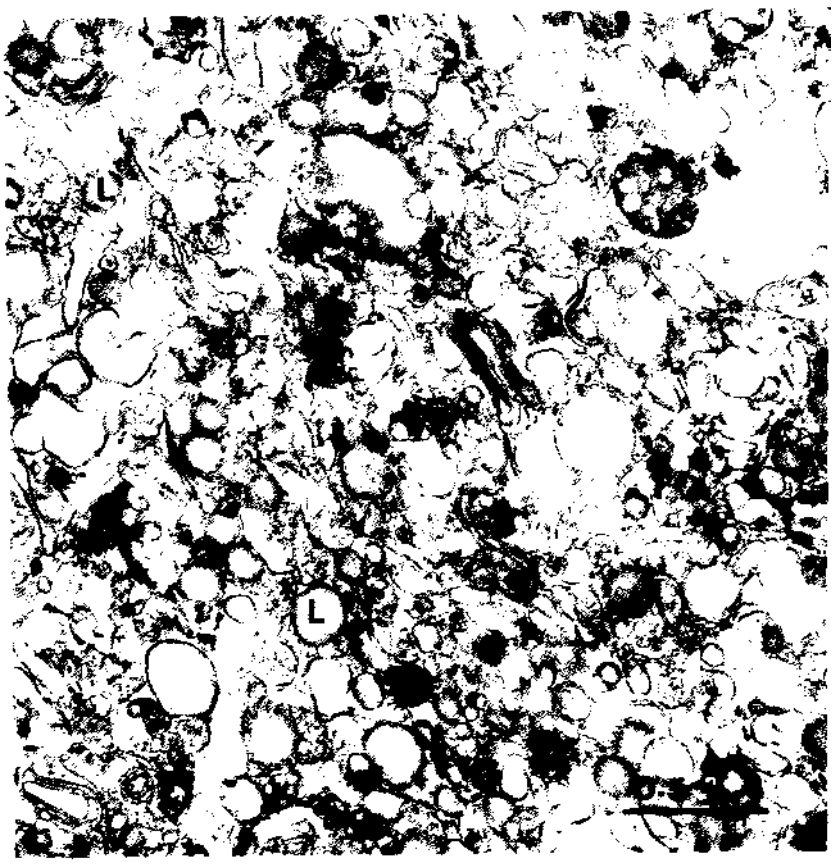


Plate 6.2 Microsomes isolated from axes of untreated unaged soybean seeds, *cv.* Davis. L = lipid droplet.

Somewhat, contradictory findings have been reported by Ferguson *et al.* (1990b). They found a slight decrease in the percentage of 18:3 fatty acids of mitochondria from axes in one cultivar of soybean accompanying a decline in seed vigour during natural ageing, despite no germination loss being noted, whereas a second cultivar did not show this effect. Gidrol *et al.* (1989) attempted to evaluate the level of conjugated dienes in microsomes of sunflower seeds to estimate lipid peroxidation in microsomal membrane fractions, but no conjugated dienes were detected from fractions of either control or accelerated aged seeds, despite the fact that lipid peroxidation was found in seed tissues. Such evidence indicates that lipid peroxidation may occur in either membrane or reserve lipid, but we are no clearer about whether lipid peroxidation is a prior cause or a subsequent effect of seed deterioration. The data of both Gidrol *et al.* (1989) and Ferguson *et al.* (1990b) suggest that peroxidation is an early event, because it was detected when sunflower lost little viability and before soybean seeds lost any germinability. Many other workers report, however, effects only after significant germination losses or even complete loss of viability (*e.g.* Stewart and Bewley, 1980). The results presented here correspond with Senaratna *et al.* (1988) in that they found no changes in fatty acid saturation in microsomal membrane fraction from soybean axes due to ageing, although they stored their seeds for 5 years and seeds had lost their viability considerably.

6.4 PRELIMINARY CONCLUSIONS

1) Amounts of total lipid extracted from both axes and cotyledons were unaffected by either ageing or seed treatments.

2) Decreases in PL contents in cotyledons (but not axes) were closely related to seed deterioration during rapid ageing

3) Pretreatments with acetone or water soaking prior to accelerated ageing caused damage to axes as well as cotyledons, and to a greater extent than AA alone.

4) There were no PL changes from either axes or cotyledons due to slow ageing, probably seed deterioration had not preceded sufficiently.

5) ME applied after slow ageing accelerated PL losses in axes but these changes were not associated with decreased seed performance, which suggests that PL losses in this tissue are either not important or that germinating seeds can repair this kind of damage, providing it is not too severe.

6) No changes in microsomal membrane lipids were observed as a result of ageing or treatment but, this may be because of high levels of contaminating storage lipid in these fractions.

7) Neither seed treatments (acetone, soaking, ME) nor ageing (AA and slow ageing) had any effects on fatty acid composition of TL from either seed tissue or from the microsomal membrane fraction, which suggests that lipid peroxidation is unlikely to be a cause of seed deterioration in the seed lot of *cv.* Davis used in this study.

CHAPTER 7

CHANGES IN THE ULTRASTRUCTURE OF CELLS DURING ACCELERATED AGEING AND EFFECTS OF PRIOR ACETONE DAMAGE OR SOAKING INJURY

7.1 INTRODUCTION

This chapter reports the results of an investigation to determine whether there was any evidence of alterations in the ultrastructure of soybean seeds *cv.* Davis resulting from accelerated ageing (AA) or its interactions with the prior seed treatments of either acetone or water soaking. Cells from axes and cotyledons were examined using transmission electron microscopy. Interpretations are made in relation to physiological changes and seed performance data (presented in Section 6.1).

7.2 RESULTS

7.2.1 **Ultrastructure of axes**

Sections from axes were confined to radicle tissue between the cotyledon attachment and the radicle tip (see details in Section 3.13). These sections contained provascular, procortical, and epidermal tissues.

7.2.1.1 *Effects of ageing*

Plates 7.1-7.2 show electron micrographs of axes from untreated, unaged material. The cytoplasm contains numerous protein bodies as well as lipid bodies (Plate 7.1A). By cross checking with the literature (*e.g.* Webster and Leopold, 1977; Chabot and Leopold, 1982), it is clear that lipid bodies are smaller than protein bodies and are scattered through the cytoplasm, lining the plasmalemma or surrounding protein bodies

(Plates 7.1-7.2). Protein bodies are generally granular in appearance (Plates 7.1B and C). The nucleus is large containing evenly staining chromatin and a prominent round nucleolus. At higher magnification (Plates 7.1B, C and D), there is evidence of the double membrane constituting the nuclear envelope. Protein bodies are of variable size and shape with a range of 0.6-3.2 μ diameter, and a few are found apparently merging to each other (Plate 7.2A). Cells from the procortical area contained more numerous and larger protein bodies than other parts of the radicle studied. Ribosomes are abundant and rough endoplasmic reticulum clearly visible (Plate 7.1D). Dictyosomes, mitochondria and proplastids with starch grains are also present (Plates 7.1D and 7.2A). The mitochondria evident in many sections are of different forms: elongated, round, dumbbell-shaped or oval and generally small. Their average diameter ranged from 0.3 to 0.6 μ, comparable in size to those found by Chabot and Leopold (1982) in the same tissue. There was no evidence that the plasmalemma was detached from the cell wall (Plate 7.2).

After ageing under 40°C, ~100% RH for 6 days (when seed viability had fallen from 99 to 85%), there are small changes in the organisation of cell organelles as shown in Plates 7.3-7.4. Plate 7.3A shows a general view of procortical cells. It is apparent that a number of protein bodies possess membrane bound vesicles (as in maize mitochondria (Berjak and Villiers, 1972)), suggesting hydrolytic damage. Evidence of this type of damage also exhibited in signs of fusion of these bodies (Plate 7.3A) and in the fact that they were not uniformly staining, many containing electron-lucent areas (Plate 7.4). Nuclei are still well defined, although patches of chromatin become more electron dense (Plate 7.3B). A few mitochondria appear to have a small area of cleared matrix (Plate 7.3C). There appears to be some plasmalemma disruption indicated by some displacement of membrane from the cell wall (Plates 7.3C and 7.4). It was observed that this and other disruption occurred mainly in the cortical region near the epidermis, decreased towards the centre, and was absent in provascular cells. In Plate 7.4, the plasmalemma of these procortical cells has withdrawn from the cell wall in many places, and the space between them is filled with a fine grainy material. In Plate 7.3C, incipient separation of plasmalemma without loss of cell contents appears to have occurred. This might represent an early stage of plasmalemma damage.

Plate 7.1 Radicle cells from provascular (A,D) and procortical tissues (B,C) of untreated, unaged soybean seed *cv.* Davis.

CW = cell wall, L = lipid body, PB = protein body, N = nucleus, M = mitochondrion, RER = rough endoplasmic reticulum, Pp = proplastid.

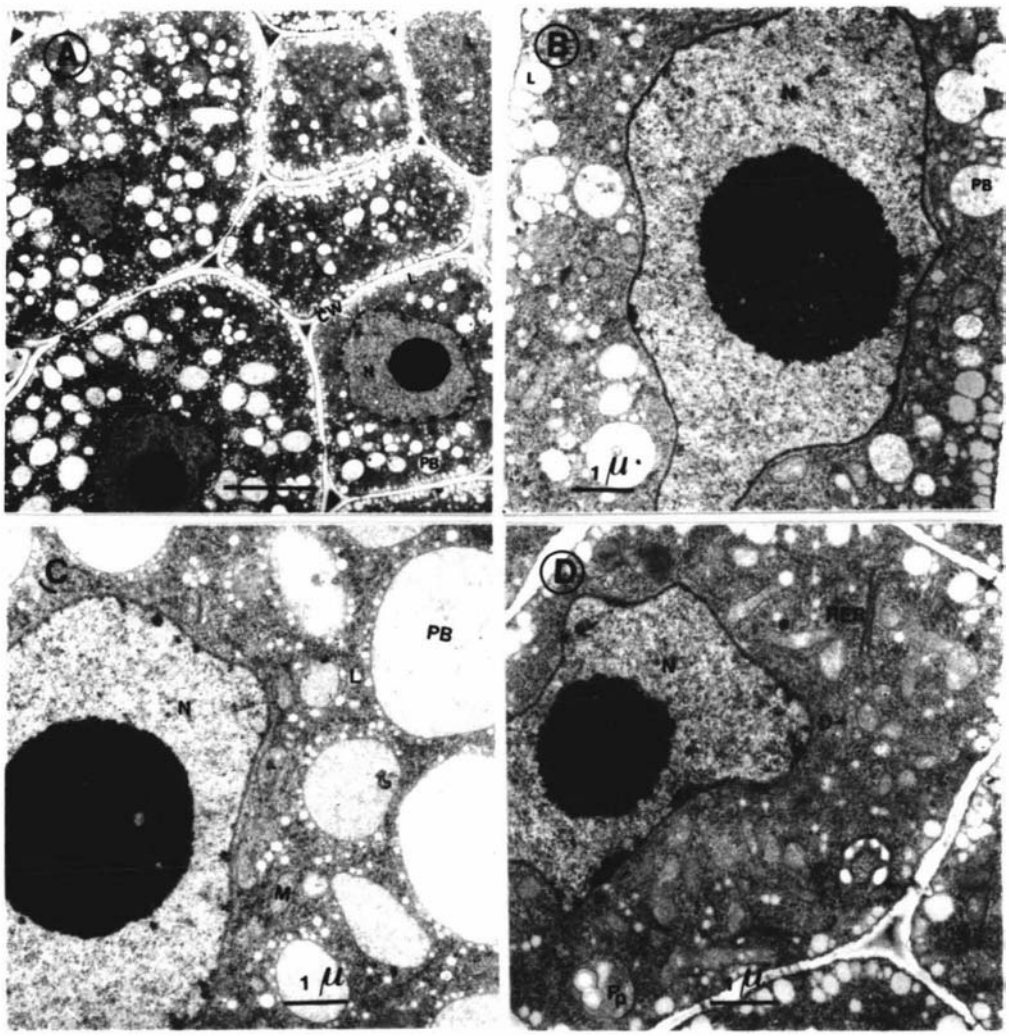


Plate 7.1

Plate 7.2 Radicle cells from procortical tissue of untreated, unaged soybean seed *cv.* Davis.

CW = cell wall, L = lipid body, PB = protein body, Arrowhead = merging protein bodies, M = mitochondrion, Pp = proplastid, PM = position of plasmalemma.

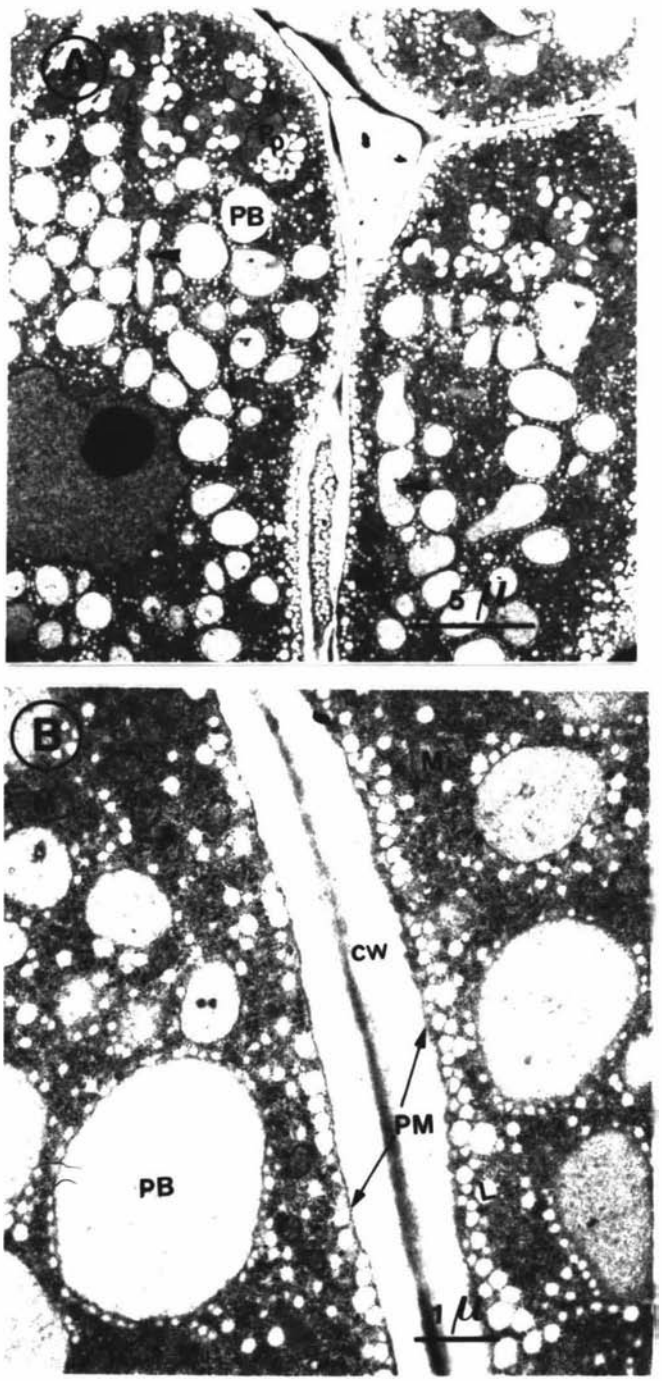


Plate 7.2

Plate 7.3 Radicle cells from procortical tissue of untreated, 6 d aged soybean seed *cv.* Davis.

CW = cell wall, N = nucleus, L = lipid body, PB = protein body, Arrowhead = merging protein bodies, M = mitochondrion, PM = position of plasmalemma.

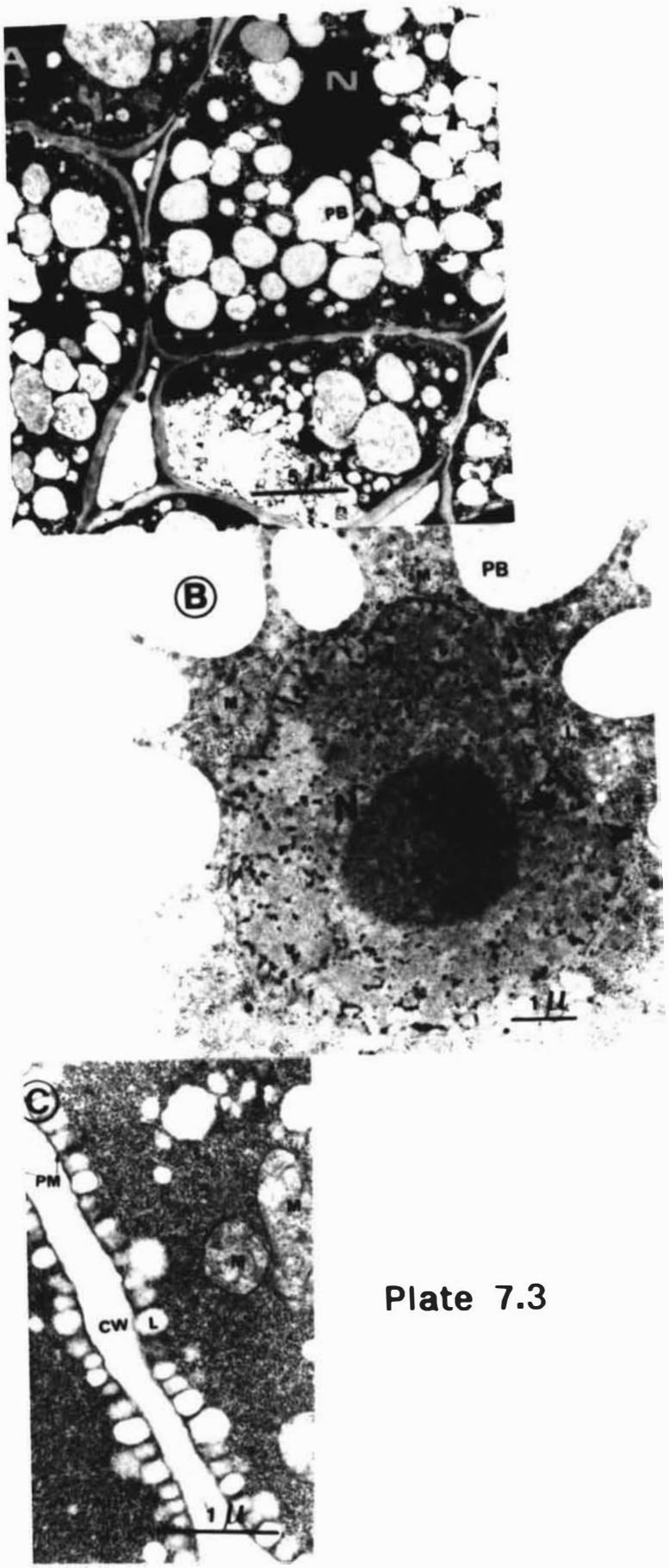


Plate 7.3

Plate 7.4 Radicle cells from procortical tissue of untreated, 6 d aged soybean seed *cv.* Davis.

CW = cell wall, N = nucleus, L = lipid body, PB = protein body, PM = position of plasmalemma.

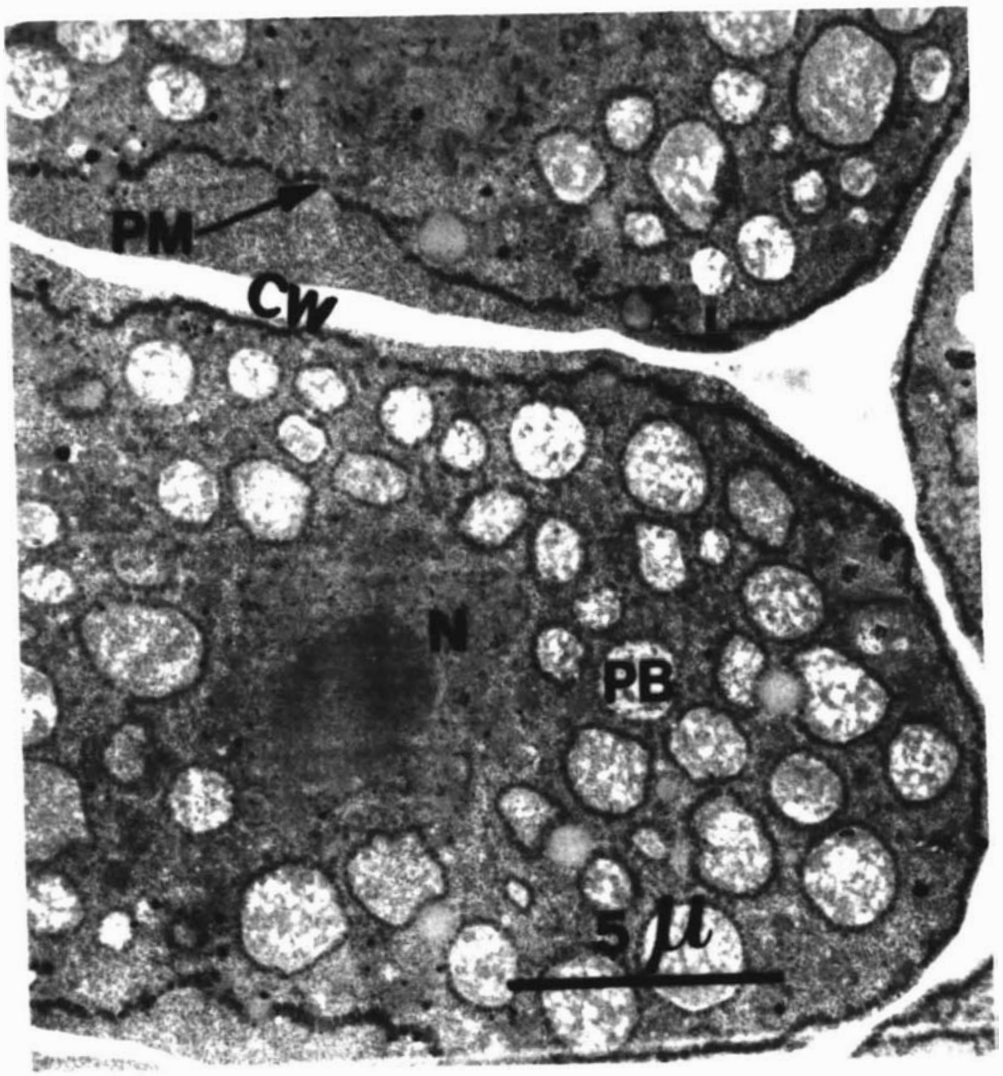


Plate 7.4

7.2.1.2 *Effects of acetone*

There was no evidence of any changes apparent in any cells in all regions examined from acetone treated, unaged seeds. As shown in Plate 7.5, nuclei, mitochondria and protein bodies appear normal. Lipid bodies are distributed through the cytoplasm or line the plasmalemma.

Plate 7.6 shows a general view (Plate 7.6A) and high magnification (Plate 7.6B-D) of the ultrastructure of provascular cells from acetone treated seeds after 6 d AA. The nucleus remains normal and is enclosed by a distinct double membrane, as is the proplastid (Plate 7.6B). Lamellar membranes within the proplastid are clearly seen in this plate. Protein bodies are less uniform than in unaged seed with both densely staining material and areas of electron transparency (Plate 7.6C). Frequent mitochondria are apparent with clearly visible boundaries, and (like untreated aged radicles, Section 7.2.1.1) many of them contained small electron lucent areas (Plate 7.6C). Under high magnification (Plate 7.7), there is more evidence of these clear areas. Fragments of rough ER are well-defined, and ribosomes are plentiful. Some withdrawal of plasmalemma from the cell wall was again apparent in those procortical cells close to the epidermis (Plate 7.6D). Evidence of extrusion of cytoplasm through the plasmalemma is indicated in the lower part of this plate. Nuclei of some cortical cells appear damaged after ageing as evidenced by condensed patches of chromatin (Plate 7.6D).

Plate 7.5 Radicle cells from acetone treated, unaged soybean seed *cv.* Davis.

N = nucleus, L = lipid body, PB = protein body, M = mitochondrion.

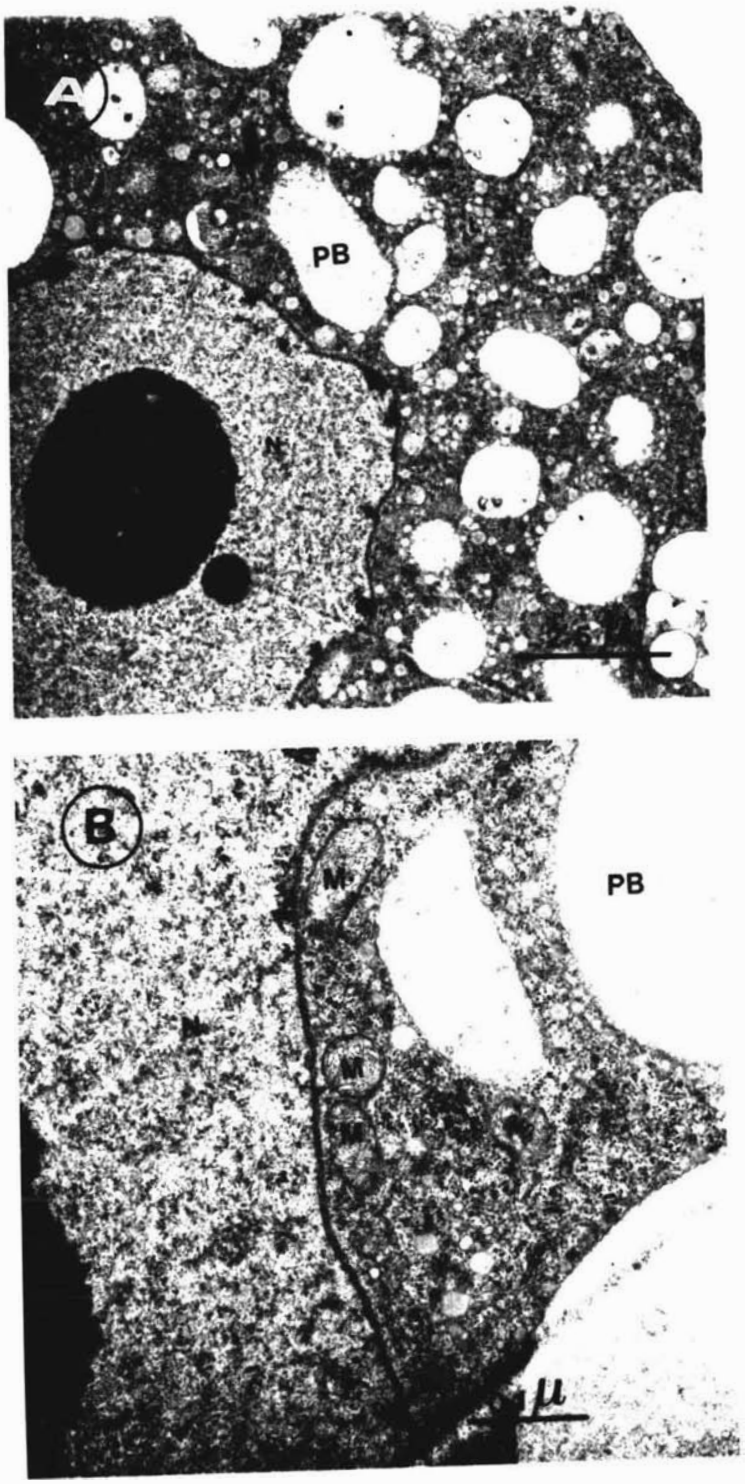


Plate 7.5

Plate 7.6 Radicle cells from acetone treated, 6 d aged soybean seed *cv.* Davis.

CW = cell wall, L = lipid body, PB = protein body, Arrowhead = merging protein bodies, N = nucleus, M = mitochondrion, ER = endoplasmic reticulum, Pp = proplastid, R = ribosome, PM = plasmalemma.

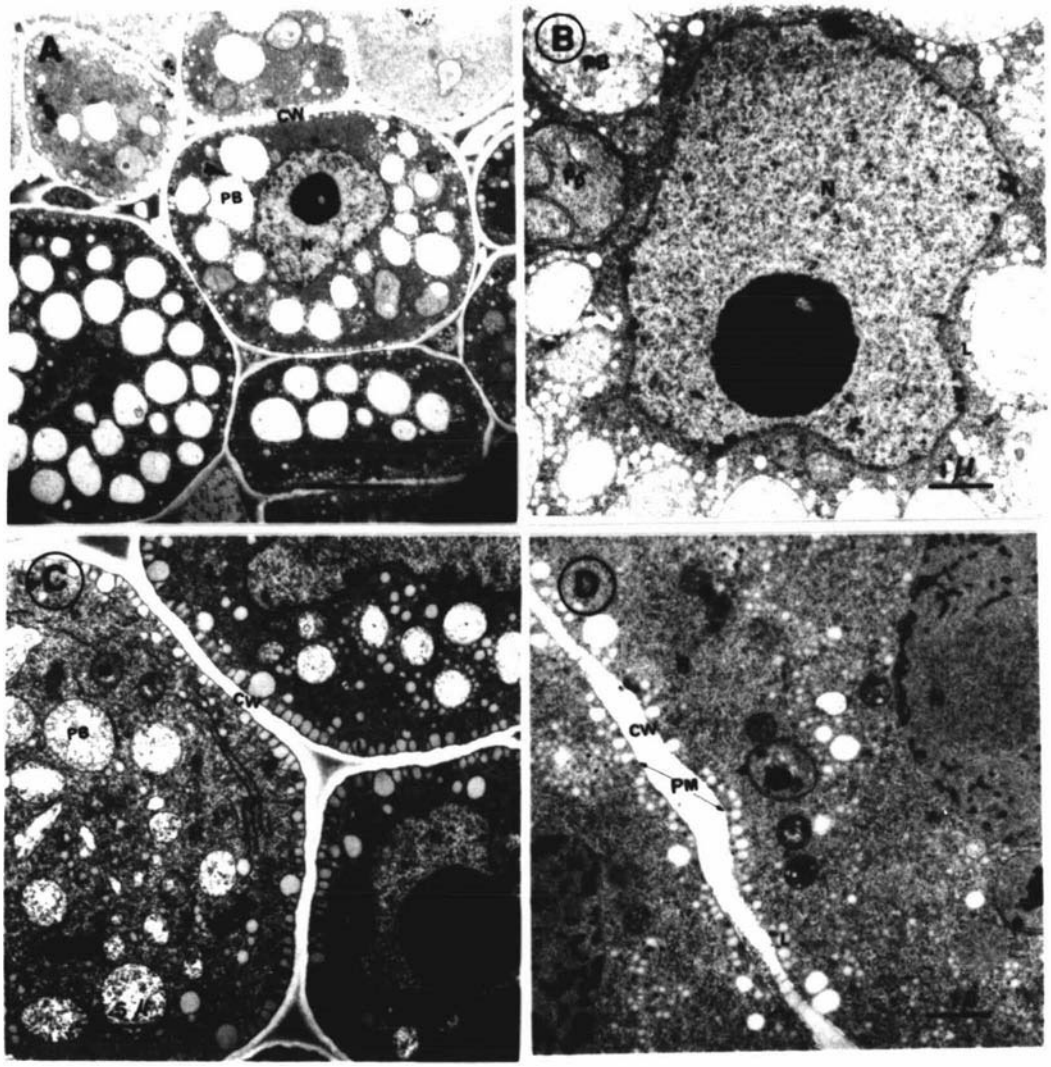


Plate 7.6

Plate 7.7 Radicle cells from cortical tissue of acetone treated, 6 d aged soybean seed *cv*. Davis.

CW = cell wall, N = nucleus, L = lipid body, M = mitochondrion. Arrow = matrix clear area.

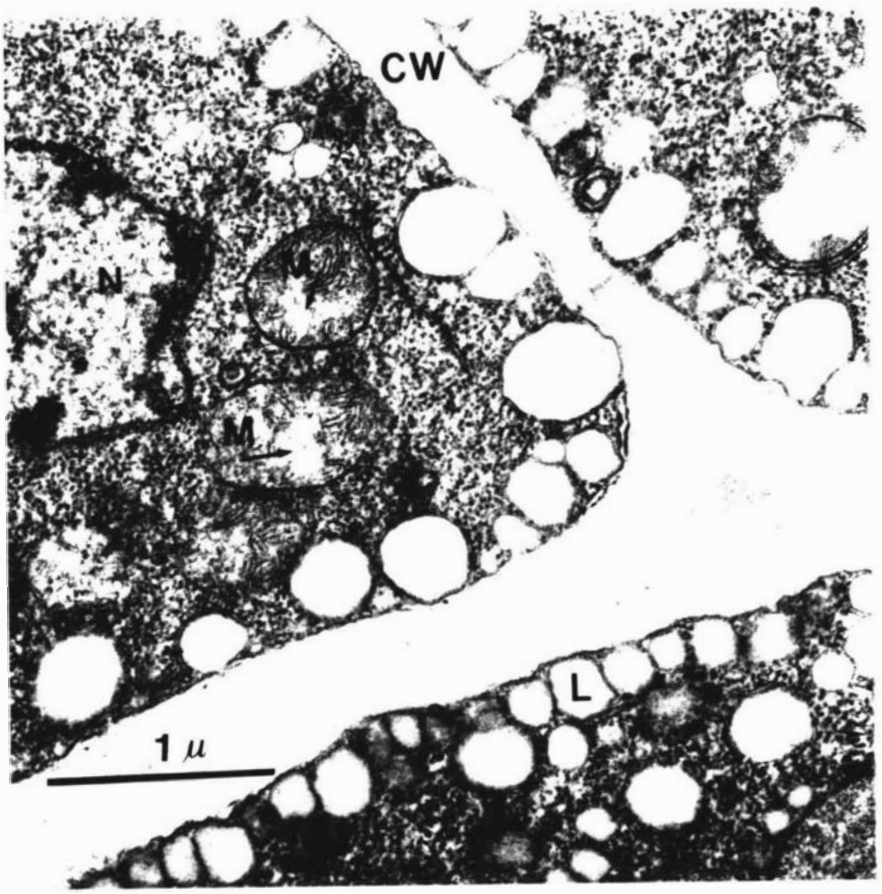


Plate 7.7

7.2.1.3 *Effect of water soaking*

Plate 7.8 shows general views of cells from provascular tissue of water soaked seeds before ageing (Plate 7.8A) and after 6 d AA (Plate 7.8B). On examination of cells from water soaked unaged axes, an obvious structural abnormality is the appearance of the protein bodies, these being non-uniform with electron dense granules. In procortical tissue (Plate 7.9A) these bodies also appear severely distorted. After ageing, there are a large number of cells where most organelles became unrecognisable (Plate 7.8B), although nuclei, protein and lipid bodies are still distinguishable, their boundaries, particularly those of nuclei, in many cases, lose their distinctness. Aged nuclei now contain dense patches of chromatin and the nucleolus appears distorted, enlarged and less electron dense. All cells appear to be plasmolysed and have leaked cytoplasmic contents. Lipid bodies are aggregated near protein bodies within the shrunken cytoplasm or found lining the plasmalemma (Plate 7.8B). Few are dispersed in the cytoplasm. Similar evidence was also observed in aged procortical cells from the same material (Plate 7.9B). Vacuolation of protein bodies is evident in both unaged and aged material (Plates 7.9A and B).

Plate 7.8 Radicle cells from provascular tissue of water soaked, unaged (A) and 6 d aged (B) soybean seed *cv.* Davis.

CW = cell wall, N = nucleus, L = lipid body, PB = protein body, PM = position of plasmalemma.

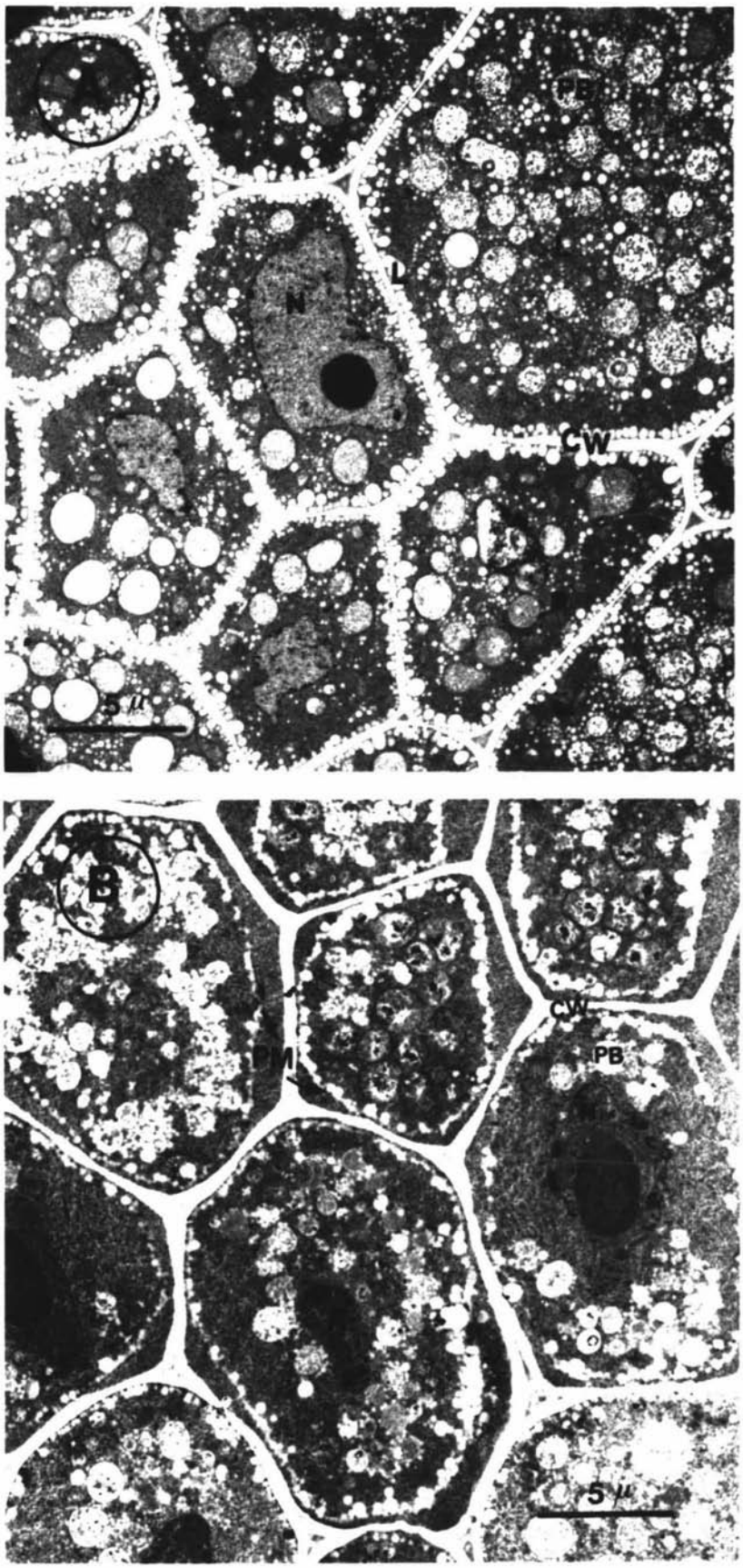


Plate 7.8

Plate 7.9 Radicle cells from procortical tissue of water soaked, unaged (A) and 6 d aged (B) soybean seed *cv.* Davis.

CW = cell wall, N = nucleus, L = lipid body, PB = protein body, Arrowhead = merging position of protein bodies, Pp = proplastid, PM = plasmalemma.

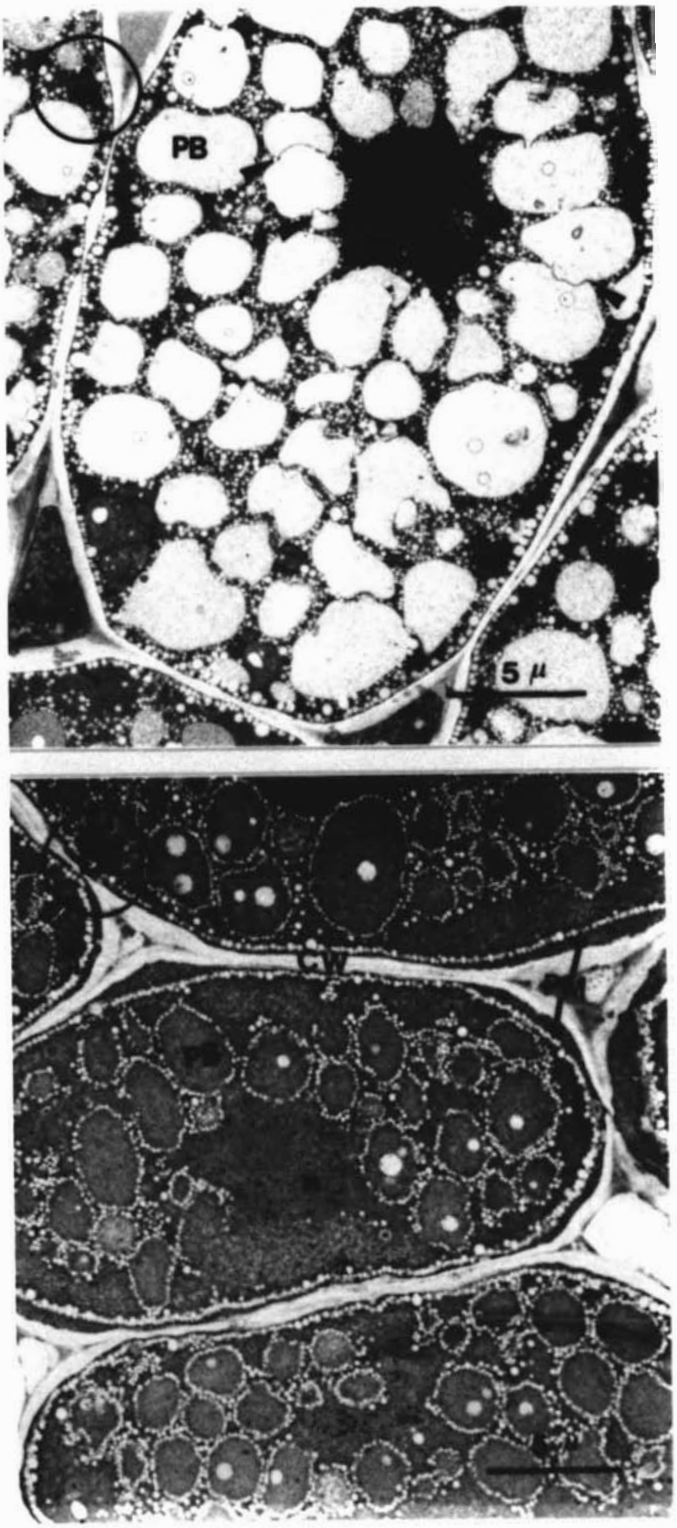


Plate 7.9

7.2.2 Ultrastructure of cotyledons

Electron micrographs of cells from cotyledons of untreated unaged and treated aged seeds are shown in Plates 7.10-7.12. It was generally noticed that cotyledonary cells were less severely damaged than those from axes (*e.g.* compare Plates 7.11 with 7.6 or Plates 7.12 with 7.8B and 7.9B). It is clear from Plate 7.10A that cells from cotyledons are packed with both protein and lipid bodies. While most of protein bodies consist of a homogeneous proteinaceous matrix, some contain possible globoid crystals (Plate 7.10B). A well-defined nucleus with its double membrane boundary is also evident in this picture. Other cell organelles such as mitochondria with normal cristae and outer membranes, short fragments of ER-like membranes and dictyosomes were frequently apparent (Plate 7.10C). There was no change affected by AA observed from cotyledons of untreated seeds (data not shown).

For acetone treated seeds, there was no evidence of any changes in unaged seeds. Electron micrographs of this material are not shown. For acetone treated, aged seeds, Plates 7.11A and B show that the nucleus contains patches of densely staining chromatin, although the nucleolus and nuclear envelope both appear unchanged. Protein bodies (Plates 7.11A, B and C) were not as granular as those from the axes from the same sample (compare Plates 7.11 A-C with 7.6).

Ultrastructural changes were not evident in cotyledons of unaged water soaked seeds, and electron micrographs are not shown. But, for aged material, Plate 7.12 shows that there are obvious changes in nuclei, protein bodies and plasmalemma. There were few mitochondria, proplastids or ER visible; and thus changes in these organelles are not described.

Nuclei from aged, soaked material contain unevenly staining chromatin, although nucleoli are still round and prominent (Plate 7.12A). The nuclear envelope is now poorly defined. Protein bodies are granular, and some are merged with one another (Plate 7.12B). It is clear from Plate 7.12C that the plasmalemma has pulled away from the cell wall. Leakage from cytoplasm appears to fill the resulting space.

Plate 7.10 Cotyledonary cells from untreated, unaged soybean seed *cv*. Davis.

CW = cell wall, PM = plasmalemma, L = lipid body, PB = protein body, N = nucleus, GC = globoid crystal, M = mitochondrion, D = dictyosome, ER = endoplasmic reticulum.

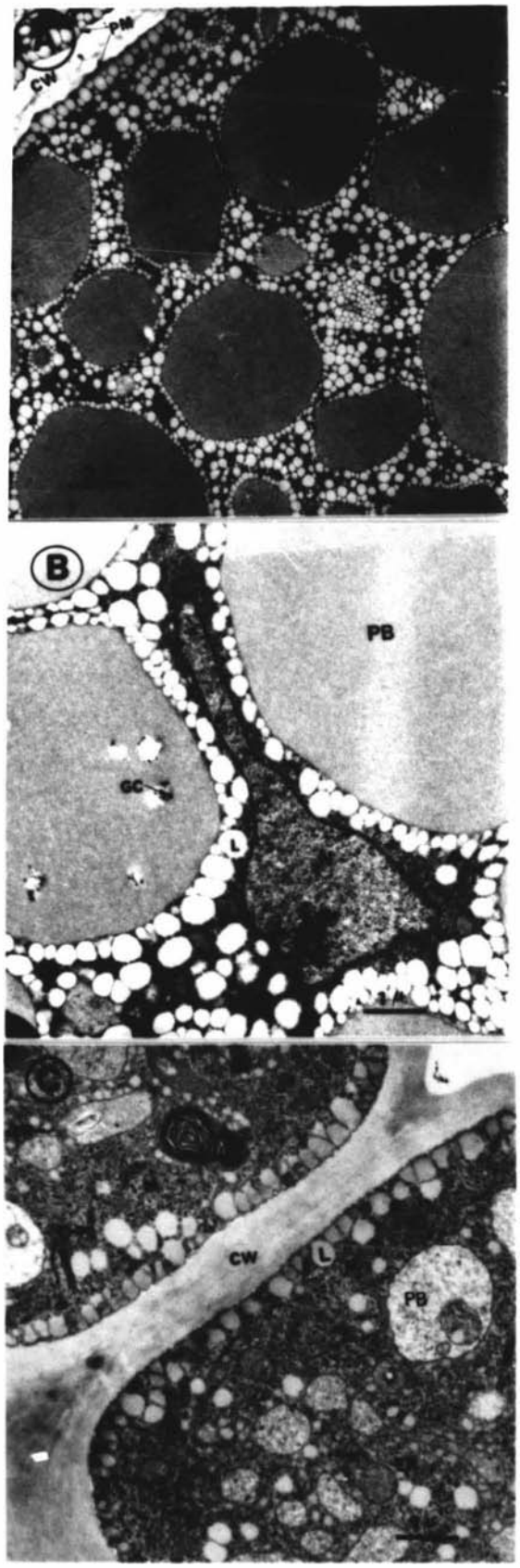


Plate 7.10

Plate 7.11 Cotyledonary cells from acetone treated, 6 d aged soybean seed *cv.* Davis.

L = lipid body, PB = protein body, N = nucleus, ER = endoplasmic reticulum.

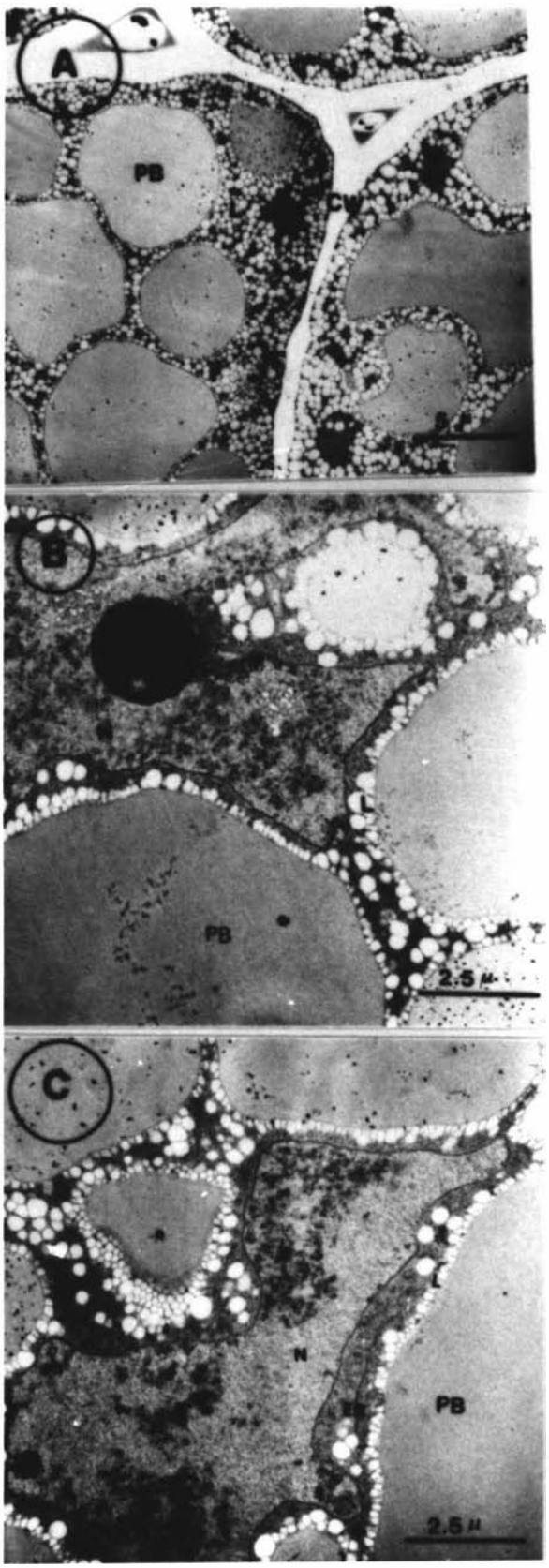


Plate 7.11

Plate 7.12 Cotyledonary cells from water soaked, 6 d aged soybean seed *cv*. Davis.

PB = protein body, N = nucleus, CW = cell wall, PM = plasmalemma, L = lipid body.

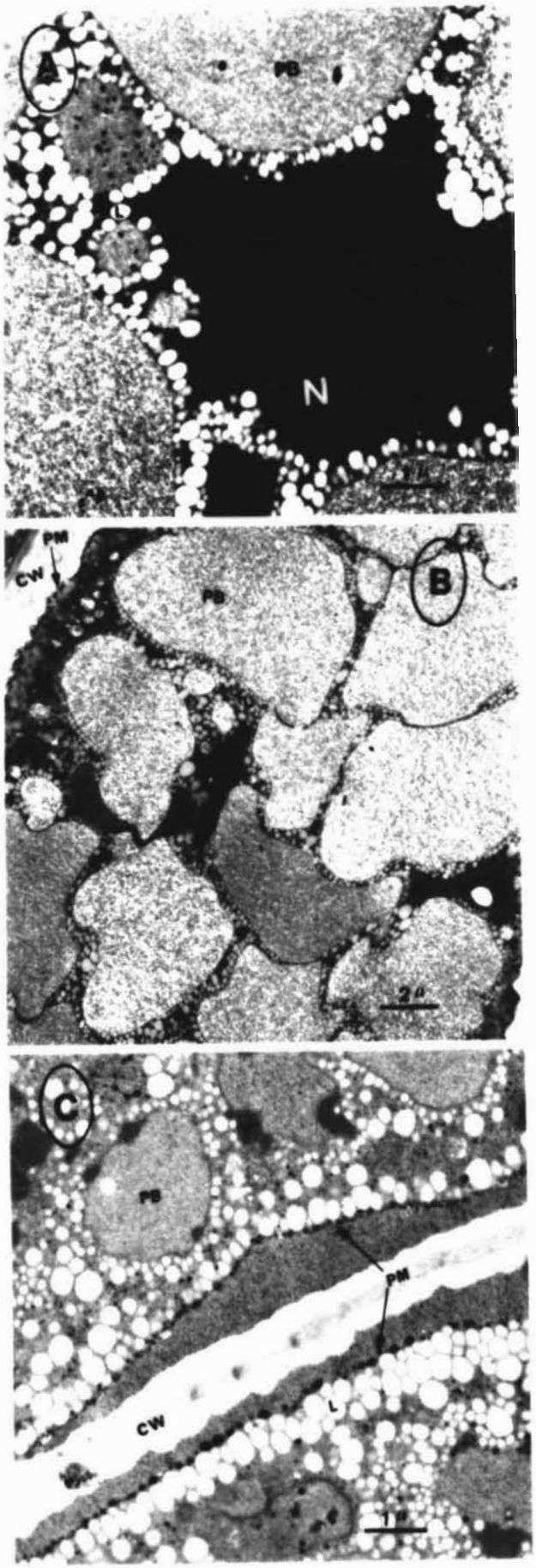


Plate 7.12

7.3 DISCUSSION

Although limited in their extent, these electron microscopic studies reveal further information about physiological changes during ageing which may be reflected in seed performance. However, care must be taken when comparisons with the literature are made, since there are two main variables involved: 1) differences in the condition of the tissues to be fixed (*i.e.* whether dry or imbibed), and 2) differences in fixation procedures. In this study, seed tissues were imbibed for 24 h and fixed in aqueous solution prior to preparation for electron microscopy (see Appendix 3.3).

Soybean seed tissues, particularly from cotyledons, contain an abundance of protein and lipid bodies. These are of different shapes, types and sizes and are found from both axes (Plate 7.1) and cotyledons (Plate 7.10). The proteinaceous matrix may be structurally homogeneous or may include small globoid crystals (Plate 7.10B). This finding corresponds with the report on this species by Lott (1981). Lipid bodies normally are electron lucent (*e.g.* Plates 7.1 and 7.2), except in some cases they become apparently electron dense (Plate 7.4). Quite why this happens is not clear, but this has also been found in the work of Chabot and Leopold (1982) and Hallam (1972) in soybean and rye, respectively. Descriptions of lipid bodies lining the plasmalemma, around protein bodies or being scattered in the cytoplasm have also been reported in unaged tissues of soybean (Webster and Leopold, 1977; Chabot and Leopold, 1982) and peas (Harman and Granett,1972). However, Hallam (1972) and Berjak (1978) found in embryo cells of rye and maize, respectively, that the lipid bodies lined the plasmalemma in dehydrated seeds, while they were often found throughout the cytoplasm after imbibition when the cytoplasmic streaming had begun. In accordance with this, Webster and Leopold (1977) found that, in imbibed cells of soybean cotyledon, lipid bodies were not as numerous along the plasmalemma as those from dry seeds. Although several reports have shown the evidence of lipid coalescence in non-viable or fungally infected material in other species (*e.g.* Vishnyakova *et al.*, 1976 in rice; Francis, 1985 in tomato; Anderson *et al.*, 1970b in wheat), this kind of change does not appear to be evident in the soybean used in this study.

The most obvious structural difference of aged seeds from unaged ones is the disruption of plasmalemma from the cell wall. Acetone and, particularly, water soaking prior to subjecting seeds to AA increased this effect. This evidence might relate to the increases in conductivity due to ageing and interactions of treatments and AA (Appendix 6.2). In many cases there was apparent leakage of cytoplasmic contents through the membrane. Withdrawal of plasmalemma has also been found in aged embryos of rye (Hallam, 1972), lettuce (Villiers, 1972), peas (Harman and Granett, 1972), rice (Öpik, 1972) and in fungally infected wheat and peas (Anderson *et al.*, 1970b; Harman and Granett, 1972, respectively). According to Öpik (1980), when dry rice coleoptiles were subjected to aqueous fixation, the plasmalemma was characteristically pulled away from the cell wall in many places, but not for the material from anhydrous fixation. Chabot and Leopold (1982) also found disrupted plasmalemmae in axes of dry soybean. Webster and Leopold (1977) have presented similar evidence for dry soybean cotyledons, but if the tissue was allowed to imbibe for 20 minutes before fixation, no disruption of plasmalemma was observed. In the present study, all tissue samples were imbibed for 24 h before fixation, therefore any disruption of membrane is likely to represent cellular damage rather than artifacts. Moreover, similar results were obtained from all types of aged tissues studied, but not from unaged ones fixed in a similar manner. Although there were some small separations found in unaged material (Plate 7.1D), these are much less than those from the aged tissue. It is also possible that the observed damage may only have occurred in aged seeds after imbibition. It may be present, but hidden in an aged dry seed.

Plate 7.6D showing axis sections from acetone treated aged seed represents an interesting stage. The plasmalemma is pulled away from the cell wall, but apparently cytoplasmic leakage has only occurred through part of the membrane. Harman and Granett (1972) found similar results in fungally infected peas and, having demonstrated that this was not an artifact, suggested that dehydration effects in infected cells may be the cause. It is possible that acetone treatment may cause similar dehydration effects as well as breakdown of the semi-permeability properties of the membrane. Leakage of cell contents into the intercellular space indicates major damage to the membrane. Hallam *et al.* (1973) working with rye, suggested that the

material which has leaked out from cytoplasm may be denatured proteins and signifies the loss of semi-permeability of the plasmalemma in the non-viable embryo. Here, the leakage material is of similar electron density and appearance to the cytoplasm suggesting membrane leakage.

We might speculate that incipient shrinkage is possibly repairable (*e.g.* Plates 7.1D and 7.3C) because seeds from which this material was taken still showed high viability (85%), whereas much more pronounced effects (Plates 7.4, 7.8B and 7.9B) are not likely to be, resulting in the death of that cell. The concept of cellular repair will be discussed further in Chapter 8. It must also be emphasised that (although they were in the same tissue) different cells behave differently (compare Plates 7.3C and 7.4). Observations in this study suggest that the cortical cells near epidermis were severely damaged by ageing, and the extent of damage decreased towards the centre.

The evidence of clear matrix areas in the mitochondria (Plates 7.3C, 7.6C and 7.7) caused by ageing or an acetone-ageing interaction is interesting. In a report on non-viable rice embryos, Vishnyakova *et al.* (1976) indicated that mitochondria of those non-viable tissues exhibited signs of degeneration as evidenced by space between the internal and external membranes increasing, the number of cristae decreasing, and the matrix clearing in the course of swelling for 24 h. Abu-Shakra and Ching (1967) also found that mitochondria isolated from 4-day-old seedlings of new soybean seeds were intact and swollen, while those of old material appeared to have dilated or inflated cristae and coagulated matrix, and some were devoid of an intact outer membrane. In accordance with this, Hallam *et al.* (1973) found swollen cristae in normal profile mitochondria of non-viable rye embryos, however the majority of mitochondria in this tissue showed distended outer membranes. These findings are consistent with alterations in the respiratory metabolism of aged seeds in soybean (Abu-Shakra and Ching, 1967; Woodstock *et al.*, 1984) as well as in other species (Hallam *et al.*,1973; Vishnyoka *et al.*, 1976). All suggest that changes in the ultrastructure of mitochondria may reflect respiratory changes resulting in alteration of the performance of seeds.

Patches of densely staining chromatin (Plates 7.3B and 7.6D), less prominent nucleoli (Plates 7.4 and 7.9B) and poorly defined nuclear membranes (Plates 7.8B, 7.9B and 7.12) all suggest nuclear damage as a result of ageing and its interactions with prior soaking in acetone or water. Villiers (1972) reported that, in lettuce embryos, normal unaged nuclei were stained fairly homogeneously, except for a fine network of heterochromatin. As ageing proceeded, the heterochromatin stained more deeply; and as seeds lost viability, dense patches of chromatin appeared, while eventually the nuclear membrane became difficult to distinguish as a continuous system. Condensed chromatin has also been reported in nuclei of non-viable tomato embryos (Francis, 1985). Nuclear damage caused by ageing has also been detected by examining chromosome damage in peas, broad beans and barley (Roberts *et al.*, 1967).

Disorganisation of cytoplasm and disintegration of cell organelles were observed in aged, water soaked seeds, which lost almost all their viability (1%), although lipid bodies were still recognisable. This corresponds with the report on embryos of non-viable rye (Hallam, 1972), where lipid bodies were not as damaged as the other organelles. This is possibly because of differences in membrane systems between the monolayer surrounding lipid bodies and the bilayer of other organelles. In contrast, other workers reported the coalescence of lipid bodies in embryos of non-viable seeds (Villiers, 1972 in lettuce; Vishnyakova *et al.*, 1976 in rice; Francis, 1985 in tomato), stored pea seeds (Harman and Granett, 1972) and in fungally infected, aged wheat seeds (Anderson *et al.*, 1970b).

Plates 7.2A, 7.3A, 7.6A and 7.12B in all indicate merged or merging protein bodies in the cortical cells of axes and cotyledons, the frequency of which increases with ageing. No increase in the size/volume of protein bodies were evident. During treatments causing loss of seed viability, protein bodies appeared to become more granular and also to contain vacuoles. Swift and O'Brien (1972) showed that this type of observation on protein bodies in wheat scutellum during germination, indicated hydrolytic activity.

The similarity in appearance of ER found in both untreated, unaged (Plate 7.1D) and acetone treated, aged embryo (Plate 7.6C) suggests that ageing or acetone treatment may have no effect on the structure of this system. Of course, no quantification of ER was attempted in this study. Although Berjak and Villiers (1972a) found abnormal ER of aged embryos in maize during early imbibition, this abnormality was corrected at later stages of germination. It is possible that the ER of viable aged seeds could be repaired under the high relative humidity conditions of AA in this study.

7.4 PRELIMINARY CONCLUSIONS

Generally, ageing caused disruption and leakage of the plasmalemma, electron dense patches of chromatin in nuclei, clearing mitochondrial matrices, and possible hydrolytic damage to protein bodies. Water soaking had more severe damaging effects, particularly after ageing, than acetone or ageing alone, as several organelles became unrecognisable (*e.g.* mitochondria, plastids, dictyosomes), and some were distorted (*i.e.* nuclei, plasmalemmae, and protein bodies). Soaking injury caused dramatic changes to protein bodies even in unaged seeds. Damage occurring in axes appeared to be greater than cotyledons. Because of limited access to facilities, only extreme treatments were examined. A much more detailed study would be necessary to determine which, if any, of these changes in ultrastructure might be a primary event in seed deterioration.

CHAPTER 8

GENERAL DISCUSSION AND SCOPE OF FURTHER WORK

8.1 INTRODUCTION

The broad aims of this study were originally to improve the storability of soybean seeds by utilising seed treatment techniques and to understand some of the factors affecting seed performance after seed storage. In this final chapter, the achievements of this work and key conclusions are discussed. Limitations and scope for further work are outlined in the last section.

8.2 ACHIEVEMENTS OF THE RESEARCH

8.2.1 **Protective effects of seed treatments**

Two types of seed treatments, *i.e.* antioxidant and hydration-dehydration treatments, have been evaluated on soybean seeds aiming to prolong seed storage life and also as a means of studying critical events in seed ageing. Contradicting several reports found in the literature, no beneficial effects of seed treatments were found in this study. However, several factors which may impinge on the effectiveness of seed treatments have been defined.

Antioxidant effects

From the literature many variables such as differences between seed species and/or cultivars, concentrations of chemicals used, techniques of application or storage conditions appear to affect the effectiveness of antioxidants (Section 2.3.2). Accordingly, in this study, an evaluation was undertaken on different types of antioxidants *i.e.* α-tocopherol, BHT, iodine and β-mercaptoethanol on different

soybean seed cultivars subjected to CD, AA or slow ageing regimes. Although this work constituted an intensive series of experiments, much more comprehensive than previously published work, none of the antioxidants assessed showed beneficial effects. Instead, high concentrations of BHT (2.2%) or iodine (1%) caused toxic effects to the seeds (Section 4.2.1). BHT reduced normal germination and seedling growth of unaged seeds, but not viability. Iodine, on the other hand, reduced viability and seedling fresh weight, regardless of ageing. These findings do not conform to several published reports in other species (*e.g.* parsley, onion: Woodstock *et al.*, 1983; pea: Gorecki and Harman, 1986; or tomato: Francis, 1985) and other cultivars of soybean (Dey and Mukherjee, 1984; Aho, 1990). This may be interpreted as differences between species or cultivars in terms of the key events limiting germination performance in deteriorated seed (Section 4.3.1).

Possible reasons to explain these effects of antioxidants may include i) lack of or variation in rates of penetration, ii) toxicity due to excess concentration of antioxidants [as when the high concentration of BHT or iodine was toxic], and iii) absence of lipid peroxidation in these seed lots under the storage conditions used. This last possible reason has been confirmed by the evidence of absence of lipid peroxidation in *cv.* Davis under either accelerated or slow ageing regimes discussed in Chapter 6. This is in contrast to most published literature on soybean, but Ferguson *et al.* (1990b) reported that lipid peroxidation was a cause of soybean seed deterioration under long-term storage in only one out of two cultivars studied. Thus, it is apparent that there may be differences between cultivars.

One key finding of this study was the demonstration that acetone used as a carrier solvent for antioxidants caused significant toxic effects on soybean seeds *per se*. This result is contradictory to several reports in the literature on a range of species (Table 2.3) and particularly with the soybean results of Lewis *et al.* (1979). A comprehensive examination of acetone toxicity was accordingly carried out as a separate study (Section 4.2.2). The results from this series of experiments demonstrated that seed moisture content (SMC), treatment duration and levels of mechanical damage are key factors determining susceptibility of soybean seeds to acetone damage. Seeds at high levels of moisture or with mechanical damage are prone to acetone toxicity. Although soaking in acetone at an initial SMC below 7.5% could avoid damaging effects to

seeds subsequently germinated immediately, hidden damage became evident after treated seeds were aged. Mechanically damaged seeds may need lower levels of SMC before soaking, so that they can avoid the toxic effects of acetone. Tetrazolium staining patterns highlighted the relationships between SMC, mechanical damage levels and responses of seeds to acetone. Examination of the sites of acetone damage by tetrazolium staining indicated that acetone damage was not limited to specific tissues, but rather increased the area of dead tissues which had been already mechanically damaged. These results are consistent with the methyl red study (Section 4.2.2.6), which showed that methyl red in acetone solvent penetrated deeper into mechanically damaged seed.

Two other points emerge from this research which may contribute to an increased understanding of varying responses of seeds to solvent treatments. Firstly, variation in responses between seed lots cannot be explained by SMC status. Secondly, toxic effects of acetone may be less apparent in unaged seeds than in aged ones. A similar kind of study has also been undertaken in peas in this laboratory (Coolbear *et al.*, 1991) and has produced similar results. It was noted that damage caused by acetone is quite different from that due to ageing because acetone tended to reduce overall viability or percentage germination rather than the vigour of remaining germinable seeds. Once again, this evidence emphasises the advantages of measuring several parameters of germination performance in this type of study.

Hydration-dehydration effects

Hydration-dehydration treatments, which have reportedly been used sucessfully to protect seeds from ageing (Saha and Basu, 1984), were also assessed. However, results showed that such methods reported in literature (soaking, ME+soaking, 24 h ME followed by drying), plus modifications attempted in this study, did not show any protective effects on the soybean seed lots investigated (Section 5.2.1). This suggests that hydration-dehydration treatment is not an appropriate technique for prolonging storability of soybean seeds. The data also illustrated that soaking treatment caused severe damage to low vigour lots immediately or reduced ageing resistance of high vigour lots. Although soaking injury could be reduced by pretreatments with moisture equilibration (ME) or imbibition, hidden damage effects were still evident in aged seeds. Moisture equilibration treatments took longer time to produce an expected seed

moisture content than that reported in the literature (Saha and Basu, 1984), but extended ME treatment increased the rate of germination loss during subsequent storage. Seed moisture contents obtained from ME treatment varied with seed lot, treatment duration, and the amount of seeds to be treated (Tables 5.1 and 3.2). Demonstrated differences in the rates of water uptake of individual seeds within a given lot, emphasise the variability of this method (Figure 5.5).

Once again, conflicts between these results and others in the literature might be explained by differences between seed lots or cultivars (Section 5.3.1). Saha and Basu (1984) worked with three seed sublots of different vigour, created from a single seed lot produced and processed in India. It may be that such a seed lot had experienced conditions that caused repairable damage. In this study, six different seed lots, produced and processed in NZ or Australia were evaluated, and no treatment showed potential for ageing protection. Although working with a seed lot produced and processed in India, Dey and Mukherjee (1986) also failed to find any treatments which gave substantial effects to protect soybean seeds from ageing. All these results emphasise that seed lot variation is a sensitive variable for this kind of treatments. Vertucci and Roos (1990) determined the physiological and physical status of different seed species with varying chemical compositions, and described that the moisture contents at which physiological changes were observed varied among the species and were correlated with the lipid content of the seed. These findings may be extrapolated to explain cultivar differences in soybean in terms of variation in seed lipid contents which range from 14-26% of seed dry weight (Trelease and Doman, 1984).

8.2.2 Hydration treatments for repair

It has been accepted that hydration treatments have the potential to repair aged seeds, provided that damage is not too great. Evidence from the present study shows that ME treatments improved germinability of soybean seed lot A-6 (Figure 5.1) and slowly aged seeds of *cv.* Davis (Figures 5.6-5.7). Moreover, this study shows that initial hydration during ageing (*i.e.* 0 d CD or 2 d AA: Chapter 4) has a potential to repair damage caused by acetone or antioxidants. It was, however, clear that not all parameters of seed performance could be repaired. Generally, normal germination, dry or fresh weights of normal seedlings were improved by hydration treatments particularly without drying back. The key question arising from these repair studies is

how to get the best benefit from such treatments. The results here suggest that pretreatment of soybean seed by moisture equilibration immediately before sowing may be the best practical method.

8.2.3 Membrane damage and seed deterioration

Although the investigations on lipid and membrane status due to ageing in soybean conducted in this study are similar to several reports in the literature, this study was more extensive and comprehensive. It has related germination behaviour (including conductivity changes) after both rapid and slow ageing to biochemical or ultrastructural changes occurring in different tissues within the seed. This study has also provided evidence for additional effects of seed treatments which impair seed storability, *i.e.* acetone, water soaking or ME and their interactions with ageing. By using different approaches to determine membrane damage, the results confirm that membrane damage did indeed occur and was associated with seed deterioration (Chapters 6 and 7).

Evidence presented here showed that there were close relationships between conductivity of seed leachates and germination performance, which suggests the possibility of membrane damage being a key event in deterioration. However, conductivity may not be a completely accurate indication of membrane damage because other factors, such as mechanical injury, the permeability of seed coat or the amount of solutes available to leak out of the seed, may alter the amount of seed leachates (Section 2.2.1.1). No changes in total lipid (TL) contents were found in either axes or cotyledons due to ageing (both rapid and slow regimes), suggesting that changes in storage lipids were not related to loss of seed germination performance. However, one of the most striking observations found in this study came from the determination of PL contents from axes and cotyledons separately. These results showed clearly that losses of PL from cotyledons occurred earlier than those from axes (Section 4.2.2), and only changes in PL content from cotyledons were correlated with loss of seed performance (*i.e.* germination or viability) due to AA. This evidence suggests that cotyledon damage might make an important contribution to seed deterioration under high temperature and high relative humidity conditions. This finding is quite surprising: changes in PL from axes would be expected to be more closely related to germination performance, because successful production of a seedling is, of course, highly dependent on the level of deterioration in the axis.

Simple correlations however do not imply cause and effect. Ageing may cause damage via other effects (Section 2.2). Moreover, the TEM study indicated that as a result of AA, damage occurring in axes was more severe than in cotyledons (Chapter 7). Evidence showed that there were changes in the ultrastructure of several cell organelles (*i.e.* mitochondria, protein bodies, nuclei, plasmmalemmae). Disruption of plasmalemmae was the most obvious indication of seed deterioration due to AA in axes (Plate 7.4), even though no depletion of PL had occurred by this time (Figure 6.5).

Another key feature found in this study was that acetone or water soaking were damaging treatments. These treatments increased the rate of loss in germination performance which was accompanied by increased PL losses or abnormalities of ultrastructure in cell organelles in both cotyledon and axes. ME treatments applied to slowly aged seeds accelerated PL loss in axes but this was unrelated to seed performance. Clearly PL losses *per se* are not a fundamental cause of deterioration and may not be the first event in membrane damage. Indeed the evidence suggests that germinating seeds might be able to repair this kind of damage.

The results from fatty acid analysis (Sections 6.2.1.3 and 6.2.2.2) indicated that lipid peroxidation was not involved in seed deterioration due to ageing or seed treatments. Losses in phospholipid are likely to be a consequence of hydrolytic damage, also suggested by the appearance of either vesicles in protein bodies or fusion of these bodies (Section 7.2.1).

8.3 KEY CONCLUSIONS

8.3.1 **Seed treatments**

Despite seed treatments being reported as effective in the literature, results from this study strongly suggest that treatments using either the antioxidant or hydration-dehydration methods employed here are unlikely to be a practical proposition for improved storage of soybean. Certainly, advantageous effects were minimal for the seed lots used in this study. It is clear that there are a large number of variables involved, some of which may be pertinent to a comparison of results reported in this research to more successful treatments in the literature. These include:

1) toxic effects of organic solvents
2) soaking injury
3) seed lot characteristics *i.e.* initial seed moisture content, level of mechanical damage or vigour status of seeds
4) way in which germination has been evaluated
5) interactions between treatments and different ageing conditions *i.e.* AA, CD, slow ageing
6) presence of oxidative damage
7) extent of seed deterioration
8) variation in SMC obtained from ME treatment
9) population variation within a seed lot
10) drying after hydration treatments
11) damaging effects due to extended ME
12) pathogens during ageing
13) penetration of antioxidants
14) presence of natural antioxidants
15) solubility of antioxidants

The ordering of the variables listed above indicates the amount of information provided by the present study. For example, toxic effects of solvent (*i.e.* acetone) have been fully investigated while the presence of natural antioxidants or solubility of applied antioxidants have not been explored at all. Although this study is unable to completely define the rest of variables on the list, it provides information which suggests directions for future work (details in Section 8.4).

8.3.2 Physiological, biochemical or structural aspects

The results showed that during AA, PL losses in cotyledon tissue occurred earlier and were more dramatic than in axes, but ultrastructural changes appeared to be more severe in axes rather than in cotyledons. Acetone or water soaking pretreatments increased rates of germination loss, conductivity of seed leachates, PL losses and ultrastructural abnormalities, particularly plasmalemma disruption, in both cotyledons and axes. All measurements, *i.e.* conductivity, PL loss and ultrastructural changes, indicated occurrence of membrane damage. Lipid peroxidation seemed unlikely to be

a primary cause of seed deterioration due to ageing or seed treatments. Similarly, PL losses do not necessarily seem to be a primary cause of membrane damage. More than this, the inevitability of loss of viability as a result of membrane damage must be questioned: subsequent ME treatment after slow ageing resulted in increased PL losses from axes although there was no evidence that this treatment increased losses of viability.

8.4 LIMITATIONS OF THE PRESENT STUDY AND SCOPE FOR FUTURE WORK

It is clear that the results found in this research are difficult to interpret because there are several variables involved (Section 8.3). Although some variables have been identified, these studies show many more aspects need to be investigated. Some of the most interesting areas for future research are as follows.

8.4.1 **Seed treatments**

1) Both information from previous reports and the results from the present experiments suggest that differences between seed cultivars/lots from India and New Zealand /Australia may be the crucial factors responsible for the effectiveness of either antioxidant or hydration-dehydration treatments. Future work therefore should aim to evaluate these kinds of treatments on a large number of cultivars and seed lots with different production and processing histories, and/or different lipid contents.

2) The damaging effects of drying after hydration treatments which, were occasionally found (Section 5.2.1), have not been characterised in this study. Different drying methods should be studied in more detail.

3) In the assessment of antioxidant effects, information about the presence of endogenous antioxidants within the seeds would be useful to confirm their role (if any) in delaying seed deterioration during ageing.

4) In this present experiment, seed storage pathogens were present during hydration-dehydration treatments even though attempts to avoid such problems by surface

sterilisation were carried out. The effects of these pathogen *per se* need to be further defined with an emphasis on identifying which parameters of germination performance are most prone to be affected by pathogen and through which tissues and/or mechanisms.

8.4.2 Mechanisms related to germination performance

1) As previously stated, at the start of this study it was hoped that pre-storage treatments might be discovered which would improve seed storability. These could be used to evaluate the relative importance of different aspects of seed deterioration and overcome some of the limitations of purely correlative evidence, enabling the key primary events in seed ageing to be identified. Since no treatments in this study improved germination performance, future experiments will be forced to rely on more carefully constructed time course studies, so that changes in some aspects of deterioration might be detected more clearly before loss of viability and even seed germination performance.

2) In the present study, an unequivocal comparison between the effects of accelerated and slow ageing regimes was not possible, because the extent of seed deterioration was not equal. For future research in this area, the extent of deterioration is needed to be controlled to similar levels under different conditions.

3) The TEM study did not show a complete sequence of seed deterioration as, because of limited time and facilities, an examination of ultrastructural changes was restricted to seeds with high germination or seeds which lost all normal germination and considerable viability. Evidence suggested clear effects of treatments, but whether or not these are a direct cause of loss of germination performance is still unclear. Once again, time course experiments may be suitable for identification of possible causes of seed deterioration.

4) The results from both lipid and TEM studies suggest that hydrolytic damage is a cause of seed deterioration due to AA, soaking, or interactions of AA with acetone or water soaking pretreatments. Relationships between seed performance due to ageing or treatments and changes in hydrolytic enzyme activities, particularly that of the lipases, may be a useful area of investigation in future research.

5) The microsomal fraction was chosen to study changes in lipid membrane *per se*, because this contains high contents of membrane which are a heterogeneous mixture of membranes originating from several subcellular organelles including endoplasmic reticulum (ER), nuclear and plasma membranes. Microsomes are also very sensitive to free radical activity (Tappel, 1973). Senaratna *et al.* (1988) reported that soybean seeds lost ~50% PL from the microsomal fraction taken from axes when accompanied with a >80% viability loss during natural ageing. In the present study, there were no changes detected in PL contents (expressed as P:L ratios) due to ageing and treatments used (Section 6.2.2). There might be high levels of storage lipid contaminants in the membrane fractions, because P:L ratios from membrane fractions are similar to those from seed tissues; and the TEM study showed a large number of lipid vesicles in microsome fractions (Plate 6.2) which confirms the above suggestion. This may be misleading in interpreting membrane properties.

In further work, besides developing an improved technique for membrane extraction in order to avoid contaminants, properties and compositions of microsome fractions are worth investigating under both rapid and slow ageing regimes. The results from microsomal membranes are needed both to confirm the finding of Senaratna *et al.* (1988) in microsome fractions, to complement the findings on changes in mitochondrial membranes reported by Ferguson *et al.* (1990b) in long-term stored soybean seeds, and to provide new information on rapidly aged seeds. Further, a similar kind of study to Ferguson *et al.* (1990b) is needed to investigate the effects of rapid ageing on mitochondria.

Therefore, the specific direction for future work should focus on investigation of ageing effects, both rapid and slow regimes with more frequent sampling, and careful examination of changes in germination behaviour accompanying the changes in properties and compositions of (i) total lipid and PL, (ii) the microsomal fraction and (iii) mitochondrial fractions obtained separately from axes and cotyledons. The results which would be obtained from such a comprehensive series of experiments may, at least, begin to provide some definitive evidence on possible causes of seed deterioration in soybean.

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APPENDICES

Appendix 3.1

Fatty acid analysis problems and their possible causes

As discussed in Section 3.6.2, there was a problem of deterioration of the DB wax column used for GLC analysis after a series of runs with fatty acid methyl esters prepared using borontrifluoride. Examples of evidence which suggested the column deterioration are shown in Traces A and B. In the initial runs (Trace A), when six standard methylated fatty acids (16:0, 17:0, 18:0, 18:1 18:2, 18:3) were injected, there were 7 well-separated peaks with an extra peak which is BF_3 contamination (confirmed by comparison with samples methylated without BF_3, data not shown). After the column had deteriorated the results from 5 standard methylated fatty acids (16:0, 18:0, 18:1, 18:2, 18:3) showed lack of resolution of 18:0 and 18:1 components (Trace B).

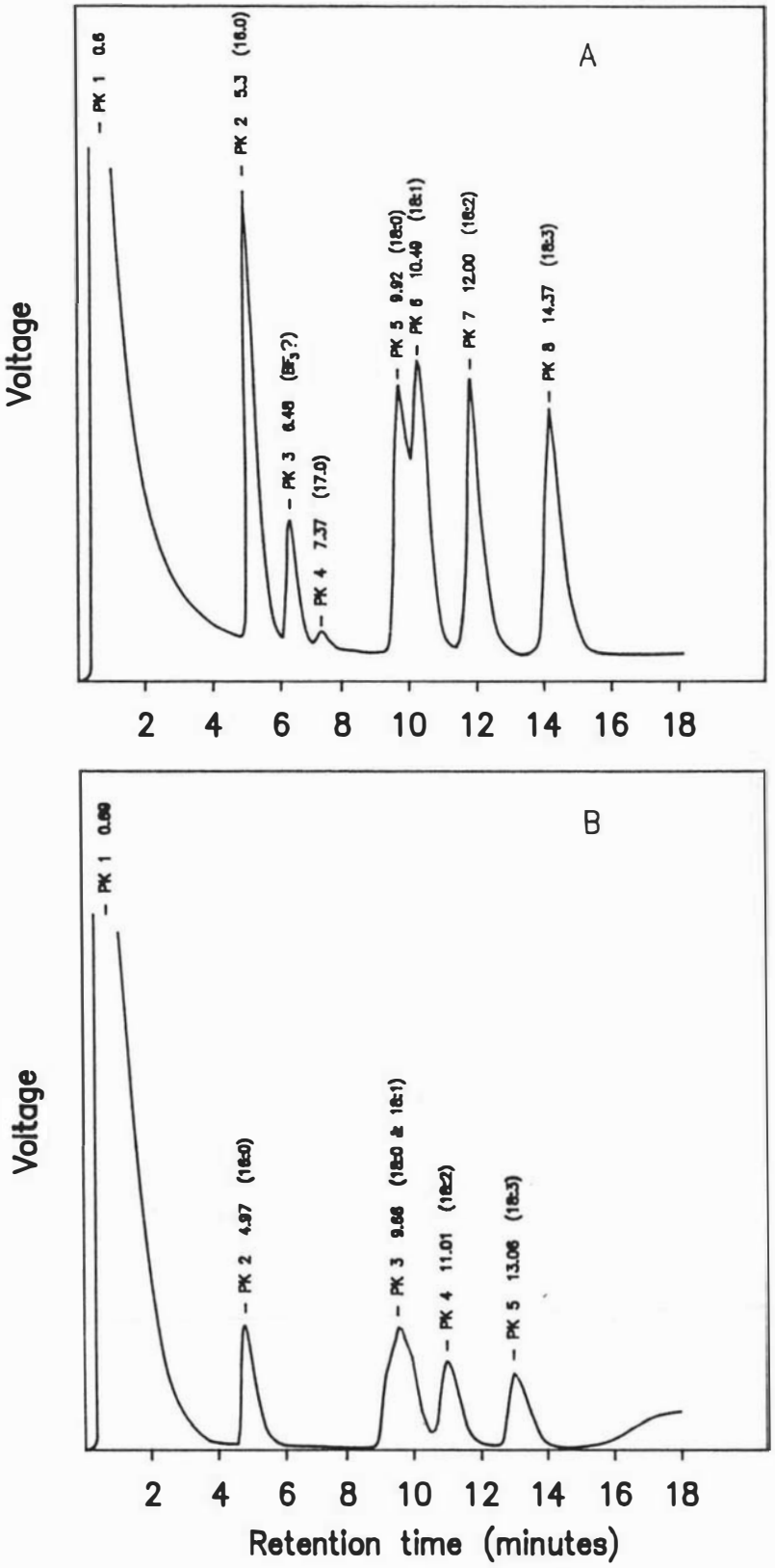
Possible causes of loss of resolution may be:

i) methylation with a combination of 14% BF_3 and high concentration of H_2SO_4 (10%) may cause loss of resolution. In a previous report, Lough (1964) found that methylation with a much higher concentration of BF_3 (50%, *w/v*), although without H_2SO_4, caused serious losses of unsaturated esters and oleic acid (18:0) and gave methoxy methyl stearate isomers in high yield, while with the low concentration (12.5-14%, *w/v*) BF_3 alone did not (Morrison and Smith, 1964). Since BF_3 alcoholates behave like strong acids, they have been used to promote methanolysis of lipid in a manner similar to HCl or H_2SO_4 added to methanol (*cf.* Morrison and Smith,1964). It is possible that when both 14% BF_3 and 10% H_2SO_4 were added into lipids for methylation, according to the preliminary method used in this study (Section 3.6.2), their effects may be similar to when the concentration of BF_3 alone was increased.

ii) extended running at the maximum temperature may also cause damage to the column because samples were run at 160-230°C oven temperature and after every 4-5 samples, the column was cleaned by baking at 230°C,

iii) running without an oxygen trap may aggravate problems of oxygen contaminants in the system which may oxidise the column confounding the damage caused by factors (i) and (ii).

These problems were overcome in the further experiments by employing a methylation procedure using a lower concentration of H_2SO_4 and with no BF_3 added after modification of the method described by Ferguson *et al.* (1990**b**). In addition to this, the esters were separated at the lower oven temperature of 160-190°C with an oxygen trap in the helium gas line (as described in Section 3.6.2).


A
PK 1 0.6
PK 2 5.3 (18:0)
PK 3 6.48 (18:2?)
PK 4 7.37 (17:0)
PK 5 9.92 (18:0)
PK 6 10.40 (18:1)
PK 7 12.00 (18:2)
PK 8 14.37 (18:3)
Voltage
2 4 6 8 10 12 14 16 18
B
PK 1 0.69
PK 2 4.97 (18:0)
PK 3 9.68 (18:0 & 18:1)
PK 4 11.01 (18:2)
PK 5 13.06 (18:3)
Voltage
2 4 6 8 10 12 14 16 18
Retention time (minutes)

Appendix 3.2

Variation between extraction replicates *vs* variation between injection replicates

There were variations both between extraction (external) replicates and injection (internal) replicates of fatty acid proportions from microsomal membrane lipids. An example of typical data to illustrate these variations is shown in Table A3.2. In general, the variation between external replications was as high as a range of 18.0% over the means on 18:3 fatty acid which was much greater than that between internal injections (8.2%). However, these variations were similar on 18:2 fatty acid (maximum variation were 9.9% between extractions and 10.6% between injections).

Table A3.2 Fatty acid composition of microsomal membrane lipid extracted from axes of soybean seeds, *cv.* Davis affected by accelerated ageing (40°C, ~100% RH). Data presented are individual injections and means of each replicate extraction.

Treatments and ageing		Fatty Acids				
	Rep.	C16:0	C18:0	C18:1	C18:2	C18:3
Untreated -	I	19.5	4.1	7.2	47.0	22.1
	I	17.9	4.1	7.6	48.9	21.5
	I	17.5	4.3	7.8	49.3	21.1
	Means	**18.3**	**4.2**	**7.5**	**48.4**	**21.6**
	II	18.9	2.8	7.9	54.6	15.8
	II	27.2	3.3	7.3	47.3	14.9
	II	27.2	2.6	7.5	48.4	14.3
	Means	**24.4**	**2.9**	**7.6**	**50.1**	**15.0**
Untreated + 4d AA	I	22.1	3.4	6.7	45.0	22.8
	I	27.5	5.4	9.2	36.3	21.8
	Means	**24.8**	**4.4**	**8.0**	**40.7**	**22.3**
	II	21.3	3.5	6.9	45.8	18.5
	II	20.3	3.4	6.4	50.9	19.0
	II	21.5	5.4	7.4	48.1	17.5
	Means	**21.0**	**4.1**	**6.9**	**49.6**	**18.3**

Note : AA = ageing at 40°C, ~100% RH

Appendix 3.3

Procedures for the preparation of seed tissue or
membrane fraction for transmission electron microscopy.

Fixation

Slices of seed tissue as shown in Figure 3.1 were fixed in primary fixative solution of 3% glutaraldehyde, 2% formaldehyde, 0.1 M phosphate buffer (pH 7.2) for 3 h and then washed 3 times for 5, 10, and 20 min respectively using 0.1 M phosphate buffer. One percent osmium tetroxide in phosphate buffer was used for a secondary fixation. After the samples were fixed for 45 min, they were washed twice for 5 and then 20 min in phosphate buffer. All procedures were conducted at room temperature.

The fixed samples were then dehydrated through a graded acetone and water series (25, 50, 75, and 95% acetone, 15 min per step) and then for 1 h in 100% acetone. Sections were then infiltrated with 50% Polarbed 812 resin: 50% acetone mixture overnight on a stirrer, and then in fresh 100% resin (on a stirrer) for 8 h. These samples were then embedded in fresh resin in silicone rubber moulds and cured at 60°C for 48 h.

Microsomal membrane fractions extracted from the axes of either untreated unaged controls or water soaked 6 d AA seeds were fixed in suspension in a primary fixative solution as used for seed tissues for 1.5 h at room temperature. The suspension was centrifuged at 3,000 g for 3 min to pellet the membranes, and the supernatant was decanted. The pelleted membranes were then mixed with a few drops of 20% Bovine Serum Albumin (BSA) in aqueous solution and recentrifuged to concentrate the membranes and to allow the BSA to coagulate with the remaining primary fixative solution. The pellets were then removed from tubes, and the excess BSA was trimmed of. The remaining pelleted samples were sliced into 0.5-1 mm slices for further cutting, as per the procedure for seed tissues.

TEM study

Initially seed tissues or membrane pellets were prepared for light microscopy in order to choose the tissue sections for TEM study. Embedded samples were first cut into 0.5-1.0 µm thick sections on a glass knife using a Reichert Ultracut E ultra-microtome and then heat mounted on to a glass microscope slide, stained with 0.05% toluidine blue in 0.1 M phosphate buffer. After drying on a hot plate, a coverslip was mounted on immersion oil and examined under the light microscope. Sections cut through epidermal, procortical and provascular tissues were chosen for TEM study (examples of tissue types are shown in Plate 3.3).

Sections for TEM study were cut on a diamond knife using a Reichert Ultracut E ultra-microtome. The sections had silver to pale gold interference colours (approximately 80 *n*m thick), and were picked up on 400 mesh copper grids. The grid mounted sections were double stained using saturated uranyl acetate in 50:50 (*v/v*) ethanol and water (3 min) followed by lead citrate for 2 min. All these procedures were carried out by Mr. D. Hopcroft of Fruits and Trees Division, DSIR. Sections were studied using a Philips 201C TEM.

Appendix 4.1 The effects of chemical treatments and subsequent controlled deterioration (40°C, 20% SMC) on dry weights of normal soybean seedlings (mg. axis^{-1}) 8 d after sowing of lot A-1, *cv.* Amsoy. Data are means of 4 replicates, unless otherwise stated.

Treatment	Ageing treatment				Treatment means
	Unaged	1d CD	3d CD	5d CD	
Expt I					
Untreated controls	39.35	44.08	37.33	44.19	41.24
Acetone alone	37.20	35.84	37.42	43.31	38.44
1% α-Tocopherol in acetone	40.30	40.25	39.55	40.81	40.19
Ageing means	38.95	40.05	38.10	42.94	
Significance level:	*Treatments x ageing durations = NS; Treatment = NS; Ageing = ** ($LSD_{0.01}$ = 3.30)*				
Expt II					
Untreated controls	52.64	42.65	37.64	-	
Acetone alone	45.95	30.81	21.79	-	
0.1 M BHT in acetone	27.87	43.17	19.50[a]	-	
$LSD_{0.05}$	7.64	NS	8.34[b]	-	

Note: *Expt I was a split plot design, treatments = main plot and ageing durations = subplot, while data in Expt II were analysed separately for each ageing time.*

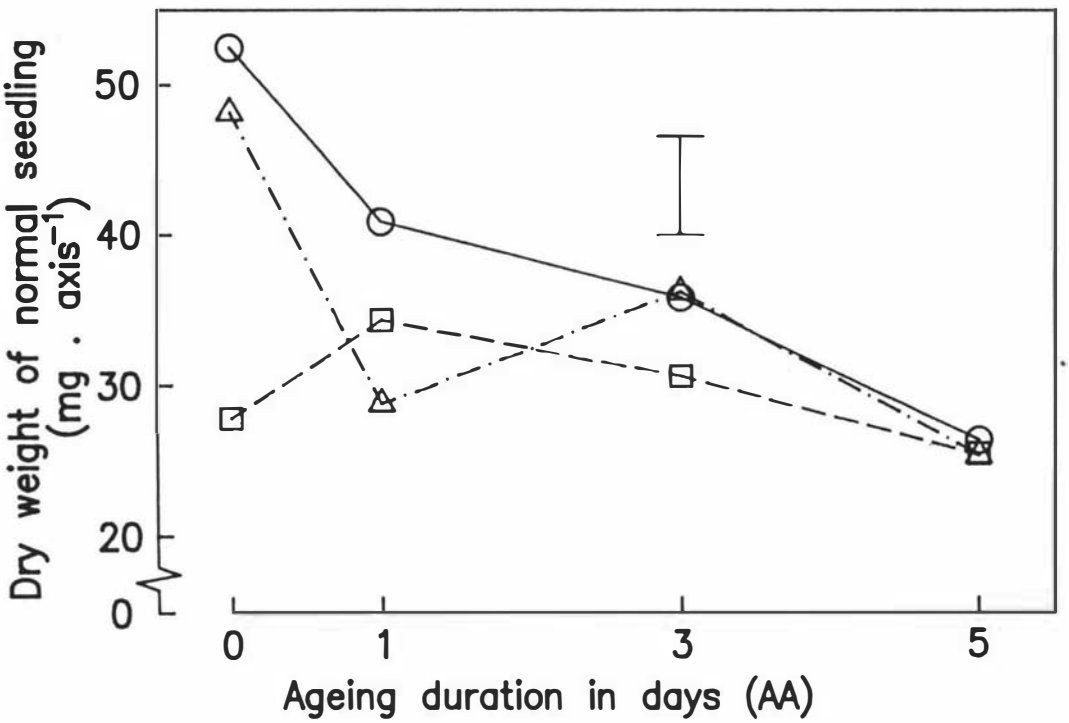
a = mean from 2 replicates

b = between untreated controls and acetone, not significant between acetone and BHT treatments

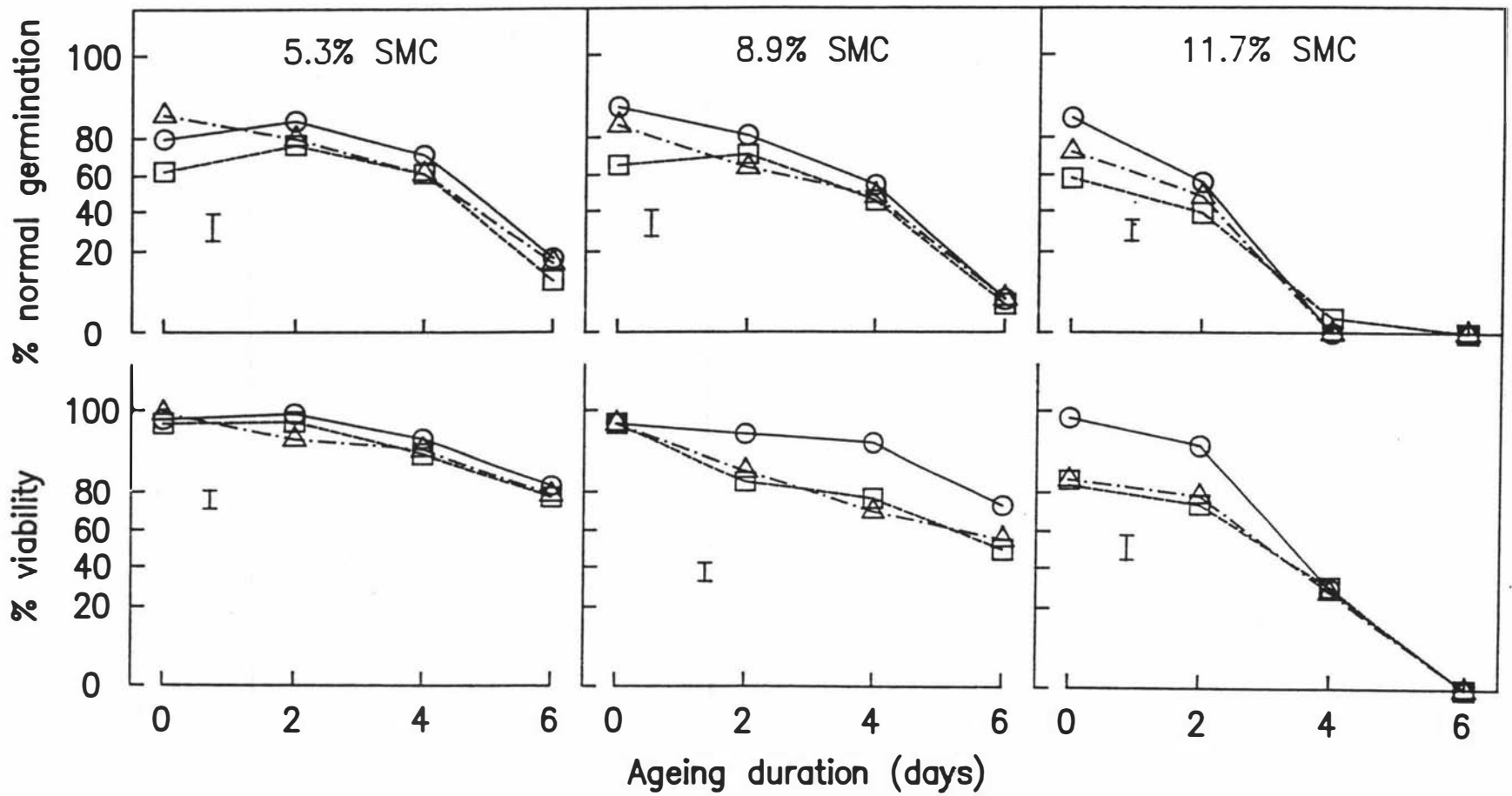
Appendix 4.2 Effects of chemical treatments and durations of accelerated ageing (40°C, ~100% RH) on time to 50% germination (T_{50}) of soybean seed lot A-1, *cv.* Amsoy (data from Expt. II).

	T_{50} (h)			
	Durations under AA			
Chemical treatments	Unaged	1 d	3 d	5 d
Untreated controls	16.5	15.8	18.3	24.3
Acetone alone	16.5	19.3	18.5	21.5
0.1M BHT in acetone	18.5	16.0	19.0	22.5

$LSD_{0.05} = 2.15$



Appendix 4.3 The effects of chemical treatments and subsequent accelerated ageing (40°C,~100% RH) on axis dry weights of normal seedlings of soybean seed lot A-1 *cv.* Amsoy 8 d after sowing. ○—○: Untreated controls; △···△: Acetone alone; □ – □ : 0.1 M BHT in acetone. Bar represents least significant difference (P=0.05).



Appendix 4.4 The effects of chemical treatments, soaking seeds at different initial moisture contents and subsequent accelerated ageing (40°C,~100% RH) on normal germination and viability of soybean seeds *cv.* Davis. Data presented are arcsin √% transformed means of 4 replicates. ○—○: Untreated controls; △-·-△: Acetone alone; □- □: 0.1 M BHT in acetone. Bars represent least significant differences (P=0.05).

Appendix 4.5 The effects of chemical treatments, soaking at 6% SMC prior to subsequent slow ageing (35°C, 12% SMC) on dry weights of normal seedlings (mg. axis^{-1}) of soybean, *cv.* Davis, after 8 d from sowing. Data presented are means of 3 replicates (Expt. V).

	Ageing duration (weeks)				
Treatment	Unaged	0	3	6	9
Untreated controls	31.12	32.74	50.03	30.82	30.9
Acetone alone	33.34	31.78	45.46	23.56	-
1% α-tocopherol in acetone	34.03	30.34	46.68	22.49	-
0.1% BHT in acetone	33.07	29.09	49.34	25.02	-
$LSD_{0.05}$	NS	NS	NS	NS	-

Appendix 4.6 The effects of different durations of soaking in acetone prior to subsequent controlled deterioration (40°C, 20% SMC) on dry weights of normal seedlings (mg. axis^{-1}) of soybean lot A-2, *cv.* Amsoy, after 8 d from sowing. Data are means of 4 replicates (Expt VII).

Soaking duration (h)	Ageing treatment			Soaking mean
	0d CD	1d CD	3d CD	
0	33.48	24.79	35.67	31.31
2	32.61	26.01	36.36	31.66
4	26.56	22.61	32.29	27.15
8	27.64	26.28	29.62	27.85
16	31.76	23.96	30.17	28.63
Ageing mean	30.41	24.73	32.82	

Significance level: *Soaking and ageing interactions = not significant.*
Soaking duration (over ageing duration) = not significant.
Ageing duration (over soaking duration) = significant at 0.1% (***) level, $LSD_{0.01}$ = 5.31.

Appendix 4.7 The effect of initial seed moisture contents on viability of untreated and acetone soaked soybean seed lot A-8, *cv.* Amsoy after 3 d controlled deterioration (40°C, 20% SMC). Data in brackets are arcsin √% transformed means of four replicates ± SE (Expt. XII).

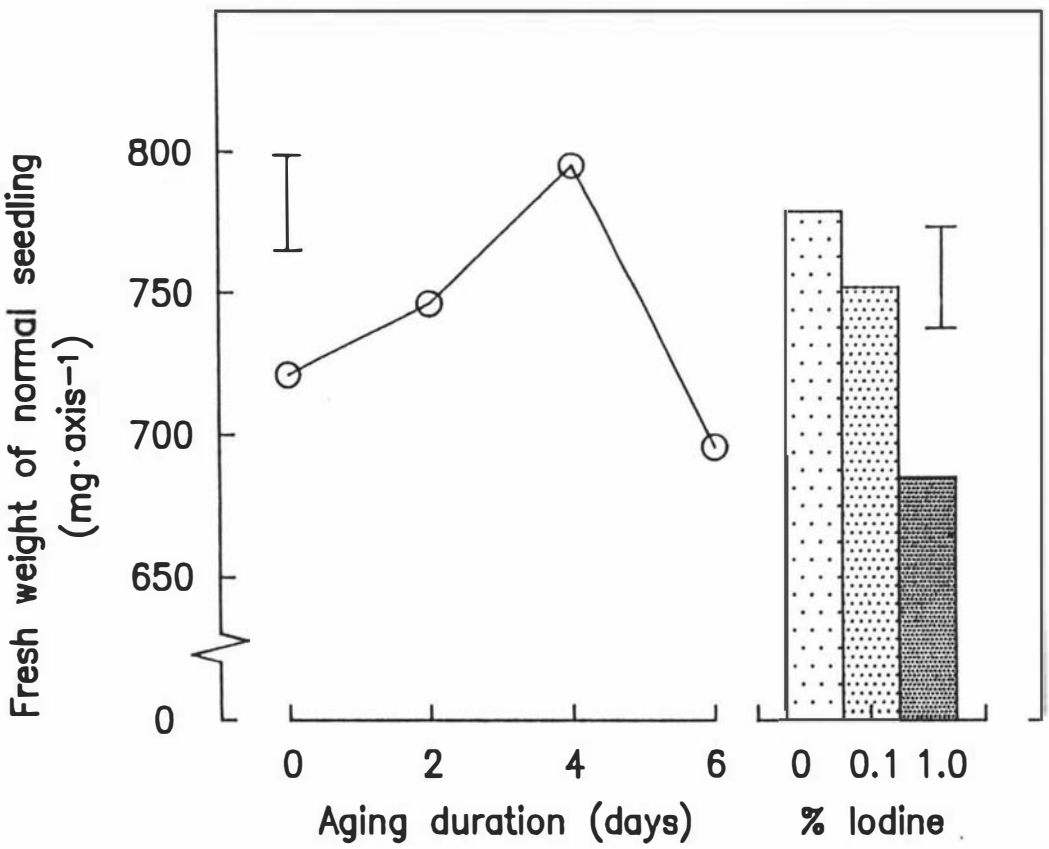
	% viability after 3 d CD	
Initial SMC (% FWT)	Untreated	Acetone
6.5	77.0 (1.071 ± 0.015)	1.2 (0.107 ± 0.069)
7.8	77.5 (1.078 ± 0.025)	1.4 (0.116 ± 0.076)
9.1	87.5 (1.213 ± 0.031)	0.75 (0.086 ± 0.051)
10.4	91.5 (1.278 ± 0.027)	0.15 (0.035 ± 0.035)
12.1	97.5 (1.413 ± 0.061)	0
12.9	99.5 (1.500 ± 0.041)	0

Appendix 4.8 The effects of different concentrations of β-mercaptoethanol treatments and subsequent controlled deterioration (40°C, 20% SMC) on normal germination of soybean seed lot A-0, *cv.* Amsoy. Data presented are means of 4 replicates. Arcsin √% transformed data are given in brackets. Mean ± SE for control is also given.

Concentration (*ppm*)	Ageing treatment				Concentration means
	Unaged	1d CD	3d CD	5d CD	
Control	88.5 (1.23 ± 0.04)	-	-	-	-
0	-	77.0 (1.07)	51.5 (0.80)	5.5 (0.24)	41.5 (0.70)
1	-	72.5 (1.01)	57.5 (0.86)	2.5 (0.16)	41.0 (0.68)
10	-	75.5 (1.05)	44.5 (0.73)	9.0 (0.30)	41.0 (0.69)
100	-	71.5 (1.01)	55.5 (0.84)	6.0 (0.25)	41.5 (0.70)
Ageing means		74.5 (1.04)	52.0 (0.81)	5.5 (0.24)	

Significance level: *Concentration and ageing interactions = not significant.*
Concentrations (over ageing durations) = not significant.
Ageing (over concentrations) = significant at 0.1% (***) level, $LSD_{0.01}$ = 0.09.

Effective concentrations of β-mercaptoethanol are 0, 0.52, 5.2 and 52 ppm.



Appendix 4.9 The effects of subsequent accelerated ageing (40°C, ~100% RH) (averaged over treatments) or iodine treatment for 24 h (different concentrations averaged over duration of AA) on axis fresh weights of normal soybean seedlings of lot A-3, *cv.* Amsoy. Data presented are means of 4 replicates. Bars represent least significant differences (P=0.05). There were no significant interactions between ageing and iodine treatments.

Appendix 4.10 The effects of chemical treatments on seed moisture contents (% FWT) of unaged seeds of soybean *cv.* Amsoy lot A-1 (Expt II) or Davis (Expts III and IV) after seeds were soaked at different initial moisture contents. Data are means of 4 replicates (± SE's).

Chemical treatment	% SMC of unaged seeds		
	Expected % initial SMC		
	6%	9%	10%
		Experiment II	
Untreated controls		10.8 ± 0.03	
Acetone alone		10.0 ± 0.05	
0.1M BHT in acetone		9.9 ± 0.00	
		Experiment III	
Untreated controls	6.2 ± 0.08	9.1 ± 0.08	12.3 ± 0.26
Acetone alone	7.2 ± 0.06	8.9 ± 0.05	9.9 ± 0.09
1% α-tocopherol in acetone	7.1 ± 0.08	8.7 ± 0.05	9.6 ± 0.04
		Experiment IV	
Untreated controls	5.3 ± 0.05	8.9 ± 0.05	11.7 ± 0.16
Acetone alone	7.7 ± 0.10	9.5 ± 0.11	10.5 ± 0.13
0.1 M BHT in acetone	7.9 ± 0.05	9.4 ± 0.05	10.4 ± 0.05

Appendix 5.1 Seedling dry weights of different seed lots of *cvs.* Amsoy and Davis as a result of hydration treatments and subsequent accelerated ageing (40°C, ~100% RH). Data presented are means of 4 replicates.

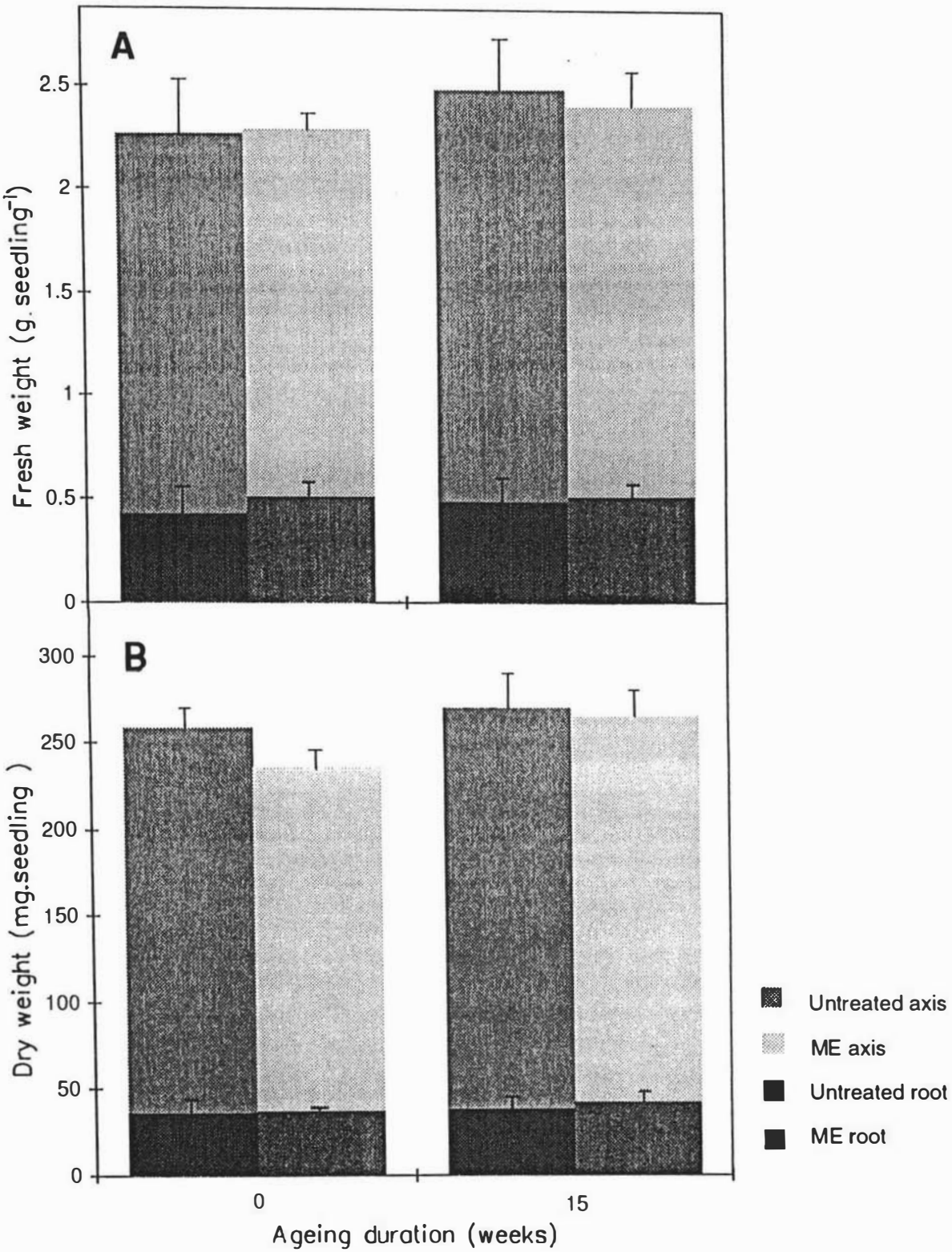
		Drying and ageing effects				
Seed lot	Treatment	BD	0d AA	2d AA	4d AA	6d AA
A-3	Untreated	36.55	36.55	34.39	21.10	-
	24 h ME	36.04	36.86	34.28	18.90	-
	48 h ME	37.58	33.35	36.92	18.21	-
	Ageing means	-	35.59	35.19	19.41	
	$LSD_{0.05}$	NS^a		2.25^b		
A-6	Untreated	35.79	35.79	39.02	34.68	19.26
	24 h ME	38.02	35.50	41.41	32.92	20.86
	48 h ME	38.40	37.46	39.23	32.40	19.28
	Ageing means	-	36.25	39.89	33.33	19.80
	$LSD_{0.05}$	NS^a		2.75^b		
A-7	Untreated	35.53	38.50	34.66	-	-
	24 h ME	35.80	39.89	33.07	-	-
	24 h ME + S	32.90	36.31	32.67	-	-
	Ageing means	-	38.23	33.47		
	$LSD_{0.05}$	NS^a	1.97^b			
A-8	Untreated	46.83	48.65	40.99	28.83	-
	24 h ME	48.48	47.26	41.51	24.23	-
	24 h ME + S	48.28	47.63	37.71	24.61	-
	Ageing means	-	47.85	40.07	23.89	
	$LSD_{0.05}$	NS^a		3.61^b		
Davis	Untreated	37.58	37.58	41.53	32.51	-
	24 h ME	46.98	38.80	49.88	32.06	-
	48 h ME	43.16	39.18	33.24	31.19	-
	72 h ME	42.90	39.03	40.62	28.57	-
	24 h ME + S	47.39	39.51	41.43	28.30	-
	Ageing means	-	38.82	40.34	30.53	
	$LSD_{0.05}$	4.79^a		3.22^b		

Note: *a = between treatments before drying (BD)*
b = between ageing durations (over treatments),
treatment and ageing interactions were not significant in all lots,
between treatments (over ageing durations) were not significant.

Appendix 5.2 The effects of accelerated ageing (40°C, ~100% RH) alone or followed by 72 h moisture equilibration (ME) with or without drying (D) on normal germination of soybean seeds, *cv.* Davis. Data are means of 3 replicates (SE's are given in brackets below each mean).

	Ageing duration			
Treatment	0 d	2 d	4 d	6 d
Untreated control	82.0 (7.02)	-	-	-
Untreated + D	85.3 (2.40)	88.0 (2.31)	64.7 (5.81)	41.33 (0.67)
72 h ME	88.0 (1.15)	86.0 (3.06)	65.3 (5.81)	39.3 (3.53)
72 h ME + D	84.7 (1.76)	82.7 (3.53)	70.7 (5.21)	38.7 (5.46)

Note: D = Drying at 20°C for 3 days followed by at ambient for 2 days to original SMC's.

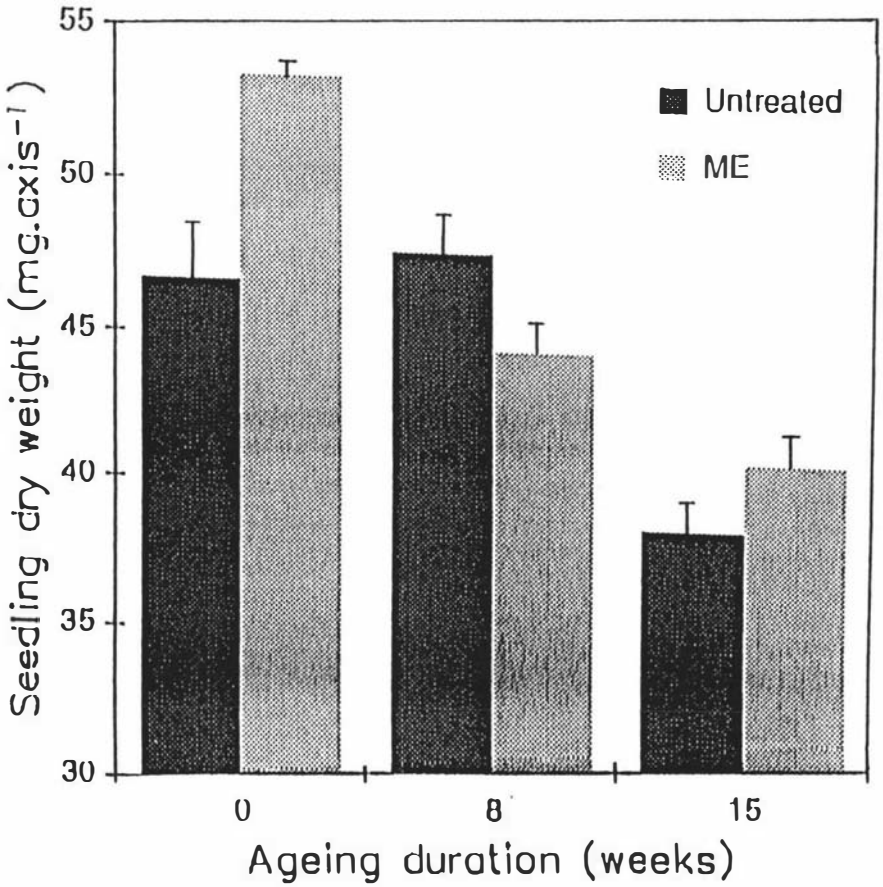


Appendix 5.3 The effects of slow ageing (35°C, 9% SMC) alone or followed by 72 h moisture equilibration on fresh (A) or dry (B) weights of normal soybean seedlings, *cv.* Davis, grown in soil 17 d after 50% establishment. Data presented are means of 3 replicates. Bars represent SE's calculated for individual means.

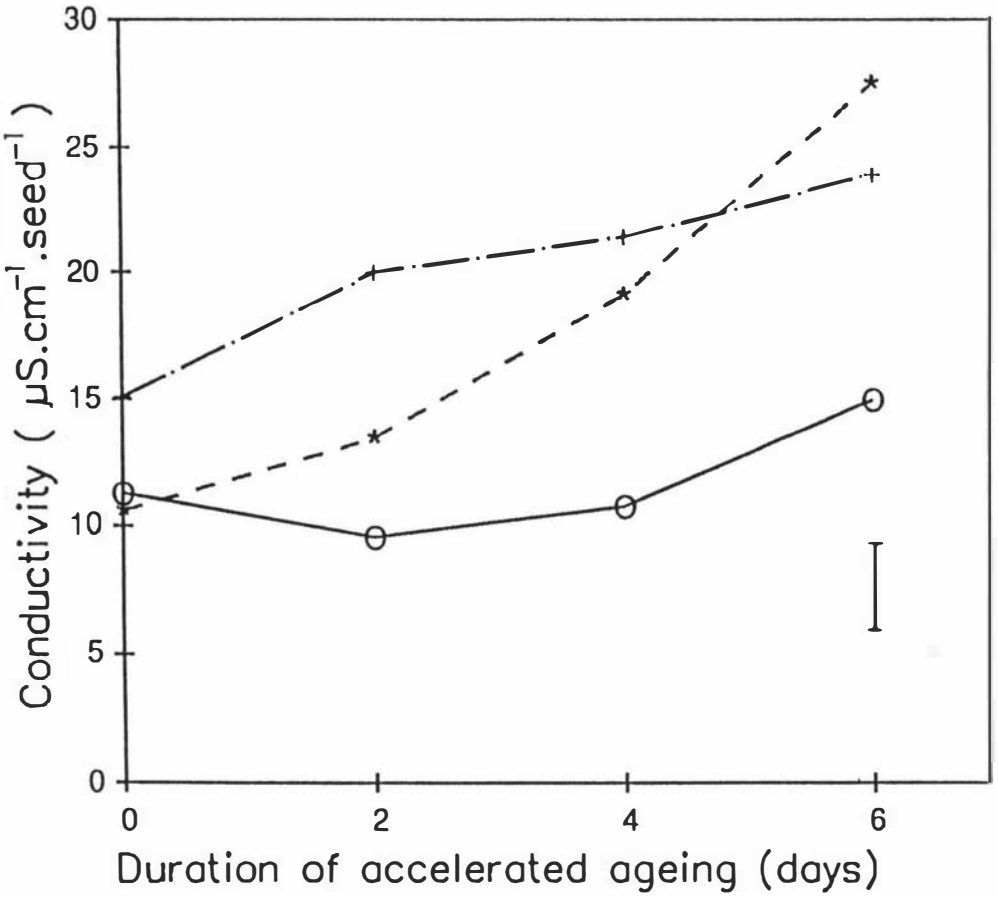
Appendix 5.4 The effects of 72 h moisture equilibration (ME) with and without drying on conductivity of leachate of accelerated (40°C, ~100% RH) or slowly aged (35°C, 9% SMC) seeds.

Treatment	Conductivity ($\mu S \cdot seed^{-1}$)	
	Accelerated aged	Slow aged
Control	14.6	14.6
ME	10.5	10.5
ME + Drying	14.8	13.5
$LSD_{0.05}$	0.7	1.17

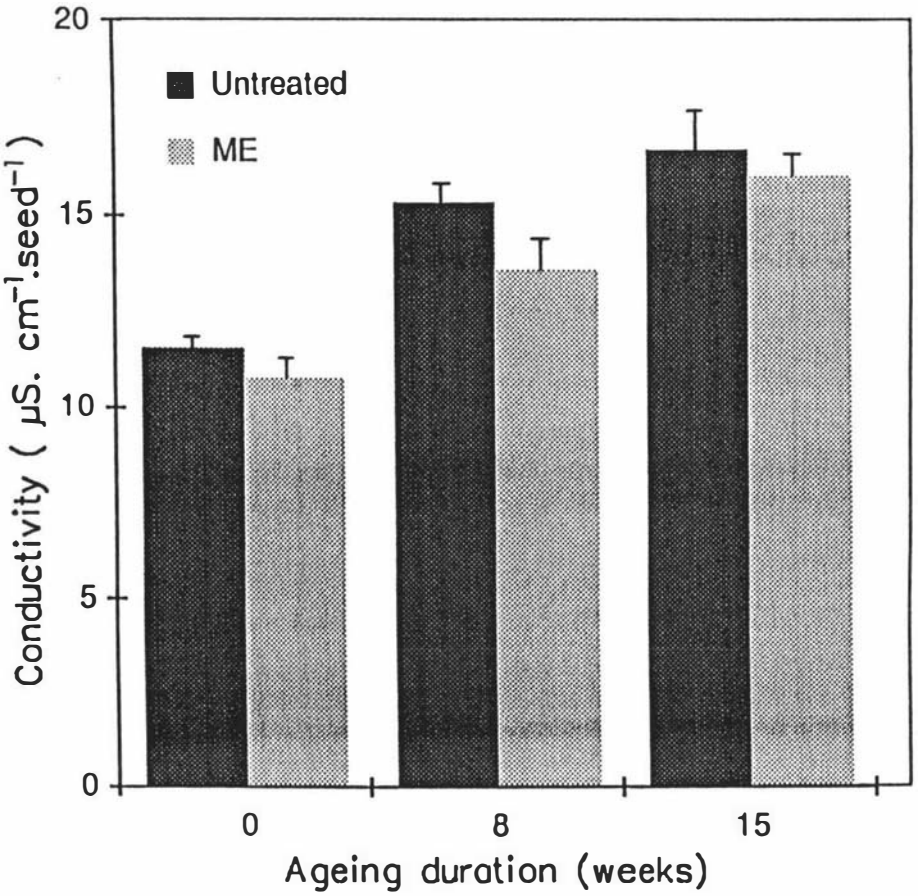
Note: Least significant differences (LSD) at 5% between two overall means of treatments irrespective of ageing duration.



Appendix 6.1 Dry weights of seedling axes of soybean seeds, *cv.* Davis, as a result of slow ageing (35°C, 9% SMC) alone or with 72 h subsequent moisture equilibration. Data presented are means of 3 replicates. Bars represent SE's calculated for individual means.



Appendix 6.2 Conductivity of leachate from soybean seeds *cv.* Davis affected by accelerated ageing (40°C,~100% RH) and interactions with preliminary acetone or water soaking. Data presented are means of 3 replicates. O—O : Untreated control; +·—·+ : Soaked in acetone for 16 h; ✱– –✱: Soaked in water for 30 min. Bars show least significant differences (P<0.05) between means.



Appendix 6.3 Conductivity of leachate from soybean seeds *cv.* Davis affected by slow ageing (35°C, 9% SMC) alone or with 72 h subsequent moisture equilibration. Data presented are means of 3 replicates. Bars represent SE's calculated for individual means.

Appendix 6.4 The correlation coefficients (r) between germinability or viability and conductivity during AA and slow ageing.

Parameters	Seed treatment		
	Untreated control	Acetone	Water soaks
AA (40°C, ~100% RH)[a]			
Germinability	-0.80**	-0.707*	-0.83**
Viability	-0.71**	-0.63*	-0.95**
	Untreated	ME	
Slow ageing (30°C, 9% SMC)[b]			
Germinability	-0.78*	-0.90*	
Viability[c]	-0.57^{NS}	-0.61^{NS}	

NS = *not significant at P = 0.05*
* = *significant at P = 0.05*
** = *significant at P = 0.01*
a df = *10 under AA*
b df = *7 under slow ageing*
c : there was no significant loss of viability under these storage conditions.

Appendix 6.5 The correlation coefficients (r) between phospholipid content and seed performance during accelerated ageing (40°C, ~100% RH).

PL	Seed treatment	Viability	Germinability	Axis FWT	Axis DWT	Conductivity
Axis	Untreated	0.44^{NS}	0.52^{NS}	0.47^{NS}	0.37^{NS}	-0.55^{NS}
	Acetone	0.49^{NS}	0.69*	-	-	-0.45^{NS}
	Water soak	0.92**	0.71**	-	-	-0.87**
Cotyledon	Untreated	0.74**	0.78**	0.80**	0.26^{NS}	-0.55^{NS}
	Acetone	0.70*	0.83**	-	-	-0.75**
	Water soak	0.89**	0.75**	-	-	-0.86**

Note: *NS* = *not significant at P = 0.05 level*
* = *significant at P = 0.05 level*
** = *significant at P = 0.01 level*
FWT = *fresh weight*
DWT = *dry weight*

Interactions between seed moisture content and solvent damage in seed treatment of soybeans

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Abstract

Acetone has often been recommended as a suitable solvent for non-aqueous seed treatment when it is desirable to introduce chemicals such as pesticides, plant growth regulators or even antioxidants into dry seed. We have, however, detected toxicity problems with this solvent in a range of species. Seeds of a high vigour lot of soybean, cv. Amsoy, showed a small decrease in germinability when soaked in acetone for between 2 and 16 hours, but the resistance of acetone treated seeds to 3 days controlled deterioration (storage at 20% seed moisture, 40°C) was markedly impaired and became more severe with increasing duration of exposure to the solvent. Susceptibility to acetone damage varied considerably between seed lots; seeds showing visible signs of mechanical damage being particularly susceptible.

Further studies with cvs Amsoy and Davis showed that there is a considerable interaction between seed moisture content (SMC) and acetone toxicity, damage being considerably reduced as SMC is lowered. Six percent is recommended as being a safer SMC for acetone treatment. Seeds soaked in acetone at higher SMC's show significant levels of phospholipid deletion from the embryo axis during artificial ageing.

Seeds at higher moisture contents show much less sensitivity to solvent damage when soaked in hexane, a solvent with a lower diclectric constant than acetone.

Additional key words: acetone, hexane, controlled deterioration, artificial ageing, phospholipid, seed germinability

Introduction

Recently there has been renewed interest in the use of organic solvents - particularly acetone - for the introduction of chemicals into dry seeds. This approach would seem to have considerable potential as a seed treatment technique where the compounds to be applied are of low water solubility or the seeds are liable to suffer from soaking injury. Successes have been reported with a variety of species using pesticides (Ekenrode *et al.*, 1974), fungicides (Shortt and Sinclair, 1980), plant growth regulators (Petruzzelli and Taranto, 1985; Persson, 1988) and (our own special interest) anti-ageing compounds (e.g., Parrish and Bahler, 1983; Gorecki and Harman, 1987; Dey and Mukherjee, 1988).

Acetone is generally the favoured choice of solvent because of its properties as a good carrier (Ekenrode *et al.*, 1974; Persson, 1988) and its low toxicity to seeds (Milborrow, 1963; Dadlani and Agrawal, 1985; Persson, 1988). However, there is some uncertainty about the possibility of acetone causing damage to seeds and the nature of other factors which might interact with the solvent. Thus, while Milborrow (1963) reported no deleterious effects after soaking peas and several other species in acetone for three months or longer and Lewis *et al.* (1979) found no problems with 24h soaks of peas and soybeans, these latter workers did find injurious effects of acetone on snapbean, an observation confirmed by Muchovej and Dhingra (1980).

In some cases toxic effects of acetone have been reported to be associated with the presence of major impurities in the solvent (such as 10-20% water, Lewis *et al.*, 1979). In other work, solvent damage has only manifested itself in aged seeds (e.g., Khan *et al.*, 1973 and Gorecki and Harman, 1987, working with lettuce and peas, respectively).

Our aim in the study reported here was to characterise the toxic effects of acetone on soybean seeds

and undertake a preliminary investigation into the nature of the mechanisms involved. Attention was focused on interactions of toxicity with soaking duration, seed lot, ageing and initial seed moisture content. Recently published work on the storage of pollen grains (Jain and Shivanna, 1988) suggests that the toxicity problems with acetone on pollen arise from its relatively high dielectric constant. Accordingly, a comparison of acetone treatment with the much less polar solvent, hexane, was also undertaken.

Materials and Methods

Seed material

Five seed lots of cv. Amsoy and one lot of cv. Davis were used in this study. All lots were harvested in 1988, with the exception of Amsoy, lot A (1987 harvest). Amsoy lots A, B and C were all of high initial germinability and were, respectively, purchased from Wrightson's Ltd., (now Challenge Seeds Ltd.) Palmerston North, N.Z.; Corson Grain Ltd., Gisborne, N.Z. or grown in the Seed Technology Centre's own experimental plots. A low germination sample of cv. Amsoy (lot D) was also gifted to us by Corson Grain Ltd. Lot E was also obtained from Corson Grain Ltd., and was visually sorted into mechanically damaged or undamaged sub-lots. The seedlot of cv.Davis was imported from Wright Stephenson & Co. (Australia) Pty. Ltd.

Solvent treatments

Analar grade acetone and Hipersolv hexane (BDH Chemicals, N.Z.Ltd.,) were used as supplied. A trial with peas had previously shown that redistilling did not ameliorate any toxicity problems found using the normal stock solvent (data not shown). Seeds were soaked by immersing each replicate in pure solvent for up to 16h at a constant 20°C. Following soaking, samples were spread out on absorbent paper and left to dry at 20°C for 24h, except in one experiment where seeds were dried at either 20°C or 35°C for up to 48h.

Ageing treatments and seed moisture adjustments

Accelerated ageing (AA) treatment was carried out using the technique described by Baskin (1987), holding seed at 100% RH, 40°C for up to 6 days. Controlled deterioration (CD) was carried out by a modification of the method of Matthews and Powell (1987). Seeds were brought up to a moisture content of 20% by adding the calculated amount of distilled water to a weighed sample of seeds of known initial moisture content and then equilibrating overnight at 10°C in heat-sealed, moisture proof, polyester-aluminium foil - polyethylene laminated packages. Following equilibration, seeds were held at 40°C for up to three days.

Where seed moisture content (SMC) required adjustment prior to acetone treatment, a similar method to the above was employed to raise the water content. Alternatively, to reduce the initial SMC, seeds were held over silica gel in desiccators at room temperature until the calculated water loss had been achieved. In all cases actual moisture contents were determined for each replicate sample in these experiments. SMC's were determined by the oven method according to International Seed Testing Association Rules (1985).

Seed germination testing

Germination trials were normally conducted on 50 seeds per replicate kept at a constant 25°C in the dark. The between paper method (ISTA, 1985) was used, recording percentage normal seedlings 8 days after sowing. Seeds were dusted with thiram prior to setting up the germination trials. Percentage seed viability (normal and abnormal germinants plus fresh non-germinated seeds) was also recorded, as were the fresh and dry weights of embryonic axes of normal seedlings 8 days from sowing.

Phospholipid analysis

The lipid fraction was extracted as described by Francis and Coolbear (1984). Twenty axes or an equivalent weight of cotyledons were boiled for 2 minutes in 1 ml water saturated butanol (WSB) containing 100 µg butylated hydroxytoluene. The tissue was then hand ground to a slurry and washed into a centrifuge tube with a further 1 ml WSB. After centrifuging for 10 min. at 1300 G, the supernatant was decanted and the precipitate re-extracted with a further 2 ml WSB and recentrifuged. The two supernatants were then combined and the volume of the fraction measured before taking a 200 µl aliquot for determining the phospholipid content according to the method of Bartlett (1959). Three replicate extractions were carried out for each treatment.

Data analysis

Four replications were used in all experiments with seed lots of cv. Amsoy, but, because of limited supplies of cv. Davis, only three replications were used. A split plot design was used to analyse the interactions between duration of acetone soaking and controlled deterioration, while randomised complete

block analyses were used for the phospholipid data. Otherwise individual standard errors of means were calculated. Where combined estimates of standard error were obtained an arcsine √% transform was employed.

Results

Effects of soaking seeds in acetone

Figure 1 shows the effects of duration of soaking on seeds of cv. Amsoy, Lot A. There was a small, but significant ($p<0.05$) toxic effect of acetone on unaged seeds after just 2h soaking, when the percentage of normal germinants fell from 95 to below 90%. Apart from that, there were no other clear toxic effects of acetone in unaged or 1d aged seeds. However, toxic effects were clearly evident on these seeds when they were subjected to 3d CD after acetone treatment. Damage increased dramatically with the duration of acetone treatment up to 16h.

In order to confirm that all the acetone in the seed was completely removed after soaking for 16h, a comparison was made between the germination behaviour of seeds dried at 20°C and 35°C for 24h or 48h after acetone treatment. Data are presented in Table 1 for unaged seeds and those subjected to 3d CD. Longer drying times or higher drying temperatures did not significantly reduce the level of acetone damage.

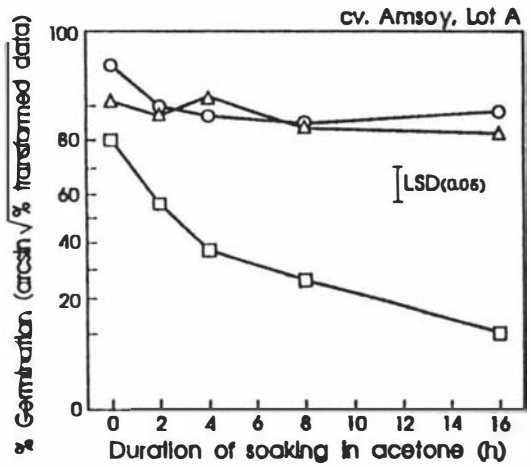

Figure 1: The effects of duration of soaking in acetone on normal germination of soybean seeds, cv. Amsoy, Lot A. Data are presented as arcsin √% transformed means of germination after 0 (O), 1 (Δ) and 3 days (□) CD following acetone treatment. The initial SMC of the seeds before treatment was 10.9%.

Seed lot variation

Figure 2 shows that, although all three high germination lots show susceptibility to acetone damage, there was considerable variation in responses between seed lots. Unaged seeds of the high vigour lots A and B do not show significant loss of germination after 16h acetone treatment. Seed lot C was of medium vigour (as assessed by preliminary germination counts and seedling growth evaluation, data not shown) and unaged seeds of this lot were severely damaged by acetone. In all three lots there were highly significant toxic effects of the solvent on soaked seeds which were then subjected to 3 days CD. Interestingly, no effects of acetone were detected on the small numbers of unaged germinable seed remaining in the poor quality seed lot D.

In another experiment (Fig. 3) evidence was obtained showing that one factor affecting the toxicity of acetone was the extent of mechanical damage in the seeds. Cracked seeds were much more sensitive to acetone damage. The addition of methyl red to the solvent showed that in intact seed there was little or no movement of dye beyond the seed coat, compared to extensive penetration in mechanically damaged material.

Table 1: The effects of extended and higher temperature drying on normal germination of seeds of cv. Amsoy, lot A after 16h soaking in acetone. Data are means of four replications and are presented as backtransformed values with arcsine √% transformed data (radians) in brackets.

Acetone treatment	Drying conditions	% normal germination unaged	After 3d CD
Control	none	98 (1.43)	86 (1.18)
Acetone	20°C, 24h	84 (1.16)	0
	20°C, 48h	85 (1.17)	0
	35°C, 24h	80 (1.11)	0
	35°C, 48h	91 (1.26)	2 (0.04)
$LSD_{0.05}$		(0.17)	(0.10)

Effect of changing seed moisture content

While it can be concluded from Figure 2 that variation between seed lots in initial seed moisture contents had little impact in determining a seed lot's behaviour, variation of SMC within a seed lot was of crucial importance in determining the resistance to acetone damage of that lot, as was the level of mechanical damage (Fig. 3). As can be seen from the figure the extent of damage caused by solvent treatment was much reduced if initial seed moisture content was lowered to around 6% before soaking in acetone. This effect has now been shown in a total of four seed lots (3 cv. Amsoy and 1 cv. Davis). Of course, the extent of damage varied between lots and

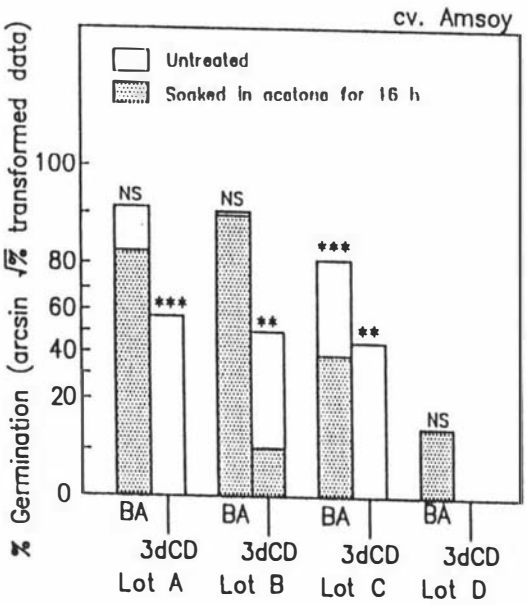

Figure 2: **The responses of four different seed lots to 16h soaking in acetone either before ageing (BA) or with a subsequent controlled deterioration treatment (CD) for 3 days at 40°C, 20% SMC. Data presented are the arcsin √% transforms of normal germination. NS: not significant; ** and *** significant differences between untreated and acetone treated seeds (p<0.01 and p<0.001, respectively). Initial SMC's of the four lots, A-D, before soaking were 15.1, 11.1, 10.1 and 11.1%, respectively.**

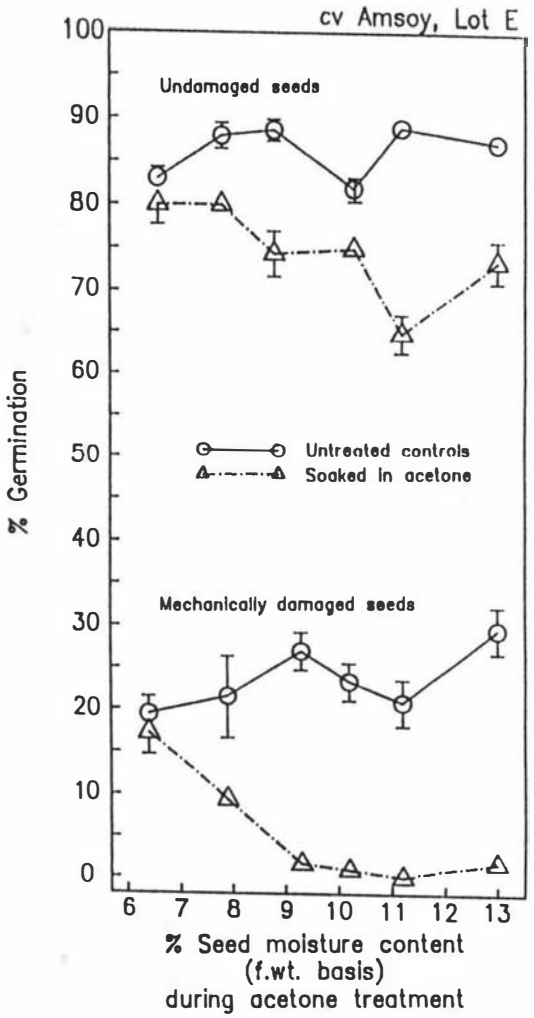

Figure 3. **The effects of initial seed moisture content and visible mechanical damage on the germinability of unaged seeds after a 16h soak in acetone. Data are the means of four replications. Individual SEM's are shown where larger than the symbols used.**

level of cracking within a seed lot, but in each case the pattern of response was similar. Careful seed drying before treatment also offered some protection to soaked seeds of cv. Davis on subsequent accelerated ageing (data not shown).

The mechanisms of acetone toxicity

Analysis of the phospholipid (PL) content of untreated or acetone soaked seeds showed that acetone treatment greatly increased the rate of PL deletion from both axes and cotyledons during accelerated ageing (AA), (Fig. 4). Untreated seeds showed no significant losses of phospholipid from axes and only a small significant ($p<0.05$) decrease (~20%) in cotyledonary PL after 6 days AA. Acetone soaked seed showed significant losses ($p<0.01$) in this tissue after 4 days AA and more than 35% deletion after 6 days. A similar rate of loss was also detected in axis tissue. Under these storage conditions, untreated seeds lost nearly 40% of their germinability after 4 days AA, whereas acetone treated material lost nearly 75% germinability (Fig. 5a). After 6 days AA untreated seeds had only 12% normal germinants, acetone treated seeds none.

Figure 5 also presents a comparison of the effects of hexane and acetone treatment on seeds of cv. Davis. Hexane is less deleterious to the seeds and there is no interaction with ageing.

Discussion

Factors affecting acetone toxicity

It is clear from these results that, despite reports to the contrary (e.g., Lewis *et al.*, 1979), there is a toxicity problem when acetone is used as a solvent to deliver chemicals to soybean seeds. This work shows that the level of damage depends on variations between seedlots, level of mechanical damage, initial seed moisture content prior to acetone treatment, duration of acetone treatment and also on subsequent storage after treatment.

The observation that mechanically damaged seeds were more prone to acetone damage is in agreement with Shortt and Sinclair (1980) who found that acetone killed cotyledon tissue in soybean with cracked seedcoats. Halloin (1977) similarly reported that while damaged cottonseed or excised embryos were injured by acetone, the germinability of intact seeds was unimpaired. Nevertheless, it is clear that this is not the only factor involved. In the studies detailed here lots A-C and cv. Davis had low levels of visible mechanical damage. Similarly, Tao and Khan (1974) reported that although acetone reached the embryos of cucumber or squash seed, it did not cause any apparent damaging effects on seeds germinated immediately after treatment.

Our data on the interaction between acetone and initial seed moisture contents complement similar results found for peas (Coolbear, McGill and Sakunnarak, unpublished data). These findings appear to be novel in that no other workers have identified this interaction. Only two relevant reports have been found in the literature, both mentioning increased toxicity of solvents when they have 10% or more water added to them (Lewis *et al.*, 1979; Persson, 1988).

Possible mechanism of action

The results in Figure 4 show that the interaction between acetone toxicity and ageing is related to losses of phospholipid from axis and cotyledon tissue, a mechanism originally suggested by Halloin (1977), although he presented no substantiating evidence apart from increased conductivity of leachate from acetone soaked embryos. It should be noted that our data present no evidence of PL losses in unaged acetone treated seed. It is worth speculating that penetration of acetone into cell membranes results in a disruption of the crystalline lipid bilayer which exists in dry tissue (Crowe *et al.*, 1989). This disruption could be exacerbated in higher SMC seeds if the solvent can bring water molecules into the apolar region of the membrane. High vigour seed may have some capacity for repair of this kind of damage, but this may become increasingly difficult for low vigour or ageing seed.

This hypothesis explains many of the observations reported here and merits further investigation. Nevertheless, the evidence obtained on PL losses to date is purely correlative, although Swanson *et al.*, (1973) reported from their electron microscope work that acetone disrupted membranes in the cells of tobacco leaves. However, it could be that acetone causes damage via other effects. For example, Meyer and Mayer (1971) found that acetone depressed oxygen uptake of lettuce seeds while Eldan and Meyer (1974) reported a decrease in invertase activity. In neither case, however, was germinability affected.

The use of a solvent with a lower dielectric constant such as hexane (Fig. 5b) could be safer than acetone because of its ability to penetrate dry membranes without disrupting the amphoteric structure of the bilayer. We are unaware of any other reports of its use as a solvent for seed treatment, but its cost and toxicity to humans may prohibit its routine use.

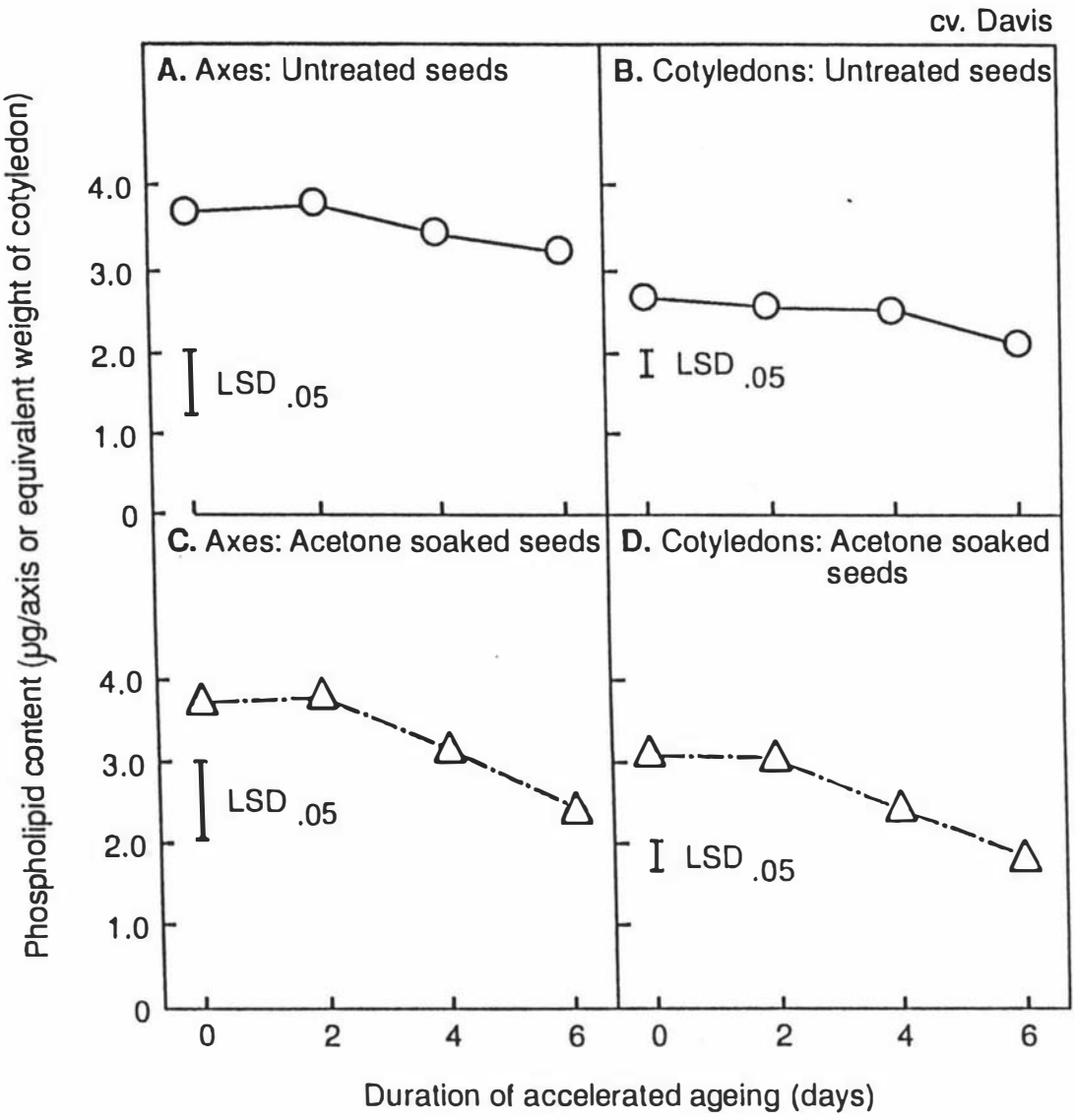

Figure 4: **The effects of acetone soaking on the phospholipid content of axis and cotyledonary tissue of soybean seeds cv. Davis subsequently subjected to accelerated ageing treatment. The initial SMC of seeds before acetone treatment was 9.3%.**

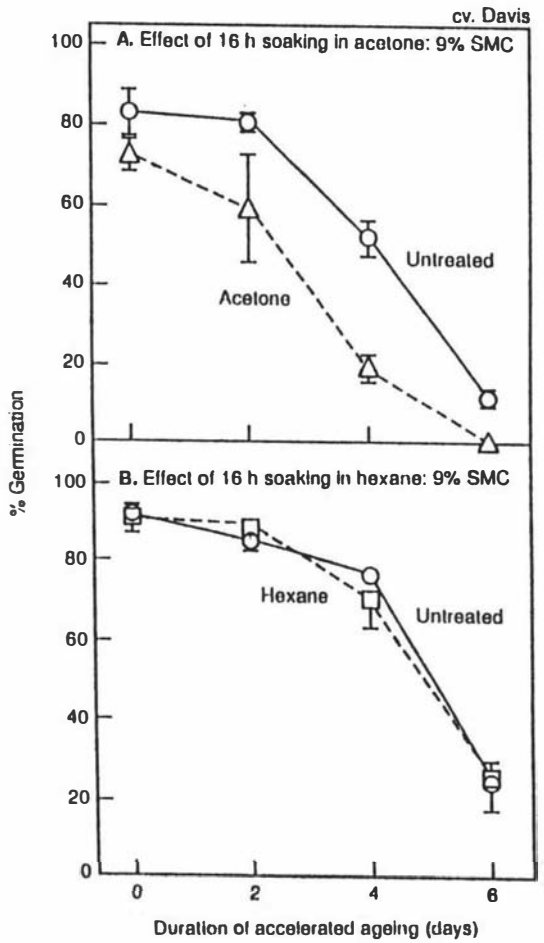

Figure 5. The effects on germination of soaking seeds of cv. Davis in either acetone (A) or hexane (B) for 16h followed by accelerated ageing treatment (100% R.H., 40°C) for up to 6 days. Individual SEM's are shown when larger than the symbols used.

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