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**CHANGES IN GERMINATION PERFORMANCE  
AND HYDROLYTIC ENZYME ACTIVITY IN  
WHEAT SEEDS (Triticum aestivum L.)  
CAUSED BY AGEING AND  
PRE-SOWING TREATMENTS**

A thesis presented in partial  
fulfilment of the requirements  
for the degree of  
**Doctor of Philosophy**  
in **Seed Technology**  
at **Massey University**  
Palmerston North  
New Zealand

**SURESH NATH**

**1991**

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## ABSTRACT

Three pre-sowing hydration-dehydration treatments were evaluated for their capacity to protect or repair wheat seeds stored under two different sets of artificial ageing conditions (accelerated ageing at 100% RH, 40°C or controlled deterioration at 15% SMC, 35°C). Although similar losses in germination capacity and decreases in radicle emergence rates occurred under both ageing conditions, differences with respect to the physiology of ageing were highlighted by changes in seedling growth and seed leakage. For example, increases in seed leakage observed during storage at 15% SMC were not found at 100% RH.

Longer hydration treatments (either 24 h at 15°C in water or 20 h at 20°C in -0.37 MPa PEG solution, followed by drying) improved the vigour of unaged seeds, but treated material deteriorated rapidly in storage compared to untreated controls. In contrast short hydration treatments (2 h at 25°C followed by drying) offered some protection of germinability during subsequent storage but did not affect the vigour of unaged seeds. When seeds were treated after storage, longer hydration periods were effective in producing substantial invigoration of viable deteriorated seeds (measured by evaluating T50 or seedling growth) compared to little or no improvement by short hydration treatments. These results support earlier suggestions from work on tomato seeds that losses in seed vigour and viability are not necessarily a continuum of the same deteriorative sequence.

The mechanisms of protection of germinability by short hydration treatments were not clear. Small decreases in T50's of unaged or aged seeds as a result of these treatments were due to leakage of germination inhibitory substances. However, the rapid germination of unaged and improved responses from aged seeds caused by longer hydration treatments suggested advances in germination processes and repair activity under these conditions. This aspect was pursued in further detail by studying changes in the hydrolytic metabolism of wheat seeds using the 20 h PEG treatment.

Although the starchy endosperm of treated seeds showed some indications of protein degradation, there were no changes in proteolytic activity (determined as 'Azocoll' hydrolysing activity at pH 6.8) as a result of ageing or pre-sowing treatment after storage. However, there were some indications of loss of control over proteolytic activity in seeds subjected to treatment before storage. Severe damage to membrane permeability in these seeds appears to be a post-mortem event as this was only found in samples showing drastic losses in seed germinability.

Pre-sowing treatment caused a buildup of germinative  $\alpha$ -amylase activity in unaged but not in aged seeds, although both showed similar radicle emergence rates. Quick resumption of  $\alpha$ -amylase production during subsequent imbibition by treated seeds, irrespective of ageing, suggests that components involved in de novo enzyme synthesis are tolerant to desiccation in wheat seeds. Increased  $\alpha$ -amylase activity in treated seeds or its maintenance during subsequent storage, surprisingly did not cause damage to stored starch. There was no relationship between increased  $\alpha$ -amylase activity and early radicle emergence.

The ageing-induced delay in germinative  $\alpha$ -amylase production appeared to be due to delayed gibberellin synthesis by the aged embryo. Pre-sowing treatment of seeds after storage effectively decreased the lag period for enzyme production in deteriorated seeds. Ageing effects on aleurone were characterised by investigating changes in the responsiveness of embryoless half seeds to gibberellic acid with respect to  $\alpha$ -amylase production in vitro. Ageing of seeds caused a significant reduction in aleurone enzyme production. These changes were at least in part, reversed by pre-sowing treatment of aged seeds.

Abbreviations: h = hours; PEG = polyethylene glycol; RH = relative humidity; SMC = seed moisture content; T50 = time to 50% radicle emergence.

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## ABBREVIATIONS

<b>AA</b>	accelerated ageing
<b>ABA</b>	abscisic acid
<b>ATP</b>	adenosine triphosphate
<b>CD</b>	controlled deterioration
<b>cv</b>	cultivar
<b>d</b>	days
<b>DNA</b>	deoxyribonucleic acid
<b>dwt</b>	dry weight
<b>EC</b>	electrical conductivity
<b>EDTA</b>	ethylenediaminetetraacetic acid
<b>Fig</b>	figure
<b>g</b>	gram
<b>GA</b>	gibberellic acid
<b>GADA</b>	glutamic acid decarboxylase activity
<b>h</b>	hours
<b>HMW</b>	high molecular weight
<b>HPLC</b>	high-performance liquid chromatography
<b>ISTA</b>	International Seed Testing Association
<b>IUPAC-IUB</b>	International union of pure and applied chemistry and the International union of biochemistry
<b>kD</b>	kilo Dalton
<b>LSD</b>	least significant difference
<b>M</b>	molar
<b>ME</b>	moisture equilibration
<b>mg</b>	milligram
<b>mM</b>	millimolar
<b>MPa</b>	megapascal
<b>mRNA</b>	messenger RNA
<b>mwt</b>	molecular weight
<b>nm</b>	nanometer
<b>OD</b>	optical density
<b>p</b>	probability of non occurrence of the event
<b>PEG</b>	polyethylene glycol

<b>PGR</b>	plant growth regulator
<b>Poly(A)<sup>+</sup> RNA</b>	polyadenylated RNA
<b>PST</b>	pre-sowing treatment
<b>RH</b>	relative humidity
<b>RNA</b>	ribonucleic acid
<b>SDS-PAGE</b>	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
<b>Sec</b>	section
<b>SH</b>	sulphydryl
<b>SMC</b>	seed moisture content
<b>SE/SEM</b>	standard error of mean
<b>T50</b>	time to 50% radicle emergence
<b>tab</b>	table
<b>v/v</b>	volume/volume
<b>w/v</b>	weight/volume
<b>μS</b>	microsiemen
<b>α</b>	alpha
<b>β</b>	beta
<b>~</b>	approximately equal to
<b>&lt;</b>	less than
<b>&gt;</b>	greater than

## CHAPTER 1

### INTRODUCTION

Agronomically, one of the surest and simplest ways of improving agricultural production is through achieving optimum and uniformly maturing plant populations. To this end, the additional investment made on high quality seed (high germinability and vigour) probably pays a higher return than any other agricultural input (see for example Woodstock, 1976; Heydecker and Coolbear, 1977). Unfortunately, however, availability of high quality seed often becomes a limiting factor, one of the important reasons being physiological deterioration, leading to vigour and viability losses in storage. Although the environmental factors causing seed deterioration are well known (for example temperature and seed moisture), the mechanisms of vigour and viability losses are poorly characterised. Studies on the physiological and biochemical changes accompanying seed deterioration are constantly being pursued in quest of identifying the primary causes of damage in seeds and their mechanisms. An understanding of the metabolism of seed deterioration may lead to better ideas for preserving or improving the quality of seeds.

In dry storage seeds deteriorate, accompanied by progressive degradation of cellular systems, including damage to membranes, nucleic acids, and cellular organelles (see Priestley, 1986). Interestingly however, longer survival of seeds buried in soil compared to normal dry storage, provided an early indication that fully imbibed seeds may have a prolonged storage life. The work of Villiers and Edgcumbe (1975) was the first to show that repair may occur in imbibed seeds which would counteract the normal deteriorative process of ageing.

Several types of seed treatments have been tried in the past for improving germination performance of stored seeds (see Heydecker and Coolbear, 1977, for a detailed review). Of these, pre-sowing hydration treatments (see Hegarty, 1978, for a review) are of special interest not only because they are simple but, in addition to improving germination performance, they provide excellent opportunities for the study of the key physiological events associated with vigour and viability losses in seeds (Coolbear et al., 1987).

### 1.1 **Pre-sowing Hydration Treatments for Protecting or Improving the Germination Performance of Stored Wheat Seeds**

Over the past two decades, a small number of papers have been published reporting attempts to invigorate cereal seeds using pre-sowing treatments (e.g. Abdul-Baki and Anderson, 1970; Hanson, 1973). A particular focus of this thesis has been on the potential of these treatments to improve the quality of stored seed by using them either as protective or repair treatments applied prior to or after storage respectively. Little coherent information has emerged from the previous work on wheat seeds, mainly because of the wide range of treatment conditions and evaluation criteria used, coupled with some contrasting and mis-interpreted reports. In particular, there appears to be some confusion about suitable hydration times for both protective and invigorative treatments. Also there is not much information with respect to effects of hydration-dehydration treatments on deteriorated seeds.

### 1.2 **Use of Artificial Ageing Techniques in Physiological Studies on Seed Deterioration.**

As natural ageing at lower seed moisture and temperature regimes is a relatively slow process, seeds are commonly exposed to harsh environmental conditions in experimental studies in order to accelerate the rates of deterioration. Recently however, there has been a growing concern about the validity of higher moisture ageing techniques for studying the metabolism of seed deterioration (Priestley and Leopold, 1983; Bewley, 1986). Accordingly, another major theme of this project is the comparison of deterioration effects of lower seed moisture (15% SMC) controlled deterioration (cf. Matthews and Powell, 1987) with those of high humidity accelerated ageing (Delouche and Baskin, 1973).

### 1.3 Ageing and/or Treatment Induced Changes to Endosperm Mobilization Processes

Although an enormous amount of work has been done on the mechanisms of endosperm mobilization in germinating cereal seeds (for a review see Laidman, 1982; Fincher, 1989) very little is known about the nature of age-induced damage to this system. Embryo and aleurone together play a key role in the production of hydrolytic enzymes which are necessary for the mobilization of stored reserves in the starchy endosperm. Although very rare, the earlier attempts separating ageing effects occurring in the embryo from those occurring in the aleurone do not seem to agree in respect to ageing damage to aleurone tissue. Whereas the work of Aspinall and Paleg (1971) in wheat shows some indication that ageing may decrease the ability of aleurone to produce  $\alpha$ -amylase, no such effects were detected by Artsruni and Panosyan (1984). Further, there are still gaps in our understanding of the relationship between hydrolytic enzyme activities and germination or seedling growth in wheat seeds. For example, higher  $\alpha$ -amylase activities are commonly associated with the problem of sprouting damage suffered by maturing wheat grains during wet harvest seasons (e.g. Sargeant, 1979). Very little information at all is available on the possible mechanisms of protection or invigoration of seeds by hydration treatments in cereals. Studies on the interactions of ageing and protective or invigorative treatments may help in identifying the key events associated with physiological deterioration of seeds.

Therefore, it was decided to investigate the possible interactions between ageing and pre-sowing treatment on the activities of two major groups of hydrolytic enzymes,  $\alpha$ -amylases and proteases, in wheat seeds. In addition to studying changes to starch and proteins quantitatively, scanning electron microscopy was utilized to examine qualitative changes in the starchy endosperm of seeds. Changes in  $\alpha$ -amylase production by aleurone tissue in response to exogenously applied gibberellic acid were also studied in an attempt to further understand the possible nature of ageing damage and to determine

whether or not pre-sowing treatment has any ability to alleviate the ageing effects on hormonal control of reserve mobilization processes.

#### 1.4 Objectives of This Study

To summarise, the main objectives of this study were as follows:

- 1) To compare the effects of slower ageing in a relatively low seed moisture regime with those of rapid ageing at saturated humidity levels.
- 2) To evaluate the utility of three of the most promising pre-sowing hydration treatments reported in the literature for protection or repair of wheat seeds stored under both sets of conditions.
- 3) To study changes in endosperm mobilization processes ( $\alpha$ -amylase and protease metabolism) during ageing and/or pre-sowing treatment.

## CHAPTER 2

### REVIEW OF LITERATURE

#### 2.1 INTRODUCTION

In order to provide a context for subsequent discussion, this review first looks briefly at the anatomy of the wheat caryopsis and some of the early events in seed germination (Sections 2.2 and 2.3 respectively). Following this there is a detailed review of the literature on two of the most important groups of hydrolytic enzymes involved in the mobilization of reserves in wheat: namely the  $\alpha$ -amylases (Sec. 2.4) and the proteases (Sec. 2.5).

It is beyond the scope of this review to cover the physiological and biochemical changes observed during ageing in various crop species in detail. However, an attempt has been made to present an overview of the diversity of ageing related changes and the complexities involved in identifying the key events of seed deterioration (Sec. 2.6). The use of artificial ageing conditions and the recent awareness concerning the validity of these techniques for studying mechanisms of seed deterioration is also highlighted briefly in Sec. 2.7. Although use of pre-sowing hydration treatments for invigoration or protection of stored seeds is not a new idea, there is some confusion in the literature, at least for wheat seeds, about the technique and the mechanisms involved. Accordingly, a critical review of the different types of pre-sowing hydration treatments used for wheat seeds is presented in Section 2.8.

#### 2.2 STRUCTURE OF THE WHEAT GRAIN

A diagram of the longitudinal and transverse sections of the wheat caryopsis, also referred to as kernel or grain or seed,

is shown in Fig. 1.1. The outermost pericarp, representing the fruit coat is tightly fused with the seed coat. The embryo lies on the dorsal side and constitutes about 2.5 - 3.6% by weight of the whole grain (MacMasters et al., 1971). The rest of the grain is occupied by the endosperm which is storage tissue. The crease region represents the site of the long vascular bundle attached to the nucellar tissue of the parent plant. It must be emphasised here that there is no direct vascular connection between the endosperm or the embryo and the mother plant, assimilates having to pass through an apoplastic phase before being taken up by the developing embryo or endosperm. The nucellar projection, a shaft like structure in the crease region, is a remnant of degenerated nucellus and is attached to a pigment strand which is a cylinder of cells with lignified walls.

### 2.2.1

#### **The Embryo**

The shoot or epicotyl consists of an apical meristem and a plumule (primary leaves) which are surrounded by a coleoptile. The radicle consists of a primary root and two pairs of lateral roots surrounded by a coleorhiza (Evers and Bechtel, 1988). Whereas, the coleoptile has two vascular bundles located laterally on opposite sides (Percival, 1921), the coleorhiza lacks a vascular system. The scutellum is a shield-like structure separating the embryonic axis from the starchy endosperm tissue. It possesses a single layer of epithelium composed of columnar cells facing the starchy endosperm; these cells are thought to function both in a secretory and absorptive role during germination. A layer of crushed cells called the fibrous region separates the scutellar epithelium from the starchy endosperm. Crushed cells are in fact formed from starchy endosperm cells that fail to develop normally, leaving only the cell walls as the cytoplasm degenerates (Evers and Bechtel, 1988). Primary food resources in the embryo are lipid droplets and protein bodies, with starch only occasionally found in mature tissue. In addition to these, protein bodies in the scutellum were shown to contain phytin reserves (Swift and O'Brien, 1972).

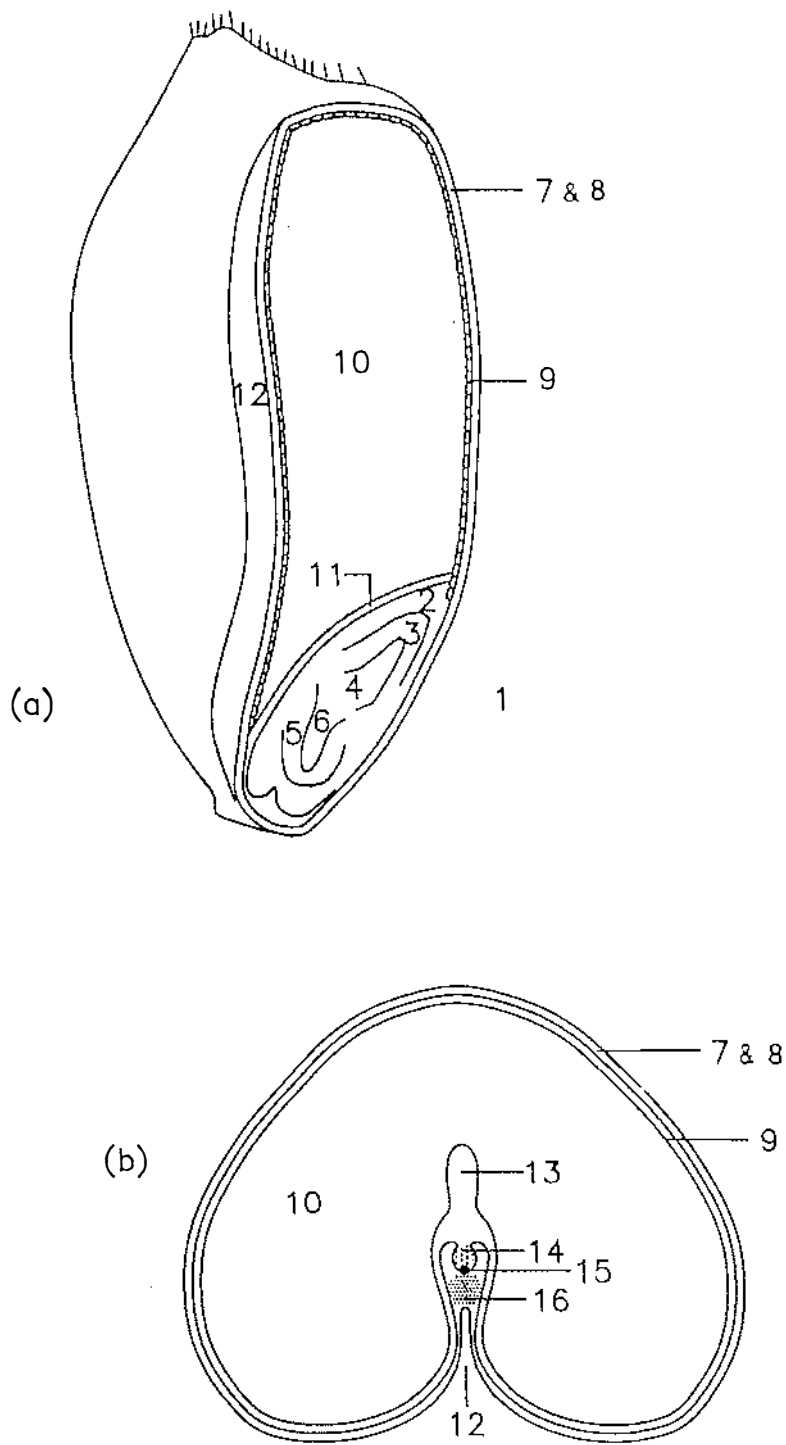


Figure 2.1 The longitudinal (a) and transverse (central plane) (b) sections of wheat seed showing some important seed tissues: 1. embryo, 2. scutellum, 3. coleoptile, 4. plumule, 5. coleorhiza, 6. radicle, 7. pericarp, 8. seed coat (testa), 9. aleurone layer, 10. starchy endosperm, 11. fibrous region, 12. crease region, 13. endosperm cavity, 14. nucellar projection, 15. pigment strand and 16. vascular bundle.

### 2.2.2 The Endosperm

Although starchy endosperm is a dead tissue at maturity, it is surrounded by a single layer of living cells called the aleurone. During later stages of development, when cell division ceases, the outer-most meristematic layer of the endosperm becomes transformed into this tissue. The aleurone cells are typically block shaped (37 to 65  $\mu\text{M}$  x 25 to 75 $\mu\text{M}$ ) when viewed in cross or longitudinal sections and have thickened cell walls (6 - 8  $\mu\text{M}$ ) (Evers and Betchel, 1988). The walls are distinctly bilayered and are uniformly autofluorescent due to the presence of a ferulic acid carbohydrate complex (Fulcher *et al.*, 1972). Aleurone proteins are stored in protein bodies or aleurone grains whereas lipids are present as droplets and do not appear to have a typical membrane surrounding them.

The nature and composition of the starchy endosperm of the wheat grain are covered in Sec. 2.3.1.

## 2.3 EARLY EVENTS DURING SEED GERMINATION

Although a seed analyst or agronomist considers the term germination to cover all the processes involved in the transformation of an embryo into a developed seedling, from a physiologist's view germination describes the process from imbibition to radicle or plumule emergence from the seed coverings (for example, see Bewley and Black, 1978; Bryant, 1985). Unless otherwise stated, it is this second definition which will be used in this thesis.

A series of events occur when a non-dormant, viable seed imbibes water and these can be broadly summarised as follows:

1. Hydration of cytoplasm accompanied by a massive structural re-organisation of cell membranes and organelles.

2. Increase in respiration and energy rich phosphates.
3. Synthesis of proteins, nucleic acids and lipids.
4. Mobilization of food reserves.

One of the early events observed upon hydration of seed is the rapid development of membrane systems (Mayer, 1977). This process involves both re-organisation of existing membranes and new synthesis for both repair processes and the formation of new organelles. Before the integrity of plasma membranes is attained there will be a passive leakage of solutes from the seeds. For normal metabolic activity, the integrity of plasma membrane and compartmentalization of various cellular organelles are essential. Time-course studies show that the rate of leakage of solutes from the seed all follow the same pattern, starting rapidly in the beginning stages of imbibition, then slowing up and finally coming to a halt (Simon and Raja Harun, 1972), as first outer cells and then all cells rehydrate and reorganise (Simon, 1974).

Respiration rates, which tend to be very low in dry seeds (Harrington, 1973), increase quickly when seeds are hydrated. The first increase in oxygen uptake is due to hydration and oxygenation of mitochondrial enzymes (Kolloffell, 1967). Respiration plays a very important role in providing biochemical energy which is essential for the metabolic processes during germination. The first hours of imbibition are accompanied by considerable mitochondrial differentiation either via modification of pre-existing ones or by synthesis of new organelles (Morohashi, et al., 1981). The levels of ATP which tend to be very low in dry seeds increase substantially during this period (Priestley, 1986), for example in wheat embryos a five fold increase was observed within 30 minutes of imbibition (Obendorf and Marcus, 1974).

Although synthesis of new messenger RNA begins earlier during imbibition (Spiegel et al., 1975), it has been suggested that

the earliest protein synthesis may occur by the activation of pre-existing messages. The work of Weeks and Marcus (1971) in wheat shows that the RNA present in the messenger fraction in the dry embryo quickly associates with ribosomes during the early stages of imbibition to form active protein synthesising polysome complexes. However, the current view concerning stored 'mRNA' is that it is probably mostly residual from the seed development phase and is believed to be degraded early during germination, much of it not being relevant to germinative metabolism (Smith and Bray, 1984). Although this aspect remains not very clear, there is evidence suggesting that protein synthesis is obligatory for germination. Exposure of germinating embryos to inhibitors of protein synthesis has been shown to prevent normal elongation of the embryonic axis (Walton and Soofi, 1969; Klein *et al.*, 1971). The work of Marcus *et al.* (1986) using wheat embryos indicates that protein synthesis during the lag phase of imbibition is essential before embryo growth can occur.

In addition to re-organisation, the other important aspect of reactivation of cellular system by hydration is the operation of extensive repair metabolism. Enough evidence has accumulated to show operation of repair to membranes, DNA, mitochondria, and other structural components during seed imbibition (Priestley, 1986).

Changes to DNA occur at a later stage during imbibition and the timing of the onset of DNA synthesis in different tissues can vary considerably depending on the progress of cell division, and can be regarded largely as a post-germination process.

As starchy endosperm of cereals is a dead tissue, mobilization of stored food reserves during germination depends on the production of hydrolytic enzymes by the aleurone and the embryo. The evidence for gibberellin production by isolated barley embryos (Radley, 1967) and the production of  $\alpha$ -amylase in response to GA<sub>3</sub> by de-embryonated barley grains (Paleg, 1960) has led

to the generally accepted view that GA released from the embryo during germination diffuses into the aleurone layer and induces de novo synthesis of the enzymes. In 1967, Filner and Varner used density labelling with  $[H_2O^{18}]$  to show that GA<sub>3</sub>-induced  $\alpha$ -amylase produced in barley aleurone layers was newly synthesised using amino acids derived from the hydrolysis of pre-existing proteins.

### 2.3.1 **Structure of Wheat Endosperm and Early Events During its Mobilization**

In cereal grains, starch is synthesised in amyloplasts. In wheat and barley each amyloplast contains one starch granule as compared to several in rice and oats (Hoseney, 1986). Wheat endosperm has two types of starch granules, the large lenticular 'A' type measuring approximately 15 - 30 $\mu$  in diameter and the small spherical, 'B' type with 5 - 8 $\mu$  diameter (Sargeant, 1980). Evers and Lindley (1977) reported that the small granules formed more than one third of the total weight of starch.

In general, starch granules are predominantly composed of two types of starch polymers depending on the arrangement of the  $\alpha$ -D-glucose units. The linear polymer is known as amylose and the highly branched polymer is known as amylopectin. In amylose starch,  $\alpha$ -D-glucose units are linked  $\alpha$ -(1 - 4) whereas the amylopectin is primarily composed of  $\alpha$ -(1 - 4) with about 4 - 5% of  $\alpha$ -(1 - 6) linkages which gives the branching structure to the polymer (Hoseney, 1986). The relative proportion of the two polymers varies among species and varieties (Fincher, 1989).

Hess (1954) first described the presence of protein matrix supporting and adhering to the starch granules in wheat endosperm, a finding later confirmed by scanning electron microscopy (Fincher and Stone, 1974; MacGregor and Matsuo, 1982). The cell walls in the starchy endosperm of wheat are obscured by

the starch granules and the protein matrix which fills the cells (Fincher and Stone, 1974), and are normally much thinner than those of the aleurone cells being devoid of lignification (Fincher and Stone, 1986). Scanning electron micrographs of both barley (Briggs, 1972) and wheat (MacGregor and Matsuo, 1982) also show that the starchy endosperm is separated from the scutellar epithelium by a layer of crushed cells (see Sec. 2.2.1).

As in barley (MacLeod et al., 1964; Gibbons, 1980), wheat endosperm cell walls are degraded at a relatively early stage followed by rapid degradation of the protein matrix (Fincher and Stone, 1974). In contrast to this, breakdown of starch granules is not apparent until 3 to 4 days after the start of imbibition (in vitro studies with de-embryonated grains).

MacGregor and Matsuo (1982) reported similar observations in sprouting damaged wheat. Studying the rate of decline in falling number in sprout damaged wheat seeds Gordon et al. (1977) also reported that endosperm degradation was well advanced, possibly by other hydrolytic enzymes before  $\alpha$ -amylase activity increased from its base level.

Several reports suggest that endosperm degradation during germination in many cereal grains including wheat (see Sec. 2.4.5.1) is initiated adjacent to the scutellum (Briggs, 1972; Okamoto et al., 1980; MacGregor and Matsuo, 1982; Marchylo and Kruger, 1987). MacGregor and Matsuo (1982) showed that initiation of starch degradation in sprouting damaged wheat appeared near the ventral crease close to the embryo and moved along the embryo-endosperm junction to the dorsal side of the grain. Some non-dormant wheat cultivars were found to exhibit protein matrix and starch granule degradation without any visible sign of germination (Marchylo and Kruger, 1987). In such cases too, endosperm degradation appeared to be initiated near the ventral crease and middle region adjacent to the layer of crushed cells.

## 2.4 $\alpha$ -AMYLASES

### 2.4.1 The Enzymes and their General Mode of Action

Cereal  $\alpha$ -amylase, defined as an endo,  $\alpha$ -1, 4-glucan 4 glucanohydrolase (E.C. 3.2.1.1.) (Meredith and Jenkins, 1973), is a group of endo-enzymes that can cleave  $\alpha$ - (1 - 4)- D-glucosidic linkages randomly within the starch polymer (Kruger and Lineback, 1987). They are known to play a pivotal role in the mobilization of starch reserves during cereal seed germination and seedling development. In contrast,  $\beta$ -amylase is an exo-enzyme, being only able to hydrolyse alternate  $\alpha$ - (1 - 4)- D-glucosidic linkages in a stepwise fashion from the non-reducing end of the starch polymer (Kruger and Reed, 1988).

By using protein separation techniques such as isoelectric focusing, the occurrence of multiple forms of  $\alpha$ -amylase has been established (Olered and Jonsson, 1970; Sargeant, 1979; Daussant *et al.*, 1979). Marchylo *et al.* (1980b) separated up to 22 different components from immature grains alone, some of which resembled those present in germinating seeds. Whereas some multiple forms are undoubtedly due to genetic diversity (Ainsworth and Gale, 1987), artifacts resulting from particular isolation procedures cannot be ruled out (Marchylo and Kruger, 1987). Strictly speaking, only those multiple forms that arise from genetically determined differences in the primary structure should be called isoenzymes as per IUPAC-IUB (1977) recommendations.

As earlier workers referred to the wheat  $\alpha$ -amylases by different names there is some confusion in the nomenclature which is generally sorted out either by referring to their salient properties (for example pI) or to their stage of appearance (seed development or germination) in seed life. The three groups and their various designations are shown in Table 2.1. In accordance with IUPAC-IUB recommendations (followed in this thesis) the group with lower-pI values is referred to as the  $\alpha$ -amylase 1 group and

Table 2.1 Different names used in the literature to describe the three groups of  $\alpha$ -amylases known to occur at different stages of wheat grain development, maturation and germination.

Authors	Low pI group pI 4.5 - 5.1	High pI group pI 6.0 - 6.5	Very high pI group pI >10.0
Olered and Jonsson (1970)	Green	Malt	
Sargeant (1979)	Group II	Group I	
Sargeant (1980)			
Daussant <u>et al.</u> (1980)	I	II	III
Daussant and Reenard (1987)			
Marchylo <u>et al.</u> (1980a)			
Marchylo <u>et al.</u> (1984)	GI GII	GIII	
Kruger and Marchylo (1985)			
IUPAC - IUB recommendation (Fincher 1989)	$\alpha$ -amylase 1 group	$\alpha$ -amylase 2 group	
Reference to respective sets of gene loci:			
Flintham and Gale (1988)	$\alpha$ -Amy-2	$\alpha$ -Amy-1	$\alpha$ -Amy-3
Baulcombe <u>et al.</u> (1987)			$\alpha$ -Amy-3
Daussant and Reenard (1987)			

the higher-pI group as the  $\alpha$ -amylase 2 group (Fincher, 1989). However, gene loci have confusingly different identifications (see Table 2.1).

The  $\alpha$ -amylase 1 group (low pI) are present during the early stages of grain development and maturation mainly in the pericarp tissue (Marchylo et al., 1980a; Daussant and Reenard, 1987). This group has a broader pH optimum (3.60 - 5.75), greater heat lability and a higher molecular weight (52 - 54 x 10<sup>3</sup>) compared to the  $\alpha$ -amylase 2 group (Marchylo et al., 1976). In germinating seeds they are less abundant than the  $\alpha$ -amylase 2 group (Marchylo et al., 1984).

The  $\alpha$ -amylase 2 group (high pI) represents about 85% of the total activity in seeds after 7 days germination (Sargeant, 1979). They are more heat stable (Marchylo et al., 1976), have a lower molecular weight (42 - 42.5 x 10<sup>3</sup>) and a narrower pH optimum (5.5 - 5.7) (Tkachuk and Kruger, 1974) compared to  $\alpha$ -amylase -1 group.

The very high pI group, recently confirmed to be controlled by a third set of genes (Baulcombe et al., 1987), essentially belongs to the grain development phase, appearing 11 days after anthesis in the pericarp tissue and disappearing more rapidly than the  $\alpha$ -amylase 1 group (Daussant and Reenard, 1987).

#### 2.4.2

#### **Mechanisms of Action on Starch Granules**

The major product of starch hydrolysis in germinating cereals is glucose which is absorbed by the scutellum and converted to sucrose (Edelman et al., 1959). Some enzyme catalysed reactions that could be involved in the degradation of starch were suggested by Duffus (1987). The products of  $\alpha$ -amylase attack on starch granules may include glucose, maltose, maltotriose,  $\alpha$ -limit dextrins and linear maltosaccharides.  $\alpha$ -limit dextrins are further converted to linear maltosaccharides and then to

maltose by the action of limit dextrinase and  $\beta$ -amylase enzymes respectively. Maltose or maltotriose sugars are eventually converted to glucose by the action of  $\alpha$ -glucosidase.

Dunn (1974) established that starch granules in maize can only be attacked by  $\alpha$ -amylase and  $\beta$ -amylase has no effect on them. Later Maeda *et al* (1978) reported similar results for barley when they investigated the effects of purified  $\alpha$ - and  $\beta$ - amylase on barley starch granules. However, differences in the ability of purified isoenzymes of  $\alpha$ -amylase themselves to degrade granular starch were shown for wheat grains by Sargeant's group (Sargeant and Walker, 1978; Sargeant, 1979). Sargeant (1979) noted that whereas enzyme adsorption onto starch granules *in vitro* appeared to be a pre-requisite for starch degradation, the  $\alpha$ -amylase 1 group (low pI) remained virtually unadsorbed in contrast to a total adsorption of  $\alpha$ -amylase 2 group (high pI) onto undamaged mature starch granules. Interestingly, however, both groups adsorbed onto immature starch granules extracted from developing grains although the affinity of adsorption decreased for the  $\alpha$ -amylase 1 group with increasing starch maturity. Kruger and Marchylo (1985) also reported that the ability of the  $\alpha$ -amylase 2 group to break down granular starch was greater than the  $\alpha$ -amylase 1 group. However they suggested that adsorption of the enzyme may not be a pre-requisite for starch granule degradation, as the non-adsorbing  $\alpha$ -amylase 1 group also showed ability to hydrolyse granular starch. They also noted that temperature can influence the adsorption characteristics because, while no binding occurred at 37°C, more than 60% binding of the  $\alpha$ -amylase 1 group onto starch granules occurred at 2°C.

Contrary to the observations made by Sargeant's group, Wesleke and Hill (1983) reported that approximately 73% of  $\alpha$ -amylase 1 group (low pI) and 34% of  $\alpha$ -amylase 2 group (high pI) adsorbed onto starch granules when incubated separately at 4°C. Also they found that the  $\alpha$ -amylase 1 group were more efficient

in solubilizing granular starch than the  $\alpha$ -amylase 2 group at 15°C. The authors however, suggested that the method of enzyme purification may have some effect on the adsorption characteristics. In addition several other factors like source material, starch granule preparation, temperature, pH and ratio of enzyme to substrate may also explain the observed discrepancies amongst different investigations (Kruger and Marchylo, 1985).

Dronzek et al. (1972) observed that during the early stages of germination of wheat grains, most of the  $\alpha$ -amylase attack was found confined to the large 'A' type granules. However, after 8 days sprouting both the large and small granules were severely eroded in the seed. Scanning electron microscope studies show that  $\alpha$ -amylases in wheat (Evers and McDermott, 1970; Dronzek et al., 1972) and barley (Lineback and Ponpipom, 1977) initially attack the equatorial groove of the large 'A' type granules, causing surface erosion and then form discrete tunnels into the granule and digest the central portions. However the mechanism of  $\alpha$ -amylase attack on small 'B' type granules appears to be different. In wheat the enzyme enters through one or two localized sites and completely digests the interior of the granules (Dronzek et al., 1972). In contrast the 'B' type granules of barley are digested only by surface erosion (MacGregor and Ballance, 1980).

#### 2.4.3 Activity in Immature Grains

During grain development and maturation, the pericarp is shown to contain a major portion of the  $\alpha$ -amylase activity in the grain and levels fall rapidly as the grains near maturity (Kruger, 1972; Meredith and Jenkins, 1973; Dedio et al., 1975; Marchylo et al., 1976; Cornford and Black, 1985; Daussant and Renard, 1987). Since the pericarp of immature grains contains massive amounts of small starch granules, it has been suggested that this  $\alpha$ -amylase activity is associated with the starch metabolism of developing grains (Kruger, 1972; Meredith and Jenkins, 1973).

However further research is needed on the significance of pericarp starch metabolism to embryo/endosperm development. In addition to the pericarp, the seed coat was also shown to contain low levels of  $\alpha$ -amylase during early stages of grain development and maturation, whereas only trace levels of activity were found in the endosperm/aleurone and in the embryo/scutellum tissues (Marchylo et al., 1980a). Interestingly, however, both the immature embryo and aleurone tissue show ability to produce the enzyme when excised from the grain and incubated in vitro. Cornford et al. (1987b) showed that excised embryos (32 days post anthesis) readily produce  $\alpha$ -amylase when incubated without GA<sub>3</sub> and that the ability to produce the enzyme increased in the presence of GA<sub>3</sub> and decreased with the inclusion of ABA in the incubation medium. The enzyme produced was predominantly  $\alpha$ -amylase 1 group and the ability to produce the enzyme was shown to increase with the age of the embryo. Interestingly, the ability to produce enzyme continued to increase between 30 and 35 days post anthesis despite a substantial drop in the germinability of the excised embryos during this period.

Unlike the immature embryo, the immature aleurone tissue of wheat and barley responds to GA<sub>3</sub> only after a sensitizing treatment, such as drying (Evans et al., 1975; Armstrong et al., 1982; Cornford et al., 1986) or pre-incubation in buffer (Cornford et al., 1986). However, the GA responsiveness of aleurone was also shown to increase with the post anthesis age of the developing grain (Armstrong et al., 1982; Cornford et al., 1986). There was some evidence that the enzyme production is controlled at the transcriptional level in the immature tissue. Cornford et al. (1986) found no induction of  $\alpha$ -amylase mRNA in freshly harvested tissue (25 days post anthesis) incubated in GA<sub>3</sub>. However, a sensitizing treatment resulted in the accumulation of  $\alpha$ -amylase mRNA.

Earlier studies by King (1976) showed that a change in responsiveness to GA<sub>3</sub> was accompanied by a decrease in the ABA content

of the grains. This, together with the responses of excised immature embryos to ABA (Cornford *et al.*, 1987b) suggests that enzyme production in the embryo of a developing grain may be prevented by an ABA control which appears to gradually decline as the grain desiccates nearing grain maturity. The presence of high levels of ABA between 25 and 40 days after anthesis (King, 1976) makes it tempting to speculate a role for the hormone in the control of  $\alpha$ -amylase production and preventing premature starch hydrolysis during wheat grain development.

#### 2.4.4

#### Activity in Mature Quiescent Grains

Normally only traces of  $\alpha$ -amylase activity are found in quiescent grains (e.g. Kneen *et al.*, 1942; Olered and Jonsson, 1970; Sargeant, 1979). It is generally believed that the  $\alpha$ -amylases found in developing grains are metabolised as the pericarp dies nearing grain maturity. Using immunochemical detection methods, Daussant *et al.* (1979) showed that losses in enzyme activity during grain maturation were probably due to degradation of enzyme proteins rather than a temporary inhibition of activity and similarly Kneen *et al.* (1942) found only small increases in extracted  $\alpha$ -amylase activity from wheat flour as a result of increased extraction time and inclusion of papain in the extraction medium.

However, a frequently cited physiological phenomenon in wheat is the occurrence of higher than normal levels of  $\alpha$ -amylase activity during the final stages of grain maturation. This may occur in the presence or absence of preharvest sprouting (e.g. Meredith and Jenkins, 1973; Sargeant, 1979; Marchylo *et al.*, 1980a; Gale *et al.*, 1983; Cornford *et al.*, 1987a). Whereas the enzyme activity in sprout damaged grains of variety Champlain was 20 times higher than normal (Sargeant, 1979), the cultivar Fenman exhibited a range of activity from 0.5 to 9.5 units/grain in different earheads without visible sprouting at maturity (Cornford *et al.*, 1987a).

Cornford et al. (1987a) also showed that the pattern of  $\alpha$ -amylase production in the non-sprouted and sprouted type of abnormalities were different from each other. In the sprouted type the enzyme first appears adjacent to the scutellum, whereas in the non-sprouted type enzyme activity was usually associated with the aleurone tissue in the ventral portion of the grain. Sprouted wheat seeds produce both high and low pI  $\alpha$ -amylases (Marchylo et al., 1980b), whereas in the non-sprouted type of abnormality, predominantly high pI  $\alpha$ -amylases are produced (Cornford et al., 1987a). Whereas sprouting damage can be related to the natural germination process of a non-dormant grain, the premature occurrence of  $\alpha$ -amylase in non-sprouted grains may be indicative of a breakdown of control mechanism(s) which serve to suppress such enzyme production during grain development and maturation (Cornford, et al., 1987a).

#### 2.4.5

#### **Control of Activity in Germinating Grains**

Increases in  $\alpha$ -amylase activity in mature germinating wheat grains occur one to six days after imbibition depending on the temperature of incubation (Kruger, 1972; Hanson, 1973; Sargeant, 1979; Marchylo et al., 1984). For example, Hanson (1973) reported that cv. Cappelle Desprez did not show any changes in  $\alpha$ -amylase activity until 4 days germination at 10°C, although the enzyme activity increased appreciably after 2 days imbibition at 15°C. Sargeant (1979) also observed similar increases in activity of variety Champlein after 3 days germination at 18°C, with the enzyme activity increasing several hundred times over the base levels after 7 days incubation. Immunochemical tests confirm that increase in activity is not due to release from bound forms (see Sec. 2.4.4).

Baulcombe and Buffard (1983) studied the effect of GA<sub>3</sub> on de-embryonated wheat grains with respect to changes in  $\alpha$ -amylase activity, total poly (A)<sup>+</sup> RNA levels and the relative changes in specific mRNA species. In vitro translation of poly

(A)<sup>+</sup> RNA indicated that  $\alpha$ -amylase mRNA and other unidentified mRNA species increased in the GA<sub>3</sub> treated tissue. Earlier studies by Higgins et al. (1976) had provided similar evidence for barley grains.

In wheat, extracts of starchy endosperm have been shown to induce the release of  $\alpha$ -amylase from aleurone tissue isolated from quiescent seeds (Tavener and Laidman, 1968). Eastwood et al. (1969) investigated this further using aleurone segments separated from wheat grains within 4 h of imbibition in order to separate the effects of starchy endosperm. Their studies revealed that addition of either kinetin or gibberellic acid alone to the incubation medium does not influence the secretion of  $\alpha$ -amylase by the aleurone tissue. However, either pre-incubation of aleurone with kinetin or soaking de-embryonated grains in water for 48 h followed by incubation in GA<sub>3</sub> causes an increase in the secretion of  $\alpha$ -amylase. This demonstrated that cytokinins are essential for  $\alpha$ -amylase secretion by the aleurone and are possibly produced by the starchy endosperm in the first 2 days of imbibition.

In barley, however, calcium ions have been shown to play a very important role in both synthesis and secretion of  $\alpha$ -amylase by the aleurone tissue in vitro (Chrispeels and Varner, 1967). Moll and Jones (1982) showed that withdrawal of calcium from the incubation medium resulted in a drastic decrease in the rate of secretion of  $\alpha$ -amylase into the medium. Calcium does not seem to influence accumulation of  $\alpha$ -amylase mRNA in the barley aleurone tissue, yet the synthesis of a high pI group of  $\alpha$ -amylase appears to be controlled by calcium at a later stage in the biosynthetic pathway (Deikman and Jones, 1986) (see Sec. 2.4.6 for effects of calcium on wheat  $\alpha$ -amylases).

Recently there has been some argument about the relative importance of scutellum and aleurone tissues in  $\alpha$ -amylase production during germination. Some studies presented in the

following sections suggest that the relative participation of the two tissues may vary among different cereal grains.

#### 2.4.5.1 *Scutellum*

Smart and O'Brien (1979) reported that the scutella of wheat, maize, barley and rice have several features in common; for example, an epithelium on the surface, facing the endosperm, whose cells elongate to varying degrees during germination. In sorghum, the scutellum appears to be the sole site of  $\alpha$ -amylase production because no other part of the grain shows a response to gibberellin application in vitro (Aisen and Palmer, 1983). Histochemical localization studies in rice have shown that  $\alpha$ -amylase appears 2 days earlier in the scutellum than in aleurone tissue (Okamoto and Akazawa, 1979). Similar studies and scanning electron micrographs showing the initiation of starch degradation adjacent to scutellum during germination (see Sec. 2.2.1) indicate that the scutellum of wheat seeds may also participate in  $\alpha$ -amylase production (Marchylo et al., 1980b; MacGregor and Matsuo, 1982). Using the starch film method, Okamoto et al. (1980) provided evidence that during early stages of wheat seed germination  $\alpha$ -amylase activity was predominantly localized in the region of scutellar epithelial cells and the enzyme activity appeared later in the aleurone region. They also showed that as germination progressed, the front of enzyme activity moved parallel to the scutellar surface in wheat seeds whereas it appeared to concentrate more along aleurone region in the case of barley seeds.

#### 2.4.5.2 *Aleurone*

The aleurone layer is one cell thick in wheat grains whereas it can be 3 to 6 cells thick in barley (Duffus, 1987). Most of the earlier work on cereal grain  $\alpha$ -amylases concentrated on the role of barley and wheat aleurone in endosperm mobilization. Isolated aleurone layers of wheat grains produce  $\alpha$ -amylase when incubated in GA<sub>3</sub> media (Rowell and Goad, 1964; Laidman

et al., 1974). The levels of  $\alpha$ -amylase produced by de-embryonated grains incubated in GA<sub>3</sub> have been shown to be lower than that in germinated whole grains (Marchylo et al., 1984). It was suggested that the shortfall in de-embryonated grains may be due to lack of enzyme production by the embryo, however in addition, optimal enzyme synthesis by the aleurone may depend on factors other than gibberellic acid supplied by the embryo (Khan et al., 1973; MacGregor, 1983). The effects of kinetin and GA<sub>3</sub> with respect to synthesis and secretion of  $\alpha$ -amylase by wheat aleurone have already been presented (Sec. 2.4.5).

#### 2.4.6

#### **Inhibitors of $\alpha$ -amylase Activity**

Cereal enzyme inhibitors can be functionally classified into two groups (Fincher, 1989):

- (i) Those that inhibit endogenous cereal hydrolases and hence participate in the regulation of grain development or germination, and
- (ii) those that act as inhibitors of exogenous enzymes and thereby provide a degree of protection against invading organisms.

In this section we are mainly interested in the inhibitors of endogenous  $\alpha$ -amylase in wheat grains. Petrucci et al. (1974) classified  $\alpha$ -amylase inhibitors in wheat albumin fraction into three main families depending on their molecular weight and physico-chemical properties. Silano et al. (1975) showed that most of these are inhibitors of exogenous  $\alpha$ -amylase. Although the occurrence of  $\alpha$ -amylase inhibitors in wheat grains has been known since 1943 (Kneen and Sadstedt, 1943), it was only recently that Warchalewski (1976, 1977) provided the first evidence for the occurrence of proteinaceous inhibitors of endogenous  $\alpha$ -amylases in wheat grains. Mundy et al. (1984) recently purified a protein inhibitor called 'wheat  $\alpha$ -amylase/subtilisin

inhibitor' (WASI) from wheat grains, that is active on endogenous  $\alpha$ -amylase. However, inhibition of  $\alpha$ -amylase activity in vitro by WASI was shown to be weaker by comparison to the 'Barley  $\alpha$ -amylase/subtilisin inhibitor' (BASI) from barley grains. Weslake et al. (1985) reported the presence of endogenous  $\alpha$ -amylase inhibitors in various cereals including wheat, but the inhibitor level in a cultivar resistant to sprouting (Columbus) was not higher than in other cultivars.

Some compounds like  $\beta$ -purothionins may cause inactivation of  $\alpha$ -amylase activity in vitro by controlling the availability of calcium to serve as a co-factor (Jones and Meredith, 1982).  $\beta$ -purothionin purified from wheat flour was shown to inhibit wheat  $\alpha$ -amylase in enzyme assays, but when calcium chloride was included in the enzyme-inhibitor mixture,  $\alpha$ -amylase activity was not inhibited. Similarly Abdul Hussain (1987) reported that proteins from sprouting resistant genotypes inhibited  $\alpha$ -amylase in standard assays, but adding EDTA to chelate calcium induced inhibitory activity in extracts of all genotypes. He concluded that proteinaceous  $\alpha$ -amylase inhibitors interact with calcium ions, but do not play a primary role in the control of sprouting, although they may have secondary effects on the process. In barley however, it has been shown that the  $\alpha$ -amylase inhibitor was an ABA induced protein and that it functions as an active mediator of  $\alpha$ -amylase activity during seed development and germination (Mundy, 1984). In wheat, King (1976) has provided correlative evidence for a possible control of  $\alpha$ -amylase production by ABA during grain development (Sec. 2.4.3).

## 2.5 PROTEOLYTIC ENZYMES IN WHEAT GRAINS

### 2.5.1 Storage Proteins of Wheat Grains

Knowledge of the types, characteristics and localization of storage proteins is essential for a better understanding of the

mechanisms of their mobilization during germination. The storage proteins of wheat, barley and rye have similar localization and structural characteristics (Pernollet and Mosse, 1983). In these species, the protein bodies disappear near grain maturity in the starchy endosperm and clusters of protein not surrounded by a membrane are found deposited between starch granules. The two major groups of storage proteins, the prolamins and glutelins, generally comprise 40 to 50% and 35 to 40% of the total protein content respectively. The prolamins of wheat are also known as gliadins and they comprise of two different sets of polypeptides, one about 30,000 and the other 60,000 daltons in molecular weight (Pernollet and Mosse, 1983).

However, with respect to glutelins of wheat, Wall (1979) reported that the average molecular weight determined by ultra-centrifuge analysis varied with pH and solvent used. Payne and Corfield (1979) showed that unreduced glutelins of wheat have a wide molecular weight distribution ranging from 200,000 to several million. There is evidence for intramolecular and intermolecular disulphide bonds in glutelins which result in an extended molecule (Ewart, 1972). With the development of SDS-PAGE techniques the understanding of the nature of glutelin polypeptides became much clearer. Bietz and Wall (1972) used 5 or 7.5% gels to obtain optimum separation of glutelin subunits including the high molecular weight compounds. Reduced glutelins were shown to consist of 15 different polypeptides ranging from 11,000 to 133,000 daltons of which high molecular weight (HMW) subunits ranged from 68,000 to 133,000 daltons. Considerable variation among wheat varieties in the pattern of glutelin subunits separated by SDS-PAGE has been reported (Payne *et al.*, 1980, 1981). In order to have some uniformity in reports Payne and Lawrence (1983) proposed a numerical system of nomenclature of 1 to 22 for the different HMW subunits identified in wheat cultivars. The subunit with lowest mobility in SDS-PAGE was designated as 'subunit 1' and further identification of others were based on their relative mobilities. Subunits of 2, 7, 8 and 12 from cv.

Chinese Spring were used as standard references in order to compare the relative mobilities of other subunits from different cultivars in a 10% gel.

### 2.5.2 The Enzymes and their General Mode of Action

In general, proteolytic activity refers to enzyme mediated hydrolysis of the peptide linkage,  $-N(H)-C(=O)-$ , resulting in the release of smaller protein/peptide fragments or free amino acids into the medium. In common usage 'protease' refers to endoproteases that cleave internal peptide linkages in a polypeptide chain, while 'peptidase' refers to exoenzymes that can hydrolyse peptide chains from one or other end. Thus the products of protease activity could be smaller peptides or amino acids whereas that of peptidase action are always amino acids.

During seed germination proteolytic enzymes play a vital role in the mobilization of stored proteins. In 1967, Jacobsen and Varner using density labelling techniques, demonstrated in vitro, de novo synthesis and secretion of protease enzymes by barley aleurone layers in response to gibberellic acid. In cereal grains a systematic sequence of proteolytic activity seems to occur in different seed tissues like embryo, aleurone, and endosperm at different stages of germination. In germinating barley for example, the degradation of reserve proteins seems to operate in 3 sequential stages (Mikola, 1983).

- (i) First some special storage proteins, mainly globulins are hydrolysed in the embryo and the aleurone layer. This primary hydrolysis provides amino acids for the synthesis of new hydrolytic enzymes in the scutellum and aleurone layer for subsequent secretion into starchy endosperm.
- (ii) The prolamins and glutelins of the starchy endosperm are rapidly hydrolysed to a mixture of amino acids and smaller peptides at an optimum internal pH of 5 (Mikola

and Mikola, 1980). Both the amino acids and the peptides are taken up by the scutellum (Sopanen, 1979; Sopanen et al., 1980).

- (iii) In the final stage, the peptides are hydrolysed further in the scutellum to amino acids before they are transported to the growing axis.

It was suggested that the general course of mobilization of grain protein reserves in other cereals may resemble that of barley. The exact number and types of different proteases and peptidases present in resting or germinating cereal grains or their specific roles are not clearly known. Apart from the difficulties in assaying specific enzymes in crude extracts (Fincher, 1989), it appears that little attention has been paid to this group of enzymes compared to amylases. However, recently Hammerton and Ho (1986) using [<sup>35</sup>S] labelled methionine confirmed gibberellic acid initiated synthesis and secretion of a protease by the isolated barley aleurone layers. The synthesis of carboxypeptidases (initially present at high levels in the tissue) was not controlled by the growth substance although secretion was shown to be clearly a GA dependent phenomenon. Further, ABA was shown to inhibit the GA induced synthesis and secretion of protease as well as the GA dependent secretion of the carboxypeptidase enzymes.

A similar kind of proteolytic activity to that of barley (Hammerton and Ho, 1986) seems to occur in the isolated aleurone tissue of wheat grains incubated in GA<sub>3</sub>. Chittenden et al. (1978) demonstrated that proteolytic activity in the aleurone tissue of wheat grains during the first two days of germination is independent of the presence of the embryo, although further increases in activity clearly depends on either the presence of embryo or GA<sub>3</sub> in the incubation medium. Thus it appears that as for barley grains some proteolytic activity is initially present in the aleurone tissue and additional activity is influenced by the

growth substance. Furthermore, the composition and levels of amino acids within the aleurone and in the incubation medium were suggestive of two types of proteolytic activity, of which only the latter was influenced by the growth substance.

### 2.5.3 Proteolytic Enzymes and their Inhibitors

#### 2.5.3.1 *Proteases of quiescent and germinating grains*

Although protease enzymes from both embryo and endosperm of quiescent wheat grains hydrolysed insulin B-chain optimally at pH 4, only the enzyme activity from the embryo was inhibited by pepstatin (Morris *et al.*, 1985). This suggested the presence of two different acid proteases in the two tissues. An earlier study by Kawamura and Yonezawa (1982) showed that wheat grains contain pepstatin and DFP (Diisopropylfluorophosphate) sensitive proteases which are mainly responsible for hydrolysis of gluten proteins *in vitro*.

In germinating wheat grains increases in protease activity were shown to coincide with the disappearance of storage proteins (Preston *et al.*, 1978). Some evidence from rice suggested that the germinative enzymes may be different from those in quiescent grains (Doi *et al.*, 1980a). The germinative groups in cereals are generally -SH proteases which show ability to hydrolyse prolamins and glutelins optimally at pH 4 to 5 and are generally inhibited by leupeptin (Mikola, 1987). Shutov *et al.* (1985) purified an -SH protease from germinating wheat grains which had a molecular weight of about 22,000. The purified enzyme preparation was shown to hydrolyse gluten proteins to a mixture of di-, tri- and tetrapeptides.

#### 2.5.3.2 *Peptidases of quiescent and germinating grains*

Recently Mikola (1986) studied carboxypeptidase activity of wheat grains in some detail and identified 5 different groups

of activity (Carboxypeptidases I to V) similar to those found in barley grains (Mikola, 1983). The quiescent grains contained high levels of carboxypeptidase II activity along with two minor groups (IV and V). The localization of this enzyme in the starchy endosperm and its wide substrate specificity (Mikola, 1986; Umetsu *et al.*, 1981) tempt speculation that this enzyme may have a key role in protein hydrolysis during early stages of germination (Mikola, 1986). Quiescent rice grains show two types of carboxypeptidase activity and one group resembling carboxypeptidase II of wheat was also found to be localized in the starchy endosperm (Doi *et al.*, 1980b).

The activity of carboxypeptidase II was found to decrease slowly during germination of wheat grains (Mikola, 1986). This was accompanied by the appearance of carboxypeptidases I and III which showed higher activity compared to groups, IV and V, which were originally present at low levels and increased slowly during germination.

Preston and Kruger (1976) have found evidence for the presence of endogenous proteolytic inhibitors in wheat grains nearing maturity and suggested that they might have a possible role in the control of *in vivo* enzyme activity in quiescent grains. In barley it has been shown that the general levels of proteolytic inhibitors decrease during seed germination with a concomitant increase in proteolytic activity (Kirsi and Mikola, 1971).

## 2.6

### PHYSIOLOGICAL AND BIOCHEMICAL CHANGES ASSOCIATED WITH SEED AGEING

A matrix of cellular events occurs during seed ageing and from the accumulated research it is now well understood that the complexity of these changes has posed several problems in studying the mechanisms of seed ageing. Firstly, the types of damage that are observed in one crop species may not be found in others

and added to this, the ageing conditions themselves appear to influence the nature of damage within each species. In this regard there is already a considerable argument about the use of high moisture artificial ageing conditions for studying the mechanisms of seed deterioration (see Sec. 2.7.1).

A second key problem lies in identifying the primary causes of ageing from secondary effects which may occur very rapidly after initial damage and are possibly interrelated events.

Ageing related damage in seeds can be broadly categorized as follows (Priestley, 1986):

1. Damage to membranes
2. Changes in enzyme activity
3. Genetic damage
4. Changes in respiration
5. Damage to protein, and RNA synthesis
6. Changes in hormones and
7. Accumulation of toxic metabolites.

A brief review of these aspects from the literature will be presented in the following sections.

#### 2.6.1 **Damage to Membranes**

Cell membranes are made up of a lipid bilayer containing both intrinsic and extrinsic proteins (Bewley, 1986). The major constituents of the lipid bilayer are polar lipids, the most abundant being phospholipids. In addition to compartmentation of different functional units within the cells, the membrane acts as a selectively permeable barrier controlling the general diffusion of materials into and out of cells. As such damage to membranes might produce a variety of secondary effects, like for example, damage to functioning of the organelles due to changes in their ultrastructure, or loss of control over compartmentalized hydrolytic activity.

#### 2.6.1.1 *Ultrastructural changes*

Despite the problems in observing the structure of cells and cellular organelles in their unimbibed state, many ultrastructural studies have proved useful to some extent in understanding the sites of lesion in aged seeds. Such studies of many species including maize (Berjak and Villiers, 1972) rice (Vishnyakova *et al.*, 1976) and rye (Hallam, 1973) have indicated damage to membranes in dry non-viable embryos. This damage increased during imbibition. The embryos of rapidly aged maize seeds were found to have cells with lobed nuclei and with distorted profiles of mitochondria, plastids and dictyosomes. The endoplasmic reticulum appeared to be damaged and polysomes were slower to form in partially deteriorated seeds. However, there was some evidence that many of these deficiencies were remedied within 48 hours of imbibition (Berjak and Villiers, 1972).

#### 2.6.1.2 *Changes in the leakage of electrolytes from seeds*

The simplest way to test damage to cell membranes is by studying changes in the leakage of electrolytes from seeds. In the dry state phospholipid membranes do not exist as an integrated layer. The recent evidence from the studies on pollen grains suggests that the dehydrated phospholipid bilayer passes through a gel to a liquid-crystalline phase upon hydration and during the transition phase it increases in permeability leading to leakage of cellular contents (Crowe *et al.*, 1989). Such leakage can occur over extended periods of time in imbibed seeds if membranes are damaged and before complete integrity is attained through the operation of repair processes.

Powell and Matthews (1977) reported that pea seeds exposed to humid or extremely dry storage conditions showed increased leakage of solutes before any significant loss in seed viability, as indicated by tetrazolium (2, 3, 5-triphenyltetrazolium chloride) staining studies. Similarly Gorecki and Harman (1987) reported

an increase in the conductivity of seed leachate as a result of ageing pea seeds at 30°C and 90% RH for up to 12 weeks. Ching and Schoolcraft (1968) observed considerable increase in the conductance of seed leachate from naturally aged crimson clover seeds, but changes were apparently small with similarly aged rye grass seeds. In cereal or grass seeds where live cells form a tiny part of the whole grain, the validity of leakage measurement for assessment of membrane damage may be questionable. It is possible that small changes in the leakage of the embryo may be masked by enormous and uncontrolled leakage by the endosperm.

In wheat, Rudrapal and Basu (1982) showed a high negative correlation between germinability and electrical conductivity of seed leachate under both natural and accelerated ageing conditions. However, their results did not show any indication of increases in conductivity before losses in seed germination and were apparently suggestive that increase in conductivity may be due to uncontrolled leakage resulting from an increase in the number of dead seeds in aged seed lots. Similar evidence comes from the recent studies of Ram and Wiesner (1988) in wheat seeds. In this study a 12 h ageing at 50°C and 100% RH did not cause a significant decrease in germination or an increase in electrical conductivity of seed leachate, and particularly in one cultivar 'Neewana' the electrical conductivity did not change despite a 44% reduction ( $p \leq 0.05$ ) in germination rate. These results provide some indication that electrical conductivity changes may not be a reliable test of vigour in wheat seeds.

### 2.6.1.3 *Changes in phospholipids*

Polyunsaturated fatty acid components of the phospholipid membrane are susceptible to oxidative damage. Thus changes either in phospholipid levels or in lipid peroxidation levels in seeds have been studied as an indication of damage to membranes. Harman and Mattick (1976) reported a decline in 18:2 and 18:3

fatty acids in both intact seed and embryonic axes of peas. This was related to loss of viability during accelerated ageing.

Although small decreases in the 18:3 fatty acid component of soybean seeds were observed during natural storage (Priestley and Leopold, 1983) such changes were not evident under accelerated ageing conditions (Priestley and Leopold, 1979). Contrary to this, substantial losses of linoleic and linolenic acids were observed in soybean axes exposed to high humidity ageing conditions (Stewart and Bewley, 1980). However, as the major losses during ageing were observed after complete loss in seed viability, the authors were doubtful if the changes were a primary cause of seed deterioration. Also in aged wheat seeds, significant increases in lipid peroxidation were found only when there was an actual fall in germination percentage (Rudrapal and Basu, 1982). In order to draw firm conclusions, further studies of this type should, perhaps, be confined to key tissues like embryo or embryonic axes while the analysis should be specific to membrane lipids.

In a well planned study, however, McDonnell *et al.* (1982), confirmed that phospholipid levels in embryos of low and high vigour wheat seeds having similar germination capacity were not significantly different although there were differences in phospholipid accumulation during germination. The results suggest that, under these circumstances at least, primary damage to membrane lipids may not occur in low vigour wheat seeds though it might later appear as a post-mortem event as suggested by Rudrapal and Basu (1982).

### 2.6.2 Changes in Enzyme Activity

As several enzymes are stored in quiescent seeds as well as being produced *de novo* during germination, changes in their activity as part of the ageing process have also been investigated. Some studies in quiescent seeds have given an indication that

changes in some enzyme activities in different crop seeds during ageing may not be similar. However, with respect to glutamic acid decarboxylase activity (GADA) there appears to be some uniformity. Delouche and Baskin (1973) reported that GADA activity was the most sensitive indication of deterioration in rice seeds. In wheat seeds enzyme activity was shown to decrease during accelerated ageing (Ram and Wiesner, 1988) and similar changes were observed in deteriorated seeds of sorghum (Perl *et al.*, 1978) and corn (Bautisa and Linko, 1962).

Systematic studies relating to changes in base levels of  $\alpha$ -amylase activity during ageing and its significance to deterioration have not been carried out for wheat seeds. This aspect of ageing may be important considering the problem of abnormal levels of  $\alpha$ -amylase activity often encountered in mature wheat seeds (Sec. 2.4.4). Warchalewski *et al.* (1985) observed a 68% increase in the base levels of  $\alpha$ -amylase activity (trace levels) after storing wheat grains for a period of 4 years at 20 °C and 74% relative humidity and this was accompanied by a 13% drop in total protein content. These results suggest that small quantities of  $\alpha$ -amylase activity held in bound form may be released as a result of possible hydrolytic breakdown of proteinaceous inhibitors during seed ageing. However in contrast Perl *et al.* (1978) reported that following an initial increase, levels of this enzyme dropped rapidly during rapid ageing of sorghum seeds. Studies in germinated wheat indicated that aged but viable seeds produced significantly lower enzyme activity compared to unaged seeds (Artsruni and Panosyan, 1984; Agrawal and Kharlukhi, 1987).

As proteolytic enzymes are present in quiescent seeds as well as being produced during seed germination the related ageing effects can be studied in 3 ways:

- (i) Changes in the base levels of enzyme activity during storage.

- (ii) Possible effects of proteolytic enzymes on storage/structural/enzyme proteins during storage.
- (iii) Changes in the ability of aged seeds to produce proteolytic enzymes during germination.

With respect to base levels, Skupin and Warchalewski (1971) showed that proteolytic activity (measured against bactohaemoglobin as a substrate) decreased in wheat seeds stored under ambient conditions. In contrast, the activity (against bovine serum albumin) was shown to increase during rapid ageing of sorghum seeds (Perl et al., 1978). Proteolytic damage in storage is considered to be one of the possible mechanisms of seed deterioration. Increased levels of amino acids in aged, but viable rye grass seeds and in sorghum have been reported (Ching and Schoolcraft, 1968; Perl et al., 1978, respectively). At the same time, decrease in activity of several enzymes was suggested to be an effect of increased proteolytic activity.

Enzyme mediated damage and alterations to membranes may be closely related events, because damage to membranes may contribute to loss of control over enzyme activity that is either membrane bound or compartmentalised in membrane bound bodies. On the other hand one of the possible mechanisms of membrane damage is that mediated by enzyme activity (e.g. by lipoxygenase and proteases).

### 2.6.3 Genetic Damage During Ageing

Damage to genetic material during ageing has been investigated in considerable detail by studying levels of chromosomal aberrations during mitotic cell division and damage to DNA.

Roberts et al. (1967) suggested that an increase in the number of aberrant cells over a critical level might result in the loss of seed viability. On the other hand, if the seeds survive with

a few aberrant cells that continue to divide, then it is likely that irregularities will appear in the developing seedlings. In some cases these may be heritable (Dourado and Roberts, 1984). However, observations by Murata, et al. (1984) in barley suggest that the incidence of irregularity greatly decreases as the plant develops, presumably because the aberrant cells fail to compete with the normal ones. Chromosomal abnormalities in aged seeds may arise due to either chromosomal or individual chromatid breaks. In durum wheat (Innocenti and Avanzi, 1971; Floris and Anguillesi, 1974), barley and peas (Dourado and Roberts, 1984), chromatid breaks have been suggested to be more characteristic of deteriorated seeds.

Roberts and Osborne (1973) found that viability loss in rye embryos was not associated with losses in total DNA content per nucleus, but the amount of spoolable, high molecular weight DNA was less in non-germinating embryos as compared with those having 95% germination. Further studies by Chea and Osborne (1978) involving separation of total nuclear DNA from rye embryos on a sucrose density gradient or polyacrylamide gel revealed that randomly sized fragments of low molecular weight accumulate in aged seeds. In an attempt to determine the effects of ageing on wheat seeds Dell'Aquila and Margiotta (1986) studied changes in DNA synthesis in embryos during germination. Ageing wheat seeds in a range of conditions resulted in decline of DNA synthetic ability in isolated embryos germinated at 20°C and decreases were shown to parallel decreases in seed germination rates associated with seed ageing. In an earlier study Dell'Aquila et al. (1980) showed that DNA polymerase activity increased more slowly during the germination of low viability wheat embryos than in those with higher viability. Apparently, however, the authors seem to have ignored the differences in the percentage of dead embryos in the two batches of seeds compared. It appears that damage to the genome in the key tissues of low vigour or abnormal seeds needs careful investigation to understand its role in the seed deterioration process.

#### 2.6.4 Changes in Respiration

It has been suggested that symptoms of damage to mitochondria seem to be one of the early events in seed ageing (Berjak and Villiers, 1972). There are a number of reports which show that a decline in oxygen uptake or an increase in respiratory quotient correlates well with seed deterioration (e.g. Woodstock and Grabe, 1967; Anderson, 1970; Anderson and Abdul-Baki, 1971). However, it must be noted that correlative evidence does not necessarily imply a cause and effect relationship.

Abu-shakra and Ching (1967) noted that oxygen uptake by aged seeds of many crop species did not decrease very much as compared to unaged material, although ATP production was greatly reduced. Such effects may be due to mitochondrial damage resulting in partial uncoupling of oxidative phosphorylation. In clover ATP content was found to be lower in aged seeds (Ching, 1973). Woodstock *et al.* (1984) working with soybean seeds also suggested that imbalances in the components of respiratory metabolism such as uncoupling for example, may be early events in the ageing process. Excess glycolysis may result in anaerobic respiration and the production of ethanol and aldehydes, both of which are toxic to seeds. The work of Woodstock and Taylorson (1981) showed that slower imbibition of aged seeds may provide some opportunity for repair of their respiratory metabolism.

One of the limitations with ATP measurements, however, is that this molecule is subject to rapid turnover in germinating seeds and there is no reason to assume that steady state levels are maintained at any given time or that the levels necessarily reflect rates of synthesis or utilization (Mazor *et al.*, 1984).

#### 2.6.5 Protein and RNA Synthesis

Protein synthesis appears to be one of the important early events during germination that may be essential for repair processes

and for the production of several enzymes which control various metabolic activities. Ageing-induced damage to protein synthesis during germination has been observed in several crop species (for e.g. Hallam *et al.*, 1973; Roberts and Osborne, 1973; Abdul-Baki and Chandra, 1977; Bray and Dasgupta, 1976).

There are some indications that long-lived mRNA is lost to some extent during extended storage. Osborne's group (Osborne *et al.*, 1977; Osborne, 1983) have shown that non-viable rye embryos yielded about half as much polyadenylated RNA (potential mRNA species) compared to those having 90% viability. A similar observation was made in 5 year-old wheat seeds, although interestingly viability was not impaired in the aged material (see Priestley, 1986). As previously mentioned (Sec. 2.3), much of the stored mRNA may not be essential for successful germination and may simply be a residual message from seed development. Messenger RNA is normally subject to extensive degradation during the first two hours of imbibition (Smith and Bray, 1982). Significantly, it also appears that degradation of polyadenylated RNA during imbibition progresses more slowly in partially deteriorated embryos than in vigorous ones (Smith and Bray, 1984).

Newly synthesised mRNA and its translation may be important for early protein synthesis during germination. In rye embryos loss of viability was associated with a decline in incorporation of radioactive precursors into all major classes of RNA (Ching, 1972; Sen and Osborne, 1977). Age induced damage to ribosomes, the sites of protein synthesis, or to the endoplasmic reticulum can also impair protein synthesis during germination (Sec. 2.6.1.1). It appears that damage to protein and RNA synthesis may be only a secondary effect of other age induced alterations to membranes, DNA or respiratory metabolism.

There are also some suggestions that DNA transcription in deteriorated seeds may be affected by alterations in the histone frac-

tion, proteins that are assumed to play a role in regulating gene expression (Priestley, 1986). Deficiencies in endogenous plant growth regulator (PGR) control in aged seeds may also affect synthesis of enzyme proteins as some of the germinative hydrolases have been shown to be under hormonal regulation (Sec. 2.4.5 and 2.5.2).

#### 2.6.6

#### Changes in Plant Growth Regulator Levels

PGR's are known to play a key role during seed development, maturity, dormancy and germination. In wheat seeds, changes in abscisic acid levels during development (see Sec. 2.4.3) and the role of kinetins and gibberellins in the production of  $\alpha$ -amylase by the aleurone (see Sec. 2.4.5) have been well documented.

However, well organised experiments to establish the role of PGR's in seed ageing are rarely found in the literature. Mierzwinska (1977) reported an improvement in the germination rates of aged wheat seeds by exogenous application of gibberellic acid. However in contrast, unaged barley seeds responded better to GA<sub>3</sub> than aged seeds (Harrison, 1977). Exogenous application of GA to aged wheat seeds was shown to have no effect on the production of  $\alpha$ -amylase by aleurone tissue (Aspinall and Paleg, 1971). The authors suggested that the aged tissue may be less efficient in responding to the hormonal stimulus as compared to the unaged tissue.

Interestingly, it was also reported that ageing effects can be slowed down by pre-application of gibberellins to durum wheat seeds before storage (Petruzzelli and Taranto, 1985), although further confirmation of these effects is lacking. Earlier studies by Khan *et al.* (1976) had also suggested that application of kinetin in acetone provided some protection against ageing by slowing the rate of deterioration in lettuce seeds, however, the effects were marginal. As the use of organic solvents for hormone

treatments is becoming increasingly popular, there is a need to evaluate the effectiveness of different solvents as carriers for growth substances into seeds and, importantly, the effects of the solvents per se.

#### 2.6.7 Accumulation of Toxic Metabolites

Many metabolites may accumulate during seed development or later in storage, but it has been difficult to establish specifically if any of these are potentially toxic to germination or cause damage during ageing. In rice seeds, however, loss of viability in storage was associated with supra-optimal concentrations of indole acetic acid (Dey and Sircar, 1968). Similarly in peanuts, loss of viability was partly found to be associated with an increase in abscisic acid like substances (Narasimhareddy and Swamy, 1977). Recently Sreeramula (1983) suggested that increase in phenolics such as vanillic, ferulic and hydroxybenzoic acids could be the cause of loss of seed viability in bambaranut seeds. Some researchers consider polyamines to be important regulators of plant growth, Mukhopadyay et al. (1983) noting that loss of viability in rice was associated with a great increase in the polyamine content of the imbibed embryo.

As toxic metabolites appear to be the by-products of various biochemical processes, their role in seed deterioration necessarily needs evaluation in relation to the primary causes of damage that might produce them. There appears to be no information on effects of this type for wheat grains.

#### 2.7 ARTIFICIAL AGEING OF SEEDS

Artificial ageing involving rapid deterioration of seeds, by exposing them to harsh relative humidity and temperature conditions, was initially developed to distinguish between seed lots with respect to their storability under ambient conditions (Delouche

and Baskin, 1973). Subsequently these techniques were adopted by researchers as a means to hasten deterioration process in seed ageing studies, which otherwise can extend over several years. In doing so, it was believed that the changes occurring during artificial ageing are similar to those occurring in natural storage except that the rates of deterioration are more rapid in the former as compared to latter.

The different types of artificial ageing techniques have been described in detail by Perry (1981) and those methods recorded for wheat are summarised in tables 2.2 and 2.3. The accelerated ageing technique (AA) involves holding seeds in a high humidity atmosphere allowing seeds to take up moisture naturally, while being held at higher temperatures for different lengths of time (Delouche and Baskin, 1973). In this method the seed moisture uptake is not precisely controlled and hence the time when seeds attain equilibrium moisture level is not known. Added to this variables like seed lot, amount and position of seeds in the humidity maintenance chamber may all contribute to reproducibility problems. On the other hand, 'controlled deterioration' (Matthews, 1980; Powell and Matthews, 1981) is basically similar to AA, but the technique has been improved to adjust the moisture content to desired levels before exposure to harsh temperature conditions (details of methods in Sec. 3.2).

Harrington (1972) suggested two rules of thumb which roughly explain the influence of the two most important environmental factors, namely seed moisture and temperature, on seed ageing. The first rule suggests that each 1% increase in seed moisture, between 5 and 14%, causes a 50% decline in storage life of seeds, and the second rule suggests similar and independent effects with every 5°C increase in temperature between 0 and 50°C. The increase in moisture content attained by accelerated ageing at 100% RH could be 15 to 20% above levels at ambient which explains the severity of expected deterioration rates.

Table 2.2 Examples of accelerated ageing conditions used for wheat seeds, their effects on germination performance and the observed physiological/biochemical changes.

Reference	Ageing Conditions	Maximum duration of ageing (d)	Initial germination (%)	Final germination after maximum ageing (%)	Other observed ageing effects	Comments
<b>Accelerated ageing of wheat seeds</b>						
Delouche and Baskin (1973)	100% RH, 40°C	6	-	-	Losses in germination	Data not shown. Positive correlation with storage under ambient conditions.
Basu (1976)	100% RH, 45°C	8	95	67	-	No replication/statistics
Rudrapal and Basu (1982)	100% RH, 42°C	7	93	42	65% increase in EC 150% increase in leakage of sugars 54% increase in lipid peroxidation	No replication/statistics
Ram and Wiesner (1988)	100% RH, 50°C	1.5	96-98	62-90	66-84% decrease in speed of germination index 10-43% increase in EC 12-29% decrease in GADA activity	-

Abbreviations: d = days; RH = relative humidity; EC = electrical conductivity; GADA = glutamic acid decarboxylase activity

Table 2.3 Examples of controlled deterioration conditions used for wheat seeds and their effects on germination performance.

Reference	Ageing Conditions	Maximum duration of ageing (d)	Initial germination (%)	Final germination after maximum ageing (%)	Other observed ageing effects	Comments
<b>Controlled deterioration of wheat seeds</b>						
Lush <u>et al.</u> (1981)	Pre-imbibed for 5 h, 50°C	0.17	89	62	-	The seed moisture before or after ageing not known
Petruzzelli and Carella (1983)	12.5-14.5% SMC, 30°C	35-42	80	50-60	-	Number of seeds per replicate was very small
Dell'Aquila <u>et al.</u> (1984)	13.5-14.0% SMC, 30°C	42	92	62	Decrease in germination rates at 20°C	-

Table 2.3 cont...

Reference	Ageing Conditions	Maximum duration of ageing (d)	Initial germination (%)	Final germination after maximum ageing (%)	Other observed ageing effects	Comments
Dell'Aquila and Margiotta (1986)	(a) 12.0-18.0% SMC, 35°C	42	92	0-78	Up to 100% increase in mean germination time	No replication/statistics
	(b) 18.0% SMC, 25 - 35°C	42	92	0-79	Up to 100% increase in mean germination time	No replication/statistics
Dell'Aquila (1987)	12.0-18.0% SMC, 25-60°C	0.08-20	89	86-90	2-52% increase in mean germination time	-
Goldsworthy <u>et al.</u> (1982)	15.0% SMC, 50°C	3.8	98	0	Decreases in coleoptile length after shorter ageing times	Replication details and statistics lacking

Abbreviations: d = days; h = hours; SMC = Seed moisture content

Roberts (1972) developed temperature and seed moisture relationships with ageing a step further and proposed that the loss of viability of orthodox seeds under a given set of storage conditions is predictable. The viability equations developed were based on observations that the frequency of individual seed deaths in a population follows a normal distribution pattern with time. Initially these equations were specific to individual seed lots, but later Ellis and Roberts (1980) using a wide variety of ageing conditions, developed improved viability equations with broader applicability at species level irrespective of seed lot or varietal considerations. In doing so it was assumed that rapid ageing under artificial storage conditions will predict seed behaviour under much less stressful natural storage.

One of the major problems in using artificial ageing techniques is interference by microbial growth, especially when high levels of seed moisture are included in the ageing conditions. The other important doubt raised in recent years is about the validity of using these techniques for studying the physiological and biochemical changes associated with seed deterioration. This will be discussed in the following section.

### 2.7.1 **Artificial vs Natural Ageing of Seeds**

Delouche and Baskin (1970) used two ageing regimes, one was harsh ageing at 45°C and 100% RH for eight days and the other, a less severe ageing at 30°C and 75% RH for up to 24 weeks. They showed that losses in germination with the two ageing regimes were similar for several crop species. Although the end results of germination and vigour losses look alike, there is little reason to assume that the causes and mechanisms of damage that lead to the observed effects are similar in each case. Such fears are strengthening mainly because of the growing evidence suggesting that the cellular metabolism of seeds in their dry and imbibed state is different.

Priestley (1986) used three sorption zones to describe the state of water in seeds. In zone I and II, moisture is tightly held by protein, lipid and starch molecules whereas in zone III water exists in a free or mobile state influenced by capillary and osmotic forces. It was also shown that the seed moisture content in the three zones may vary for different crop species. Differences in equilibrium moisture content among species are primarily due to variations in quantity of storage lipid, a component that interacts with water poorly (Cromarty, 1984).

In the air-dry state (sorption zone I and II), seeds lack a well developed or active cellular metabolism that might allow for self repair of ageing damage as demonstrated, for example, in lettuce (Villiers, 1974) or maize seeds (Barnes and Berjak, 1978). Thus it is suggested that ageing damage may accumulate in dry stored seeds ultimately leading to losses in vigour and viability. On the other hand there is some evidence both from buried seed experiments (see Priestley, 1986) and from studies on lettuce seeds that fully imbibed seeds (in sorption zone III) may survive longer than dry stored seeds. Classical studies by Villiers and Edgcumbe (1975) showed that dormant lettuce seeds stored under fully imbibed conditions maintained full viability for 12 months as compared to dry stored seeds (13.5% SMC) which lost all viability by the end of the second month. As may be expected such protective effects were shown to be dependent on the availability of oxygen (Ibrahim *et al.*, 1983).

Thus critical questions are being raised about the use of rapid ageing techniques for studying the physiology of seed deterioration and especially the use of high moisture (sorption zone III) ageing conditions (Priestley, 1986). To support this view there is also some evidence suggesting that the mechanisms of ageing damage may be somewhat different between lower moisture natural and high moisture rapid ageing conditions. With respect to changes in the free-radical status of seeds it is suggested

that hydration leads to enhanced molecular motion and as a result the free radicals get destabilized (Randolf et al., 1968). Studies on Hibiscus cannabinus with respect to free-radical mediated damage showed that the seeds were most susceptible to radiation damage in their dry state (sorption zone I) compared to those with higher seed moisture content (Mahama and Silvy, 1982). Surprisingly, Priestley et al., (1985b) found increases in free radical levels in soybean axes only under high humidity ageing, and not under natural storage although earlier similar work (Buchvarov and Gantcheff, 1984) showed increases in levels under both types of ageing conditions. However elevated free radical levels under high humidity ageing were not associated with lipid peroxidation. Thus evidence for the interactions of free radical mediated damage with moisture content is not very clear.

Abdul-Baki and Anderson (1970) reported that accelerated ageing of barley seeds at 40°C and 100% RH did not cause an increase in leakage of sugars during the early stages of deterioration and the results were in contrast with naturally aged seeds which showed significant increases compared to unaged seeds. In cucumber seeds, Koostra and Harrington (1969) found that the phospholipid levels dropped markedly under high humidity ageing conditions in contrast to no changes under dry storage despite losses in seed germinability.

However, some studies on changes in the proportion of polyunsaturated to total fatty acids do not reveal clear differences between the two ageing conditions. For example, in peanuts the proportion of polyunsaturated to saturated fatty acids did not change either under high humidity or under lower humidity ageing conditions (Pearce and Abdul Samad, 1980). However, in soybean axes the proportion remained constant during high moisture ageing (Priestley and Leopold, 1979; Ohlrogge and Kernan, 1982; Priestley et al., 1985b), while there were small decreases in the linoleate (18:2) and linolenate (18:3) fractions in dry stored seeds.

Although further studies are needed to resolve these issues, it is clear that questions have been raised. Nevertheless, practicalities of research timetables often mean that the use of some form of accelerated ageing techniques is often inevitable. It may also be necessary to give similar attention to the effects of the elevated temperature regimes commonly used in artificial ageing techniques.

## 2.8 PRE-SOWING TREATMENTS FOR WHEAT SEEDS

In recent years there have been a number of papers (Tables 2.4 and 2.5) published on the effects of hydration treatments either for invigoration of wheat seeds or for improving their storability. However, little coherent information has emerged, due in the part to variation in responses between cultivars and seed lots, and also due to the wide variety of treatments and evaluation conditions used. The confusion on the topic has been compounded by problems of mis-interpretation of experimental results. In the following paragraphs the range of invigorative and protective treatments tried for wheat seeds has been reviewed to assess our understanding of the different variables that are involved in these treatments.

### 2.8.1 Pre-sowing Treatments for Invigoration of Wheat Seeds (treatments applied on unaged or aged seeds)

The advantages of invigorative treatments can be assessed both on unaged and aged seeds. As earlier workers (e.g. Hanson, 1973; Lush *et al.*, 1981) did not evaluate the effects of hydration—dehydration treatments on unaged and aged seeds separately, clear information on the possible change in responses due to ageing are not known.

Table 2.4 The effects of short imbibition treatments on wheat grains

Reference	Treatment method	Drying-back method	Evaluation method	Cultivars	Ageing method	Result	Comments
<b>A Short imbibition treatments of unaged seeds</b>							
Goldsworthy, <u>et al.</u> (1982)	2h, 35°C	Dried-back, 35°C	Coleoptile growth at 10°C	Various seed lots of cultivars: Flanders Maris Hobbit Sicco	-	up to 45% increase	Effects very variable between cultivars and seed lots. Some lots showing no response. 5 - 30 minutes imbibition is effective in some cases.
Rudrapal and Basu (1982)	2h (ambient?)	Dried-back	Germination after 72h (?)	cv. Sonalika	-	No response	A decrease in leaching from the seeds was noted.
<b>B Short imbibition treatments on aged seeds</b>							
Goldsworthy, <u>et al.</u> (1982)	2h, 35°C	Dried-back, 35°C	Coleoptile growth at 10°C	cv. Flanders	24h at 50°C, 15% SMC (before treatment)	38% increase	This seed lot had shown no response before ageing.

Table 2.4 cont...

Reference	Treatment method	Drying-back method	Evaluation method	Cultivars	Ageing method	Result	Comments
<b>C Short imbibition treatments applied to seeds before ageing</b>							
Basu (1976)	3h, 25°C	Dried-back, 35°C	Germination after 72h at 28°C	cv. Sonalika	8d, 100% RH, 45°C (after treatment)	22% increase	Additional effect on growth by various chemicals in aqueous solution.
					5 months ambient storage (after treatment)	35% increase	No statistics presented.
Rudrapal and Basu (1982)	2h (ambient?)	Dried-back	Germination after 72h(?)	cv. Sonalika	7d, 100% RH, 42°C (after treatment)	92% increase	Same seed lot as unaged seed showing no response.

Abbreviations: d = days; h = hours; SMC = seed moisture content; RH = relative humidity.

Table 2.5 The effects of longer hydration treatments on wheat grains

Reference	Treatment method	Drying-back	Evaluation method	Cultivars	Ageing method	Result	Comments
<b>A Longer treatments on unaged seeds</b>							
Hanson (1973)	24h, 15°C	Dried-back 24h, 0% RH, 20°C	Coleoptile emergence from vermiculite at -0.3 MPa, 22°C	cv's Cappelle Desprez and Maris Nimrod	-	9.5-22% increase in emergence rate	Variation between seedlots. No statistics presented.
				cv. Joss Cambier	-	No effects	
Rudrapal and Basu (1982)	24h M.E in 100% RH, 25°C	Dried-back	Germination after 72h(?)	cv. Sonalika	-	No response	
Dell'Aquila, <u>et al.</u> (1984)	48h, 20°C at -0.38 MPa PEG	No drying-back	T50 at 20°C	cv. Appulo	-	60% increase in germination rate	
Dell'Aquila (1987)	36h, 20°C at -0.37 MPa PEG	No drying-back	T50 at 20°C	cv. Appulo	-	20% increase in germination rate	

Table 2.5 cont...

Reference	Treatment method	Drying-back	Evaluation method	Cultivars	Ageing method	Result	Comments
Lush, <u>et al.</u> (1981)	48h at 31% SMC and 20°C	Dried-back for 2-7 days at 20°C	Time to 40% germination in a range of tempera- tures from 4 to 36°C	cv. Egret	-	Increase in germination rate at 4 to 24°C with a maximum of >50% at 4°C	No statistics presented.
<b>B Longer treatments on aged seeds</b>							
Dell'Aquila, <u>et al.</u> (1984)	48h, 20°C at -0.38 MPa PEG	No drying-back	T50 at 20°C	cv. Appulo	6 weeks at 30°C, 13.5-14% SMC before treatment	77% increase in germination rate	
Dell'Aquila (1987)	36h, 20°C at -0.37 MPa PEG	No drying-back	T50 at 20°C	cv. Appulo	up to 20d at 25- 18% SMC before treatment	23-45% increase in germination rate	

Table 2.5 cont...

Reference	Treatment method	Drying-back	Evaluation method	Cultivars	Ageing method	Result	Comments
<b>C Longer treatments applied to seeds before ageing</b>							
Rudrapal and Basu (1982)	24h ME in 100% RH, 25°C	Dried-back	Germination after 72h(?)	cv. Sonalika	7d at 100% RH, 42°C after treatment	81% increase	The same lot showed no response before ageing.
					Natural ageing after treatment	Similar good response	24h ME validated as the best time.
Lush <u>et al.</u> (1981)	48h at 31% SMC and 20°C	Dried-back for 2-7 days at 20°C	Germination after 14 days	cv. Egret	3d, at 50°C	74% higher losses in germination compared to control	Replication details not shown.
					5h soaked seeds exposed to 50°C for 4h	91% higher losses in germination compared to control.	

Table 2.5 cont...

Reference	Treatment method	Drying-back	Evaluation method	Cultivars	Ageing method	Result	Comments
Dell'Aquila and Tritto (1990)	36h, 20°C at -0.37 MPa PEG	Dried-back 24h at 25°C	Germination, cv. Appulo mean germination time.		2 weeks at 15% SMC and 30°C	17-23% Protection of germination rate. No effect on germination.	The germination data prior to ageing not shown.

Abbreviations: h = hours; ME = moisture equilibration; MPa = mega pascals; PEG = polyethylene glycol; SMC = seed moisture content; T50 = times to 50% radicle emergence; RH = relative humidity

### 2.8.1.1 *Cultivar and seed lot responses to pre-sowing treatments*

Cultivar responses to hydration treatments are often different. Hanson (1973) reported that one wheat cultivar (cv. Joss Cambier) did not show consistent invigoration effects in response to longer hydration treatments. In contrast, all the cultivars tested by Goldsworthy *et al.* (1982) appeared to respond to short hydration treatments. Within a cultivar longer hydration treatments were shown to produce invigoration effects in all the seed lots tested (Hanson, 1973; Dell'Aquila, 1987), whereas only some seed lots responded to short hydration treatments (Goldsworthy, *et al.*, 1982). It is difficult to explain from these studies as to why cultivars or seed lots respond differently to hydration treatments, yet there are some indications that the mechanisms of invigoration by short and longer hydration treatments could be different.

### 2.8.1.2 *Treatment method and conditions used for invigorative pre-sowing treatments*

A variety of imbibition methods, times and other conditions like temperature and drying back were used by earlier workers for the invigoration of wheat seeds. The different methods involved hydration of seeds by soaking in excess water (Goldsworthy *et al.*, 1982), or on the surface of blotters with a free (Hanson, 1973) or restricted water supply (e.g. Dell'Aquila, 1987), or by adding a known amount of water with gentle mixing to allow uniform absorption (Lush *et al.*, 1981). It is evident that aeration was not limiting in all the reported methods except the soaking treatment of Goldsworthy *et al.* (1982). Although artificial aeration was initially provided during the short soaking treatment, the authors finally concluded that aeration was not required for producing invigoration effects.

Dell'Aquila (1987) used different osmotic potential to restrict the water supply and found that imbibition with -0.37 MPa poly-

ethyleneglycol (PEG) solution produced better invigoration effects as compared to those imbibed in distilled water or in -1.0 MPa PEG solution. It was also reported that the lower concentration of PEG avoided the problem of fungal contamination during seed treatment. Lush et al. (1981) found that raising seed moisture to 31% produced maximum invigoration effects compared to 20 or 26% moisture levels. Moisture losses from hydrated seeds were prevented by holding them in sealed containers for desired periods. Though considerable invigoration effects were reported by all treatment methods, the data from short soaking treatments (Goldsworthy et al., 1982) appear highly variable. In his studies on longer hydration treatments, Hanson (1973) attempted to increase uniformity of response by arranging wheat seeds crease down over wet blotters.

In most of the methods reported above, the seeds were dried back after the hydration period although Dell'Aquila's group (Dell'Aquila et al., 1984; Dell'Aquila, 1987) was an exception to this; at the end of PEG treatment period, the seeds were thoroughly washed to remove the PEG and then transferred directly to distilled water for germination. Recently Dell'Aquila and Tritto (1990) showed that some portion of the beneficial effects gained by hydration may be lost if the seeds are dehydrated. Although such effects are to be expected, drying-back procedures are usually included in hydration treatments for the sake of convenience in handling of seeds both after treatment and during subsequent sowing. One important pre-requisite for drying back is that there should be no pre-germination during the imbibition phase as these seeds will be damaged by desiccation (cf. May et al., 1962; Berrie and Drennan, 1971; Sen and Osborne, 1974). Both Hanson (1973) and Dell'Aquila (1987) reported a small proportion of pre-germination during their hydration treatments. Losses of such seed may represent losses of the highest vigour component of the seed lot.

For both short and longer hydration treatments, very little information is available on the mechanisms of invigoration. Hanson's (1973) work shows some indication that treated seeds produce  $\alpha$ -amylase activity earlier compared to untreated seeds during germination. The data on [ $^{14}\text{C}$ ] leucine incorporation into protein revealed higher synthetic activity in the aleurone tissue of treated compared to untreated seeds, but similar effects were not noticed in the embryo. It was suggested that the main effect of invigoration could be the result of an advancing effect on the aleurone tissue.

In general, there is no clear consensus about the optimum range of temperatures for pre-sowing hydration treatments of wheat. Goldsworthy *et al.* (1982) suggested that repair processes may be more rapid at higher temperatures of 35°C although their reported results with radicle emergence rates did not clearly support this view. However, other workers have used either the ISTA (1985) recommended optimal germination temperature of 20°C or a slightly lower regime of 15°C for the invigoration of wheat seeds (Table 2.5). The use of near optimal germination temperatures for pre-sowing seed treatments is often recommended for a range of crops (Akers and Holley, 1986).

### 2.8.2 **Pre-sowing Treatments for Protection of Stored Wheat Seeds** (treatments applied before ageing)

There is not much information at all in the literature about cultivar or seed lot responses to protective pre-sowing treatments; for example all the short hydration treatments (Table 2.4) have been tried on a single wheat cultivar, each time using a single seed lot. Although Dell'Aquila and Tritto (1990) used several seed lots, the ageing time after the treatment was not sufficient to draw useful conclusions.

Protective treatments, like the invigorative ones, vary widely with respect to hydration time. Obviously, in all protective

treatments reported, seeds were dried back to approximately their original moisture levels after the hydration times. Short hydration treatments involved soaking of seeds in water for 2 to 3 h at room temperature or at 25°C (Table 2.4). For longer hydration treatments, Dell'Aquila and Tritto (1990) and Lush *et al.* (1981) used the same method and conditions as described for invigoration treatments (Sec. 2.8.1.2), whereas Rudrapal and Basu (1982) used a 24 h moisture equilibration at 25°C and 100% RH.

All short hydration treatments were shown to protect the germination capacity (Table 2.4) both under artificial (Basu, 1976; Rudrapal and Basu, 1982) and natural ageing conditions (Basu *et al.*, 1975; Basu, 1976). Basu (1976), in addition, reported small increases in the root and shoot length of seedlings of treated seeds as compared to untreated seeds, although it is not known whether the changes were significant.

However for the longer hydration treatments there seems to be no agreement in the results obtained by the three earlier workers. For example, a 24 h moisture equilibration was shown to protect the germination capacity (Rudrapal and Basu, 1982) as compared to a 36 h hydration which did not show any effect (Dell'Aquila and Tritto, 1990). In complete contrast, 48 h hydrated seeds (Lush *et al.*, 1981) suffered severe losses in germination as compared to untreated controls as a result of exposing them to different types of artificial ageing conditions. On the other hand, with respect to changes in seed vigour there seems to be some agreement in reports, as treated seeds showed lower conductivity of seed leachate (Rudrapal and Basu, 1982) and higher germination rates (Dell'Aquila and Tritto, 1990) compared to untreated controls after subsequent storage under artificial ageing conditions.

The initial experiments in this thesis (Chapters 4 and 5) constitute an extensive study which attempts to resolve some of the ques-

tions and disagreements with respect to artificial ageing and pre-sowing treatments discussed in sections 2.7 and 2.8 respectively.

## CHAPTER 3

### MATERIALS AND METHODS

This chapter contains details of all the material and methods used during this project. The results of some of the preliminary tests conducted on pre-sowing treatment methods and on optimisation or validation of enzyme assay techniques are also presented here.

#### 3.1 SEED MATERIAL

A total of seven seed lots from five different wheat cultivars was used. All were procured harvest fresh (1987 or 1988 harvest) except Oroua 1985 which was 2 years old. The source, percentage germination and experimental use of these seed lots are shown in table 3.1. Seeds were stored in sealed plastic containers at 5°C and 12 - 13% seed moisture content (SMC) until further use. If the seed was treated or aged during the experiment and not used immediately, it was stored in sealed aluminium foil packets (12/20/50 µM polyster/aluminium/polythene, manufactured by Printpak - UEB, N.Z.) at 5°C until required.

#### 3.2 ARTIFICIAL AGEING TECHNIQUES

Two types of ageing conditions were used, one was a harsh accelerated ageing at 100% RH (Delouche and Baskin, 1973) and the other was a relatively slow controlled deterioration at 15% SMC (Matthews and Powell, 1987).

##### 3.2.1 Accelerated Ageing

Appropriately sampled seed material (ISTA, 1985) was placed in small muslin bags and subjected to accelerated ageing (AA) at  $40 \pm 1^\circ\text{C}$  and  $\sim 100\%$  relative humidity for up to 6 days in sealed glass jars containing distilled water at the bottom.

Table 3.1 Details of the different seed lots used in the experiment.

Lot No.	Cultivar	Harvest date	Source	Germination %	Experiments
1	Karamu	1987	Wrightson-Dalgety Ltd, New Zealand	97	Chapter 4
2	Oroua	1987	" "	85	
3	Oroua	1985	Manawatu Mills, New Zealand	63	
4	Unknown (Australian)	1988	" "	96	
5	Pegasus	1988	" "	92	
6	Rongotea	1988	Hodder and Tolley Ltd, New Zealand	89	Chapters 5 6 and 7
7	Karamu	1988	" "	96	

Note: None of the seed lots were chemically treated on arrival at the Seed Technology Centre.

After ageing, seeds were spread out thinly on blotters and air dried at 25°C for 48 hours to moisture levels within the range 12.2 to 13.8%. An example of changes in SMC's during AA of cv. Karamu 1988 is shown in table 3.2. Within a single seed lot there was up to 4% variation in seed moisture after 6 days AA, between different experiments (data not shown) which indicates the problem of reproducibility with this method. Such variations are to be expected as there is no precise control over moisture uptake by seeds during ageing. Increases in the quantity of seed in each muslin bag generally caused small but significant decreases in moisture uptake during AA (data not shown).

However within an experiment, neither changes in seed lots, as in the case of cv. Oroua, nor use of different pre-sowing hydration treatments (see Sec. 3.3) prior to storage in cv. Karamu, significantly affected seed moisture uptake during exposure to AA conditions (data not shown).

### 3.2.2 Controlled Deterioration

Weighed seed samples of known moisture content were placed in aluminium foil packets and calculated amounts of distilled water added to raise the seed moisture levels to 15%. The quantity of water to be added was calculated as follows:

$$V = \left[ \frac{100 - MCO}{100 - MCR} \times W \right] - W$$

where      V      = volume of water to be added (ml)  
             MCO = original moisture content (%)  
             MCR = required moisture content (%)  
             W      = weight of seeds (g)

Following addition of water, the foil packets were heat sealed and the seeds allowed to equilibrate with the added water for

Table 3.2 Changes in seed moisture content during accelerated ageing (40°C, 100% RH) or controlled deterioration (35°C, 15% SMC) of wheat seeds (cv. Karamu, 1988)

Accelerated Ageing		Controlled Deterioration	
days	SMC %	days	SMC %
0	12.4 ± 0.20	0	15.0 ± 0.04
2	19.6 ± 0.31	10	15.0 ± 0.05
4	22.6 ± 0.22	20	15.0 ± 0.16
6	25.3 ± 1.30	30	15.2 ± 0.11
		40	15.3 ± 0.13
		50	15.3 ± 0.17

The data represent means of 4 replicates, ± standard errors.

Abbreviations: RH = relative humidity; SMC = seed moisture content

24 h at 5°C. Preliminary moisture tests confirmed that the method was reliable and the desired seed moisture levels could be attained with reasonable accuracy. After equilibration the seed packets were incubated at 35°C for periods of up to 60 days. Following ageing, the packets were opened and the seed moisture contents checked before being used for further experimentation. No attempt was made to dry the seeds further. Overall, the seed moisture levels were uniformly maintained irrespective of the length of ageing or the treatments received by seeds prior to controlled deterioration (CD). A typical example of changes in seed moisture content during CD using cv. Karamu, 1988 harvest is shown in table 3.2.

### 3.3 PRE-SOWING HYDRATION TREATMENTS

Two types of treatments were used to either protect or repair stored seeds as follows:

#### 3.3.1 Short Hydration Treatments

This method involved soaking of seeds for short periods (normally up to 2 h), followed by drying and was similar to the methods used by Basu (1976) and Goldsworthy *et al.* (1982). The seeds were soaked in one-and-a-half to twice their weight of distilled water either at 25°C or at 35°C. At the end of the soaking period, the water was drained off and seeds were blotted dry and spread out in thin layers to air dry for 48h at 25°C. By this method the moisture levels were reduced to within the range 12.2 to 13.7%.

#### 3.3.2 Longer Hydration Treatments

In contrast to short soaking (Sec. 3.3.1), longer hydration treatments were intended to provide more time for physiological repair and other advances in germination processes, and accord-

ingly they also differ in other treatment conditions and methods. The temperatures used were similar or close to those recommended for normal germination (ISTA, 1985) and the methods allowed for sufficient aeration of hydrated seeds. In one of the treatments (Sec. 3.3.2.1), temporary suppression of radicle emergence was achieved by using an inert osmotic solution as the germination medium (Heydecker, 1974). The hydration times were chosen in such a way that they did not permit any visible radicle emergence during treatment.

### 3.3.2.1 *Hydration in water*

This method involved imbibing seeds in single layers over two Whatman 1 filter papers soaked in distilled water in petri dishes at 15°C after the method of Hanson (1973), except that no attempt was made to orientate the seeds crease down over blotters. A preliminary study on unaged Karamu seeds showed that a 24h imbibition followed by drying was optimal for obtaining maximum reduction in times to 50% radicle emergence (T50's) at 10°C (Fig. 3.1). At the end of the imbibition period, seeds were blotted dry before air drying as at Sec. 3.3.1. An example of moisture uptake by unaged and differently aged seeds is shown in table 3.3.

### 3.3.2.2 *Hydration in PEG solution (PEG treatment)*

The treatment used was modified from that used by Dell'Aquila, et al. (1984). A -0.37 MPa polyethylene glycol (PEG 6000, BDH Ltd, Poole, England) solution in distilled water was prepared according to the formula suggested by Michel and Kaufmann (1973). The seeds were hydrated over PEG soaked filter papers as in Sec. 3.3.2.1. Preliminary studies on unaged seeds showed that a 20h hydration at 20°C was optimal for obtaining maximum reduction in T50's at 10°C (Fig. 3.1). Further increases in hydration times were not beneficial and a 30h hydration resulted in 10 to 12% radicle emergence. Upon drying-back total

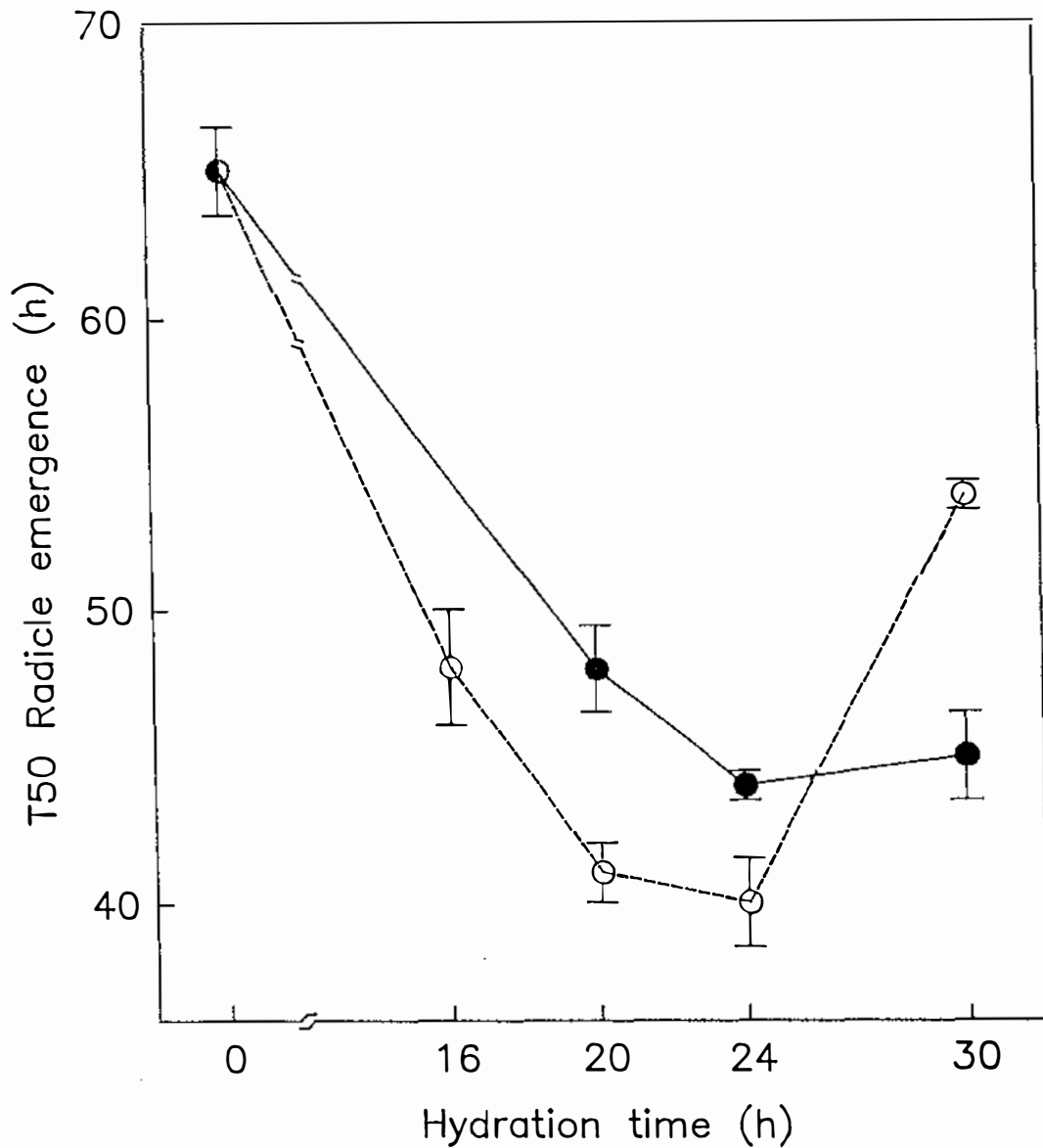


Fig. 3.1 Effect of different hydration times, in two treatment methods, on times to 50% (T50) radicle emergence ( $10^{\circ}\text{C}$ ) of wheat seeds. ●, hydration in distilled water at  $15^{\circ}\text{C}$  followed by drying-back seeds; ○, hydration in  $-0.37$  MPa Polyethylene glycol solution at  $20^{\circ}\text{C}$  followed by drying-back seeds. Data represent means of 4 replications  $\pm$  standard errors.

Table 3.3 Comparison of seed moisture levels attained by unaged and differently aged seeds of cv. Karamu during short and longer hydration treatments.

Hydration Treatment	Accelerated ageing at 40°C, 100% RH		Controlled deterioration at 35°C, 15% SMC	
	0 d	6 d	0 d	50 d
2 h soaking at 25°C in distilled water	26.8 ± 0.15	26.0 ± 0.30	27.0 ± 0.28	24.9 ± 0.09
20 h hydration at 20°C in -0.37 MPa, PEG	34.0 ± 0.67	33.0 ± 0.50	34.3 ± 0.30	32.4 ± 0.24
24 h hydration at 15°C in distilled water	36.9 ± 0.80	35.2 ± 0.42	36.5 ± 0.44	35.4 ± 0.45

Abbreviations: h = hours; d = day; RH = relative humidity; SMC = seed moisture content; PEG = polyethylene glycol

radicle emergence decreased significantly from 98% ( $\pm 0.5$ ) for untreated to 88% ( $\pm 1.0$ ) for these treated seeds, presumably because of damage to seeds which had lost their desiccation tolerance after radicle protrusion (May et al., 1962). T50's were also increased significantly ( $p < 0.01$ ) compared to 20 or 24 h hydrated seeds.

At the end of imbibition period, seeds were thoroughly washed several times in distilled water to remove any PEG adhering to the surface of seeds followed by drying as previously described (3.3.2.1). In earlier studies by Dell'Aquila, et al. (1984) seeds were not dried after this type of treatment, but set to germinate immediately after removal from PEG.

#### 3.4 SEED MOISTURE DETERMINATION, GERMINATION AND SEEDLING GROWTH TRIALS

Seed moisture content was determined by drying ground seeds for 2h at 130°C as recommended by ISTA (1985). A two stage drying was followed whenever the seed moisture exceeded 17% (ISTA, 1985); as for example after accelerated ageing or after hydration of seeds.

All germination trials were conducted in the dark, except for short periods of counting. Although trials were conducted at both 10 and 20°C initially, a 10°C incubation was generally used throughout the study to emphasise changes in germination performance (Heydecker et al., 1975) due to ageing or pre-sowing treatments. In this study the term 'germination' means visible radicle emergence from seeds (Sec. 2.3), whereas normal germination refers to seedlings with well developed plumule and radicle as defined by ISTA (1985).

### 3.4.1 Normal Germination and Seedling Growth Trials

Twenty five seeds arranged horizontally in a single row were germinated by using the between paper method (ISTA, 1985). After 10 (20°C) or 15 (10°C) days incubation normal and abnormal seedlings were evaluated as recommended by ISTA (1985). Roots and shoots of all normal seedlings were then separated from the seeds at the point of attachment and dried separately at 65°C for 4 days. Dried roots and shoots were cooled in a desiccator, weighed and the mean weights were expressed as mg, shoot or root per normal seedling.

### 3.4.2 Time to 50% Radicle Emergence (T50)

Fifty seeds were germinated at 10°C over two layers of Whatman 1 filter papers in 9 cm petri dishes. Radicle emergence was counted at very frequent intervals (less than 6h at times of peak activity). The median radicle emergence time (T50) was calculated by linear interpolation according to the formula suggested by Coolbear et al. (1984).

$$T50 = t_i + \left[ \frac{(N + 1)/2 - n_i}{n_j - n_i} \right] \cdot (t_j - t_i)$$

where N is the final number of seeds germinating and  $n_i$ ,  $n_j$  the total number of seeds germinated by adjacent counts at times  $t_i$  and  $t_j$  respectively where  $n_i < (N + 1)/2 < n_j$ .

## 3.5 GENERAL APPROACH TO EXPERIMENTAL DESIGN

Where the major emphasis was to evaluate several pre-sowing treatments precisely over a range of seed deterioration levels, it was decided to use the approach of a split plot design using ageing times as main plots and imposing various pre-sowing treatments on differently aged seeds as sub plots. The experi-

ments were replicated fully right from the beginning of each set of treatments by ageing or treating each of the 4 replicates separately under identical conditions. Analysis of variance for the data was done as recommended for split plot design using arcsine transformations for percentages.

The effects of ageing and/or pre-sowing treatments with respect to biochemical changes in seeds were studied using 3 or 4 replications and comparing data by simple 't' tests.

### 3.6 SEED LEACHATES

#### 3.6.1 Electrical Conductivity of Seed Leachate

Conductance of seed leachate was determined after soaking fifty seeds in 100 ml de-ionised water for 24h at 25°C, using a CDM 83 digital conductivity meter (Radiometer, Copenhagen). After deducting blank conductance values, the results were expressed on a per gram of seed basis.

#### 3.6.2 Soluble Sugars in seeds and Seed Leachate

Changes in the soluble sugar content of seeds and of seed leachates were studied to assess the possible damage to stored starch and to the integrity of cell membranes respectively. Total soluble sugars were extracted from seeds using 80% (v/v) hot ethanol following the method of Adams *et al.* (1980), 200 mg of finely ground seed powder being extracted thrice in hot ethanol. The combined ethanol fractions were evaporated and the resultant water extract diluted to 200 ml. To a 3 ml aliquot of diluted extract was added 6 ml fresh anthrone reagent (2 g anthrone reagent in 1 litre of cold 95% sulphuric acid). All operations were done on ice and after mixing thoroughly the mixture was heated for 7.5 minutes at 100°C in a water bath. The reaction was terminated by cooling the mixture rapidly

to 25°C and the absorbance read at 630 nm. A set of glucose standards with concentrations ranging from 0 to 60 µg/ml were run simultaneously along with seed extracts.

Sugars in seed leachate were determined similarly, but by using a 3 ml sample of seed leachate collected by soaking 25 seeds in 50 ml de-ionised water for 24 h at 25°C.

### 3.6.3 **Studies on Leakage of Germination Inhibiting Metabolites from Seeds**

In order to test the presence of germination inhibiting substances, the effect of washing seeds in running water for 2 h, on T50's and coleoptile growth was studied. In addition, both washed and unwashed seeds were germinated over seed leachate media and their responses were studied. The seed leachate for this study was collected in advance by soaking seeds of the original seed lot for 2 h at 25°C in a volume of distilled water twice the weight of the seeds.

## 3.7 **ELECTRON MICROSCOPY**

Scanning electron micrographs of the starchy endosperm in the region formed by the scutellum-crease junction in a mid-longitudinal plane were taken by the Electron microscope unit, DSIR Fruit and Trees, Palmerston North (details in Appendix 1).

## 3.8 **STUDIES ON PROTEOLYTIC ACTIVITY**

Increases in proteolytic activity during ageing has been suggested to be one of the causes of damage in aged seeds (Perl *et al.*, 1978, Agrawal and Kharlukhi, 1987). However, increases in proteolytic activity during germination helps in reserve mobilization as well as turnover of proteins. In the present study changes

in proteolytic activity as influenced by ageing or pre-sowing treatment were assessed by looking at changes in the seed proteins themselves using SDS-PAGE or HPLC and by using a commercial protease substrate.

### 3.8.1 SDS-Polyacrylamide Gel Electrophoresis of Total Seed Proteins

Initially qualitative changes in total seed proteins were studied using the Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) technique. Ten seeds per replicate were ground to fine powder and the proteins were extracted for 1 h in centrifuge tubes using an extraction medium (2.3% SDS; 5% 2-mercaptoethanol; 10% Glycerol in 62.5 mM Tris HCl, pH 6.8) at the rate of 1 ml/0.1 g seed powder. The extract was then centrifuged at 10,000 g and the supernatant was used as the protein solution for evaluation.

The details of buffers and polyacrylamide gel recipes are as given by Hames (1981). A discontinuous buffer system with a 3.8% stacking gel and a 7.5% resolving gel was used to resolve the proteins. The advantages of using polyacrylamide gels are that in addition to obtaining maximum resolution between separated components, they remain chemically inert during separation and are uniform in their physical properties throughout the gel.

After electrophoresis, the gels were first stained by silver staining technique using a procedure described by Merrill *et al.* (1981) and then transferred to de-ionised water before staining with coomassie brilliant blue G.250 (Hames, 1981). Changes in seed proteins were studied using both photographs and densitometric scans (Bio-Rad model 1650 Transmittance/Reflectance Scanning densitometer).

Specific details of the technique including gels, buffers and stains used are as given by Srivastava(1989).

### 3.8.2 High-performance Liquid Chromatography

An HPLC analysis of seed proteins in selected samples was conducted by the Wheat Research Institute, Department of Scientific and Industrial Research, Christchurch, New Zealand (Details in Appendix 2 ).

### 3.8.3 Protease Activity

Protease activity in seeds was determined using a modified procedure from the method described by Ragster and Chrispeels (1979) in which the authors used 'Azocoll', a buffer-insoluble commercial substrate to estimate the endopeptidase activity in soybean leaves. Azocoll consists of a red dye bound to collagen and the dye is released by cleavage of internal peptide bonds and the intensity of solubilised colour can be measured by reading absorbance at 520 nm.

#### 3.8.3.1 *Choice of assay conditions*

As different groups of proteases with different pH optima are known to occur in seeds, (Sec. 2.5.3.1), a preliminary study was conducted to determine the pH optimum for 'Azocoll' hydrolysing activity of wheat seed extract.

For each replicate 10 ungerminated seeds (unaged-untreated) were ground and extracted in 5 ml extraction buffer (50 mM Sodium/Potassium Phosphate buffer pH 7.5 with 0.1% [v/v] 2-mercaptoethanol and 1% [w/v] polyvinyl-pyrrolidone [Sigma]) using a pestle and mortar. This was done on ice and using ice cold solutions. The extract was centrifuged at 10,000 g (4°C) and the resultant supernatant used as an enzyme source. The 'Azocoll' hydrolysing activity was studied at pH 5.0 (100 mM Acetate buffer), pH 6.8 (100 mM Sodium/Potassium Phosphate buffer) and pH 8.5 (100 mM Tris-HCl buffer). The reaction mixture consisted of 10 mg 'Azocoll', 0.5 ml enzyme extract

and 4.5 ml of desired buffer solution. The mixture in a 25 ml sealed Erlenmeyer flask was incubated with agitation in darkness for a known period at 37°C using an orbital shaker set to run at 60 oscillations per minute. At the end of incubation period, the reaction was stopped by cooling the mixture rapidly to 4°C. The mixture was centrifuged at 10,000 g (4°C) and the absorbance of supernatant read at 520 nm. As shown in Figure 3.2 'Azocoll' was found to be best hydrolysed at pH 6.8 compared to pH 5.0 or 8.5.

Following this a time-course study of enzyme activity was carried out to determine optimum assay time at pH 6.8 (Fig. 3.3). A 16 h incubation period falling well within the linear phase of the increasing enzyme activity was chosen for further experimental work.

#### 3.8.3.2 *'Azocoll' hydrolysing activity of seeds*

'Azocoll' hydrolysing activity of ungerminated wheat samples (10 seeds/replicate) was determined following the extraction procedure described in Sec. 3.8.3.1 and incubating the reaction mixture containing 10 mg 'Azocoll', at pH 6.8 and 37°C for 16 h. The levels of absorbance at 520 nm after deducting blank values were used to compare relative enzyme activities in different seed samples.

### 3.9 **α-AMYLASE ACTIVITY**

#### 3.9.1 **Determination of Enzyme Activity in Seeds**

Enzyme activity was determined according to a method described by Cornford et al. (1987b) in which a dye conjugated-limit dextran was used as a substrate (Phadebas blue starch powder, Pharmacia Diagnostics Ltd). For each assay ten whole seeds were ground and extracted for 10 minutes, using a pestle and mortar, in

Fig.3.2

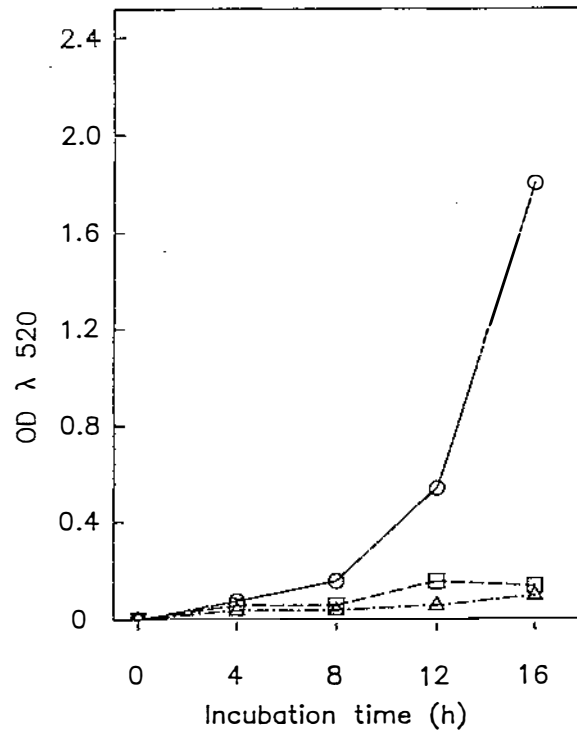


Fig.3.3

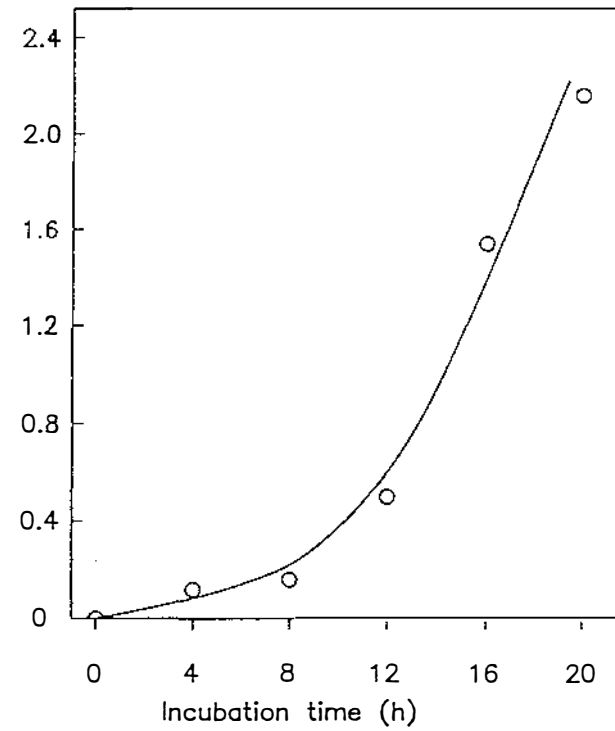


Fig. 3.2 The effect of different pH levels on the 'Azocoll' hydrolysing activity (OD  $\lambda$  520) of extracts from unaged-untreated (ungerminated) wheat seeds. ( $\Delta$ , pH 5.0; O, pH 6.8;  $\square$ , pH 8.5). The data represent means of two assay replications.

Fig. 3.3 Time-course of 'Azocoll' hydrolysing activity (OD  $\lambda$  520) of extracts from unaged-untreated (ungerminated) wheat seeds at pH 6.8. The data represent means of two assay replications.

5 ml 50 mM Tris-maleate buffer, pH 6.2 containing 10 mM calcium chloride. The extract was centrifuged at 2000 g for 10 minutes and the supernatant used as an enzyme source. One ml of phadebas substrate (10 mg/ml in buffer) was added to 1 ml enzyme extract and 4 ml buffer and the mixture incubated at 37°C for 20 minutes. The reaction was terminated by adding 1 ml 0.5 M sodium hydroxide, centrifuged at 2000 g for 10 minutes and the absorbance of supernatant read at 620 nm. The test was standardised against pure barley malt  $\alpha$ -amylase (2.3 units activity/mg, Sigma Chemical Co.) using an activity range of 0.02 to 0.2 units, one enzyme unit being defined as the amount of enzyme that will hydrolyse 1.0 mg maltose from starch at pH 6.9 in 3 minutes at 20°C.

#### 3.9.1.1 *Enzyme extraction efficiency*

As there were large differences in enzyme activities among treatments, it was decided to check whether this variation was caused by any differences in enzyme extraction efficiency. Treatments showing very low and high enzyme activities were chosen and seeds were extracted as described previously (Sec. 3.9.1). Following this, the pellet was subsequently re-extracted thrice using fresh buffer each time and supernatants collected separately. Then the enzyme activity was estimated in each of the four extractions (Table 3.4). The results show that the extraction efficiency does not differ significantly between low and high activity seedlots, with approximately 50% of the total activity (total of 4 extractions) being present in the first extraction. Thus, in view of practical consideration of handling larger number of samples, a single extraction procedure (Sec. 3.9.1) was followed as a relative measure of enzyme activity for comparing the differently treated seed lots. Figure 7.1 was, however, an exception to this, where the pellet was re-extracted once subsequent to extraction of ground seed. Extraction conditions were kept as constant as possible between samples and between experiments.

Table 3.4 Efficiency of  $\alpha$ -amylase extraction determined by following the normal extraction procedure by 3 subsequent extractions of the pellet. Samples of seed showing high ( $193 \pm 19.7$  milli units/seed) or low ( $15.0 \pm 1.8$  milli units/seed) enzyme activity were used for comparison. Data presented are means of 3 replications  $\pm$  standard errors.

	% total $\alpha$ -amylase activity extracted	
	Low activity seed sample	High activity seed sample
Extraction 1 (Extraction of ground seed)	53 $\pm 4.2$	51 $\pm 1.7$
Extraction 2 (1st extraction of pellet)	26 $\pm 0.8$	28 $\pm 1.0$
Extraction 3 (2nd extracton of pellet)	16 $\pm 2.2$	12 $\pm 1.3$
Extraction 4 (3rd extraction of pellet)	5 $\pm 2.6$	9 $\pm 2.0$

### 3.9.1.2 *Inhibition of $\alpha$ -amylase activity by possible co-extraction of inhibitors*

In order to find out if there were any differences in levels of possible co-extracted enzyme inhibitors, a small study was undertaken using four samples of differently aged and treated seeds.

A known amount of barley malt  $\alpha$ -amylase was added to each of the different seed extracts and the total activity was measured simultaneously, under similar conditions along with those of extracts alone and the added enzyme activity. The data in Table 3.5 shows that there was no inhibition of the added enzyme activity in any of the seed extracts, in fact there were increases in the total activity. This was surprising and the reasons for such effects were not known. However this provided some indication that any inhibitors did not influence the extracted activity, although the presence of wheat specific endogeneous  $\alpha$ -amylase inhibitors cannot be completely ruled out.

## 3.9.2 **Localization of Enzyme Activity in Seeds**

### 3.9.2.1 *Substrate-film technique*

Preliminary studies on localization of  $\alpha$ -amylase activity in grains were conducted using a histochemical blotting procedure on a starch agar plate (Okamoto and Akazawa, 1979). Starch agar plates were prepared in Petri dishes using 2% w/v soluble starch, 2% w/v agar, 10 mM  $\text{CaCl}_2$  in 50 mM succinic acid buffered to pH 5. Wheat grains soaked for short periods (2 h at 25°C) were cut longitudinally from the dorsal to the ventral crease, halving both the embryo and the endosperm. One half of each grain was incubated in a sealed container over agar plates, with the cut surface down, for 60 minutes at 37°C. Following this, the half grains were removed and the agar plates were flooded with potassium iodide solution (4.02% w/v potassium

Table 3.5 The effect of wheat seed extracts from differently aged or treated seeds on the activity of added barley malt  $\alpha$ -amylase. Data presented are means of 3 replications  $\pm$  standard errors.

$\alpha$ -amylase activity in milli units/seed				
	Unaged		40 d aged (15% SMC, 35°C)	
	Seed extract	Seed extract + 100 milli-units barley malt $\alpha$ -amylase	Seed extract	Seed extract + 110 milli-units barley malt $\alpha$ -amylase
Untreated	16 $\pm$ 2.1	140 $\pm$ 8.7	19 $\pm$ 4.0	157 $\pm$ 7.0
Treated (20 h PEG treatment)	81 $\pm$ 6.5	215 $\pm$ 9.7	21 $\pm$ 3.5	163 $\pm$ 4.1

Abbreviations: d = day; SMC = seed moisture content; PEG = polyethylene glycol

iodide with 0.09% w/v iodine). The starch agar stains purple leaving clear zones where starch degrading enzymes have been secreted from the grain.

### 3.9.2.2 *Quantification of enzyme activity in different parts of the grain*

From soaked wheat grains (2 h at 25°C), the embryo was first dissected and separated carefully without any contamination of outer layers (seed coat, pericarp) and the aleurone. Then the rest of endosperm along with outer layers was cut transversely to divide it into 3 equal parts (Fig. 7.2). The enzyme activity in the embryo and the 3 endosperm sections was then determined separately using the assay described in Sec. 3.9.1.

### 3.9.3 **Studies on Aleurone Responses to Exogenously Applied GA<sub>3</sub>**

Aleurone responsiveness to GA<sub>3</sub> was assessed by measuring the  $\alpha$ -amylase activity produced by the de-embryonated grains after the method of Cornford et al. (1986). The experiment was completely set-up under aseptic conditions in order to avoid microbial interference during the study.

For each determination, ten seeds were surface sterilized by soaking in 1% w/v silver nitrate solution for 2 minutes followed by a 2 minute soak in 1% w/v sodium chloride solution to remove any adhering silver nitrate. The seeds were then thoroughly washed in sterile distilled water and cut transversely in the middle to remove the embryo containing portion of the grain. The resultant endosperm halves were further sterilized for 20 minutes in sodium hypochlorite solution (1% available chlorine and with 2 drops of Tween 80) and were then washed several times in distilled water.

The sterilized endosperm halves were transferred to 25 ml Erlenmeyer flasks and 2 ml of incubation medium (consisting of 15

$\mu\text{M}$  chloramphenicol, 20 mM  $\text{CaCl}_2$  in 2 mM sodium acetate buffer, pH 5.5 and with or without an appropriate concentration of  $\text{GA}_3$ ) added. The flasks were closed with sterile cotton wool and incubated with agitation in darkness at  $25^\circ\text{C}$  for known lengths of time using an orbital shaker set to run at 60 oscillations per minute.

At the end of incubation times,  $\alpha$ -amylase activity in the half grains and the secreted activity in the incubation medium were determined separately as described in Sec. 3.9.1. Enzyme extracts were appropriately diluted when the levels of activity exceeded the working range of 0.02 to 0.2 units. The  $\alpha$ -amylase produced in response to applied  $\text{GA}_3$  was determined by adding together the activities within the endosperm halves and in the incubation medium and subtracting from this the activity detected in similarly incubated endosperm halves without  $\text{GA}_3$ .

## CHAPTER 4

### EVALUATION OF SHORT SOAKING TREATMENTS FOR IMPROVING THE GERMINATION PERFORMANCE OF AGED WHEAT SEEDS

#### 4.1 INTRODUCTION

Although short soaking treatments of seeds have been tried in several crops as protective treatments before storage (e.g. Basu et al., 1975; Basu, 1976), there are very few reports regarding their use for invigoration of physiologically deteriorated seeds. Goldsworthy et al. (1982) reported that "flash imbibition" can re-invigorate aged wheat seeds. Such simple, short treatments look a highly attractive option for handling larger quantities of cereal grains. However, in the above investigation, the choice of seed lots used, vigour test procedures adopted and the high variability in test results did not seem to provide clear evidence for the invigoration of deteriorated seeds by the treatment.

Thus an attempt was made to evaluate the efficiency of these short soaking-drying treatments with respect to:

- (a) differences in seed lot responses
- (b) responses from artificially or naturally aged seeds and
- (c) differences in cultivar responses.

In addition, one of the possible mechanisms of invigoration, i.e. leaching of germination inhibiting substances from seeds during soaking treatment, was also investigated briefly.

The seed lots, ageing conditions and pre-sowing treatments used in this study are as follows:

**Cultivars/Seed lots**

One seed lot each of cv. Karamu and cv. Oroua from 1987 harvest (harvest fresh) and a 2 year old lot of cv. Oroua from 1985 harvest year were used. In addition, cvs Rongotea, Pegasus and an Australian cultivar were tested for the presence of germination inhibitory substances in seeds.

**Ageing Conditions**

Accelerated ageing (AA) at 40°C, 100% RH for up to 6 days.

**Pre-sowing Treatments**

(on unaged seeds or after seed ageing)

- i) 2 h soaking in distilled water at 25 or 35°C.
- ii) 12 h soaking in distilled water at 35°C.

After soaking, seeds were dried back to approximately their original moisture contents in the range of 13 - 14%.

The details of methods are given in Chapter 3.

## 4.2

## RESULTS

## 4.2.1

**Responses of the Three Wheat Seed Lots to Accelerated Ageing Conditions**

Prior to any artificial ageing treatment, the three seed lots used in this study differed in their capacity for normal germination and also in times to 50% radicle emergence (T50's). The Karamu 1987 seed lot had a significantly higher normal germination (Fig. 4.1) whereas Oroua 1987 showed faster radicle emergence (Fig. 4.2) than the others.

Although significant increases in times to 50% radicle emergence at 10°C, were found in all the 3 seed lots after exposure to accelerated ageing conditions for up to 6 days (Fig. 4.2), significant losses in radicle emergence (data not shown) or normal germination (Fig. 4.1) capacity occurred only in the two Oroua seed lots. Losses in radicle emergence and normal germination followed a similar pattern. Germination responses were essentially similar when the seeds were germinated at 20°C (data not shown).

Accelerated ageing did not affect coleoptile growth of normal seedlings in both the Oroua lots when germinated at either 10 or 20°C (data not shown). But surprisingly, in Karamu 1987, although ageing effects were not evident at 20°C, when germinated at 10°C, the mean coleoptile lengths of 4 and 6 day aged seeds ( $8.6 \pm 0.36$  and  $8.1 \pm 0.41$  cm, respectively) were significantly reduced in comparison to unaged controls ( $9.7 \pm 0.24$  cm).

Prior to ageing the electrical conductivity (EC) of seed leachate for Karamu 1987 was  $23.0 (\pm 0.88) \mu\text{S}\cdot\text{cm}^{-1}\text{g}^{-1}$  seed and there was a larger and significant difference between the two Oroua lots with Oroua 1985 showing a higher conductivity of  $44.5 (\pm 1.56) \mu\text{S}\cdot\text{cm}^{-1}\text{g}^{-1}$  compared to  $19.6 (\pm 0.45)$

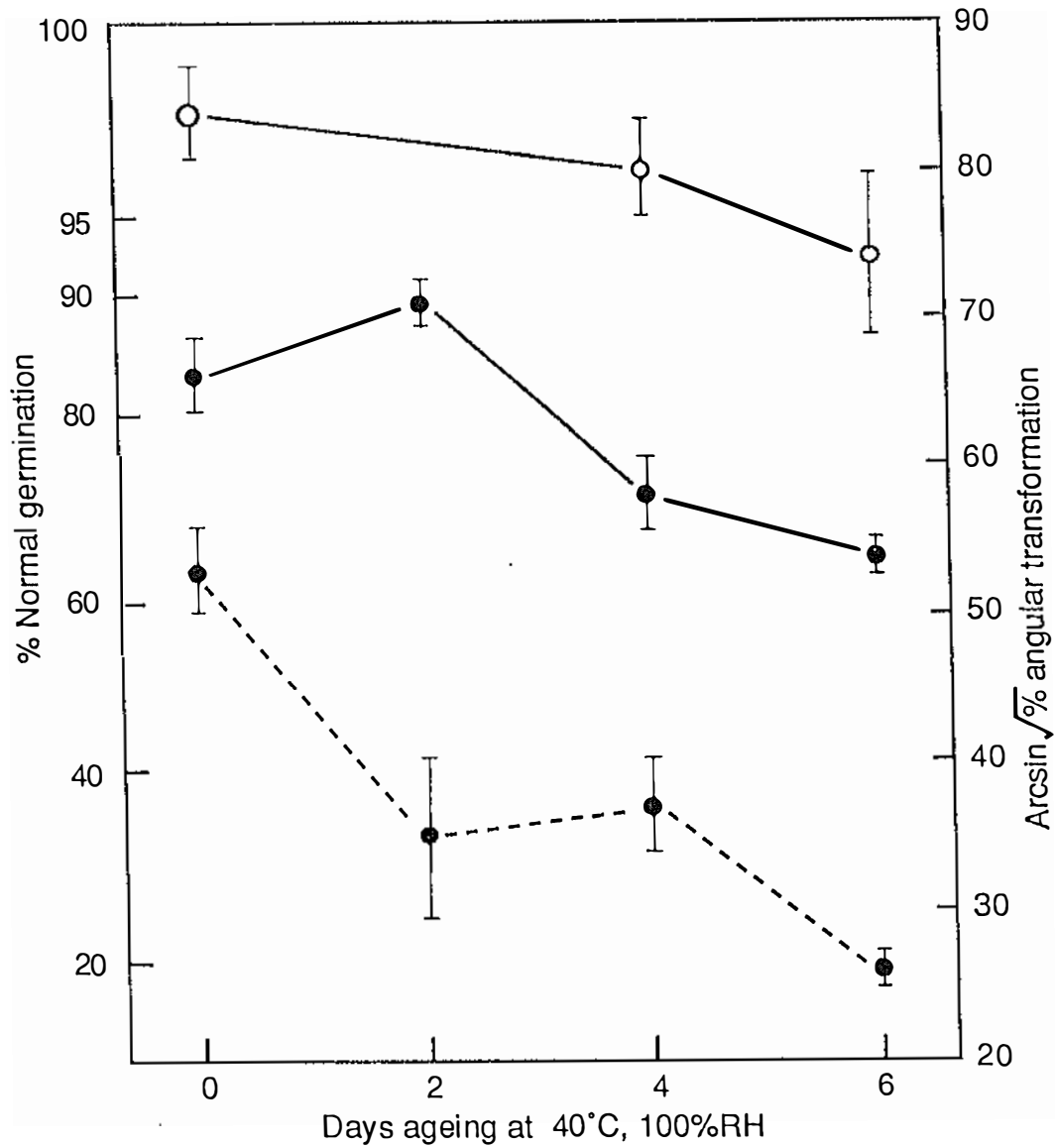


Figure 4.1

The effect of accelerated ageing on the normal germination of Karamu 1987 (O-O), Oroua 1987 (●-●) and Oroua 1985 (●- -●) seed germinated at 10°C. Each data point represents the mean of 4 replicates. Standard error bars for transformed data are also shown.

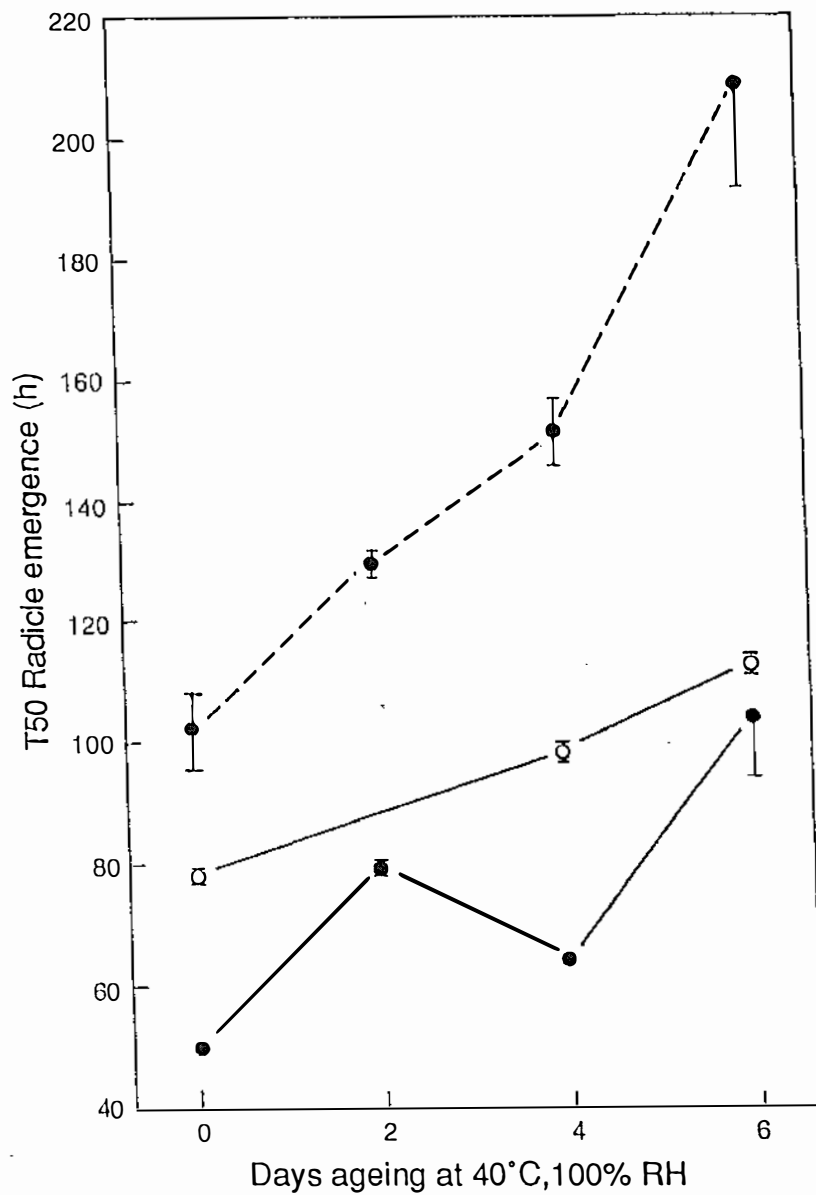


Figure 4.2

The effect of accelerated aging on times to 50% radicle emergence (T50) of Karamu 1987 (O—O), Oroua 1987 (●—●) and Oroua 1985 (●--●) seed lots germinated at 10°C. Each data point represents the mean of 4 replicates with standard error bars.

$\mu\text{S.cm}^{-1}\text{g}^{-1}$  seed for Oroua 1987. However, in none of the seed lots was EC affected by the accelerated ageing treatments (data not shown).

#### 4.2.2

#### **The Effect of 2 or 12 h Soaking Treatments at 35°C on the Germination Performance and Electrical Conductivity of Unaged and Aged Karamu 1987 Wheat Seeds**

Although both 2 and 12 h soaking treatments caused significant losses in percentage radicle emergence of 6 d aged seeds (Fig. 4.3), the normal germination percentage of either unaged or any of the artificially aged seeds at 10°C remained unaffected. Losses in radicle emergence appear to be exaggerated compared to normal germination data which, over all ageing times, averaged 95% ( $\pm 1.8$ ) and was not affected significantly after 2 or 12 h soaking treatments ( $95 \pm 3.0\%$ , and  $93 \pm 3.7\%$  respectively). It is possible that this anomaly may be due to over watering in the radicle emergence trials. When treated seeds were germinated at 20°C, there was no evidence of damage either to radicle emergence or to normal germination capacity in any of the unaged or aged seeds (data not shown).

Both 2 and 12 h soaking treatments were equally effective at reducing the T50's of unaged or 4 d aged seeds germinated at 10°C (Fig. 4.4). However in contrast, 6 d aged seeds did not respond to the 2 h treatment whereas T50's were significantly increased by the 12 h treatment. The results were basically similar when seeds were germinated at 20°C (data not shown).

The mean coleoptile lengths of normal seedlings at 10 or 20°C were not significantly influenced by any of the soaking treatments either in unaged or aged seeds (data not shown).

The EC averaged over all ageing times decreased significantly ( $p < 0.01$ ) from  $22.8 \mu\text{S.cm}^{-1}\text{g}^{-1}$  for untreated controls to

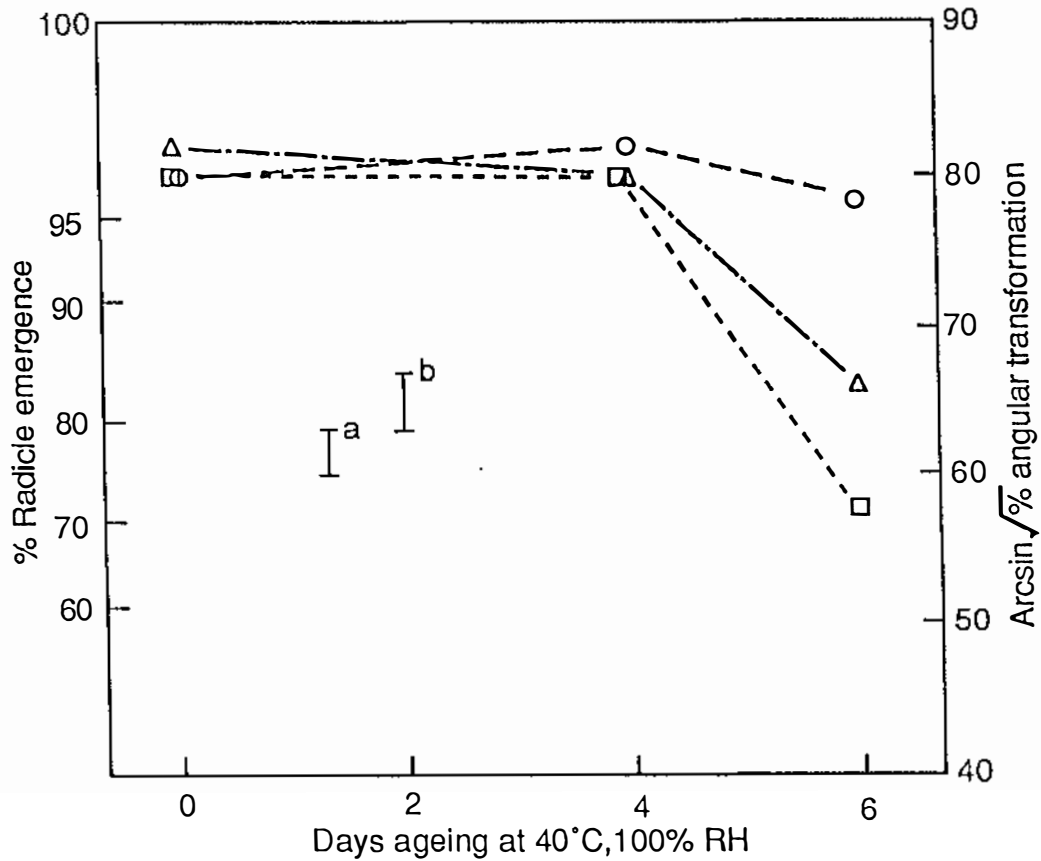


Figure 4.3 The effect of short soaking (at 35°C) and drying treatments on the radicle emergence capacity of unaged and rapidly aged Karamu 1987 wheat seeds germinated at 10°C. O, Control; Δ, 2 h soaking; □, 12 h soaking treatments. Each data point represents the mean of 4 replicates. Vertical bars a =  $LSD_{0.05}$  within ageing times and b =  $LSD_{0.05}$  between ageing times for transformed data.

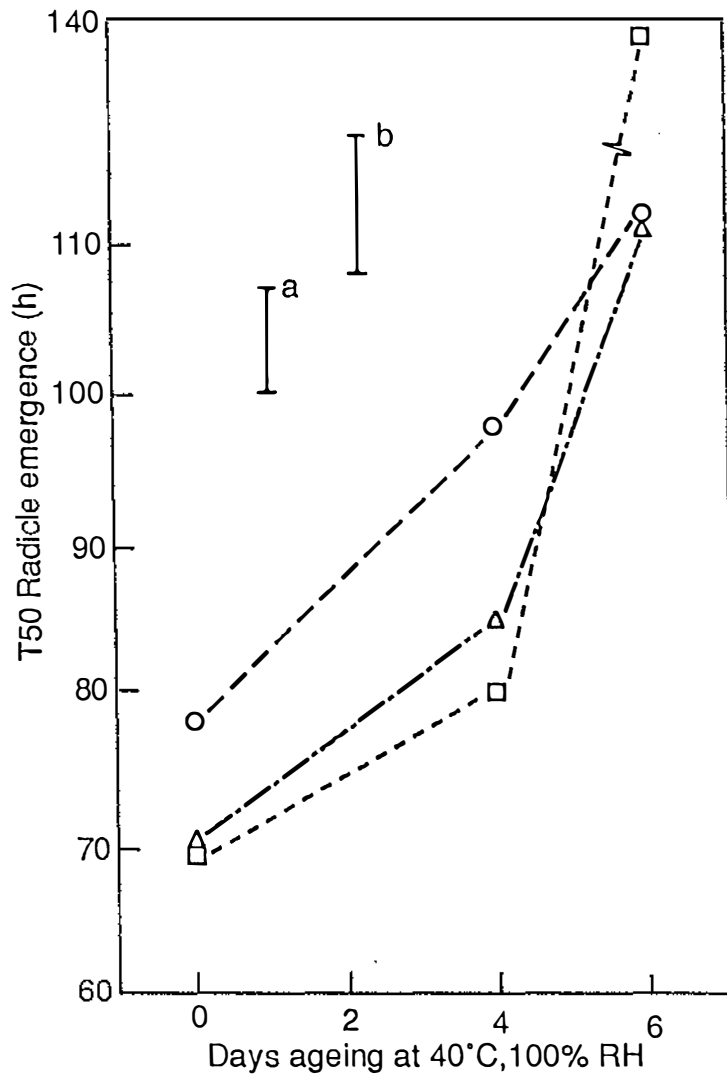


Figure 4.4

The effect of short soaking (at 35°C) and drying treatments on times to 50% radicle emergence (T50) of unaged and rapidly aged Karamu 1987 wheat seeds germinated at 10°C. O, Control; Δ, 2 h soaking; □, 12 h soaking treatments. Each data point represents the mean of 4 replicates, vertical bars a =  $LSD_{0.05}$  within ageing times and b =  $LSD_{0.05}$  between ageing times.

12.8 and 9.2  $\mu\text{S}\cdot\text{cm}^{-1}\text{g}^{-1}$  after 2 and 12 h soaking, respectively. There was no interaction between duration of seed ageing and soaking treatment.

#### 4.2.3

#### **The Effect of 2 or 12 h Soaking Treatments at 35°C on the Germination Performance and Electrical Conductivity of Unaged and Aged 'Oroua' Seed Lots**

The effect of soaking treatments on radicle emergence capacity of Oroua 1987 was broadly similar to that observed for Karamu 1987. There was no significant effect of soaking treatments on final percentage radicle emergence at 10°C, except on seeds aged for six days when 12 h soaking significantly ( $p < 0.05$ ) decreased radicle emergence from 61% for untreated controls to 29% for treated seeds. Results at 20°C were very similar (data not shown). However in Oroua 1985 radicle emergence at 10°C was significantly ( $p < 0.01$ ) decreased by the 12 h treatment, over all ageing times, from an overall mean of 47% for untreated controls to 27% for treated seeds, whereas the 2 h treatment did not have any effect. The treatment effects were similar when seeds were germinated at 20°C.

In contrast to Karamu 1987 (Sec. 4.2.2), the 12 h soaking treatment was deleterious to normal germination capacity of both the Oroua lots and germination losses (Figs 4.5 and 4.6) followed similar patterns to losses in radicle emergence capacity. The results were basically similar at both 10 and 20°C.

Both the soaking treatments produced marginal but significant decreases in T50's in unaged and 2 or 4 d aged Oroua 1987 (data not shown), similar to those reported for Karamu 1987 (Sec. 4.2.2). However neither of the treatments were effective for any of the unaged or artificially aged seeds of Oroua 1985 and on the contrary the 12 h soaking treatment was

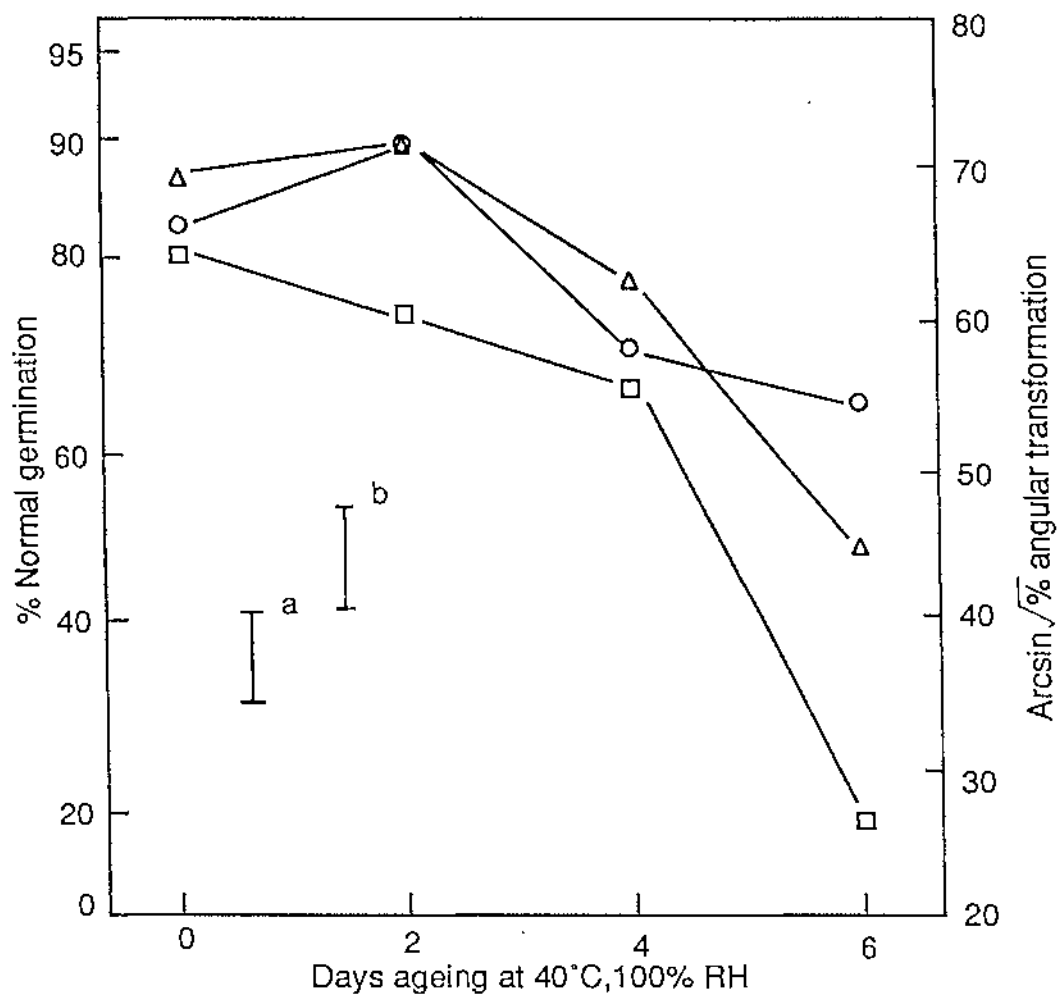


Figure 4.5 Effect of short soaking (at 35°C) and drying treatments on the normal germination capacity of unaged and rapidly aged Oroua 1987 wheat seeds at 10°C. O, Control; Δ, 2 h soaking; □, 12 h soaking treatments. Each data point represents mean of 4 replicates. Vertical bars a =  $LSD_{0.05}$  within ageing times and b =  $LSD_{0.05}$  between ageing times for transformed data.

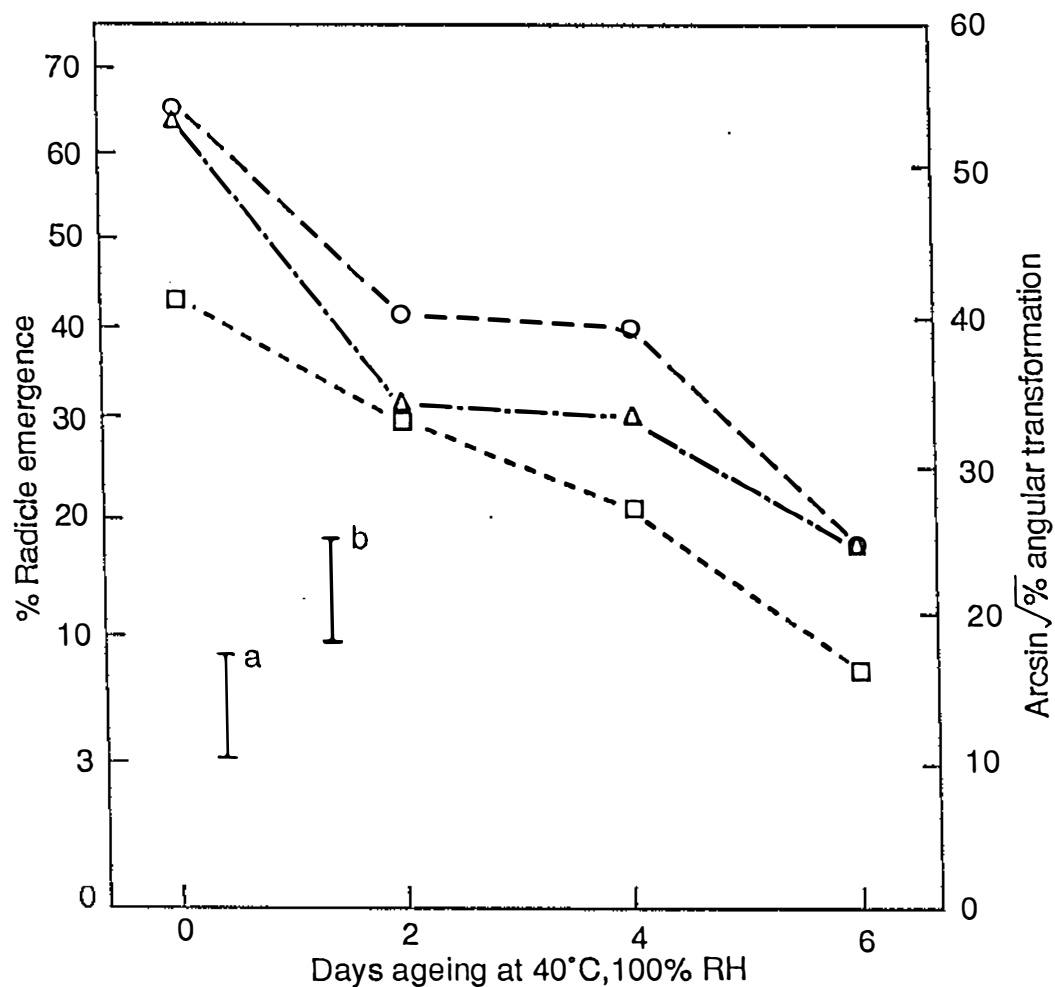


Figure 4.6 Effect of short soaking (at 35°C) and drying treatments on the normal germination capacity of unaged and rapidly aged Oroua 1985 wheat seeds at 20°C. O, Control; Δ, 2 h soaking; □, 12 h soaking treatments. Each data point represents mean of 4 replicates. Vertical bars a =  $LSD_{0.05}$  within ageing times and b =  $LSD_{0.05}$  between ageing times for transformed data.

clearly detrimental to all of them, increasing the T50's (10°C) significantly ( $p < 0.05$ ) over all ageing times from 147 h for untreated seeds to 181 h for treated seeds. The 6 d aged seeds of Oroua 1987 also showed similar susceptibility to damage with their T50's being increased significantly over controls by the 12 h treatment (data not shown).

As observed in Karamu 1987 (Sec. 4.2.2) none of the soaking treatments had any significant influence on the coleoptile growth of normal seedlings of both the Oroua seed lots. Similarly, decreases in electrical conductivity of the two Oroua lots as a result of soaking treatments followed a similar pattern to Karamu 1987.

#### 4.2.4 **Effect of Reducing the Soaking Temperature on the Efficiency of Invigoration by 2 h Soaking Treatment of Karamu 1987**

In an unreplicated comparison of the effects of soaking temperature, Goldsworthy *et al.* (1982) reported only a marginal advantage of treatment for 2 h at their preferred temperature of 35°C compared to 25°C. Time to 50% radicle emergence was advanced by only 7 h at 4°C, a temperature which might be expected to amplify any differences in effectiveness of different treatments. In this study, a comparison of the effectiveness of 25°C and 35°C was made on unaged and aged seeds of the Karamu lot germinated at 10°C. The results (Table 4.1) show no significant differences between the two temperatures when used for a 2 h treatment.

#### 4.2.5 **Effect of Washing Seeds in Water and/or Germinating Over Seed Leachate Media on the Germination Performance of Four Wheat Cultivars**

As invigoration effects following short soaking treatments were small (Sec. 4.2.2 and 4.2.3) and not influenced either by changes in temperature (Sec. 4.2.4) or by changes in soaking

Table 4.1 The effect of soaking temperature on times to 50% radicle emergence (T50) at 10°C of unaged and artificially aged Karamu 1987 seeds.

	T50 (h)			
	Days ageing at 40°C, 100% RH			
	0	2	4	6
Control	68 ± 0.6	77 ± 0.6	116 ± 1.1	154 ± 3.5
2 h soaking at 25°C	63 ± 0.3	72 ± 0.7	113 ± 1.9	148 ± 4.9
2 h soaking at 35°C	61 ± 1.3	70 ± 0.7	110 ± 3.2	156 ± 2.3

Each value represents mean of 4 replicates ± standard errors.

Abbreviations: RH = relative humidity; h = hours

times up to 6 h (Goldsworthy et al., 1982), it was speculated that the effects could simply be due to leaching of germination inhibiting substances. To test this hypothesis, seeds of different cultivars were washed in running water before being germinated over their own leachate media (Sec. 3.6.3) and changes in germination performance were studied.

In all the four cultivars tested, neither washing nor germination over leachate media had any effect on percentage radicle emergence. A significant inhibitory effect of leachate medium on T50's could be seen in both control and washed seeds in most cases (Table 4.2). It was also interesting that decreases in T50's by washing of seeds, were completely reversed by subsequent germination over leachate media in the case of Karamu and the Australian cultivar. However such responses to leachate medium were only partial in cv. Rongotea and not found in cv. Pegasus. In the Australian cultivar, root growth was also inhibited by the leachate medium (Tab. 4.3). However such effects were not found in the other cultivars (data not shown).

Table 4.2 The effect of washing seeds for 2 h in water and/or germination over seed leachate on times to 50% radicle emergence (T50) of wheat seeds at 10°C.

T50's (h) at 10°C								
	Karamu 1987		Unknown (Australian Cultivar)		Rongotea		Pegasus	
	Distilled water medium	Seed leachate medium	Distilled water medium	Seed leachate medium	Distilled water medium	Seed leachate medium	Distilled water medium	Seed leachate medium
Control	65 ± 0.2	69 ± 0.6	70 ± 1.2	75 ± 0.7	77 ± 1.2	80 ± 0.6	91 ± 1.8	97 ± 1.6
Washed	62 ± 0.3	65 ± 0.5	64 ± 0.7	70 ± 0.6	69 ± 0.4	73 ± 0.6	78 ± 2.0	80 ± 1.2

Each value represents mean of four replicates ± standard errors.

Abbreviation: h = hours

Table 4.3 Effect of washing seeds and/or germination in seed leachate on the mean root length (5 d) of Australian cultivar germinated at 10°C.

Mean root length (mm)		
	Control (not washed)	Washed (2 h in water)
Distilled water medium	12 ± 0.4	14 ± 0.7
Seed leachate medium	6 ±0.2	7 ±0.3

Each value represents mean of 4 replicates ± standard errors.

Abbreviations: d = day; h = hours

### 4.3 DISCUSSION

#### 4.3.1 Responses of the Three Seed Lots to Accelerated Ageing Conditions

The AA technique is useful in producing a number of deteriorated sublots from a single seed lot in shorter periods of time than 'natural' ageing and facilitates simultaneous comparison of unaged with aged material under identical conditions. However evidence is accumulating that the events occurring during artificial and 'natural' ageing conditions may not be similar (see Sec. 2.7.1). The seed lot of Karamu 1987 appeared to be of high vigour as its normal germination was not affected by ageing for up to 6 days. The two year old Oroua 1985 had a poor germination and was confirmed to be of low vigour as its storability was poor compared to a freshly harvested lot of Oroua 1987. Ram and Wiesner (1988), who compared seed vigour in wheat by different tests including germination index and accelerated ageing, reported that the normal germination of an unaged high vigour seed lot of cv. Lew (97%) was not affected by exposure to AA (41°C, ~ 100% RH) for up to 6 days. On the contrary, the germination of a low vigour and low germinability (88%) seed lot dropped to 44% after exposure to similar ageing conditions.

Compared to changes in normal germination (Fig. 4.1), increases in T50's (radicle emergence) evident from the earliest ageing times in all the 3 seed lots (Fig. 4.2) were a better indication of the progress of deterioration (cf. similar findings by Dell'Aquila, 1987; Ram and Wiesner, 1988). There were differences in cultivar responses with respect to coleoptile growth, with the Oroua lots showing no response to AA (compare 4.2.1). It is possible that in some cultivars root and shoot may not be affected similarly by AA. Earlier ageing studies in wheat seeds, using short soaks in hot water at

58°C, have suggested that radicles may be more susceptible to damage than plumules (Bhattacharya *et al.*, 1985). Although Goldsworthy *et al.* (1982) reported decreases in coleoptile growth in response to controlled deterioration (50°C, 15% SMC) of wheat seeds, the class of seedlings evaluated (i.e. normals only or all germinants) was not stated. Because the seedlings were grown in the dark and did not photosynthesise, coleoptile growth may be limited by the rapid decrease in stored reserves during the later stages of development, when differences among lots of different vigour may not be apparent. Some evidence for this comes from Karamu 1987 where although aged seeds germinated at 10°C showed decreased coleoptile growth compared to unaged seeds, such effects were not apparent in more advanced seedlings germinated at 20°C (Sec. 4.2.1).

The use of seed leakage as a measure of age-induced damage to cell membranes and vigour loss is coming under close scrutiny as doubts have been raised that enormous leakage by the dead seeds in the population (Priestley, 1986), and/or by the large non-viable areas of storage tissue in the case of cereal grains, might mask small leakage changes due to ageing in the viable parts of seeds (see Sec. 2.6.1.2). Thus earlier attempts to relate vigour loss with seed leakage in wheat seeds during AA (Rudrapal and Basu, 1982; Ram and Wiesner, 1988) were not conclusive as changes in EC were always accompanied by losses in viability, suggesting a possibility that increased seed leakage might be only a post-mortem event. During the present study, neither vigour losses measured as T50's in Karamu 1987, nor losses in viability of the two Oroua lots during AA caused any significant change in seed leakage. This finding supports the suggestion that EC may not be a reliable test of vigour for wheat seeds. Any differences in the EC of leachate from unaged Oroua 1987 and naturally aged Oroua 1985 may simply be a function of the numbers of non-viable seeds. Further work is needed

in this area to elucidate the effects of 'natural'/slow and accelerated ageing conditions on seed leakage and vigour loss in wheat seeds. This topic will be explored in the next chapter of this thesis.

#### 4.3.2

#### Responses of Seeds to Short Soaking Treatments

During the present study several wheat cultivars showed only marginal invigoration effects in response to short soaking treatments (Sec. 4.2.2, 4.2.3 and 4.2.5) which appear to be of little practical value in improving the germination performance of deteriorated seeds. Although Goldsworthy et al. (1982) claimed that short soaking treatments are useful in invigoration of aged wheat seeds, their T50 results in an apparently unreplicated trial at a minimum germination temperature of 4°C (Mayer and Poljakoff-Mayber, 1989), which might be expected to amplify any differences, showed only small effects. Also their observations, collected at only daily intervals do not seem realistic for evaluating treatment differences amounting to no more than hours.

The small decreases in T50's in this study were found to be similar in both unaged and artificially aged Oroua 1987 (Sec. 4.2.3) which contradicts Goldsworthy et al.'s (1982) suggestion that invigoration effects can be found only in aged seeds. These authors did not, however, make a systematic comparison of responses of unaged with aged materials and the seed lots chosen were from undefined storage conditions. This adds to the previously mentioned ambiguities resulting from the fact that the authors did not replicate some trials sufficiently for statistical analysis and many reported effects were only marginal.

Measured invigoration effects appeared to be highly variable; for example compare the T50 decreases in Karamu 1987 shown in Fig. 4.4 with those in Table 4.1. A similar kind

of variability was observed by Goldsworthy et al. (1982) when they measured changes in response to different short soaking times. For example, in one of their experiments increases in coleoptile length observed after a 30 minute soaking treatment was not found with a 60 minute treatment. The authors omitted to make note of such crucial inconsistencies in treatment responses.

Extending soaking times to 12 h at 35°C during this study in an attempt to improve invigoration effects was not beneficial and, on the contrary, proved deleterious to low vigour seed. In addition to losses in germination capacity, the remaining viable seeds of both 6 d artificially aged seeds of Karamu or Oroua 1987 and naturally aged seeds of Oroua 1985 suffered losses in seed vigour measured as T50's, as a result of the treatment (Sec. 4.2.2 and 4.2.3). Goldsworthy et al. (1982) did not report any such effects on low vigour wheat seeds subjected to similar treatment for up to 6 h.

The optimal germination temperature reported for wheat by several workers falls within the range of 15 to 31°C and temperatures above 30°C may be damaging depending on cultivar (Mayer and Poljakoff-Mayber, 1989). ISTA (1985) has recommended 20°C for rapid and complete germination of wheat seeds. Thus it is doubtful if supra-optimal temperatures like the 35°C suggested by Goldsworthy et al. (1982) can be ideal for repair of aged seeds (see also Sec. 4.3.3) and is likely that these higher temperatures may retard the complex metabolic pathways and synthetic activity necessary for such processes (Koller, 1972).

#### 4.3.3

#### **Evaluation of the Suggestion of Physiological Repair (Goldsworthy et al., 1982) During Short Soaking Treatments**

In general improved germination performance of seeds by hydration treatments could be due to any of the following:

- (i) leaching of germination or growth inhibiting substances
- (ii) softening of the seed coat
- (iii) quenching of free radicals
- (iv) advances in germination processes
- (v) operation of physiological repair

Although most of these can operate on a reasonably short time scale, it is doubtful if physiological repair and advances in early germination processes (e.g. synthesis of enzymes, proteins, nucleic acids, etc.) are possible in such short imbibition times as appear effective. Although Goldsworthy et al. (1982) argued that age induced damage to membranes, DNA etc., are repairable by soaking for as little as 5 or 30 minutes, they provided no data to support this view. In their own study artificial aeration during soaking did not improve the invigoration effects, which probably suggests that active repair metabolism involving energy requiring synthetic processes may not operate on such short time scales. Also contrary to their suggestion that repair activity may be efficient at an 'ideal' temperature of 35°C, neither from their own study, nor from the present investigations was there any evidence that soaking at 35°C could be superior to 25°C (Table 4.1). Further, if repair processes were to operate efficiently at 35°C, then we might expect longer treatments to be more efficient and also gain better responses from aged as compared to unaged seeds. Such results were not obtained, as responses were small and similar in both unaged and aged seeds (Sec. 4.2.2 and 4.2.3).

On the other hand, work done by the same group (Fielding and Goldsworthy, 1982) showed some indication that wheat

seeds may contain volatile compounds inhibitory to coleoptile growth. One of the simplest ways of testing the presence of germination inhibiting substances is by imbibing seeds in inhibitor extract (Ketring, 1973). In the present study there was some evidence for the presence of germination inhibitory substances in all of the cultivars tested (Sec. 4.2.5). In cv. Karamu and the Australian cultivar germination times for washed seed in leachate were the same as unwashed controls in water. For cv. Rongotea the inhibitory effects of leachate were less marked. This may be due to differences in absorption efficiency of inhibitory substances or possible loss of volatile inhibitory substances from seed leachate. The lack of response to leachate by washed seeds of cv. Pegasus may possibly suggest that other alternative mechanisms are also involved, like for example softening of the seed coat by the washing treatment. These details including possible interactive effects of seed ageing were not investigated further during the course of this study but may be a useful area for future research.

## CHAPTER 5

### EVALUATION OF DIFFERENT PRE-SOWING HYDRATION TREATMENTS FOR THE PROTECTION OR REPAIR OF STORED WHEAT SEEDS, CV. KARAMU

#### 5.1 INTRODUCTION

As short hydration treatments proved to be of little use for the invigoration of aged wheat seeds (Chapter 4), it was decided to make a general evaluation of some of the most promising pre-sowing hydration treatments reported in the literature. As most of the earlier work was confined to unaged seeds (e.g. Hanson, 1973; Lush *et al.*, 1981) there is no clear information about the usefulness of pre-sowing hydration treatments for invigoration of aged wheat seeds. Further, systematic studies have not been conducted to determine the relative efficiency of various types of pre-sowing treatments for protection of wheat seeds in storage. The few reports available are controversial (see Sec. 2.8.2). Hence the aim of this study was to compare different treatment effects before or after the storage of seeds.

Earlier workers evaluated ageing effects and treatment responses using either very rapid or relatively slow, artificial ageing systems. As the process of seed deterioration may not be the same under different storage conditions (see Sec. 2.7.1), it was decided to compare harsh accelerated ageing conditions with a less severe controlled deterioration with respect to both ageing effects and treatment responses. This detailed study was conducted on a single high quality seed lot of wheat, cv. Karamu obtained harvest fresh in 1988.

The ageing conditions and pre-sowing treatments used in this study are as follows (for details see Sections 3.2. and 3.3).

#### **Ageing conditions**

- (i) Accelerated ageing (AA) at 40°C, 100% RH for up to 6 days.
- (ii) Controlled deterioration (CD) at 35°C, 15% SMC for up to 50 days.

#### **Pre-sowing treatments**

(before or after exposure of seeds to ageing conditions)

- (i) 2 h soaking in distilled water at 25°C
- (ii) 24 h hydration in distilled water at 15°C
- (iii) 20 h hydration in -0.37 MPa Polyethylene glycol (PEG) solution at 20°C.

After the above treatments seeds were dried back to approximately original moisture contents in the range of 13 to 14%.

## 5.2 RESULTS

### 5.2.1 **The Effects of Accelerated Ageing or Controlled Deterioration on Karamu 1988 Wheat Seeds**

Increases in times to 50% radicle emergence (T50's) began to occur from the earliest ageing times under both ageing regimes (Fig. 5.2), whereas normal germination capacity began to decline only after exposure to 30 days controlled deterioration (Fig. 5.1A) or 6 days accelerated ageing conditions (Fig. 5.1.B). In contrast to significant decreases in the mean root dry weights (dwt's) of normal seedlings after 50 days, there was no effect on the dwt's of shoots as a result of exposure to controlled deterioration conditions for up to 50 days (data not shown). However significant losses in both root and shoot dwt's of normal seedlings were evident under the much more severe accelerated ageing conditions after 4 and 6 days respectively (Fig. 5.3).

It was interesting to note that changes in electrical conductivity (EC) of seed leachate as a result of ageing seeds by the two methods were quite different (Fig. 5.4). Despite the fact that losses in germination (Fig. 5.1) were of a similar magnitude, significant increases in seed leakage were found only during controlled deterioration of seeds, and not in the shorter accelerated ageing treatment.

### 5.2.2 **The Effects of Different Pre-sowing Hydration Treatments on Unaged Karamu 1988 Wheat Seeds and Interactions with Ageing.**

#### 5.2.2.1 *Effect on unaged seeds*

None of the hydration treatments had any influence on the germination capacity of unaged seeds (Tab. 5.1). Both longer hydration treatments, 24 h in water and 20 h in PEG solution,

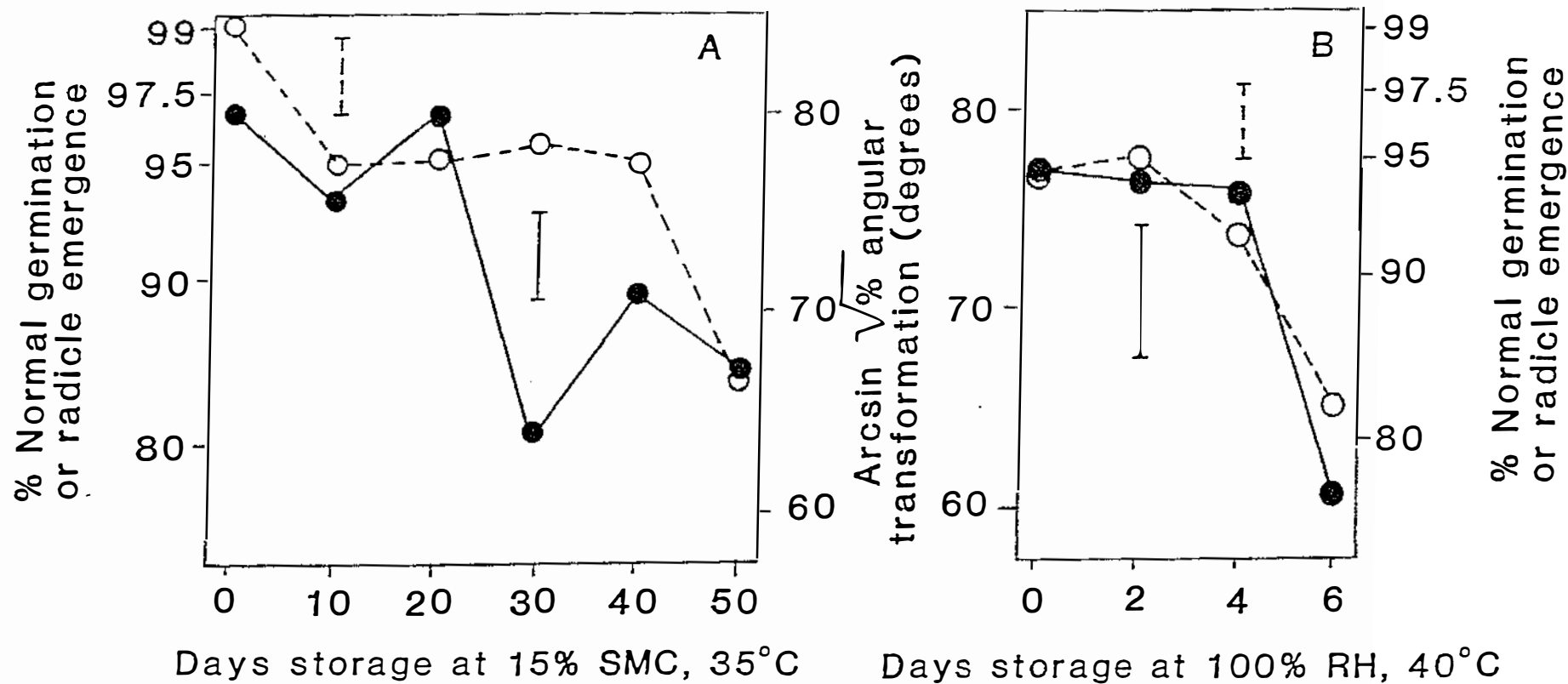


Figure 5.1 A, B. Final percentage (square root arcsin transformed values) radicle emergence (O - - - O) and normal germination (●—●) of wheat seeds, cv. Karamu at 10°C after exposure to two different storage regimes. Vertical bars represent combined estimates of standard errors of differences between means (15 and 9 degrees of freedom, respectively).

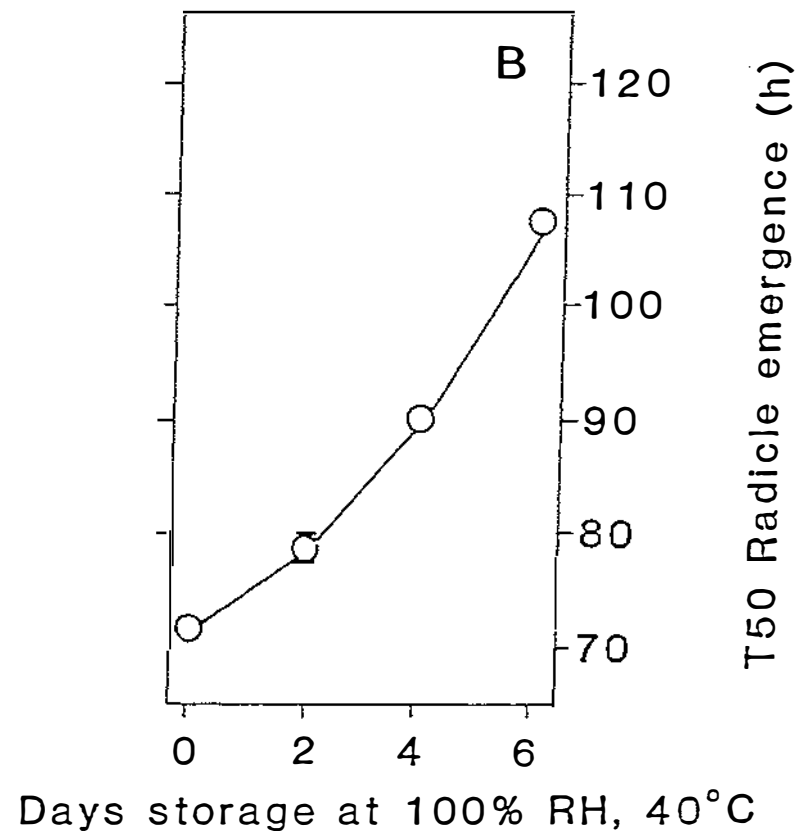
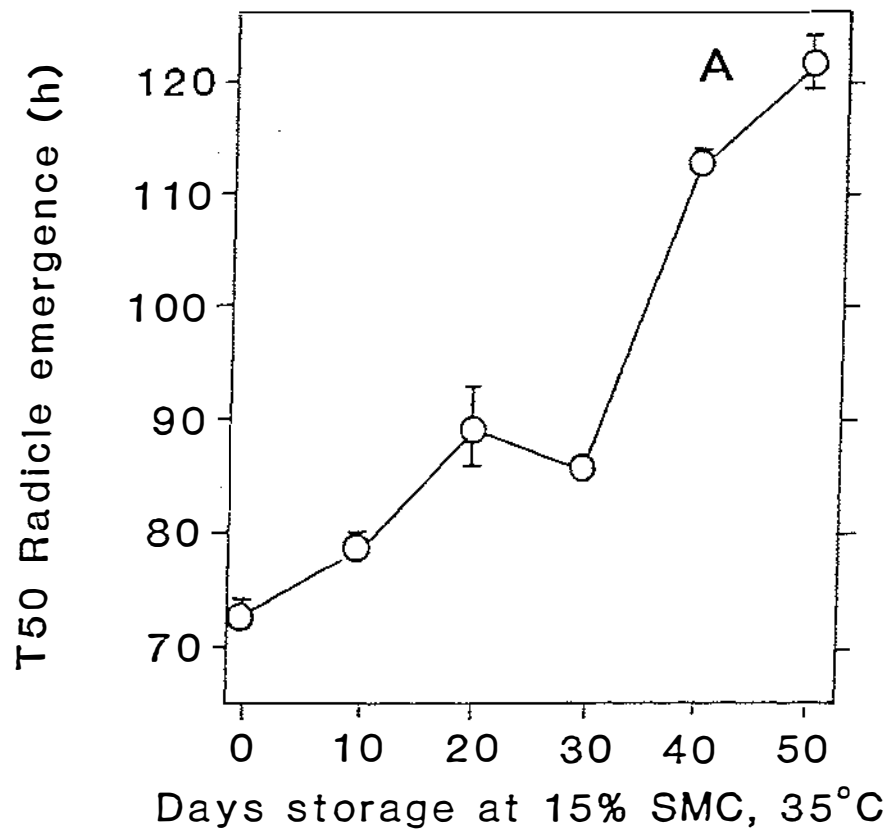


Figure 5.2 A, B. Times to 50% radicle emergence (T50) of wheat seeds, cv. Karamu at 10°C after exposure to two different storage regimes. Data presented are means of four replications and vertical bars representing SE's are shown where larger than the symbols used.

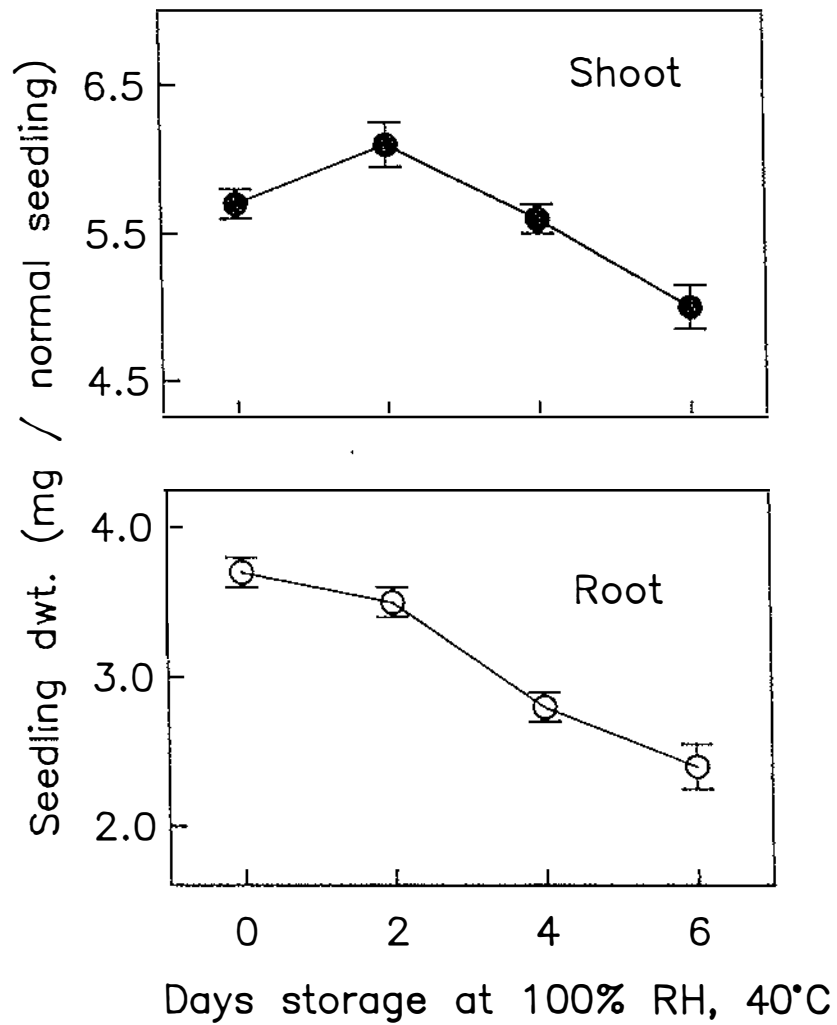


Figure 5.3 The effect of accelerated ageing on mean dry weights of shoots (●) and roots (O) of normal seedlings germinated at 10°C. Data presented are means of four replications and vertical bars represent SE's.

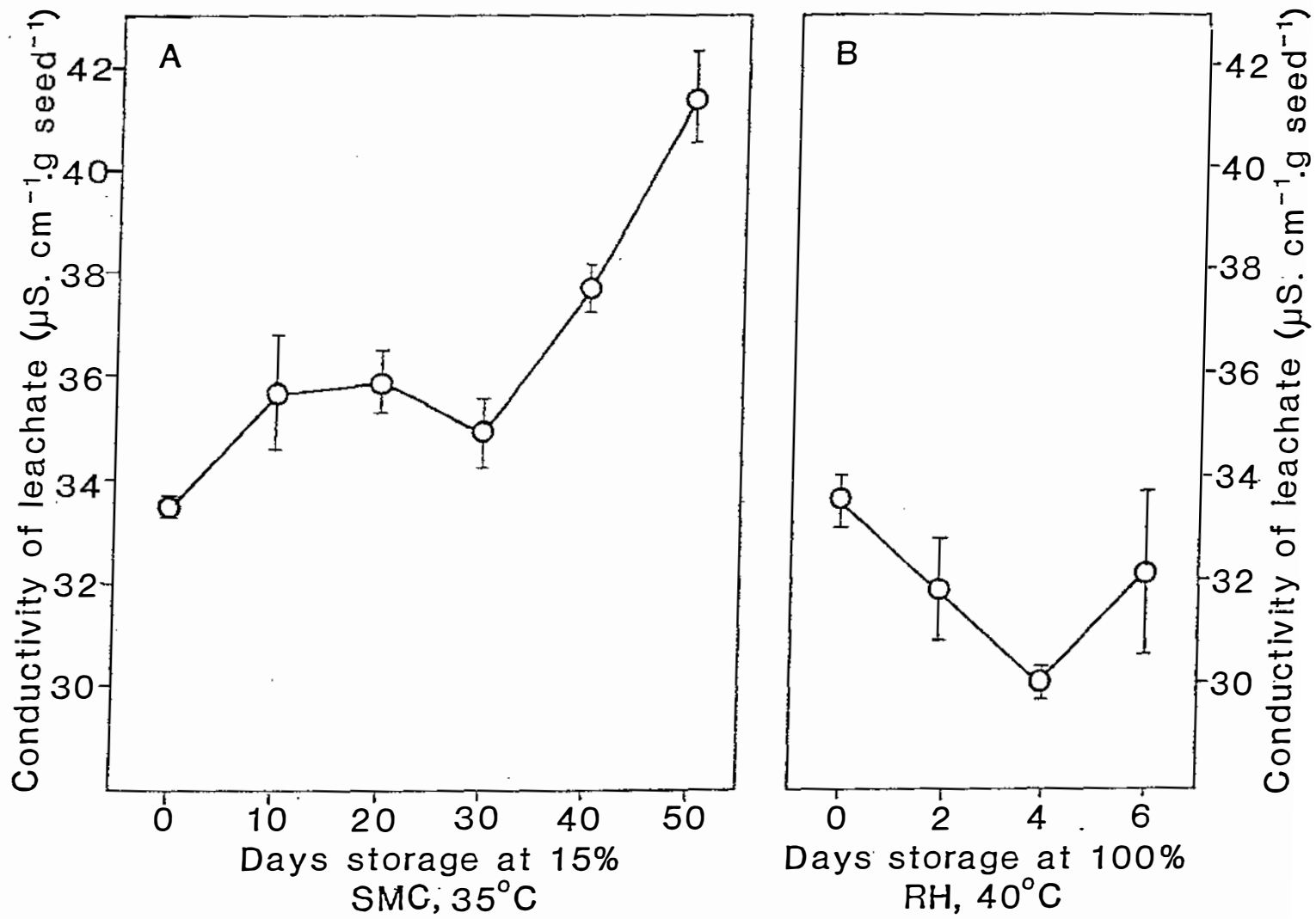


Figure 5.4 Electrical conductivity of seed leachate from wheat seeds, cv. Karamu stored under two different ageing regimes. Data presented are means of four replications; vertical bars represent SE's.

Table 5.1 Effects of different hydration treatments on the vigour and electrical conductivity of unaged wheat seeds, cv. Karamu. (All seeds were dried after treatments). Data are means of four replications. (Figures in brackets denote standard errors).

Treatment	Radicle emergence*	Normal germination*	Time to 50% Radicle emergence (h)	Seedling growth test after 15 days at 10°C		Electrical conductivity of leachate ( $\mu\text{S}\cdot\text{cm}^{-1}\cdot\text{g seed}^{-1}$ )
				<u>Dry weight roots</u> (mg/normal seedling)	<u>Dry weight shoots</u>	
Untreated Control	76.8 ( $\pm 3.05$ )	77.1 ( $\pm 4.96$ )	73 ( $\pm 1.3$ )	3.7 ( $\pm 0.10$ )	5.7 ( $\pm 0.09$ )	33.5 ( $\pm 0.51$ )
2 h Imbibition at 25°C	81.0 ( $\pm 3.45$ )	77.2 ( $\pm 1.22$ )	69 ( $\pm 0.8$ )	4.0 ( $\pm 0.13$ )	5.6 ( $\pm 0.05$ )	15.2 ( $\pm 0.36$ )
24 h Hydration at 15°C	85.1 ( $\pm 2.92$ )	81.8 ( $\pm 4.74$ )	46 ( $\pm 1.0$ )	4.1 ( $\pm 0.10$ )	6.3 ( $\pm 0.16$ )	17.9 ( $\pm 0.44$ )
20 h Hydration in PEG at -0.37 MPa, 20°C	83.1 ( $\pm 2.45$ )	79.2 ( $\pm 4.16$ )	48 ( $\pm 1.2$ )	4.1 ( $\pm 0.09$ )	6.4 ( $\pm 0.07$ )	17.5 ( $\pm 0.48$ )

Note: \* Arcsin  $\sqrt{\%}$  angular transformed data presented in degrees.

Abbreviations: h = hours;  $\mu\text{S}$  = microsiemen.

caused large and significant ( $p < 0.001$ ) decreases in times to 50% radicle emergence, whereas the 2 h soaking treatment produced only marginal effects. The dry weights (dwt's) of both roots and shoots of normal seedlings grown at 10°C were significantly ( $p < 0.05$ ) increased by the two longer hydration treatments compared to untreated controls. All hydration treatments caused large decreases in solute leakage from treated seeds.

#### 5.2.2.2

#### *The effects of pre-sowing hydration treatments applied to seeds before exposure to ageing conditions*

The 2 h imbibition treatment offered some protection against deterioration in both harsh and less severe storage regimes (Fig. 5.5). Seeds treated this way maintained a significantly higher percentage radicle emergence after 40 and 50 d CD or 4 and 6 d AA in comparison to untreated controls. The normal germination capacity was also similarly protected by the treatment under both the ageing conditions. For example, the normal germination capacity of 2 h hydrated seeds, after exposure to different periods of controlled deterioration was significantly higher ( $p < 0.05$ ) by up to 12% compared to similarly aged untreated controls (data not shown). The results were basically similar under the much more rapid AA conditions. However the 2 h treatment did not protect the vigour of stored seeds when this was measured as T50, shoot dry weight (data not shown) or root dry weight (Fig. 5.6).

In complete contrast, the two longer hydration treatments dramatically increased the rates of deterioration of stored seeds (Fig. 5.5). There was a total loss of germinability in treated seeds after 40 d controlled deterioration and less than 15% germination after 6 days rapid ageing at 100% RH and 40°C. Also the remaining germinable seeds from the treated lots exhibited damage, with their T50's showing

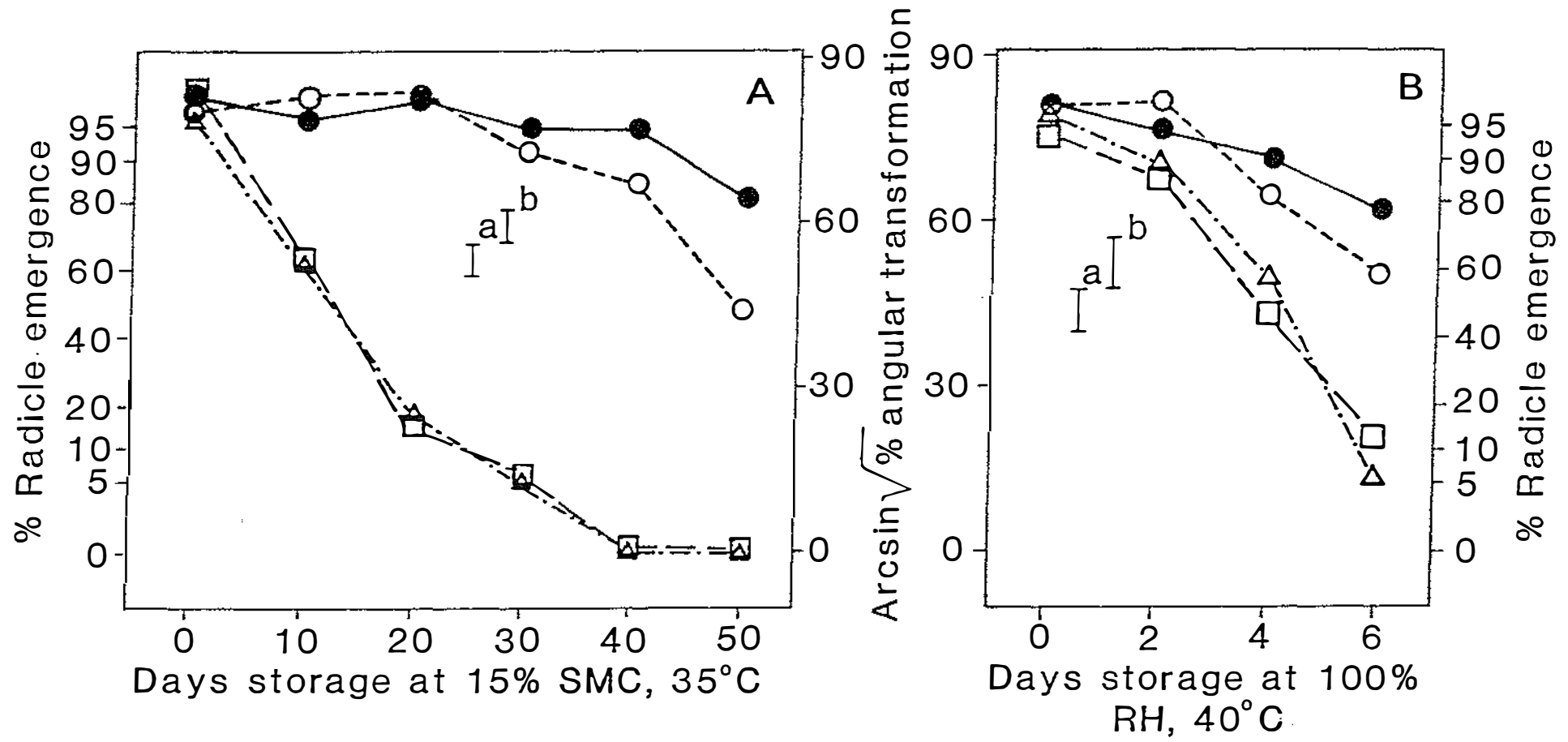


Figure 5.5 A, B. The effect of pre-storage hydration treatments on percentage radicle emergence of wheat seeds, cv. Karamu, stored under two different ageing regimes. O, untreated controls; ●, imbibed for 2 h in distilled water at 25°C; Δ, imbibed for 24 h in distilled water at 15°C; □, imbibed for 20 h in -0.37 MPa PEG at 20°C. a, b are LSD's for means (a) within ageing times and (b) between ageing times, (p = 0.05) are shown. Each data point is the mean of four replications.

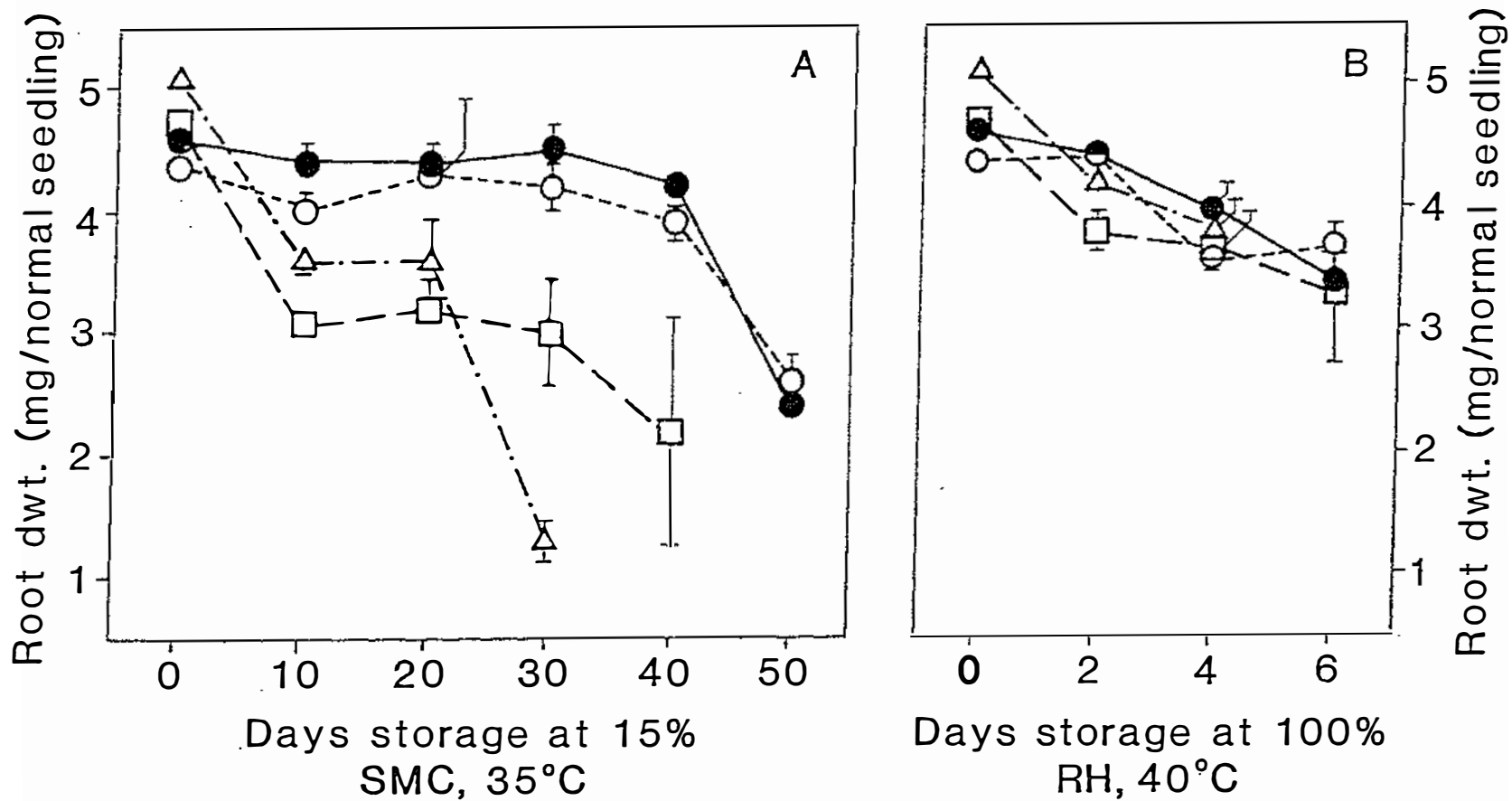


Figure 5.6

A, B. The effect of pre-storage hydration treatments on mean root dry weights of normal seedlings from wheat cv. Karamu stored under two different ageing regimes. Each data point is the mean of four replications. Individual SE bars are shown where larger than the symbols used. O, untreated controls; ●, imbibed for 2 h in distilled water at 25°C; Δ, imbibed for 24 h in distilled water at 15°C; □, imbibed for 20 h in -0.37 MPa PEG at 20°C.

drastic increases over untreated controls after storage under either of the conditions (data shown for CD in Tab. 5.2). Under the less severe CD conditions, decline in seedling root dry weights tended to follow changes in germination capacity for the two longer hydration treatments (Fig. 5.6A), but after exposure to the much harsher AA conditions following treatment, normal seedlings did not exhibit impaired root growth compared to similarly aged untreated controls (Fig. 5.6B). The electrical conductivity of leachates from seeds given the two longer hydration treatments increased rapidly as germination capacity fell under both ageing regimes (Fig. 5.7).

#### 5.2.2.3 *The effects of pre-sowing hydration treatments applied to seeds after exposure to ageing conditions*

When applied after storage, none of the hydration-dehydration treatments had any effect on the germination capacity of the seeds (data not shown). The 2 h soaking treatment resulted in a small reduction in the T50 of aged seeds (Figs 5.8 A and B), but under more severe AA conditions this disappeared after 4 d. In contrast the benefits of longer hydration treatments are much more evident for both storage regimes. T50's of seeds treated after various periods of controlled deterioration at 35°C were close to or better than that of unaged controls. As the duration of ageing increased, the PEG treatment emerged as consistently the more effective of the two longer hydration methods.

The longer hydration treatments induced a significant increase in shoot growth over untreated control seeds stored under high humidity AA conditions (Fig. 5.10), but there were no significant changes in shoot dwt's of normal seedlings resulting from treatment of seeds aged under the much lower moisture controlled deterioration conditions (Fig. 5.9). However root growth from seeds exposed to either

Table 5.2 The effect of different pre-storage hydration treatments on the median radicle emergence times (T50) of wheat seeds cv. Karamu stored at 35°C and 15% SMC. Data are means of four replications (Figures in parenthesis represent standard errors).

	Days ageing at 35°C and 15% SMC					
	0	10	20	30	40	50
Untreated Control	72 (±0.9)	89 (± 1.3)	86 (± 1.0)	99 (±1.1)	137 (±2.0)	121 (±4.9)
2 h Imbibition at 25°C	66 (±0.5)	85 (± 1.4)	83 (± 1.2)	93 (±2.5)	137 (±3.5)	117 (±3.4)
24 h Hydration at 15°C	46 (±0.9)	225 (±30.3)	223 (±51.2)	- *	-	-
20 h Hydration in PEG at -0.37 MPa, 20°C	49 (±1.4)	313 (±23.4)	304 (±28.1)	-*	-	-

Note: \* T50's not shown for seed lots having less than 10% germination.

Abbreviations: SMC = seed moisture content; PEG = polyethylene glycol; MPa = mega pascal.

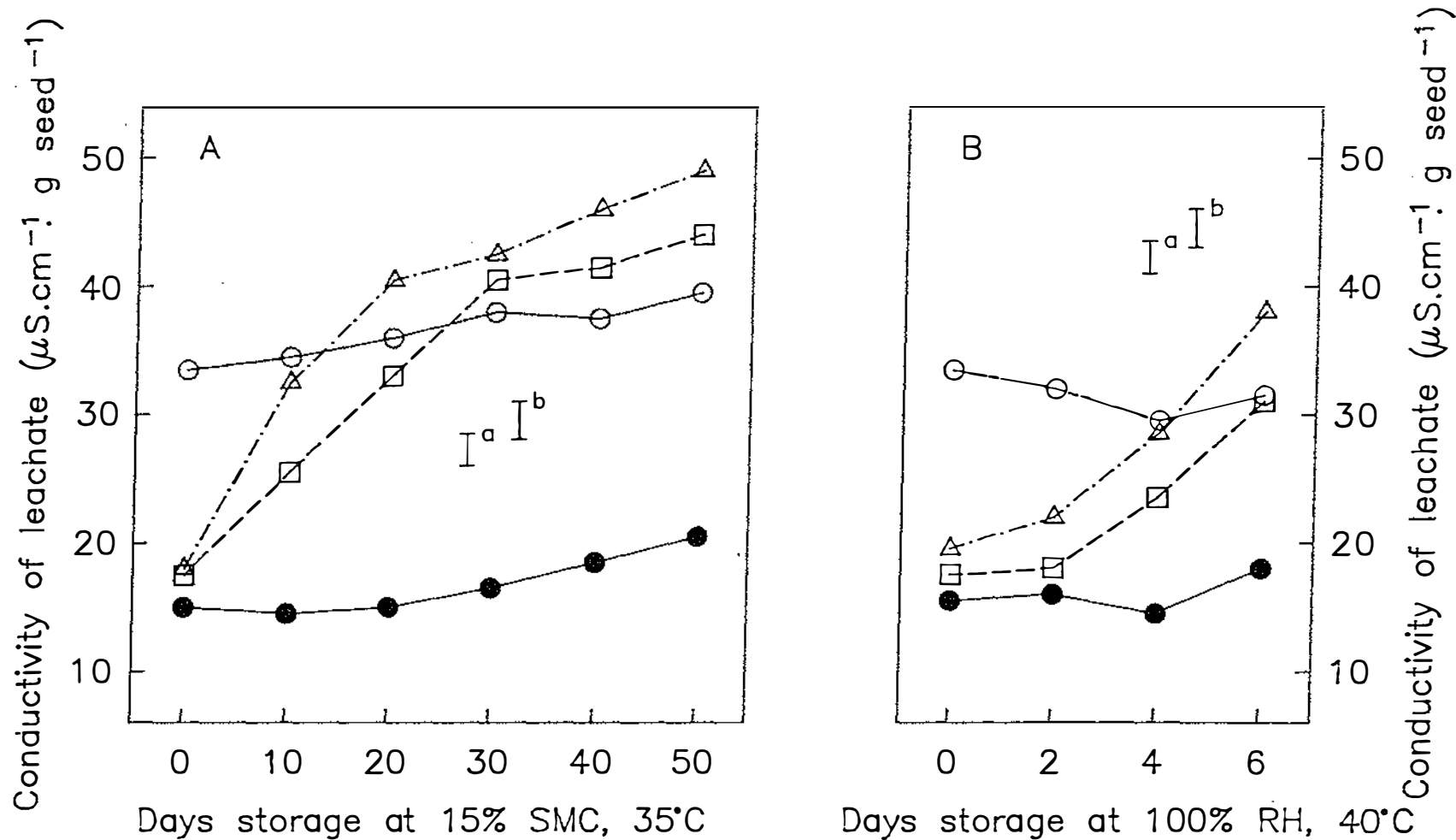


Figure 5.7 The effect of pre-storage hydration treatments on the electrical conductivity of seed leachate from wheat seeds, cv. Karamu, stored under two different ageing regimes. O, untreated controls; ●, imbibed for 2 h in distilled water at 25°C, Δ, imbibed for 24 h in distilled water at 15°C; □, imbibed for 20 h in -0.37 MPa PEG at 20°C. a, b are LSD's for means, (a) within ageing times and (b) between ageing times, calculated at  $p = 0.05$  level. Each data point is the mean of four replications.

Figure 5.8 The effect of different post-storage hydration treatments on the median radicle emergence time (T50) of wheat seeds cv. Karamu held under two different ageing regimes. O, untreated controls; ● imbibed for 2 h in distilled water at 25°C; △, imbibed for 24 h in distilled water at 15°C; □, imbibed for 20 h in -0.37 MPa PEG at 20°C. Each data point is the mean of four replications. Individual SE bars are shown where larger than the symbols used.

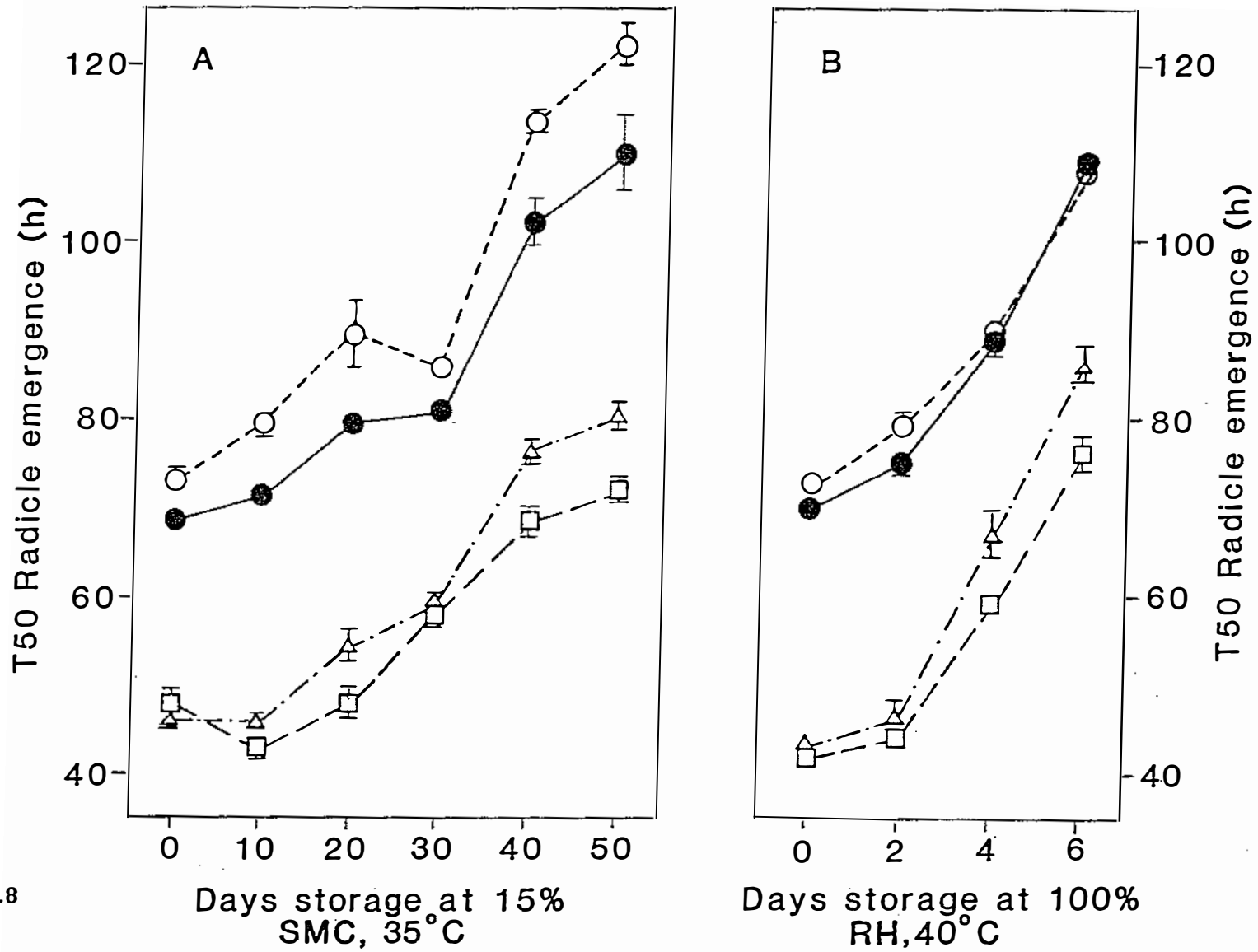


Fig. 5.8

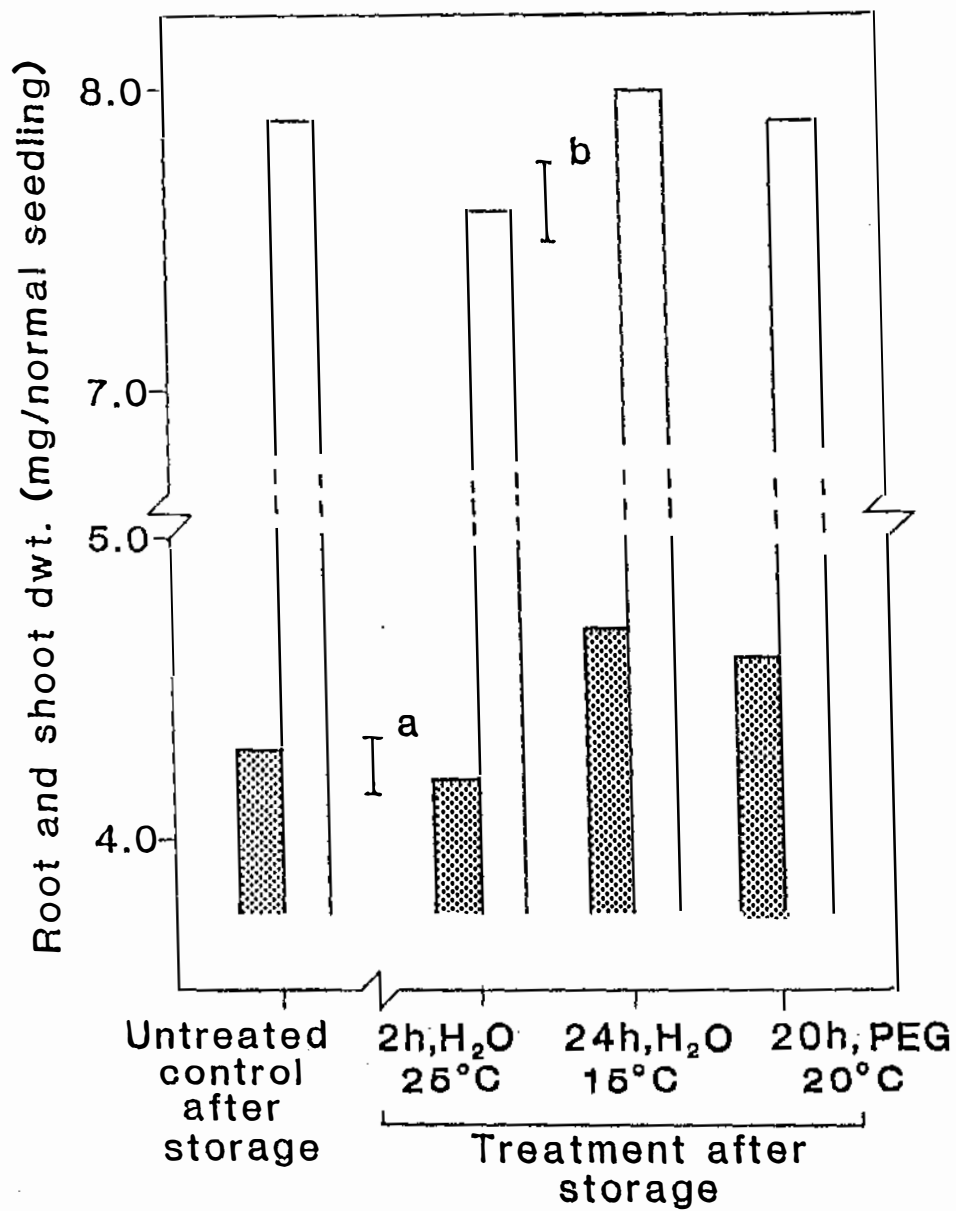


Figure 5.9

Root (▨) and shoot (□) dry weights of normal wheat seedlings cv. Karamu grown from seeds aged at 15% SMC, 35°C for up to 50 d and the effect of post-ageing treatments. There were no significant interactions between treatment and duration of ageing. So data presented are overall means of six ageing times (four replications for each treatment). a, b are LSD's calculated at  $p = 0.05$  level for roots and shoots respectively.

Figure 5.10 The effect of different post-storage hydration treatments on the dry weights of (A) shoot and (B) roots of normal seedlings from seeds aged at 40°C and 100% RH for up to 6 days. O, untreated controls; ●, imbibed for 2 h in distilled water at 25°C; △, imbibed for 24 h in distilled water at 15°C; □, imbibed for 20 h in -0.37 MPa PEG at 20°C. Each data point is the mean of four replications and vertical bars representing standard errors are shown where larger than the symbols used.

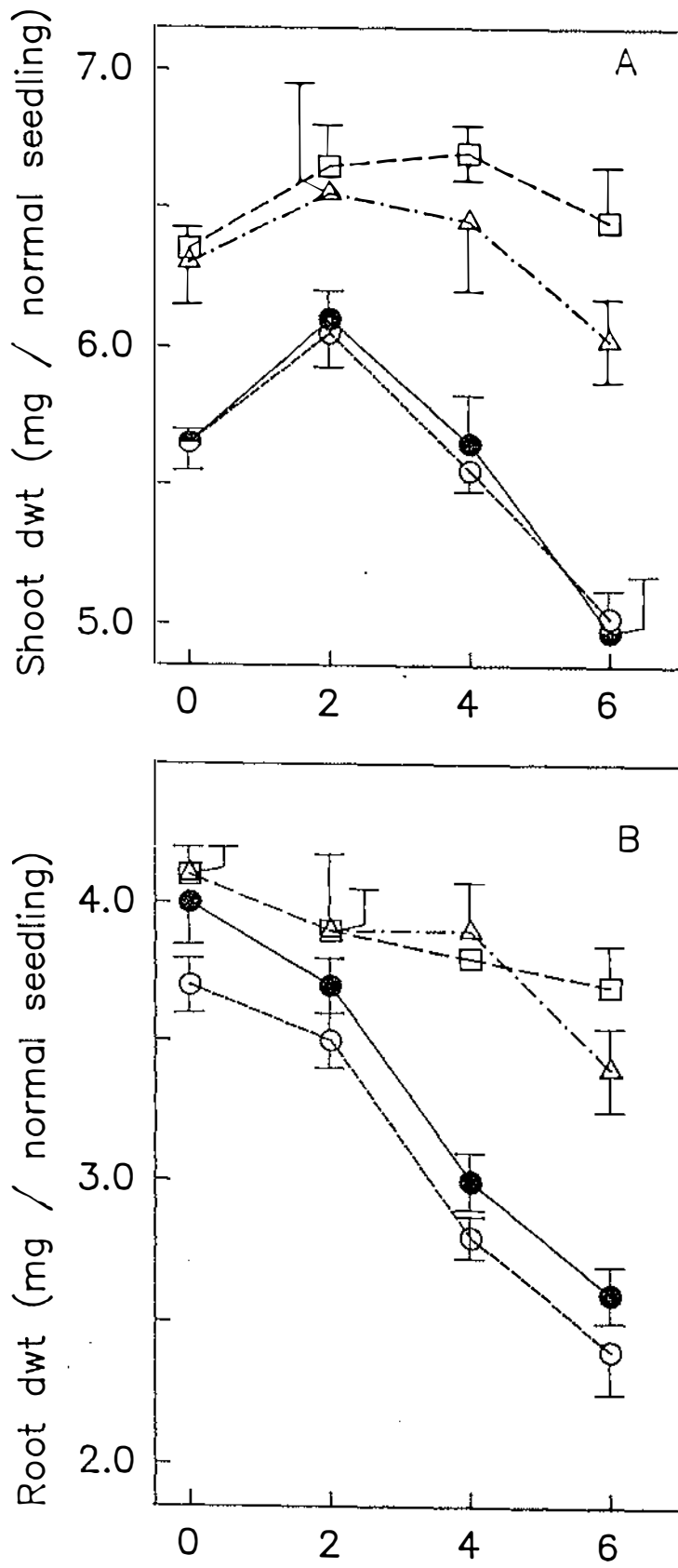


Fig. 5.10 Days storage at 100% RH, 40°C

of the storage regimes (Figs 5.9, 5.10) was significantly improved by the two longer hydration treatments.

Significant increases in conductivity of leachate from untreated control seeds as a result of exposure to CD conditions, disappeared after subjecting both unaged and aged seeds to longer hydration treatments (Tab. 5.3). On the other hand, high humidity AA conditions did not affect leakage of control seeds, as we have already seen (Sec. 5.2.1) but after the two longer hydration treatments, aged seeds showed a small but significant reduction in conductivity of seed leachate compared to similarly treated unaged seeds (Tab. 5.3).

Table 5.3 Changes in the electrical conductivity of leachate from wheat seeds, cv. Karamu, subjected to different hydration treatments after storage under two different ageing regimes.

All data are the means of four replications  $\pm$  standard errors.

	Controlled deterioration, 15% SMC, 35°C		Accelerated ageing, 100% RH, 40°C	
	Od <sup>a</sup>	50d	Od	6d
	$\mu\text{S. cm}^{-1}.\text{g seed}^{-1}$			
Untreated	33.5 $\pm$ 0.19	41.4 $\pm$ 0.88	33.5 $\pm$ 0.51	32.1 $\pm$ 1.56
2 h Imbibition at 25°C	15.5 $\pm$ 0.27	22.7 $\pm$ 1.30	15.2 $\pm$ 0.36	15.7 $\pm$ 0.93
24 h Hydration at 15°C	19.3 $\pm$ 0.49	19.7 $\pm$ 0.52	17.9 $\pm$ 0.44	13.8 $\pm$ 0.39
20 h Hydration in PEG at -0.37 MPa, 20°C	17.5 $\pm$ 0.69	17.8 $\pm$ 0.72	17.5 $\pm$ 0.48	14.4 $\pm$ 0.82

<sup>a</sup> Seeds equilibrated at 15% SMC, but not placed at 35°C

Abbreviations: SMC = seed moisture content; h = hours; RH = relative humidity; d = day;  $\mu\text{S}$  = microsiemen; MPa = megapascal; PEG = polyethylene glycol

## 5.3

## DISCUSSION

## 5.3.1

**Responses of Wheat Seeds, cv. Karamu, to Different Storage Regimes**

The pattern of changes in T50's and germination capacity under both AA and CD conditions seems to be in agreement with the earlier suggestion by Ellis and Roberts (1981), that losses of seed vigour precede losses in viability during storage. However, differences in T50's and normal germination between the two storage regimes, when the germinability (radicle emergence capacity) begins to decline (Figs 5.1, 5.2. Compare 40 d CD and 4 d AA), does not support their suggestion that seed death is a continuum of discrete deterioration events expressed as losses in vigour. Coolbear et al. (1984) working on deteriorated tomato seeds instead argued that seed deterioration is a matrix of inter-related events each of them being susceptible to different environmental constraints.

In the present study the 2 h hydration treatment prior to storage was found to slow down losses in germination (Fig. 5.5) without protecting seed vigour (Sec. 5.2.2.2). On the contrary, despite their apparently high vigour levels (measured as T50's), unaged seeds subjected to longer hydration treatments (Tab. 5.2) suffered severe losses in both vigour and germination (Fig. 5.5) in storage compared to similarly aged control seeds. This is clearly opposed to the general observation that seeds with higher vigour have a prolonged storage life (Roberts, 1986; Matthews, 1980; Matthews and Powell, 1987). These results provide additional support to the earlier suggestion by Coolbear et al. (1984) that the mechanisms governing germination and aspects of vigour loss may not be the same (cf. Alvarado and Bradford, 1988, for a similar discussion on priming effects in tomato seeds).

Recently there has been a growing awareness among workers that the physiological and biochemical changes that occur in seeds during slow, 'natural' ageing at lower seed moisture levels may not be simulated by rapid artificial ageing at higher moisture regimes (see Sec. 2.7.1). In the present study differences in the relative patterns of decline in radicle emergence and normal germination capacity under the two sets of storage conditions (Figs 5.1, A and B), support reservations about the validity of using rapid ageing techniques for studying the metabolism of seed deterioration (Abdul-Baki and Anderson, 1970; Bewley, 1986; Priestley, 1986). This fear seems to be strengthened by contrasting changes in the electrical conductivity of leachate from seeds under the two storage regimes (Figs 5.4, A and B). Similar kinds of differences were observed by Abdul-baki and Anderson (1970) with respect to leakage of sugars from naturally and artificially aged barley seeds. A very highly significant ( $P < 0.001$ ) positive correlation between T50's and EC changes (Fig. 5.11) suggests possible damage to cell membranes during low moisture controlled deterioration of seeds. However such a relationship did not exist under high moisture AA conditions, where T50's increased without significant changes in seed leakage. It is possible that the higher seed moisture content attained by seeds stored at 100% RH and 40°C may allow some degree of membrane re-organisation and repair. Ward and Powell (1983) reported that onion seeds stored at 25% SMC, 45°C showed slower rates of deterioration compared to those at 15% SMC, 45°C. In the present study, wheat seeds attained moisture levels of up to 25% during accelerated ageing at 100% RH and 40°C (Tab. 3.2).

Under the more severe accelerated ageing system, damage to coleoptile growth of normal seedlings was evident (Fig. 5.3), suggesting these conditions are either capable of impairing synthetic mechanisms required for growth or disrupting the associated reserve mobilization required for that growth

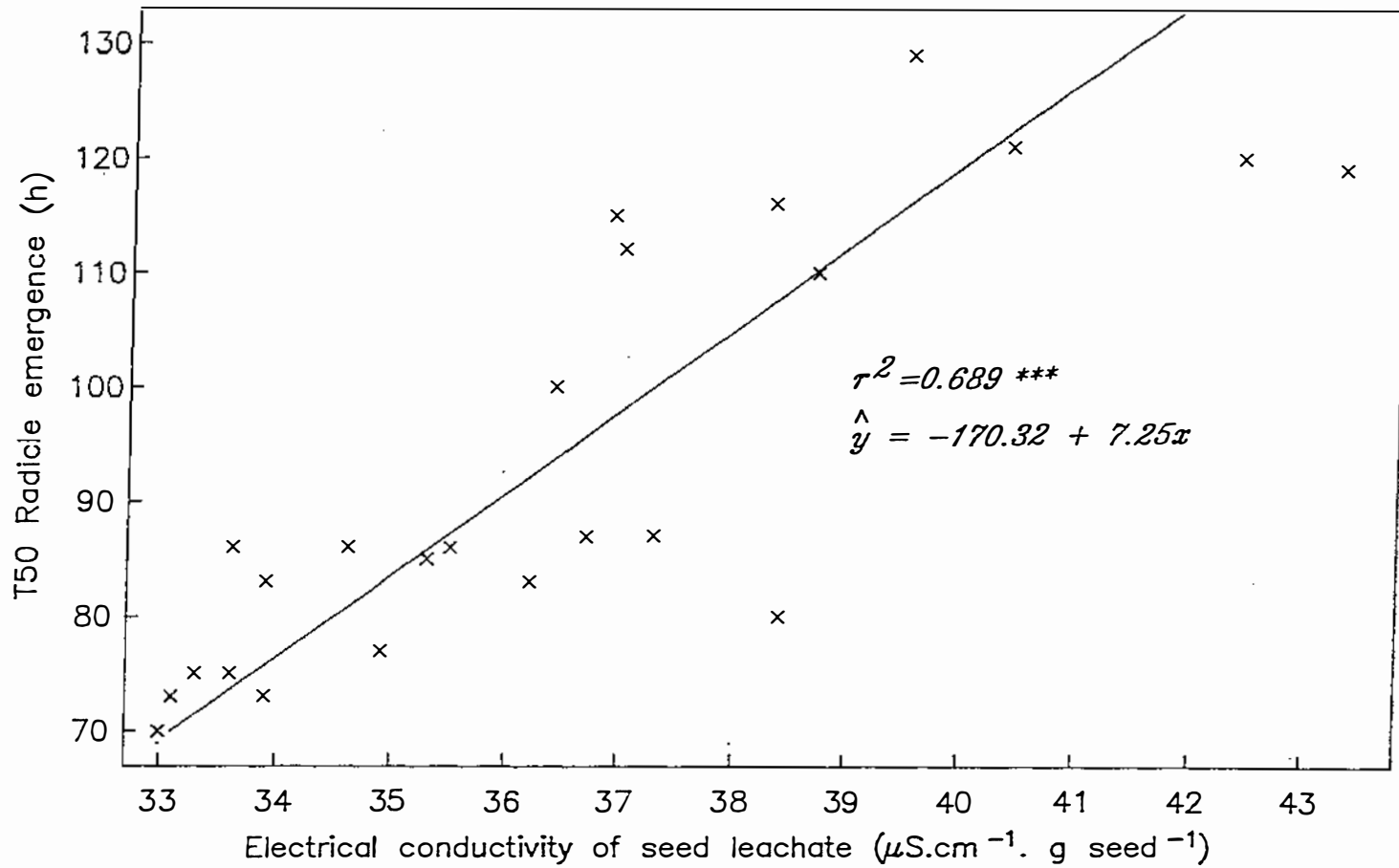


Figure 5.11 The relationship between electrical conductivity and times to 50% radicle emergence as a function of controlled deterioration at 35°C and 15% SMC.

in otherwise healthy seeds. However, there was no evidence of damage to shoot growth of normal seedlings under less severe CD conditions (Sec. 5.2.1). There were also differences between the two ageing conditions with respect to seedling growth when seeds were subjected to longer hydration treatments prior to storage (Fig. 5.6, A and B).

### 5.3.2

#### **Effect of Different Hydration Treatments on Unaged and Deteriorated Wheat Seeds, cv. Karamu**

The responses to different hydration treatments show that longer hydration times are required for obtaining a clear improvement in the germination performance of wheat seeds. Contrary to the deleterious effects of 12 h hydration at 35°C (Chapter 4, Sec. 4.3.2), these results show that temperatures of 15 to 20°C are suitable for longer hydration treatments of wheat seeds. Considerable advancement in the germination (Tab. 5.1) of unaged seeds by the two longer hydration treatments confirm earlier findings for wheat by Hanson (1973) and Lush *et al.* (1981). Also this study has demonstrated that the longer treatments have potential for improving the vigour of aged wheat seeds (Figs 5.8, 5.9 and 5.10), an effect which has been shown earlier for vegetable crops (cf., for example, Coolbear *et al.*, 1984; Dearman *et al.*, 1986). This result also demonstrates that, the benefits reported by Dell'Aquila's group for aged wheat seeds given hydration treatments without subsequent drying (Dell'Aquila *et al.*, 1984; Dell'Aquila, 1987), are retained after drying back. After the experiments in this thesis were completed, the same group (Dell'Aquila and Tritto, 1990) have reported that the benefits of osmotic priming in PEG solution were partially retained upon drying seeds to 15% seed moisture content. In the present study a 20 h PEG treatment was found to produce maximum invigoration effects in unaged seeds of cv. Karamu (see Fig. 3.1), whereas Dell'Aquila's group used a 36 h treatment for cv. Appulo under similar

temperature and osmotic conditions. Thus it is possible that the optimal hydration times may vary depending on the cultivar.

One of the aims of pre-sowing treatments is to advance germination time by allowing completion of early germination processes before sowing (Heydecker and Coolbear, 1977). In the present study reduction in T50's of both unaged and 10 or 20 d aged seeds (Fig. 5.8A) to similar levels by the PEG treatment, suggest that repair metabolism may also operate in aged seeds in addition to advances in germination processes. In comparison to better responses from 50 d aged seeds (Fig. 5.8A), however, seeds aged for 6 days under harsh AA conditions responded poorly to the longer hydration treatments, relative to the treatment effects on unaged seeds (Fig. 5.8B). It is possible that differences in the nature of damage under the two sets of ageing conditions may be the probable cause of such effects and that the damage sustained after 6 d AA is partly irreversible.

The additional effectiveness of PEG treatment at 20°C over distilled water at 15°C, can be explained on a day-degree imbibition basis, if it is assumed that any water stress imposed by PEG is insignificant. Thus PEG treated seeds receive 13.3 d.°C imbibition compared to 11.0 d.°C for distilled water treated seeds, if the minimum functional temperature for germination of wheat grains is taken as 4°C (Mayer and Poljakoff-Mayber, 1989). However the additional advantage of PEG treatment over 24 h hydration was found only in aged but not in unaged seeds. For example compare T50 decreases in 50 d aged (Fig. 5.8A) or 6 d aged seeds (Fig. 5.8B) with those of unaged seeds. This probably suggests that physiologically deteriorated seeds may need longer hydration times compared to unaged seeds. Dearman *et al.* (1986) also reported similarly that extending priming treatment from 10 to 14 days produced larger decreases in mean germination times of artificially aged onion seeds, whereas such additional benefits were not found in unaged seeds.

Treatment induced changes in seedling growth after storage seem to be largely a function of advancement of radicle emergence. For example, increases in root dry weight as a result of treatment were closely correlated with median radicle emergence times (T50's) for both storage regimes ( $r = -0.852^{**}$  for 35°C, 15% SMC;  $-0.901^{**}$  for 40°C, 100% RH). It is interesting, however, that shoot growth of normal seedlings from seeds stored at 35°C, was not correspondingly increased by the longer term hydration treatments (Fig. 5.9). This supports the earlier suggestion by Das and Sen-Mandi (1988) that root growth is indeed more plastic than shoot growth. Their results showed that root growth suffers preferential damage as compared to shoot growth, during exposure to accelerated ageing conditions; whereas unaged wheat seeds germinate by radicle protrusion, the deteriorated seeds produce radicles after the emergence of plumules.

### 5.3.3

#### **Responses of Wheat Seeds, cv. Karamu, to Hydration Treatments Given as Protection Before Storage**

Although effects of the short hydration treatment on unaged seeds are marginal (Tab. 5.1), they have a clear protective value on germinability if given prior to ageing (Fig. 5.5), a confirmation of earlier Indian work in wheat (Basu, 1976; Rudrapal and Basu, 1982) and in other crop seeds, as for example rice, sunflower, chillies and carrot (Basu *et al.*, 1975). As discussed before (see Sec 4.3.3) leaching of germination inhibiting substances and quenching of free radicals are some of the possible events that might occur in seeds soaked for shorter periods. As cv. Karamu has been found to contain germination inhibitory metabolites, it may be speculated that leaching of these during short soaking might be the cause of protective effects in stored seeds. However, reports of similar protective effects obtained by a 24 h moisture equilibration of wheat seeds (Rudrapal and Basu,

1982) do not support this view, unless this treatment allowed time for these compounds to diffuse from their site of action. Despite confusion in the literature (Priestley, 1986), one of the prime candidates for age-induced seed deterioration is possible damage caused by dangerous chain reactions initiated by free radicals. Although the nature and levels of free radicals in seeds are poorly understood (Priestley, 1986), radiation induced free radical levels have been shown to decline due to hydration of seeds (Conger *et al.*, 1969). Accordingly, Rudrapal and Basu (1982) suggested that quenching of free radicals by hydration treatments could be the possible cause of reduced lipid peroxidation and the accompanied protective effects in stored wheat seeds. Extension of storability of maize seeds exposed to an electric field also indicates a possible role for free radicals in seed deterioration (Pammenter *et al.*, 1974). However literature on changes in free radical levels in stored seeds has led to some confusion, mainly because of differences in responses obtained under different storage conditions and the problems in relating free radical levels to actual damage in seeds (Priestley, 1986). Thus clear explanations on the mechanism(s) of protection of stored seeds by the short hydration treatments are yet to come.

In contrast, both longer hydration treatments, applied before storage, were severely detrimental to the germination capacity under both storage regimes (Figs 5.5, A and B). This was unexpected in view of successes with vegetable seeds using broadly equivalent systems (Coolbear *et al.*, 1984; Dearman *et al.*, 1986), although recent work of Bradford's group (Alvarado and Bradford, 1988; Argerich *et al.*, 1989) has shown that primed tomato seeds have reduced storability despite improved germination rates. However, for wheat, there are some conflicting reports with respect to storability of seeds hydrated for longer periods. Whereas a 24 h moisture equilibration protected germination capacity (Rudrapal

and Basu, 1982), a 36 h (Dell'Aquila and Tritto, 1990) or 48 h (Lush et al., 1981) hydration had no effect or caused severed damage respectively in stored seeds (see Section 2.8.2). Such discrepancies have been suggested to be due to possible differences in the type of germinative metabolism advanced under various treatment conditions (Argerich et al., 1989).

Under both storage regimes, seeds hydrated for longer periods exhibited drastic increases in leakage (Figs 5.7, A and B) suggesting either increased levels of solutes or damage to membranes or both. One of the major changes observed during seed germination is an increase in the activity of several enzymes. It is possible that the increase in leakage of treated seeds may be mediated by hydrolytic activity, considered to be involved in seed deterioration (cf., Perl et al., 1978). Hanson (1973) has shown early induction of  $\alpha$ -amylase activity (and by inference other hydrolases) in germinating wheat grains as a result of pre-sowing treatment. Similarly in oats there was an early increase in the proteolytic activity during subsequent imbibition of treated seeds (Berrie and Drennan, 1971). Although increases in enzyme activity related to reserve mobilization and synthetic processes are likely to be essential for rapid germination of treated seeds, they are highly undesirable in stored seeds. Within the embryo, enzyme mediated breakdown of essential substrates or damage to cellular compartmentalization can be lethal, while reduction in seedling growth of normal seedlings from seeds treated for longer periods before storage (Figs 5.6, A and B) suggests possible damage either to reserve mobilization or to synthetic processes. The final chapters of this thesis report studies on some aspects of this problem.

## CHAPTER 6

### CHANGES IN SEED PROTEINS AND PROTEOLYTIC ACTIVITY DURING AGEING OR PRE-SOWING TREATMENT OF WHEAT SEEDS

#### 6.1

#### INTRODUCTION

Wheat seeds subjected to longer hydration treatments showed considerable advances during subsequent germination, but deteriorated faster in storage (Chapter 5). Berrie and Drennan (1971) reported that pre-sowing treatment of oat grains induces early protease activity during subsequent imbibition. Thus it was speculated that changes in and/or possible loss of control over proteolytic enzyme activity might be involved in the damage of treated-stored seeds.

Initially, the appearance of the endosperm of untreated and treated grains was examined using a scanning electron microscope. Following this, qualitative changes in seed proteins were assessed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Although it is difficult to assess overall changes in seed proteolytic enzyme activity by using a single substrate (see Chapter 2, Sec. 2.5.3) an attempt was made using 'Azocoll', a commercial dye-labelled protease substrate. In order to assess quantitative changes in seed proteins, high-performance liquid chromatography (HPLC) analysis of supplied material was conducted by the Wheat Research Institute, Department of Scientific and Industrial Research, Christchurch, New Zealand (Details of methods and results in Appendices, 2, 3 and 4).

Seeds subjected to the analysis reported in this chapter were aged by controlled deterioration (35°C, 15% SMC) for up to 60 days while a 20 h PEG treatment (imbibing seeds in

-0.37 MPa PEG solution at 20°C, followed by drying back), being the most effective for invigoration of aged seeds, was used as a pre-sowing treatment either before or after ageing (details of methods in Chapter 3).

## 6.2 RESULTS

### 6.2.1 Scanning Electron Microscope (SEM) Studies of the Starchy Endosperm

Because of limited resources, SEM examination of the starchy endosperm was confined to only the scutellum-crease region, this being chosen as MacGregor and Matsuo (1982) had reported initiation of protein matrix degradation in this part of the endosperm in sprouting damaged wheat seeds. The starchy endosperm of unaged-untreated seeds (Plate 6.1A) showed no changes after 40 days storage (Plate 6.1B). Starch granules could not be seen clearly in either of these preparations as they were embedded in a dense layer of protein matrix. However, when unaged or aged seeds were subjected to the pre-sowing treatment (after storage), this region showed some scattered patches where starch was clearly visible with well defined margins (compare Plates 6.1A and B, and Plates 6.2A and B respectively). This suggests that some degradation of the surrounding matrix proteins had occurred. There was, however, no evidence of further changes to matrix proteins during the subsequent storage of unaged-treated seeds (Plate 6.2C).

### 6.2.2 Studies on Qualitative Changes in Seed Proteins Using SDS—Polyacrylamide gel electrophoresis (SDS—PAGE)

Plate 6.3 shows changes in total seed proteins as influenced by ageing and/or pre-sowing treatment of wheat seeds. Exposure of untreated wheat seeds to controlled deterioration

Plate 6.1

Scanning electron micrographs (x 1500) of the starchy endosperm of unaged and aged (40 days storage at 35°C, 15% SMC) wheat seeds. The endosperm was scanned in the region near scutellum-crease junction with special attention on locations showing apparent changes to matrix proteins.

6.1 A, unaged seeds (untreated)

6.1 B, 40 d aged seeds, (untreated)

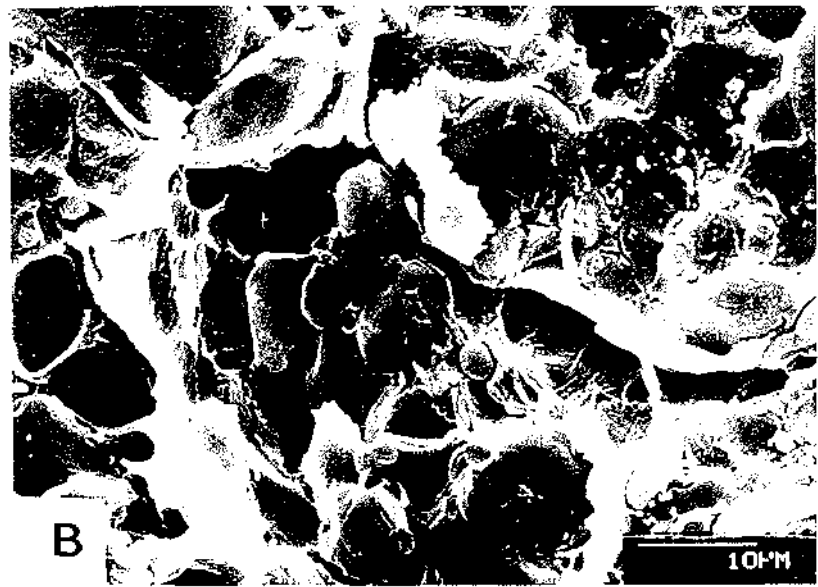
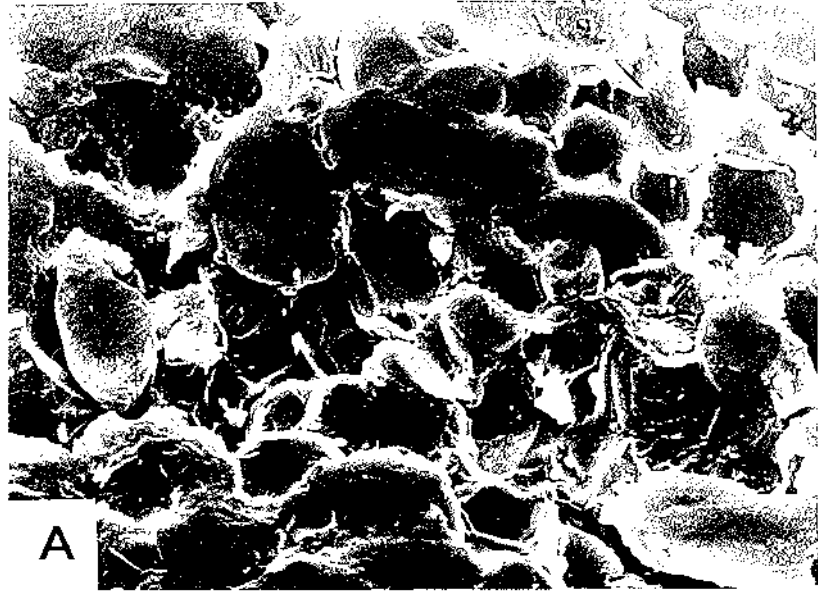


PLATE 6.1

Plate 6.2

Scanning electron micrographs (x 1500) of the starchy endosperm of wheat seeds subjected to pre-sowing treatment (20 h hydration in -0.37 MPa PEG solution at 20°C followed by drying-back), before or after storage (40 days at 35°C and 15% SMC). The endosperm was scanned in the region near scutellum-crease junction with special attention on locations showing apparent changes to matrix proteins.

6.2 A, unaged seeds, treated

6.2 B, 40 d aged seeds, treated after storage

6.2 C, 40 d aged seeds, treated before storage.

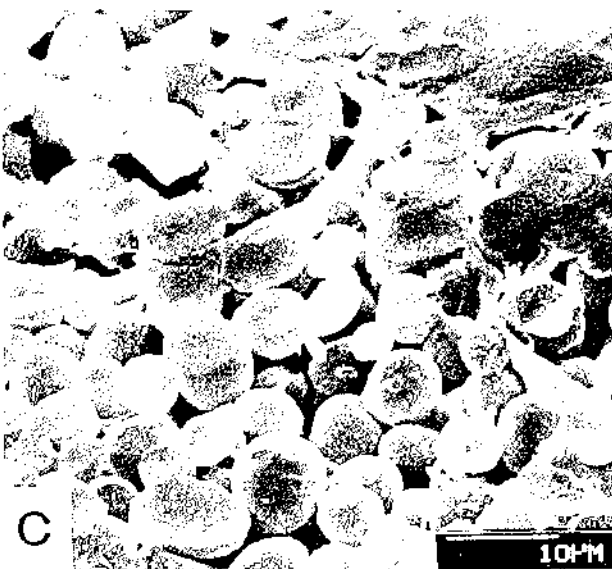
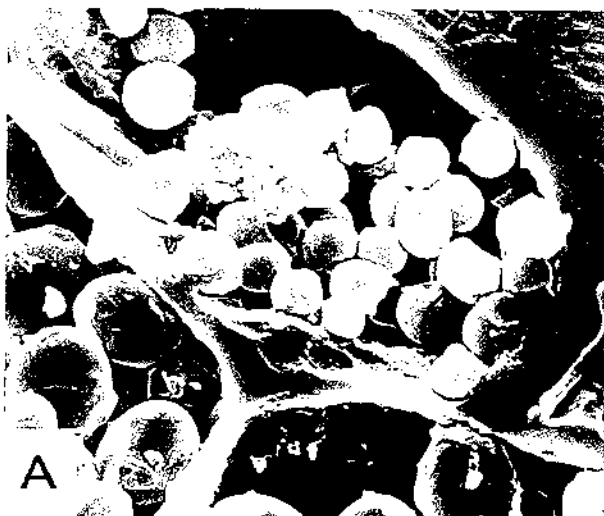


PLATE 6.2

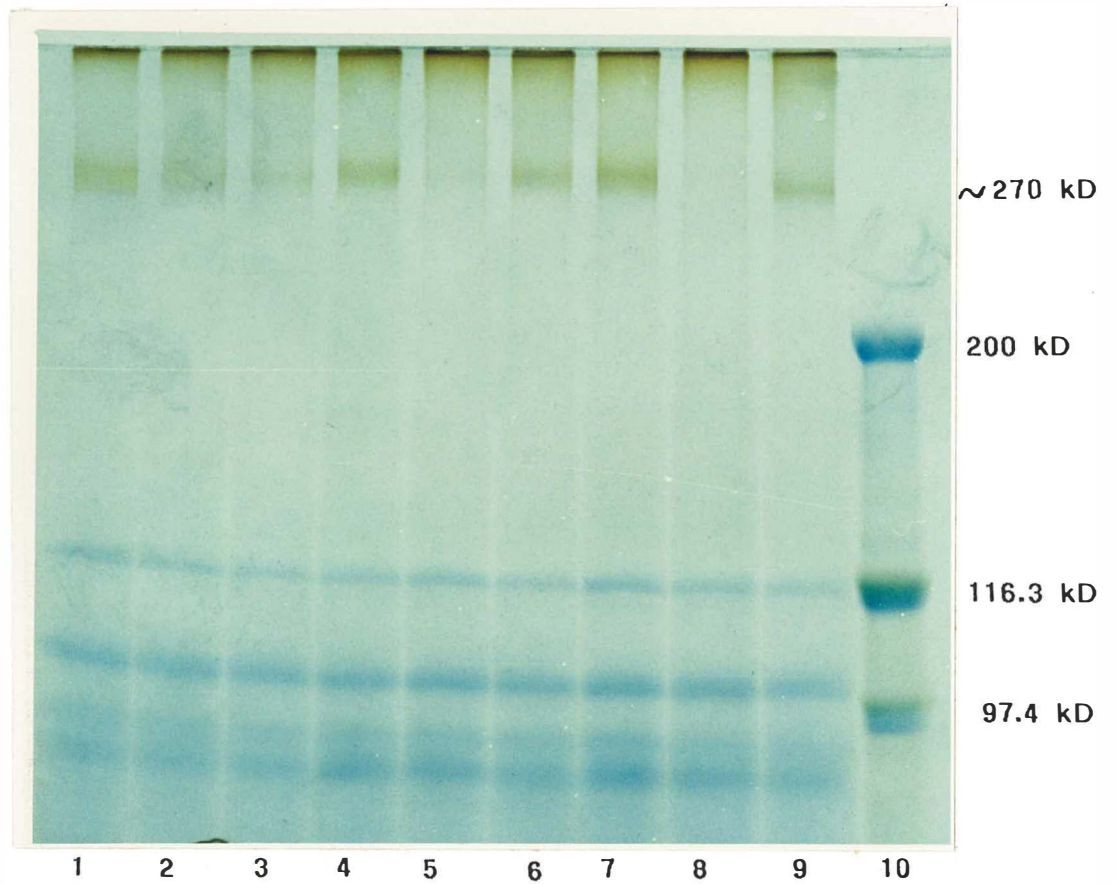


Plate 6.3 SDS-PAGE of total seed proteins using 7.5% gel and a dual staining process (Silver stain-Coomassie blue). Changes in protein banding patterns as influenced by ageing (20 or 40 d controlled deterioration at 35°C and 15% SMC) and/or pre-sowing treatment (20 h hydration in -0.37MPa PEG solution at 20°C followed by drying back) of wheat seeds. The sample details of each track are as follows:

	1	Untreated control
Unaged	2	Treated
	3	Treated
	4	Untreated control
20 d Aged	5	Treated before ageing
	6	Treated after ageing
	7	Untreated control
40 d Aged	8	Treated before ageing
	9	Treated after ageing
	10	Molecular weight standard (kilo Dalton)

at 35°C and 15% SMC for 20 or 40 days does not produce any apparent changes in the protein banding pattern (Plate 6.3, compare lane 1 with lanes 4 and 7). Also when unaged and 20 or 40 d aged seeds were subjected to pre-sowing treatment (treatment after ageing), there were no qualitative changes (Plate 6.3, compare lane 1 with 2, 3; lanes 4 and 7 with lanes 6 and 9 respectively). However, when unaged-treated seeds were exposed to subsequent ageing, there was an apparent loss of a protein band with an estimated molecular weight (mwt) of 270 kilo Dalton (kD) (Plate 6.3 lanes 5 and 8, representing 20 and 40 d aged seeds respectively). ☆

### 6.2.3 **Studies on Quantitative Changes in Seed Proteins Using High Performance Liquid Chromatography (HPLC)**

HPLC analysis for seed proteins was conducted on submitted samples by the Wheat Research Institute, Christchurch, New Zealand. Changes in the four high molecular weight (HMW) polypeptide subunits of glutenins as a result of pre-sowing treatment and/or 40 d slow ageing are shown in Appendix 3. Both treatment and subsequent storage of seeds appeared to cause losses in all the four HMW glutenin subunits. However, a second HPLC run, using freshly treated and aged samples albeit from the same original seed lot, did not show differences in HMW glutenins during storage of treated seeds (Appendix 4).

### 6.2.4 **Changes in the Azocoll Hydrolysing Activity of Seeds in Response to Ageing or Pre-sowing Treatment of Seeds**

Table 6.1 shows changes in azocoll hydrolysing activity of wheat seeds as a result of controlled deterioration (15% SMC and 35°C) or pre-sowing treatment of differently aged seeds. Despite high variation between replicates, clear trends emerge. There was a significant drop in enzyme activity of untreated seeds after 60 d controlled deterioration when almost all the seeds were dead. Pre-sowing treatment did not influence

☆ Apparent differences shown in the photograph in the intensity of bands from lanes 1 to 3 have been disregarded as these were not consistent between the three experimental replications of seed extracts.

Table 6.1 Changes in the azocoll hydrolysing activity due to ageing and/or pre-sowing treatment (20 h hydration in -0.37MPa PEG at 20°C followed by drying-back) of wheat seeds. Extracts were prepared from ungerminated seed and incubated with azocoll for 16 h at pH 6.8 (details in Sec. 3.8.3). The data represent changes in absorbance at 520 nm, due to hydrolysis of azocoll; OD. seed<sup>-1</sup>, reaction volume 5 ml. Data are means of 3 replicates ± Standard errors and figures in parenthesis represent % radicle emergence capacity of the seed samples.

	Days ageing at 35°C and 15% SMC			
	0	20	40	60
Control	1.62 ± 0.056 (98%)	1.33 ± 0.218 (95%)	1.22 ± 0.449 (95%)	0.36 ± 0.248 (1%)
Treated before ageing	1.61 ± 0.083 (98%)	1.79 ± 0.198 (6%)	0.71 ± 0.504 (8%)	1.79 ± 0.062 (1%)
Treated after ageing	1.61 ± 0.083 (98%)	1.23 ± 0.314 (98%)	0.77 ± 0.450 (93%)	0.80 ± 0.187 (2%)

Abbreviations: h = hour; MPa = megapascal; PEG = polyethylene glycol;  
OD = optical density; SMC = seed moisture content.

enzyme activity either in unaged or aged seeds when treated after storage. However, when seeds were treated before exposure to ageing the enzyme activity levels remained high even after 60 days ageing.

### 6.3 DISCUSSION

#### 6.3.1 **Changes in Protease Activity (Azocoll Substrate) and Seed Proteins During Ageing of Wheat Seeds**

Azocoll hydrolysing activity of ungerminated seeds was not significantly affected until germination losses occurred during storage (Tab. 6.1). Thus decline in azocoll hydrolysing activity after 60 d controlled deterioration is likely to be a post-mortem event. Although germination data were not provided in their paper, Skupin and Warchalewski (1971) reported that the protease activity measured against bacto-haemoglobin substrate, decreased gradually to less than 50% at the end of a 13 month storage of wheat seeds. In contrast, protease activity of sorghum seeds was shown to increase during storage at 30°C and 17% SMC, which caused a 10% decrease in germination capacity (Gelmond *et al.*, 1978). This was suggested to be the cause of damage to other enzyme proteins (Perl *et al.*, 1978).

In the present study, examination of the starchy endosperm near the scutellum-crease region (Plates 6.1 A and B) and SDS-PAGE studies (Sec. 6.2.2) did not show evidence of qualitative changes to seed proteins during storage of untreated wheat seeds. Protein changes in the embryo, if any, are likely to be masked when assessed along with the large amounts of storage proteins of the starchy endosperm. In future work it might be worthwhile to investigate protein changes separately in the embryo as they might be more closely related to vigour and/or viability losses in seeds.

#### 6.3.2 **Changes in Seed Proteins and Protease Activity (Azocoll Substrate) Due to Pre-sowing Treatment of Unaged and Aged Seeds**

The apparent losses of matrix proteins in the starchy endosperm of unaged or aged seeds due to pre-sowing treatment (Plate 6.2),

suggests the initiation of storage protein degradation. But owing to limited resources, the examination of starchy endosperm was necessarily confined to a small area near the scutellum-crease region and hence the extent of these changes is not clear. HPLC studies, however, did not show major quantitative changes in gliadins and glutenins (Appendix 4) which largely make up the storage proteins in the starchy endosperm of wheat seeds (Pernollet and Mosse, 1983). This perhaps suggests that appreciable hydrolysis of storage proteins may not occur as a result of pre-sowing treatment of wheat seeds. In parallel with these findings for treated seeds (Plate 6.2A and B), sprouting damaged wheat seeds also show protein matrix degradation near the scutellum-crease region (MacGregor and Matsuo, 1982). In vitro studies by Fincher and Stone (1974) have demonstrated that degradation of cell walls and protein matrix occur earlier than degradation of starch during hydrolysis of wheat endosperm. As the storage proteins in the starchy endosperm of wheat grains are not completely surrounded by membrane bound bodies (Pernollet and Mosse, 1983), and thus comprise matrix protein (Hess, 1954), they appear more vulnerable to proteolytic degradation. However an early degradation of these proteins and cell walls in the starchy endosperm would seem essential for both efficient degradation of starch and its mobilization to the embryo.

The absence of any significant change in azocoll hydrolysing activity of treated seeds (Tab. 6.1 compare enzyme activities in unaged and aged controls with those treated after ageing) also suggests that significant proteolysis may not occur during pre-sowing treatment of wheat seeds. The relative insensitivity of the assay method may prevent it detecting small localised changes in enzyme activity, while the possibility of changes occurring in other protease enzymes, not detected by azocoll substrate cannot be ruled out. Although comparable references are few, Preston et al. (1978) found no changes in the levels of protease activity (azocasein substrate, pH 6.0) of wheat

seeds during either a 24 h steeping treatment or subsequent germination for 24 h at 18.5°C. As already mentioned, however, a preliminary study by Berrie and Drennan (1971) in oats showed increases in seed protease activity (edestin substrate) as a result of pre-sowing treatment.

Further work seems necessary to confirm, both qualitatively and quantitatively, the extent of protein degradation in the starchy endosperm during pre-sowing treatment of wheat seeds. In such studies it must be remembered that any enzyme extraction from seeds causes de-compartmentalization and possibly break-up or formation of enzyme-inhibitor complexes and hence the estimated activity may not truly represent in situ activity. In this regard, the usefulness of histochemical localization techniques (e.g. Okamoto et al., 1980) and immunological tests for assessing in situ enzyme activity needs evaluation.

### 6.3.3

#### **Effects on Seed Proteins and Protease Activity Due to Treatment of Seeds Prior to Storage**

In contrast to the significant drop in azocoll hydrolysing activity after 60 d storage of untreated seeds (Tab. 6.1), the enzyme levels appear to be maintained in seeds treated before storage, despite severe losses in germination capacity. Although reasons for this are not known, it may be speculated that some mechanisms responsible for the control of enzyme activity are lost as a result of pre-sowing treatment and subsequent ageing of wheat seeds.

Although unreduced glutenins representing the high molecular weight (HMW) proteins of wheat have mwts ranging over a million (Sec. 2.5.1), their constitutive HMW polypeptides have reported mwts in the range 68 - 133 kD (Bietz and Wall, 1972). Under the reducing conditions of the electrophoretic analysis used in this study (see Sec. 3.8.1), it would be expected

that the glutenin polypeptides (HMW) disaggregated, but the protein band observed at 270 kD (Plate 6.3) seems to represent a partially reduced or unreduced HMW glutenin residue fraction. In the literature, SDS-PAGE studies normally do not show this protein band either because this HMW residue fails to enter the resolving gel (when the pore size is small) or because it does not stain by the commonly used coomassie blue staining technique, which is much less sensitive than the silver staining technique (Switzer et al., 1979) also used in this study. The problem of resolving unreduced protein (when 1%  $\beta$ -mercaptoethanol was excluded from the extraction buffer) fractions of wheat has been highlighted by Lukow and Bushuk (1984) who found heavy staining at the slots and extensive streaking of protein in the high molecular weight region of gels.

Apparent loss of this protein band ( $\sim$ 270 kD) during storage of treated seeds (Plate 6.3 lanes 5 and 8) may simply indicate breakdown of glutenin aggregates into their constituent polypeptides, because quantitatively there seems to be no change in the HMW glutenin polypeptides levels (Appendix 4). Further studies on unreduced glutenins may be more useful in understanding the extent of losses in glutenin aggregation, if any, during the storage of treated seeds. During germination however, glutenins seems to be degraded preferentially compared to gliadins (Lukow and Bushuk, 1984; although only the low molecular weight unreduced glutenins that resolved clearly were examined). The mechanisms involved in the preferential degradation of glutenins or its importance in germinating wheat seeds are not known. There is clearly scope for further work in this area.

## CHAPTER 7

### THE EFFECTS OF AGEING AND PRE-SOWING TREATMENT ON THE $\alpha$ -AMYLASE ACTIVITY OF WHEAT SEEDS

#### 7.1

#### INTRODUCTION

In cereals where starch is stored in abundance, amylases, especially  $\alpha$ -amylases, are some of the most important and abundant enzymes found during seed germination (see Sec. 2.4). As the starchy endosperm of the mature cereal grain is a dead tissue,  $\alpha$ -amylase necessary for starch degradation has to be synthesised and secreted by the embryo and aleurone tissue. Lack of co-ordination of enzyme production with germination may affect mobilization of starch reserves and consequently seedling development. On the other hand, during wet harvest conditions, early increases in  $\alpha$ -amylase activity in wheat seeds, associated with various degrees of sprouting at maturity causes damage to stored starch (commonly referred to as sprouting damage, cf., for example, MacGregor and Matsuo, 1982).

In light of this, the effects of ageing and pre-sowing treatment (PST) on possible changes in the  $\alpha$ -amylase activity of quiescent or germinating wheat seeds were investigated. Further, the effect of  $\alpha$ -amylase on stored starch was studied by examining the starchy endosperm in the scutellum-crease region using a scanning electron microscope. The effects of enzyme activity on stored starch were also assessed by studying quantitative changes in the total soluble sugar content of seeds.

In order to understand the mechanisms of these enzyme activity changes in more detail, possible changes in the responsiveness of aleurone tissue to gibberellic acid (GA) as a result of ageing

or pre-sowing treatment, were investigated by measuring the  $\alpha$ -amylase activity produced by embryoless half grains in response to exogenously applied  $GA_3$ .

For this study, seeds were aged either by accelerated ageing (rapid ageing at 40°C and 100% RH for up to 6 days) or by controlled deterioration (slow ageing at 35°C and 15% SMC for up to 60 days) techniques. The effects of pre-sowing treatment, either before or after storage, were assessed by using the 20 h PEG treatment (imbibing seeds in -0.37MPa polyethylene glycol solution at 20°C, followed by drying back) as this was the most effective among different treatments in improving the vigour of aged wheat seeds (Chapter 5). For details on methods see Chapter 3.

In the following sections, the  $\alpha$ -amylase activity in quiescent untreated seeds (unaged or aged) has been referred to as the base levels.

## 7.2 RESULTS

### 7.2.1 The Effect of Pre-sowing Treatment (PST) on the $\alpha$ -amylase Activity of Unaged Wheat Seeds

#### 7.2.1.1 *Changes in $\alpha$ -amylase activity in response to pre-sowing treatment*

The base levels of  $\alpha$ -amylase activity in unaged, untreated 'Karamu' wheat seeds were very low (Tab. 7.1). Pre-sowing treatment resulted in a large increase in the  $\alpha$ -amylase activity of ungerminated seeds. Figure 7.1 shows the time-course of changes in  $\alpha$ -amylase activity of seeds during pre-sowing treatment. Increases in  $\alpha$ -amylase activity occurred between 15 and 20 hours of imbibition (20°C), and continued over to the early phase of seed drying. Most of the observed increases in enzyme activity appeared to be retained in seeds upon drying them to lower moisture levels.

Further, when subsequently germinated at 10°C, treated seeds showed an ability for quick resumption of  $\alpha$ -amylase production (increase of 58 milli-units/seed) while untreated controls showed only very small increases (5 milli-units/seed) over base levels after 24 h imbibition (Tab. 7.1).

#### 7.2.1.2 *Localization of $\alpha$ -amylase activity in untreated and treated seeds*

Initially changes in  $\alpha$ -amylase activity of treated seeds were studied using a starch agar blotting technique. In comparison with untreated controls (Plate 7.1A), the large cleared zones on the starch agar plates left by treated seeds (Plate 7.1B) were not only indicative of the considerable  $\alpha$ -amylase activity but also suggest their possible localization either in the embryo itself or in aleurone tissue adjacent to the embryo.

Table 7.1 The effect of pre-sowing treatment on the  $\alpha$ -amylase activity and germination performance of unaged seeds.

	$\alpha$ -amylase activity, milli-units per seed			Germination Performance at 10°C	
	ungerminated	24 h germinated at 10°C	't' test for $\alpha$ -amylase activity	Radicle emergence %	T50 Radicle emergence h
Untreated	3 ± 1.2	8 ± 1.2	*	98 ± 1.2	76 ± 0.4
Treated	76 ± 16.6	134 ± 11.4	*	98 ± 1.2	54 ± 2.5
't' test	*	***		NS	***

Note: Data represent the means of 3 replicates ± standard errors

Abbreviations: T50 radicle emergence = time to 50% radicle emergence; h = hours

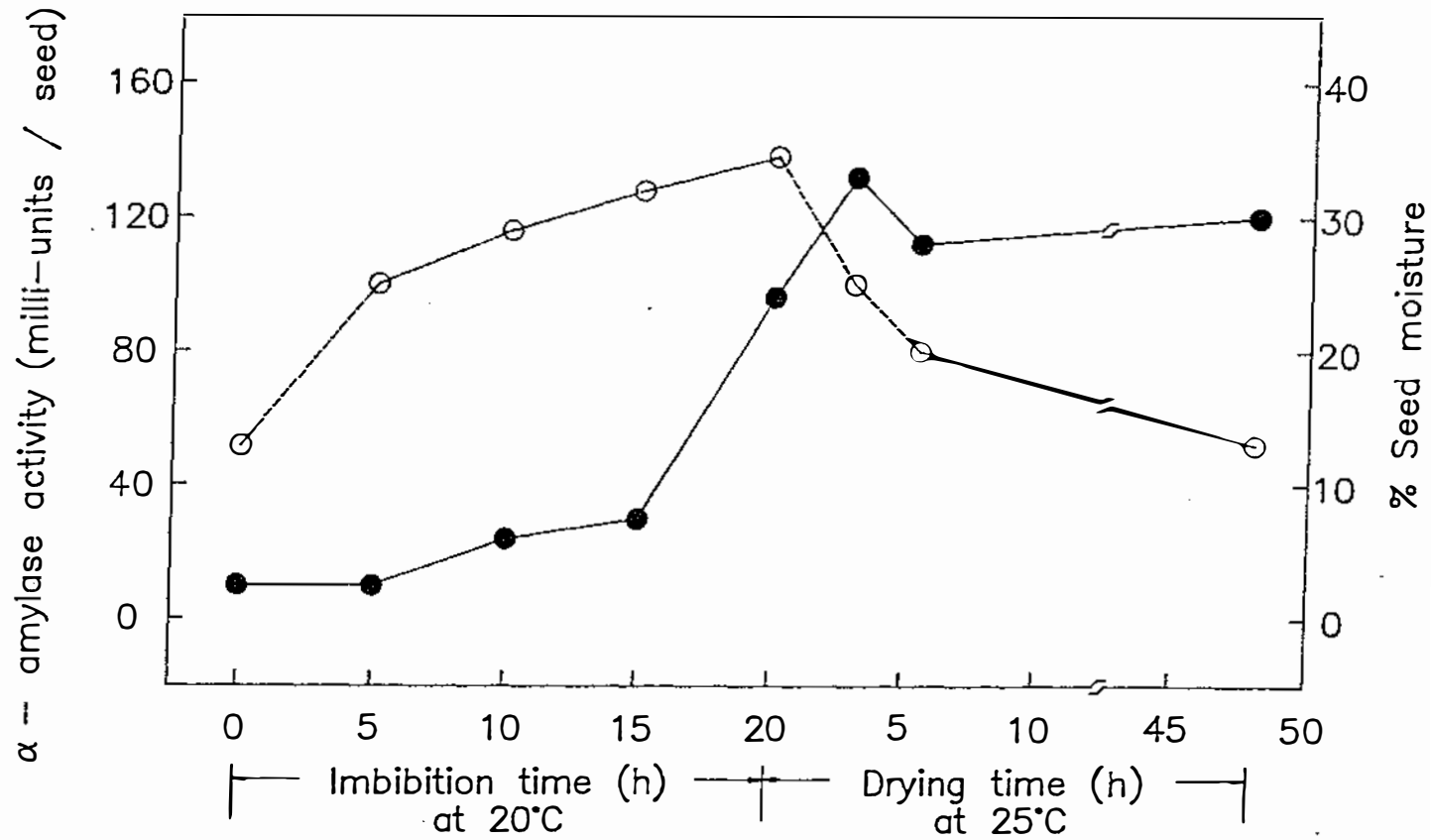
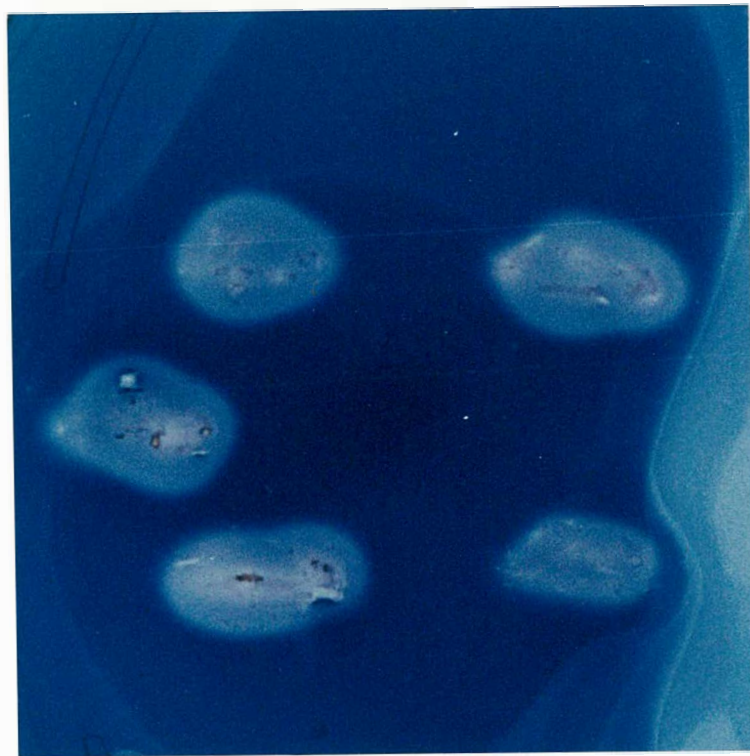
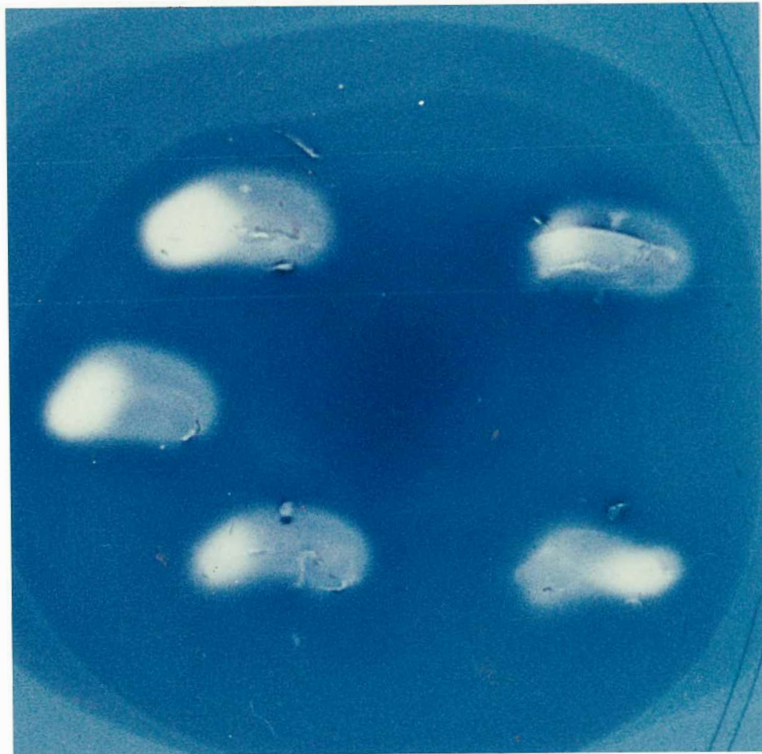


Figure 7.1 Changes in seed moisture content and  $\alpha$ -amylase activity during pre-sowing treatment of unaged wheat seeds cv. Karamu. Open circles (○) represent seed moisture and closed circles (●)  $\alpha$ -amylase activity. Each point represents the mean of two replicate assays following a double extraction procedure (see Section 3.9.1.1 for methods).

Plate 7.1      Starch agar plates showing localization of  $\alpha$ -amylase activity in ungerminated wheat seeds. Seeds soaked for 2 h at 25°C were longitudinally bisected and one half of each seed incubated on agar plate for one hour at 37°C. A, unaged-untreated; B, unaged-treated seeds. Cleared zone impressions from treated seeds (B) on agar surface developed with potassium iodide, represent starch degrading activity and its localization.



A



B

PLATE 7.1

Assays of dissected grains showed that traces of  $\alpha$ -amylase activity present in untreated grain are localized in the endosperm with its outer covers (seed coat and pericarp), while none was detected in the embryo (Tab. 7.2). Dissection of endosperm parts revealed that this activity was uniformly spread throughout the endosperm (Fig. 7.2). After treatment however,  $\alpha$ -amylase activity appeared in the embryo of ungerminated seeds for the first time while the activity in the endosperm quadrupled, all this increased activity being localized in tissue proximal to the embryo (Fig. 7.2; Tab. 7.2).

## 7.2.2 The Effect of Ageing and Pre-sowing Treatment on $\alpha$ -amylase Activity

### 7.2.2.1 *Pre-sowing treatment induced changes in $\alpha$ -amylase activity of slow aged seeds (treatment after ageing)*

Trace levels of  $\alpha$ -amylase activity found in quiescent unaged-untreated seeds (base levels), increased significantly during exposure to controlled deterioration conditions (see changes in untreated seeds in Fig. 7.4). However, these increases (9 to 10 milli-units/seed) were very small compared to large increases occurring during germination (Fig. 7.3, for e.g. 510 milli-units after 3 d germination of unaged seeds). The effect of controlled deterioration on germinative  $\alpha$ -amylase production itself was to delay the start of enzyme production during germination. Whereas unaged seeds showed a lag phase of one day, 40 d aged seeds began to produce  $\alpha$ -amylase only after 2 days imbibition at 10°C (Fig. 7.3).

Changes in  $\alpha$ -amylase activities over base levels in unaged and aged seeds (ungerminated) due to pre-sowing treatment (PST) are shown in Figure 7.4. Unaged seeds showed a large increase (73 milli-units/seed) in  $\alpha$ -amylase activity as a result of PST. In contrast either no significant change or small increases (10 milli-units/seed) over base levels were

Table 7.2 Localization of  $\alpha$ -amylase activity in unaged wheat seeds, cv. Karamu, with or without pre-sowing treatment.

	$\alpha$ -amylase activity, milli-units/seed	
	Embryo (without seed coat and pericarp)	Endosperm (inclusive of seed coat and pericarp)
Untreated	0	13 $\pm 1.7$
Treated	16 $\pm 2.1$	81 $\pm 4.0$
't' test	* *	* * *

Note: Data represent the means of 3 replicates  $\pm$  standard errors.

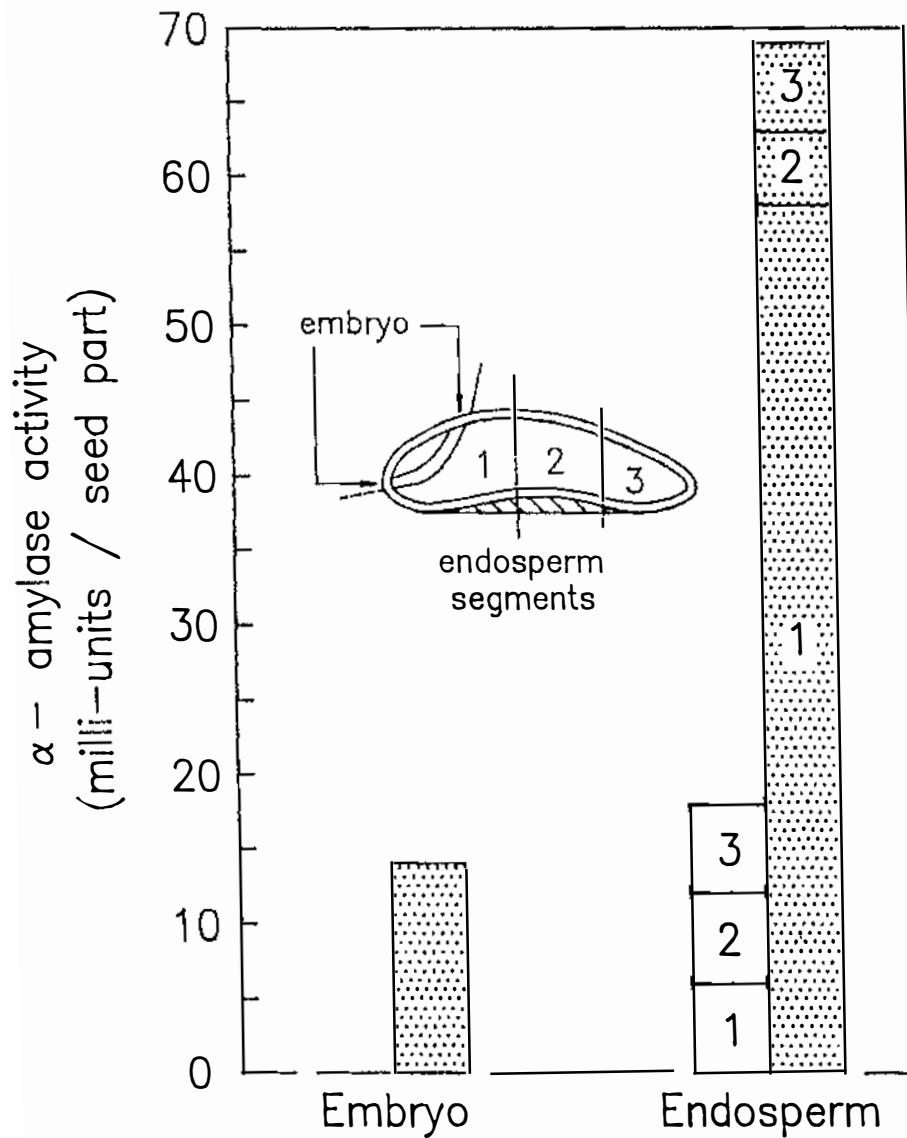


Figure 7.2  $\alpha$ -amylase activity from different parts of unaged seeds before (open histogram bars) or after (shaded histogram bars) pre-sowing treatment (20 h hydration in  $-0.37\text{MPa}$  PEG solution at  $20^\circ\text{C}$ , followed by drying-back). Data are means of two replicate assays. The inset diagram shows how the grains were dissected. Note that no activity was detected in embryos from untreated seeds.

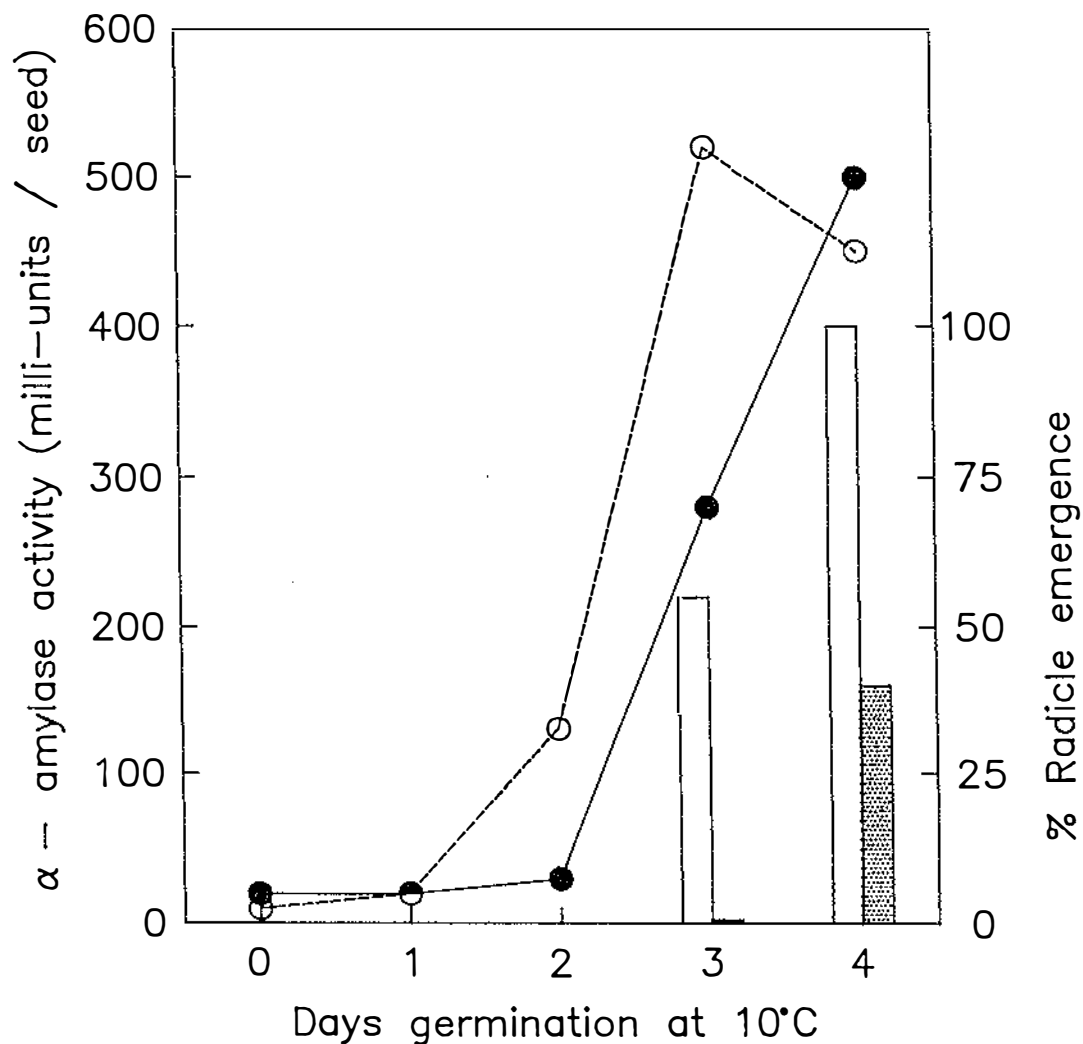


Figure 7.3 Time course of  $\alpha$ -amylase production by unaged and 40 d slow aged seeds during germination at 10°C. Each point represents the mean of two replicates with open circles (O) representing unaged and closed circles (●) representing 40 d aged seeds. The open and shaded histograms represent cumulative radicle emergence for unaged and aged seeds respectively.

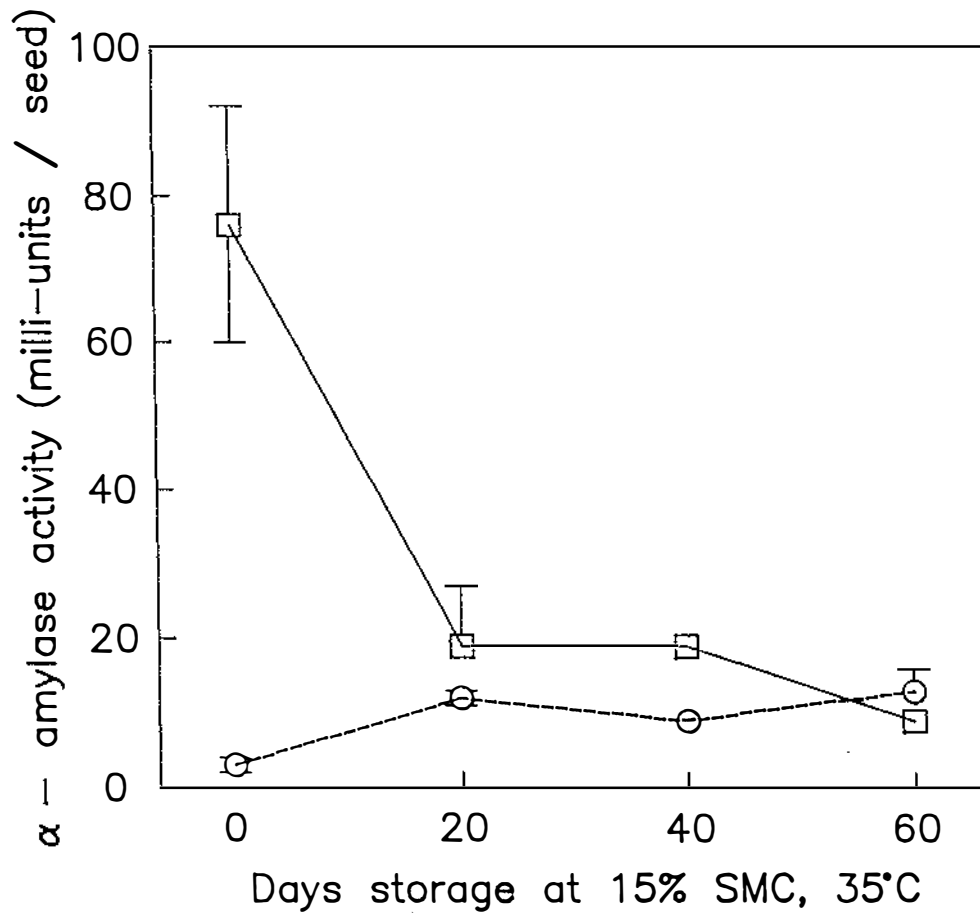


Figure 7.4 The effect of pre-sowing treatment (after ageing) on the  $\alpha$ -amylase activity of slow aged seeds (ungerminated). Each point represents mean of three replicate assays. Individual SEM's are shown where larger than symbols used (O, untreated;  $\square$ , treated seeds).

found in 20 or 40 d aged seeds respectively as a result of PST. However, when untreated and treated (PST) seeds from different ages were germinated at 10°C all viable treated seeds, both unaged and aged, produced  $\alpha$ -amylase rapidly during a 24 h imbibition compared to their respective untreated controls which failed to begin enzyme production (Fig. 7.5). The data on germination performance of these seeds are shown in Table 7.3. It should be noted that 60 d aged control seeds were almost completely dead.

Localization of enzyme activity in 40 d aged, untreated or treated seeds (ungerminated) followed similar patterns (data not shown) to those observed for treated unaged seeds (Sec. 7.2.1.2). Embryos of 40 d aged, untreated seeds showing no  $\alpha$ -amylase activity contained 4 milli-units of activity per seed after pre-sowing treatment, and the activity increased from 6 to 18 milli-units/seed in the endosperm tissue proximal to the embryo.

#### 7.2.2.2

#### *Changes in $\alpha$ -amylase activity due to pre-sowing treatment of rapidly aged seeds (treatment after ageing)*

In contrast to slow ageing there was no significant change in the base level of  $\alpha$ -amylase activity, in seeds exposed to rapid ageing at 40°C and 100% RH for up to 6 days (Fig. 7.6). The effects of pre-sowing treatment on the  $\alpha$ -amylase activity of rapidly aged seeds, ungerminated or germinated at 10°C are presented in Figures 7.6 and 7.7 respectively. The treatment effects on 2 d rapidly aged seeds appeared to be broadly similar to those on 20 or 40 d slow aged seeds (Figs 7.4 and 7.5) in that increase in  $\alpha$ -amylase activity was small compared to unaged seeds. It was interesting that the T50's of these three aged lots before treatment, were of a similar order falling in the range of 90 to 98 h (compare T50's of 2 d AA and 20 or 40 d CD, untreated seeds in Tabs 7.3 and 7.4). On the other hand 4 and 6 day rapidly aged

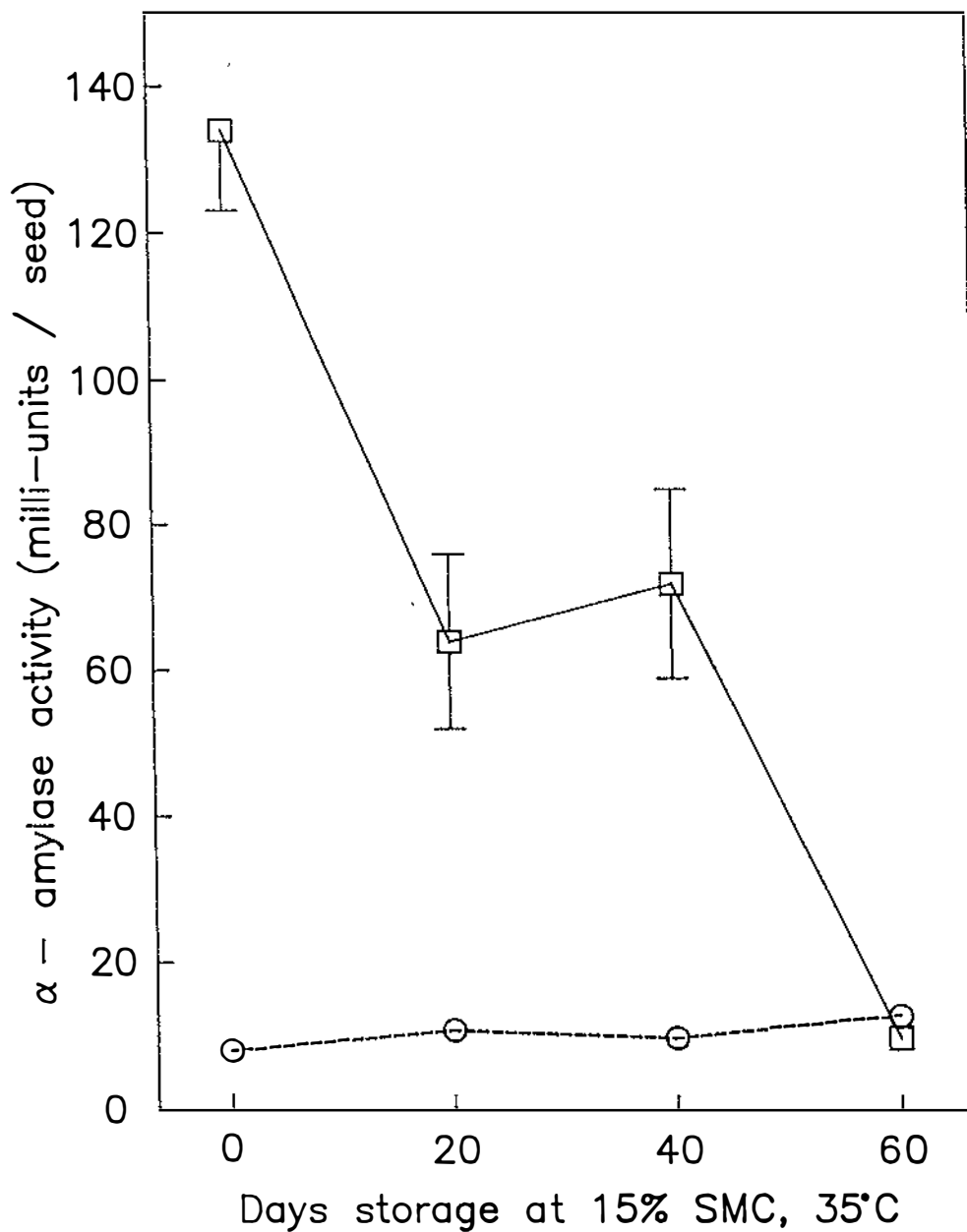


Figure 7.5

The effect of pre-sowing treatment (after ageing) on the ability of slow aged seeds to produce  $\alpha$ -amylase during germination for 24 h at 10°C. Each point represents the mean of three replicate assays. Individual SEM's are shown where larger than symbols used (O, untreated; □, treated seeds). The levels of activity before germination are as illustrated in Figure 7.4.

Table 7.3 The effect of pre-sowing treatment on germination performance of unaged and slow aged seeds (CD at 35°C, 15% SMC).

Slow ageing days	Radicle emergence at 10°C (%)		T50 Radicle emergence at 10°C (h)	
	Untreated	Treated (After ageing)	Untreated	Treated (After ageing)
0	98 ± 1.2	98 ± 1.2	76 ± 0.4	54 ± 2.5
20	95 ± 2.4	98 ± 1.2	98 ± 0.5	60 ± 0.7
40	95 ± 1.8	93 ± 0.7	96 ± 2.7	63 ± 3.7
60	1 ± 0.7	2 1.2	-	-

Note: Data represent the means of 3 replicates ± standard errors.

Abbreviations: CD = controlled deterioration; T50 radicle emergence = time to 50% radicle emergence; h = hours; SMC = seed moisture content

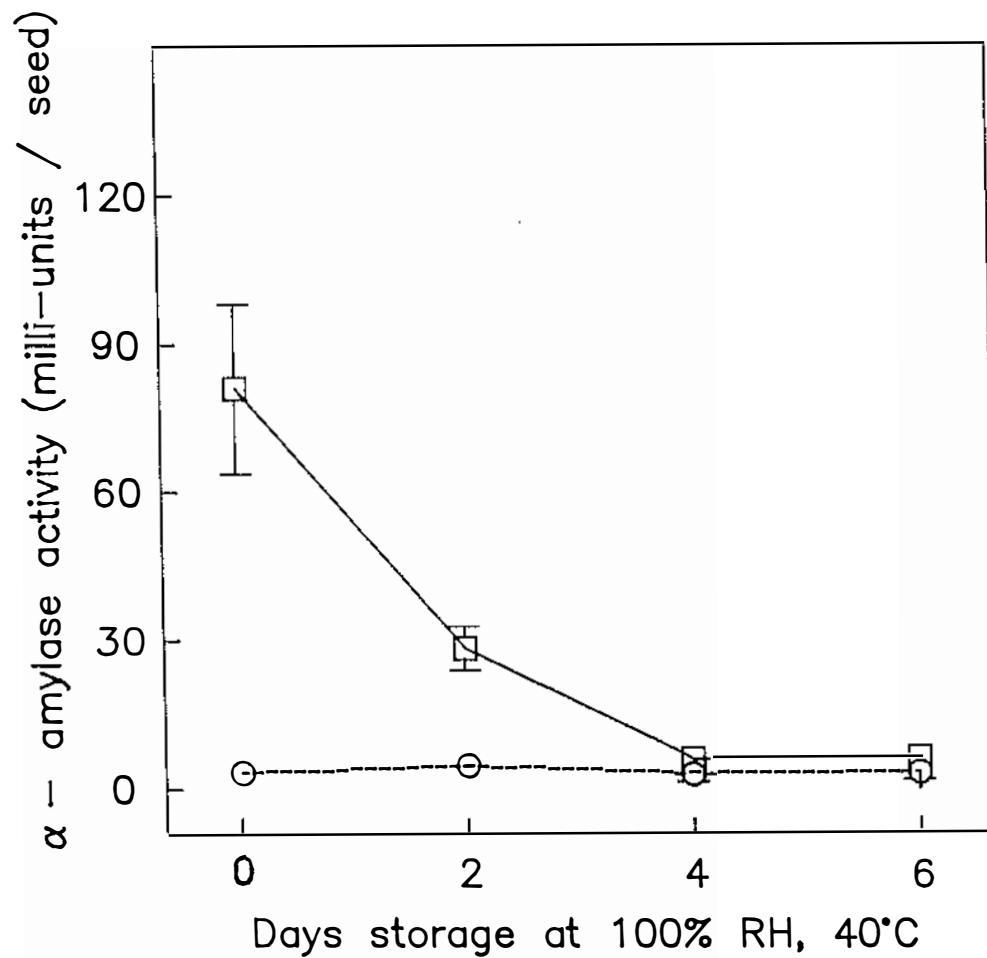


Figure 7.6 The effect of pre-sowing treatment (after ageing) on the  $\alpha$ -amylase activity of rapidly aged seeds (ungerminated). Each point represents mean of three replicate assays. Individual SEM's are shown where larger than symbols used (O, untreated;  $\square$ , treated seeds).

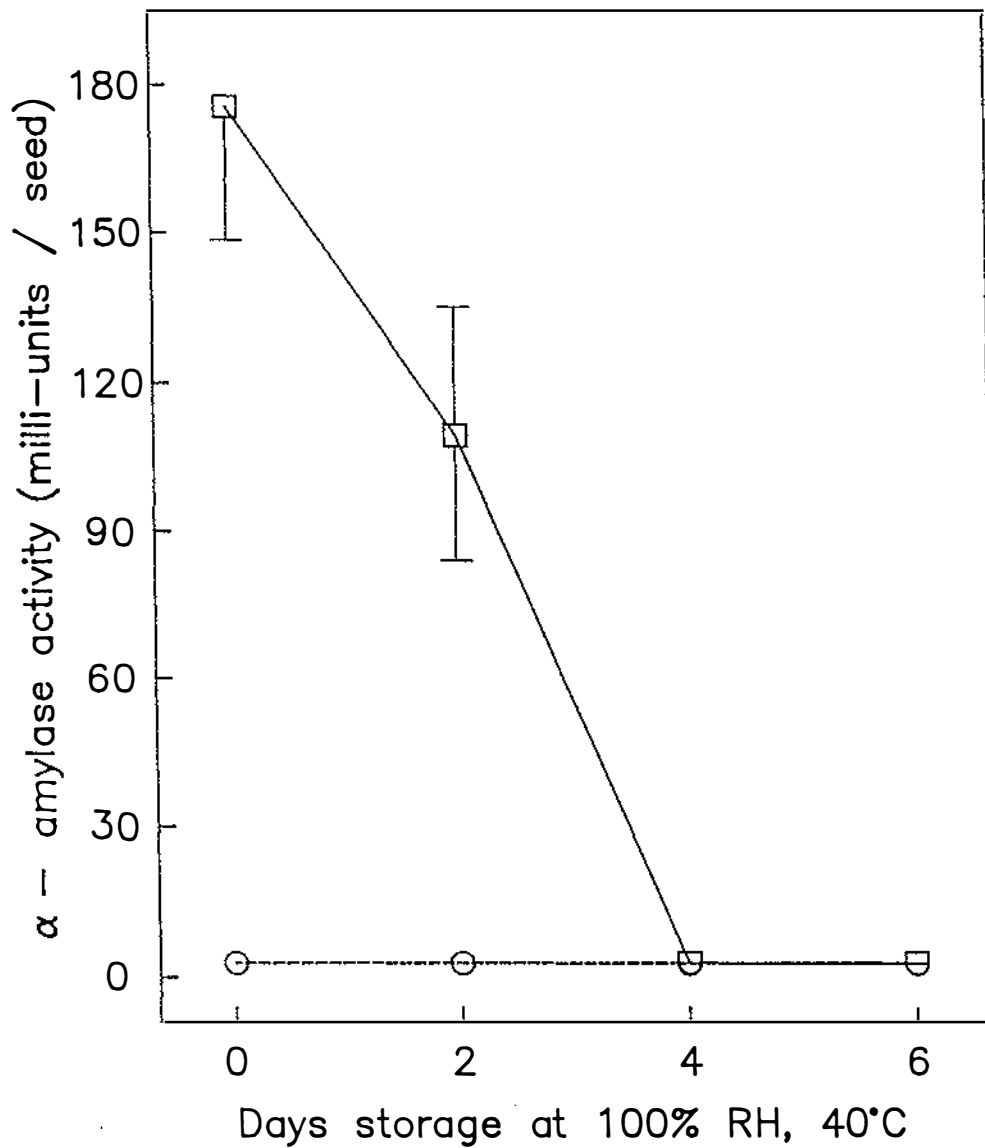


Figure 7.7 The effect of pre-sowing treatment (after ageing) on the ability of rapidly aged seeds to produce  $\alpha$ -amylase during germination for 24 h at 10°C. Each point represents the mean of three replicate assays. Individual SEM's are shown where larger than symbols used (O, untreated; □, treated seeds). The levels of activity before germination are as illustrated in Figure 7.6.

Table 7.4 The effect of pre-sowing treatment on germination performance of unaged and rapidly aged seeds (AA at 40°C, 100% RH).

Rapid ageing days	Radicle emergence at 10°C (%)		T50 Radicle emergence at 10°C (h)	
	Untreated	Treated (After ageing)	Untreated	Treated (After ageing)
0	97 ± 1.3	97 ± 1.3	81 ± 0.6	51 ± 3.2
2	97 ± 0.7	95 ± 0.7	90 ± 1.3	65 ± 3.5
4	92 ± 2.3	85 ± 2.9	128 ± 2.7	93 ± 5.2
6	57 ± 3.5	49 ± 1.8	150 ± 7.1	164 ± 19.1

Note: Data represent the means of 3 replicates ± standard errors.

Abbreviations: AA = accelerated ageing; RH = relative humidity; T50 radicle emergence = time to 50% radicle emergence; h = hours

seeds which have much higher T50's (Tab. 7.4) did not respond to treatment with respect to  $\alpha$ -amylase production (Figs 7.6 and 7.7).

### 7.2.2.3 *Changes in the $\alpha$ -amylase activity during storage of treated seeds (treatment before ageing)*

The high levels of  $\alpha$ -amylase activity observed in unaged treated seeds were not significantly affected when they were exposed to either slow (Fig. 7.8) or rapid (Fig. 7.9) ageing conditions. The changes in germination capacity and T50's of these seeds are presented in tables 7.5 and 7.6. There was almost complete loss in the germinability of treated seeds stored under slow ageing conditions (Tab. 7.5). Although treated seed samples exposed to 2 or 4 days rapid ageing conditions retained 76 and 51% viability respectively (Tab. 7.6), they were unable to re-start  $\alpha$ -amylase production during germination for 24 h at 10°C (Tab. 7.7). This contrasts with the similarly aged seeds subjected to treatment after exposure to 2 d rapid ageing conditions (Fig. 7.7).

### 7.2.3 **The Effect of Ageing or Treatment Related Changes in $\alpha$ -amylase Activity of Seeds on Stored Starch**

The following studies were conducted on ungerminated seed samples from differently aged or treated lots.

#### 7.2.3.1 *Electron microscopic study of the starchy endosperm*

In the untreated seeds, whether unaged or aged, the starch granules in the starchy endosperm tissue were not clearly visible as they were embedded in a layer of protein matrix (see Plate 6.1). Irrespective of ageing, however, in all treated grains (Plate 7.2 A, B and C) there were scattered patches of starch grains in the scutellum-crease region, which appeared free from matrix proteins. Starch granules appeared cleaner

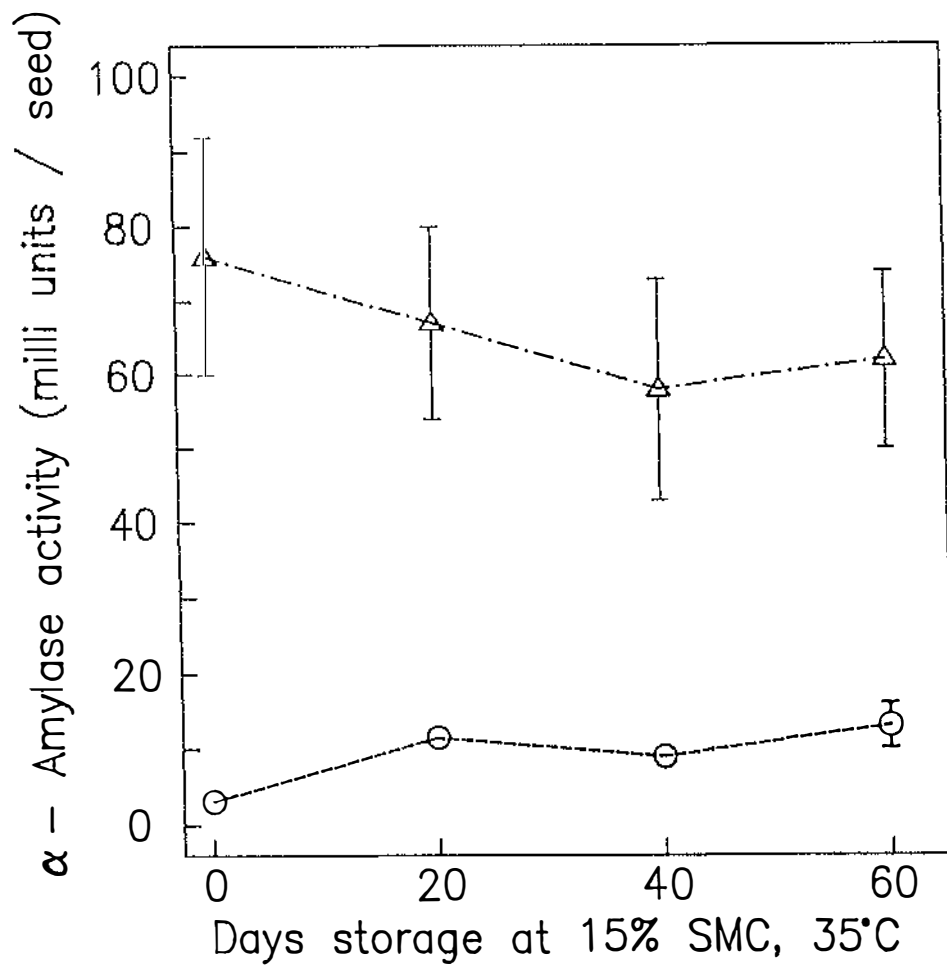


Figure 7.8 Changes in the  $\alpha$ -amylase activity of ungerminated seeds which had been subjected to pre-sowing treatment before storage under slow ageing conditions. Each point represents the mean of three replicate assays. Individual SEM's are shown where larger than symbols used (O, untreated;  $\Delta$ , treated seeds).

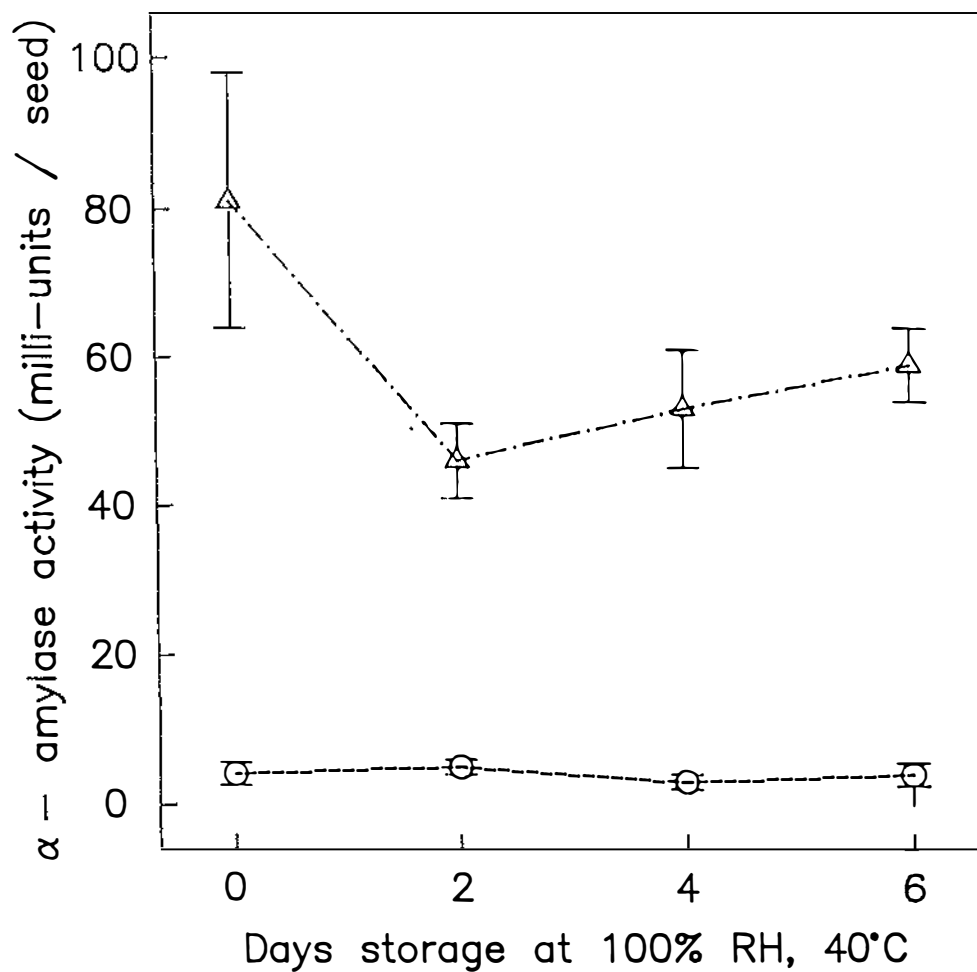


Figure 7.9 Changes in the  $\alpha$ -amylase activity of ungerminated seeds which had been subjected to pre-sowing treatment before storage under rapid ageing conditions. Each point represents the mean of three replicate assays. Individual SEM's are shown where larger than symbols used (O, untreated;  $\Delta$ , treated seeds).

Table 7.5 Effects of slow ageing on the germination performance of wheat seeds subjected to pre-sowing treatment prior to storage (controlled deterioration at 35°C and 15% SMC).

Slow ageing days	Radicle emergence at 10°C (%)		T50 Radicle emergence at 10°C (h)	
	Untreated	Treated (Before ageing)	Untreated	Treated (Before ageing)
0	98 ± 1.2	98 ± 1.2	76 ± 0.4	54 ± 2.5
20	95 ± 2.4	6 ± 2.0	98 ± 0.5	-
40	95 ± 1.8	8 ± 3.1	96 ± 2.7	-
60	1 ± 0.7	1 ± 0.7	-	-

Note: Data represent the means of 3 replicates ± standard errors.

Abbreviations: SMC = seed moisture content; h = hours; T50 radicle emergence = time to 50% radicle emergence

Table 7.6 Effects of rapid ageing on the germination performance of wheat seeds subjected to pre-sowing treatment prior to storage (accelerated ageing at 40°C and 100% RH).

Rapid ageing days	Radicle emergence at 10°C (%)		T50 Radicle emergence at 10°C (h)	
	Untreated	Treated (Before ageing)	Untreated	Treated (Before ageing)
0	97 ± 1.3	97 ± 1.3	81 ± 0.6	51 ± 3.2
2	97 ± 0.7	76 ± 2.0	90 ± 1.3	157 ± 7.0
4	92 ± 2.3	51 ± 8.1	128 ± 2.7	295 ± 21.5
6	57 ± 3.5	9 ± 2.4	150 ± 7.1	-

Note: Data represent the means of 3 replicates ± standard errors.

Abbreviations: RH = relative humidity; h = hours; T50 radicle emergence = time to 50% radicle emergence

Table 7.7 Changes in  $\alpha$ -amylase activity during germination of seeds subjected to pre-sowing treatment prior to rapid ageing.

Seeds subjected to pre-sowing treatment before rapid ageing	$\alpha$ -amylase activity (milli-units/seed)			
	Days ageing at 40°C, 100% RH			
	0	2	4	6
Ungerminated	81 ± 17.6	46 ± 5.5	53 ± 8.5	59 ± 4.7
Germinated for 24 h at 10°C	176 ± 27.1	57 ± 2.6	36 ± 1.6	54 ± 7.4
't' test	*	NS	NS	NS

Note: Data represent the means of 3 replicates ± standard errors.

Abbreviations: RH = relative humidity; h = hours

Plate 7.2

Scanning electron micrographs (x1500) of the starchy endosperm of wheat seeds subjected to pre-sowing treatment (20 h hydration in -0.37 MPa PEG solution at 20°C followed by drying—back), before or after storage (40 days at 35°C and 15% SMC). The endosperm was scanned in the region near scutellum-crease junction with special attention on locations where starch grains were clearly visible.

7.2 A, unaged seeds, treated;

7.2 B, 40 d aged seeds, treated before ageing;

7.2 C, 40 d aged seeds, treated after ageing.

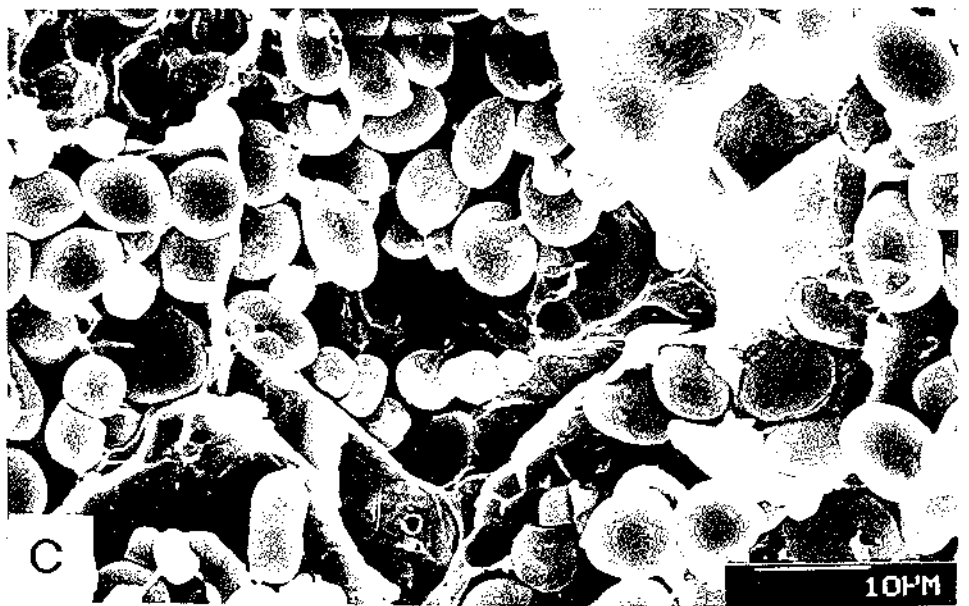
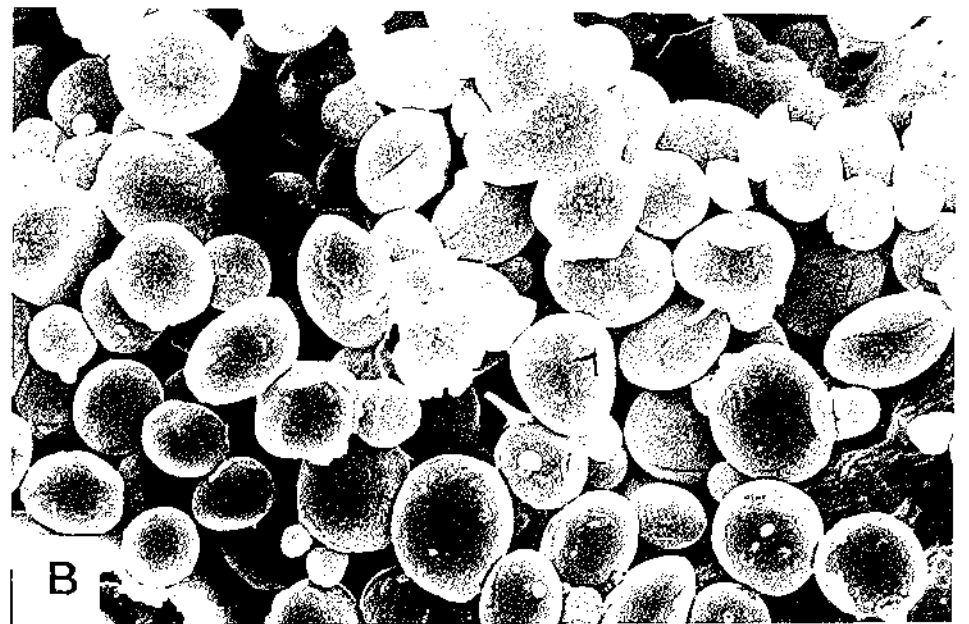
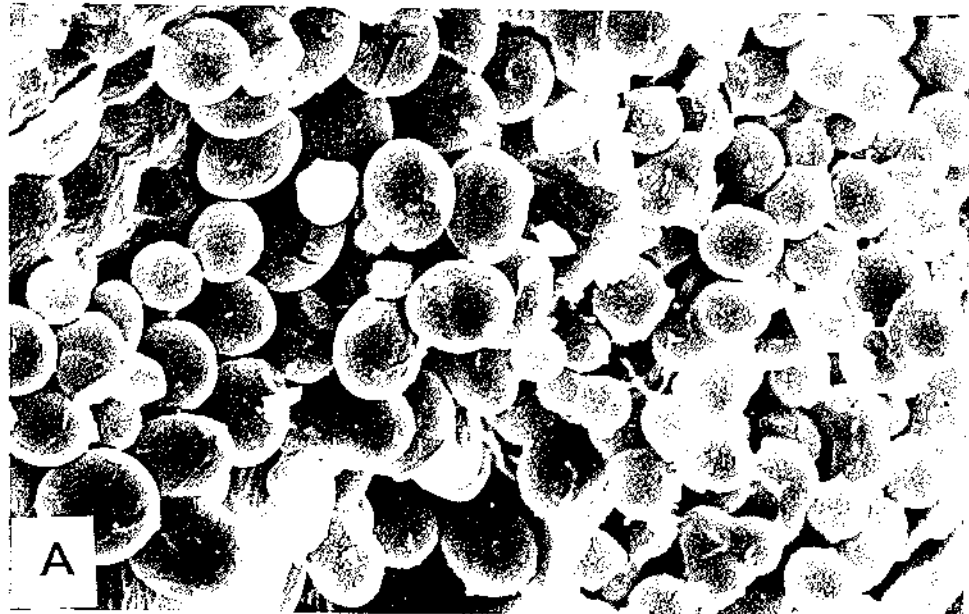


PLATE 7.2

and smoother in these patches but, there were no apparent signs of starch degradation. If there was any damage to starch by amylase attack, then we would expect to find pitting or erosion on the surface of starch granules (see Sec. 2.4.2).

#### 7.2.3.2 *Changes in soluble sugar levels in seed or seed leachate*

Pre-sowing treatment of unaged seeds did not cause any change in the total soluble sugar concentration of unaged seeds (Tab. 7.8). Also storage of either untreated controls or treated seeds, for 40 days at 35°C and 15% SMC did not cause any significant change in the total soluble sugar concentration of seeds.

Analysis of seed leachate showed that pre-sowing treatment has no effect on leakage of sugars from unaged seeds (Tab. 7.8). However, storage of either untreated or treated seeds for 40 days (at 35°C and 15% SMC) significantly increased sugar leakage, with the latter showing drastic increases in comparison to the former.

#### 7.2.4 **The Effect of Ageing and Pre-sowing Treatment on the Responses of Aleurone Tissue (Endosperm Halves) to Exogenous Application of Gibberellic Acid**

After pre-sowing treatment, aged wheat seeds were able to produce  $\alpha$ -amylase in a similar fashion to unaged-treated seeds during germination (Figs 7.4 and 7.5). Actual increase in enzyme activity during 24 h imbibition was 58( $\pm$  15.5) milli-units/seed for unaged-treated seeds and similar increases of 45( $\pm$  5.4) and 53( $\pm$  11.8) milli-units/seed were found in 20 and 40 d aged-treated seeds respectively. As the aleurone tissue is a major source of enzyme, it was decided to determine the effects of seed ageing and pre-sowing treatment on the ability of this tissue to produce  $\alpha$ -amylase in response to gibberellic acid (GA).

Table 7.8 Changes in soluble sugar concentration of seeds or in seed leachate as influenced by pre-sowing treatment given prior to storage (slow ageing at 35°C, 15% SMC).

	Soluble sugars in seed (mg/seed)		Soluble sugar in seed leachate (µg/seed)	
	Untreated	Treated (Before ageing)	Untreated	Treated (Before ageing)
Unaged	1.79 ± 0.132	1.75 ± 0.138	13.73 ± 1.033	12.33 ± 2.228
40 d aged	1.75 ± 0.145	1.67 ± 0.157	38.30 ± 8.266	188.47 ± 10.663
't' test	NS	NS	*	* * *

Note: Data represent the means of 3 replicates ± standard errors.

Abbreviations: SMC = seed moisture content; d = day

#### 7.2.4.1 *The effect of ageing*

Figure 7.10 shows a time-course of  $\alpha$ -amylase production by endosperm halves of unaged and 40 d slow aged seeds following incubation in  $2 \times 10^{-6} \text{M}$ ,  $\text{GA}_3$  at  $25^\circ\text{C}$ . After different periods of incubation, aleurone tissue of 40 d slow aged seeds were found to produce significantly lower levels of  $\alpha$ -amylase activity compared to responses from the aleurone tissue of unaged seeds.

#### 7.2.4.2 *The effect of pre-sowing treatment on the aleurone tissue of unaged or slow aged seeds*

In general, aleurone tissue of unaged-treated seeds did not produce significantly higher  $\alpha$ -amylase activity when compared to responses from the tissue of unaged-untreated seeds, following incubation in  $2 \times 10^{-6} \text{M}$   $\text{GA}_3$  for upto 42 h (Fig. 7.11). However, after 40 days slow ageing ( $35^\circ\text{C}$ , 15% SMC), aleurone tissue from treated seeds (treated after ageing) produced significantly higher  $\alpha$ -amylase activity compared to those from similarly aged but untreated controls, during a time-course incubation in  $\text{GA}_3$  (Fig. 7.12).

#### 7.2.4.3 *Responses of aleurone tissue from aged or treated seeds to changes in $\text{GA}_3$ concentration*

The data presented for this study represent data from a single unreplicated experiment. Essentially similar results were obtained with a second experiment although in a third attempt there were shifts in  $\text{GA}_3$  concentrations that produced maximum and minimum responses as compared with the first two trials. Hence these results are presented only as a preliminary report. Figure 7.13 shows  $\alpha$ -amylase activity produced by unaged and 40 d slow aged aleurone tissue in response to changes in  $\text{GA}_3$  concentration in the incubation medium. The threshold levels of growth substance required to evoke

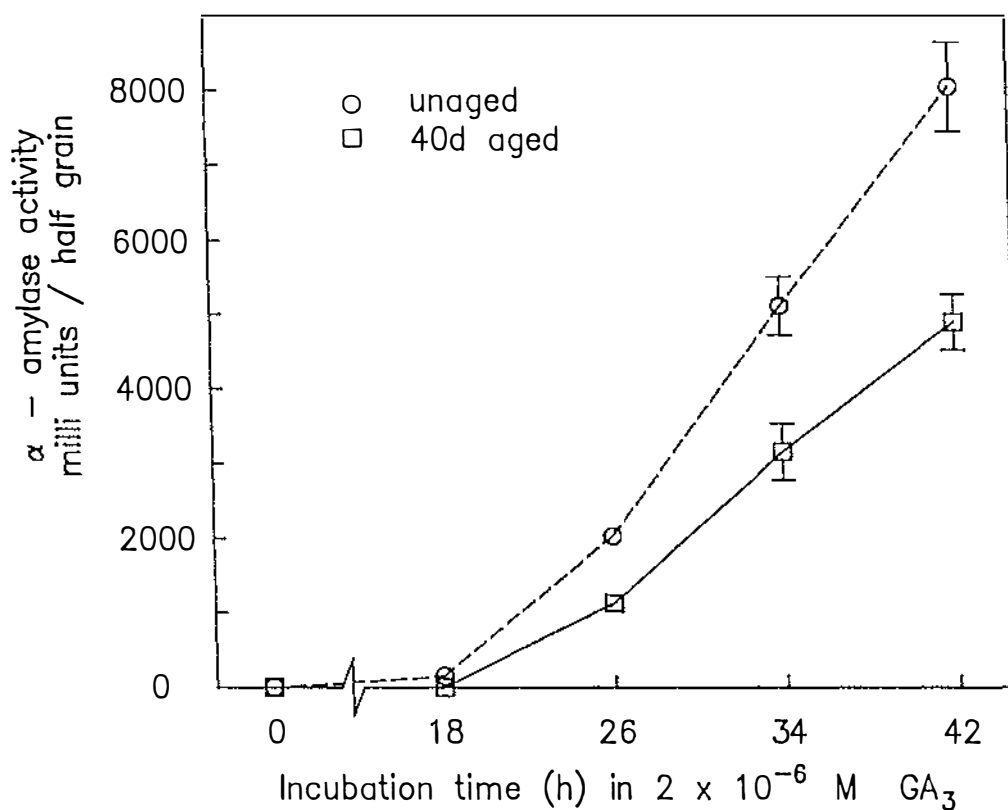


Figure 7.10 Time-course of  $\alpha$ -amylase production, by the endosperm halves (total activity) cut from unaged ( $\circ$ ) and 40 d slow aged ( $\square$ ) seeds, in response to exogenous application of  $2 \times 10^{-6}$  M  $GA_3$  at  $25^\circ\text{C}$ . Each point represents the mean of four replicate assays; SEM's are shown where larger than symbols used. (See Chapter 3 for details of methods).

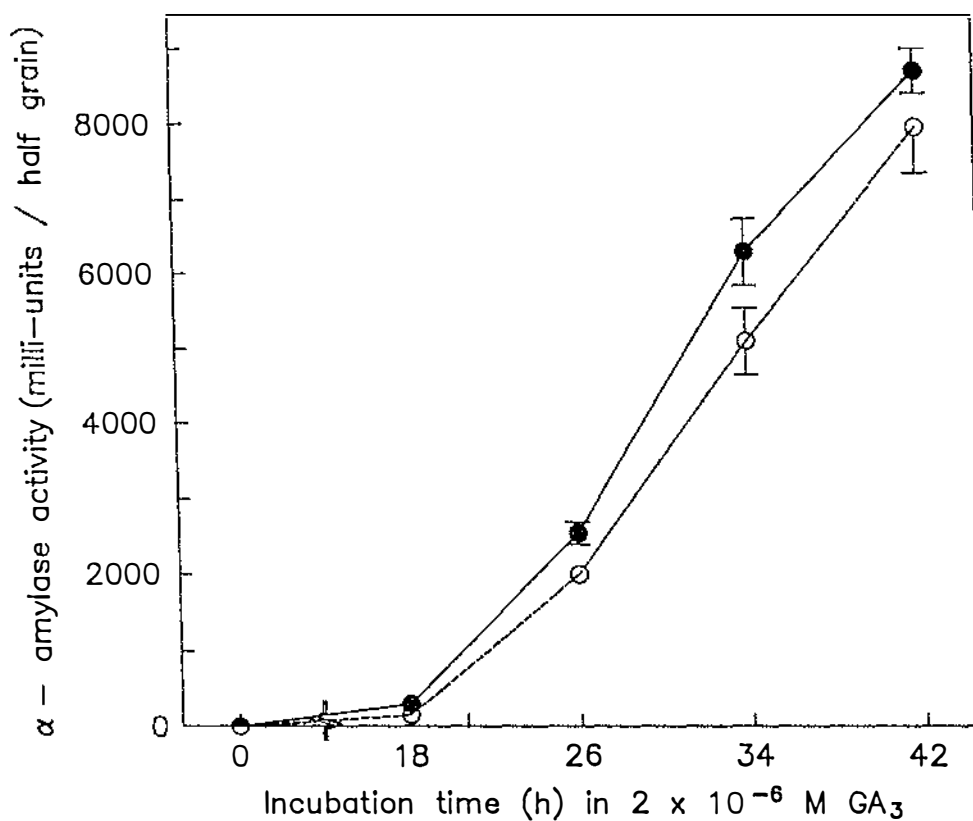


Figure 7.11 Time-course of  $\alpha$ -amylase production by endosperm halves (total activity) cut from unaged seeds, with (●) or without (○) pre-sowing treatment prior to dissection, in response to incubation in  $2 \times 10^{-6} \text{M GA}_3$  at  $25^\circ\text{C}$ . Each point represents the mean of four replicate assays; SEM's are shown where larger than symbols used. (See Chapter 3 for details of methods).

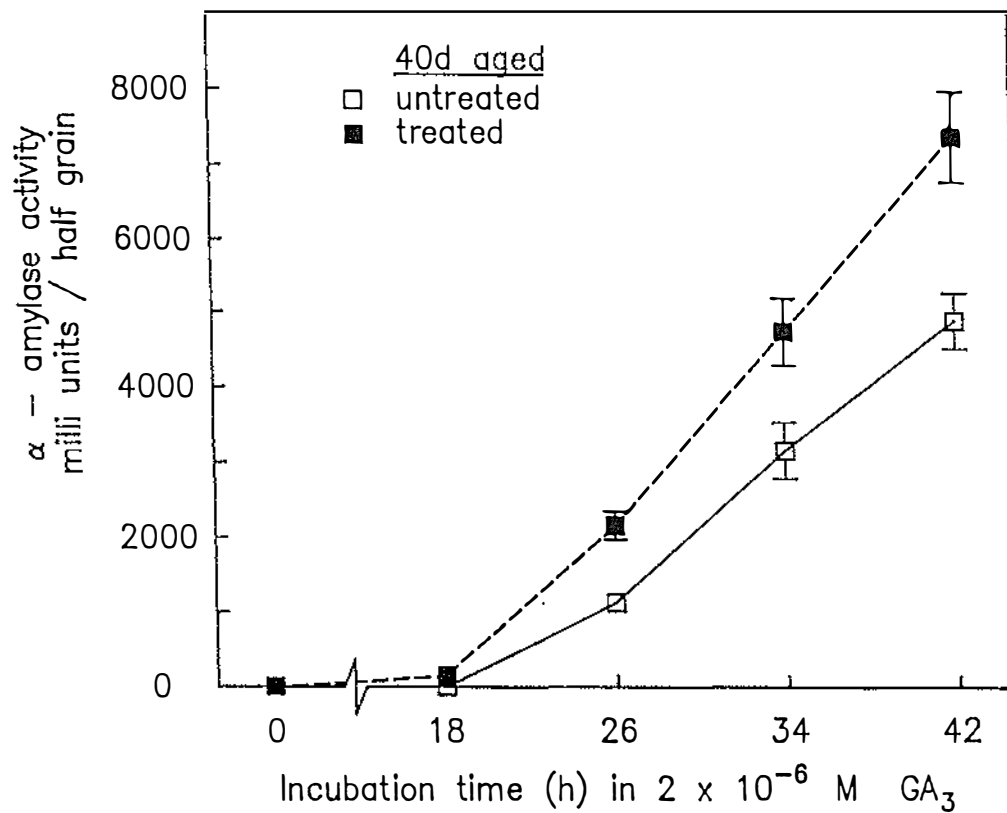


Figure 7.12 Time-course of  $\alpha$ -amylase production by endosperm halves (total activity) cut from 40 d aged seeds, with (■) or without (□) pre-sowing treatment prior to dissection, in response to incubation in  $2 \times 10^{-6}$  M  $GA_3$  at  $25^\circ\text{C}$ . Each point represents the mean of four replicate assays; SEM's are shown where larger than symbols used. (See Chapter 3 for details of methods).

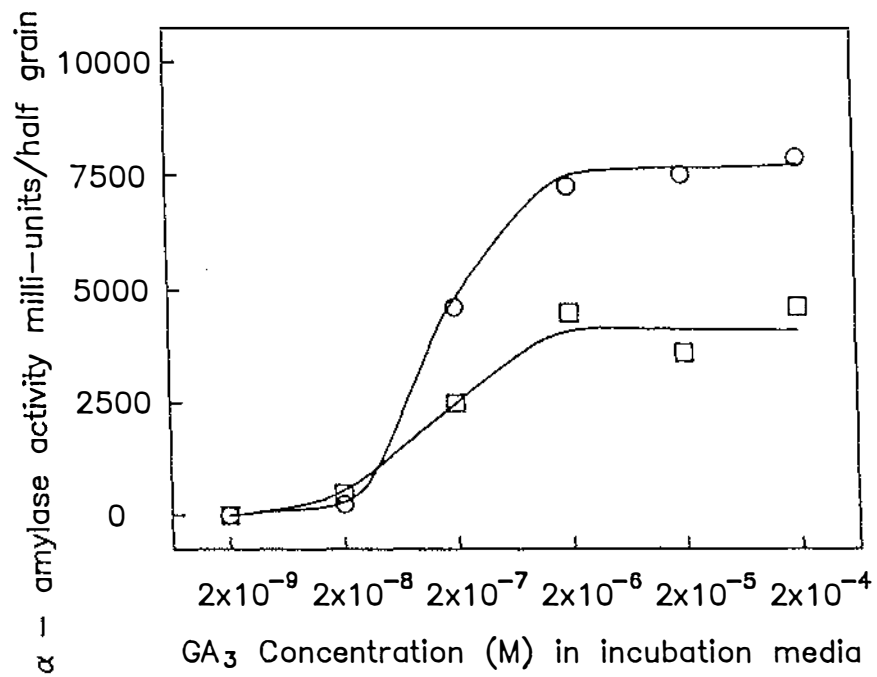


Figure 7.13  $\alpha$ -amylase production (total activity) by endosperm halves cut from unaged (O) and 40 d slow aged ( $\square$ ) seeds in response to changes in the concentration of GA<sub>3</sub> (42 h incubation at 25°C). The data are from a single unreplicated experiment. (See Chapter 3 for details of methods).

response appears to be similar for the unaged and aged aleurone tissue as both began to produce  $\alpha$ -amylase after incubation in  $2 \times 10^{-8} \text{M}$   $\text{GA}_3$ . Also both the tissues produced maximum response at a concentration of  $2 \times 10^{-6} \text{M}$   $\text{GA}_3$ , showing no changes in response with further increase in  $\text{GA}_3$  concentrations. The maximum levels of enzyme produced, however, appears to be lower for aged aleurone compared to unaged tissue.

Pre-sowing treatment of seeds, both unaged and aged, did not have any effect on the aleurone tissue with respect to either threshold levels of  $\text{GA}_3$  ( $2 \times 10^{-8} \text{M}$   $\text{GA}_3$ ) required to evoke a response or on the levels ( $2 \times 10^{-6} \text{M}$   $\text{GA}_3$ ) required to produce maximum response (Figs 7.14A and B). However treatment of seeds resulted in some improvement in the maximum response capacity of both unaged and aged aleurone tissue.

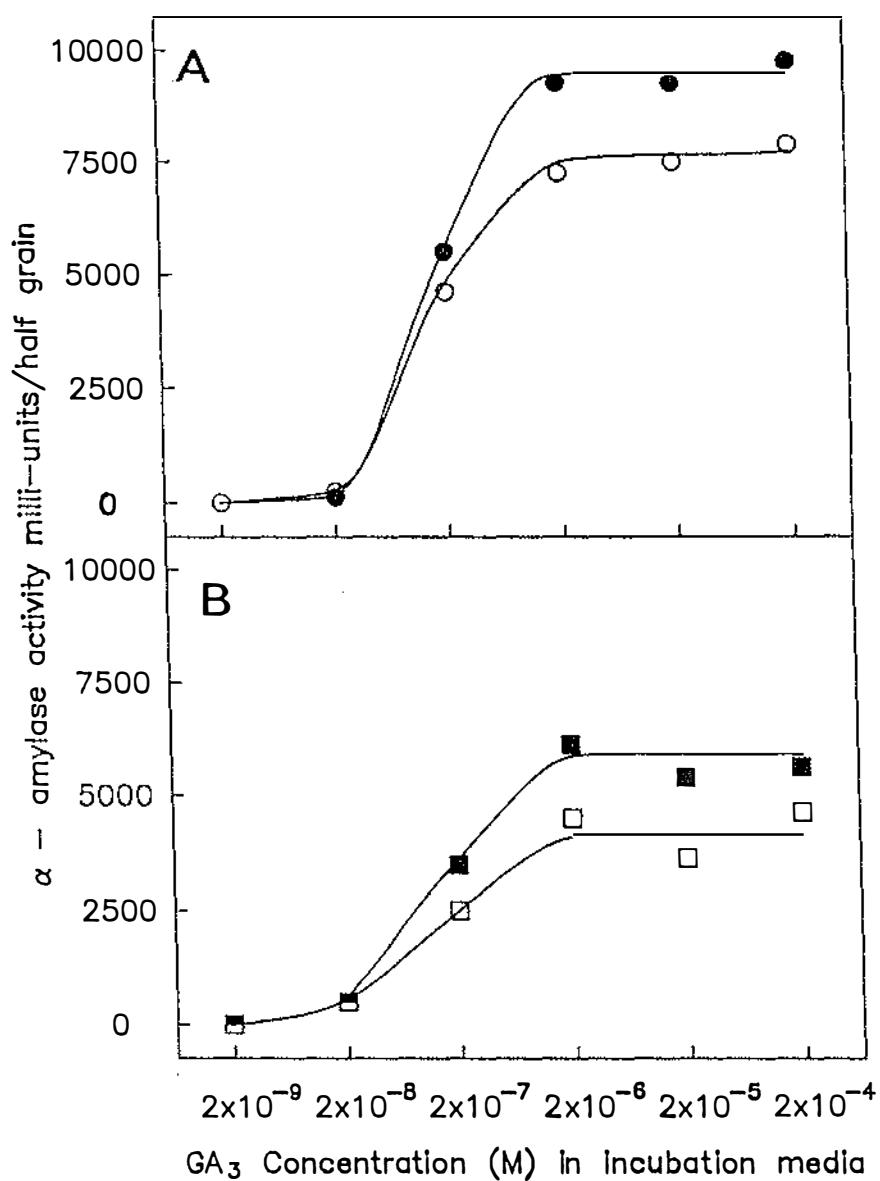


Figure 7.14  $\alpha$ -amylase production (total activity) by endosperm halves cut from treated (●) or untreated (○), unaged seeds (Fig. 7.14A) and from treated (■) or untreated (□), 40 d slow aged seeds (Fig. 7.14B), in response to changes in the concentration of  $GA_3$  (42 h incubation at 25°C). The data are from a single unreplicated experiment. (See Chapter 3 for details of methods).

### 7.3 DISCUSSION

#### 7.3.1 Effects of Ageing and Pre-sowing Treatment on the $\alpha$ -amylase Activity of Wheat Seeds

##### 7.3.1.1 *Effect of ageing on base levels of activity*

In the literature, base levels of  $\alpha$ -amylase activity in mature, quiescent wheat grains are generally shown as traces (Sec. 2.4.4). However, levels can vary considerably among cultivars. For example, Marchylo and Kruger (1987) reported that mature kernels of a white wheat contained about fifty times more activity than those present in three red wheat cultivars although they were all grown under similar conditions and did not suffer any visible sprouting damage.

Compared to the high levels of enzyme activity produced by germinating seeds (Fig. 7.3), very low base levels, such as those detected in ungerminated-untreated seeds of cv. Karamu (Tab. 7.1) have been suggested to be due to residual activity from the developmental phase of the grain and are thought to be largely located in the pericarp (see Sec. 2.4.4). Absence of any of this activity in the embryo and its uniform distribution in the endosperm region which was assayed with its seed coat and pericarp (see Fig. 7.2), seems to support this suggestion.

The small increases in base levels during storage (Fig. 7.4, untreated seeds) correspond with the findings of Warchalewski *et al.* (1985) during longer storage of wheat seeds (see Sec. 2.6.2). Such increases were suggested to be due to release of enzyme activity from its bound forms. However, during the present study inhibition of exogenous  $\alpha$ -amylase activity could not be detected in the extracts of either unaged or aged wheat seeds (Sec. 3.9.1.2). In sorghum seeds  $\alpha$ -amylase activity has been shown to increase initially followed by rapid

decline to much lower levels during storage at 30°C and 17% SMC (Perl et al., 1978).

#### 7.3.1.2 *Effect of pre-sowing treatment on the $\alpha$ -amylase activity of unaged and aged seeds*

The increases in  $\alpha$ -amylase activity of unaged wheat seeds observed as a result of PST (Tab. 7.1, ungerminated seeds) were not detected in the earlier work of Hanson (1973). Such differences in results are likely to be the function of either the relative sensitivities of the different assay systems used or differences in cultivar responses to the treatment. If the increase in enzyme activity was due simply to release of bound forms, the increases expected might be between two to three fold over the base levels (cf. Kneen et al., 1942; Warchalewski et al., 1985). On the contrary, the 25 fold increase over base levels in treated seeds (Tab. 7.1) are similar in magnitude to abnormal increases in  $\alpha$ -amylase activity reported in sprouting damaged wheat grains (Sargeant, 1979). Moreover, appearance of  $\alpha$ -amylase for the first time in the embryo (Tab. 7.2) and large increases in proximal endosperm levels as a result of treatment (Plate 7.1; Fig. 7.2) suggests that the activity is due to germinative metabolism.

Although slow or rapidly aged seeds do not produce  $\alpha$ -amylase in similar levels to unaged seeds during PST (Figs 7.4 and 7.6), they show similar ability as unaged seeds to produce the enzyme rapidly during the first 24 h of germination as a result of pre-sowing treatment (Figs 7.5 and 7.7, Sec. 7.2.4). This suggests that the age-induced delay in  $\alpha$ -amylase production (Fig. 7.3) has been effectively reduced by PST.

Increased enzyme levels in unaged seeds as a result of treatment were maintained without significant loss during their subsequent exposure to either slow or rapid ageing conditions (Figs 7.8 and 7.9), suggesting the stability of enzyme activity.

in stored seeds. However, the inability of treated-stored seeds (of those treated before ageing, only 2 and 4 d rapidly aged seeds retained some viability; see Tabs 7.5 and 7.6) to re-start  $\alpha$ -amylase production during the first 24 h of germination (Tab. 7.7) indicates possible ageing damage to the synthetic processes involved in increased enzyme production completed during the pre-sowing treatment.

### 7.3.1.3 *Age-induced reduction of aleurone tissue sensitivity to GA<sub>3</sub> and the effects of pre-sowing treatment*

It must be emphasised that the enzyme levels produced in the in vitro studies using endosperm halves (e.g. Figs 7.10, 7.11 and 7.12) are several fold higher than that found in intact germinating seeds (Fig. 7.3), and hence a direct comparison cannot be made between them. These differences probably reflect large differences between in vitro and in vivo GA concentrations at this stage of germination (cf. Artsruni and Panosyan, 1984). However, the in vitro studies using endosperm halves are useful in understanding some of the possible physiological changes that might occur in aleurone tissue as a result of ageing or pre-sowing treatment of wheat seeds. They may also provide a useful system for studying the mechanisms of sensitivity changes in tissues to growth substance (details in Sec. 7.3.4).

There is no clear indication of any difference in a lag period before enzyme production either due to ageing or due to pre-sowing treatment. In all cases endosperms began to produce  $\alpha$ -amylase after 18 h incubation in GA<sub>3</sub> (Figs 7.10, 7.11 and 7.12). Earlier studies have shown that wheat aleurone exhibits a 12 h lag period for  $\alpha$ -amylase production in vitro of which the first 6 h do not need the presence of GA<sub>3</sub> (Collins *et al.*, 1972), although it may be dangerous to assume that all cultivars (and even seed lots) show precisely the same time frame. Some early events like induction of phospholipid

synthesis and proliferation of endoplasmic reticulum in the wheat aleurone occur independently of the embryo or GA (Colborne *et al.*, 1976). Thus, presence of GA may not be necessary until later stages of the lag period when the transcription of  $\alpha$ -amylase mRNA is initiated in the aleurone cells (see Sec. 2.4.5).

Low levels of enzyme production by the aged aleurone compared to unaged tissue suggests possible damage to the ability of aleurone to produce  $\alpha$ -amylase in response to GA. Earlier works by Aspinall and Paleg (1971) in wheat and Jackson (1971) in barley, similarly showed a decrease in the release of reducing sugars by aged endosperms incubated in GA<sub>3</sub>. In contrast, Artsruni and Panosyan (1984) found no evidence of damage to the aleurone of aged wheat seeds. In all these earlier studies unaged and aged seed lots were chosen from different years of harvest and hence the observed differences in aleurone performance may also be due to seed lot effects. Moreover, there were also differences in the germination capacity of seed lots compared. In the present study, however, unaged and aged seeds were derived from the same original lot and had similar germination capacity.

Hanson (1973) reported some improvement in oxygen uptake and protein synthetic ability of unaged aleurone tissue due to pre-sowing treatment, but the effects on  $\alpha$ -amylase producing ability were not investigated. Results from this study show little change in aleurone response to GA<sub>3</sub> due to treatment (Fig. 7.11). Interestingly however, some of the ageing damage to aleurone tissue seems amenable to repair as the endosperm halves of aged seeds produce significantly higher enzyme activity as a result of PST (Fig. 7.12).

### 7.3.2 **The Relationship Between Changes in $\alpha$ -amylase Activity and Germination Performance of Wheat Seeds**

There seems to be no direct relationship between  $\alpha$ -amylase levels and seed germination rates. For instance, 20 or 40 d slow aged seeds showed no substantial increases in  $\alpha$ -amylase activity due to PST (Fig. 7.4), yet germinate at similar rates to unaged-treated seeds (Tab. 7.3) having higher levels of enzyme activity. On the other hand 2 or 4 d rapidly aged seeds, treated before storage contain higher levels of  $\alpha$ -amylase activity than untreated controls (Fig. 7.9), but their germination rates are slower (Tab. 7.6). Aspinall and Paleg (1971) found no improvement in germination rates of aged wheat seeds by pre-incubating them in sucrose solution and hence suggested that sugar release by the endosperm may not be an important cause of slower germination. However, both germination rates and  $\alpha$ -amylase metabolism appear to be influenced similarly during pre-sowing treatment of wheat seeds. Irrespective of ageing, seeds respond to treatment by showing both increased germination rates (Tabs 7.3 and 7.4) and early enzyme production during germination (Figs 7.5 and 7.7). Thus improved seedling growth observed in response to PST (see Section 5.2.2.3) may be partly due to rapid mobilization of endosperm reserves to the embryo, consequent to rapid development of  $\alpha$ -amylase and possibly other hydrolytic enzyme activities during the germination of treated seeds.

### 7.3.3 **Effects of Ageing or Treatment Related Changes in $\alpha$ -amylase Activity on Stored Starch**

Comparison of soluble sugar levels of unaged and 40 d aged, untreated seeds (Tab. 7.8) indicate that increases in base levels of  $\alpha$ -amylase activity during storage (Fig. 7.4) may not cause damage to stored starch. This may well be expected considering the relatively small magnitude of increases in

$\alpha$ -amylase activity during storage in comparison to those occurring during germination (see Fig. 7.3).

Higher levels of  $\alpha$ -amylase activity were induced as a result of pre-sowing treatment of unaged seeds (Tab. 7.1). These increased enzyme levels were maintained in active form (Figs 7.8 and 7.9), accompanied by severe losses in vigour and viability (Tabs 7.5 and 7.6) during subsequent storage of treated seeds. Yet, the starchy endosperm apparently showed no evidence of  $\alpha$ -amylase attack (for example pitting or surface erosion) on those starch granules near the scutellum-crease region which were no longer surrounded by protein matrix (Plate 7.2, A and B; see also Sec. 7.2.3.1).

The in vitro studies of Fincher and Stone (1974) in wheat, showed that erosion of starch granules is a relatively late event during endosperm degradation. They reported that, although rapid dissolution of protein matrix was seen in the sub-aleurone region after 24 h incubation of endosperm halves in GA<sub>3</sub>, the starch granules remained unaffected. Absence of any quantitative change in the total soluble sugar content of treated seeds as a result of storage (Tab. 7.8) would appear to confirm that  $\alpha$ -amylase levels are not a primary cause of deterioration of stored seeds. It is possible that  $\alpha$ -amylase may not be secreted into the starchy endosperm either during treatment or during subsequent storage of seeds. Secondly, studies in vitro suggest that all isoenzyme groups of  $\alpha$ -amylase may not have similar ability to degrade granular starch (see Sec. 2.4.2). Thus identification of the  $\alpha$ -amylase group in unaged-treated seeds may be helpful in explaining the absence of damage to starch.

On the other hand, very large increases in leakage of sugars from treated-stored seeds (Tab. 7.8) are evidence that other types of hydrolytic damage (particularly that affecting membrane permeability) are occurring in these rapidly deteriorating seeds (see also data on protease activity in Chapter 6).

#### 7.3.4 Possible Mechanisms of Changes in $\alpha$ -amylase Metabolism During Ageing or Pre-sowing Treatment

The increase in  $\alpha$ -amylase activity during treatment of unaged seeds (Tab. 7.1) and the ability of all treated grains, irrespective of ageing, to resume  $\alpha$ -amylase production quickly upon re-imbibition (Fig. 7.5) suggest possible completion and preservation, during drying back (PST), of all the essential metabolic processes required for commencement of enzyme production. This includes production of GA by the embryo and its signalling of mRNA induction by the target tissues (see Sec. 2.3). Concentration of increased  $\alpha$ -amylase activity in the endosperm adjacent to the embryo suggests embryo control over the enzyme production (Fig. 7.2).

Intact aged untreated wheat seeds express damage to  $\alpha$ -amylase metabolism by showing a longer lag period before the start of enzyme production during germination (Fig. 7.3). This may be due to delayed GA production by the embryo and/or delayed response from the target tissues. However, in vitro studies with exogenous GA<sub>3</sub>, show that ageing does not affect the aleurone lag period for  $\alpha$ -amylase production (Fig. 7.10). Thus, it appears that delayed signalling by GA may be the cause of increased lag periods for  $\alpha$ -amylase production in aged seeds. Recently Petruzzelli and Taranto (1990) have shown that differences in the levels of enzyme production between unaged and aged wheat seeds, during germination, disappears when GA<sub>3</sub> is included in the germination media. But the authors did not show whether the GA<sub>3</sub> effects are mediated by changes in lag periods before enzyme production.

Apart from lag phase effects, the levels of  $\alpha$ -amylase produced in germinating wheat seeds may also be affected either due to deficiencies in the growth substance or due to losses in the ability of responding tissue to produce the enzyme.

This aspect has already been mentioned in Section 7.3.1.3 and was investigated in a little more detail in Section 7.2.4.3 in order to understand the possible mechanisms involved.

After considerable debate (Trewavas and Cleland, 1983), it is now generally accepted that plant growth responses are controlled by changes in the sensitivity of the responding tissue as well as changes in the concentrations of plant growth regulators. Although the molecular mechanism of hormone action in plant cells is not clearly understood (Roberts and Hooley, 1988), it is assumed that the observed metabolic changes are a consequence of interactions between the molecules of the growth substance and receptors within the cell. Firm (1986) suggested that three factors can be used to explain more clearly the sensitivity changes in plant cells:

- a) the number of receptors in the cell
- b) the affinity of receptor for the growth substance
- c) the overall response capacity of the cells.

Additionally, the observed response can also depend on the uptake efficiency of the growth substance as well as its stability within the responding cells.

In general, in this study, there was no indication of changes in the affinity of receptors to growth substance either in differently aged (Fig. 7.13) or differently treated aleurone tissue (Figs 7.14A and B) as the threshold concentration required to evoke response ( $2 \times 10^{-8} \text{M}$ ,  $\text{GA}_3$ ) remained the same in all cases, as well as the concentration giving maximum response ( $2 \times 10^{-6} \text{M}$ ,  $\text{GA}_3$ ).

However, endosperm halves of 40 d aged wheat seeds showed a reduction in the maximum response capacity compared to those from unaged seeds (Fig. 7.13). Reduced capacity of aged tissue to produce the enzyme may be due to either

a decrease in the number of receptors and/or a decrease in the overall capacity of the cells to produce the enzyme. A decrease in the number of receptors would be expected to result also in a reduced response at all concentrations below that which gave the maximum response level. Accordingly, an apparent reduction in the slope of the curve for aged aleurone compared to unaged tissue (Fig. 7.13) may be a possible indication of loss in receptivity, but it may be premature to draw such conclusions because of problems with reproducibility of these results (see Sec. 7.2.4.3).

Pre-sowing treatment appears to improve the maximum response capacity of the aleurone tissues of both unaged and aged seeds (Figs 7.14, A and B). Such effects may be mediated by changes in overall response capacity of the cells rather than changes in receptivity as there were little or no changes in the slope of curves between untreated and treated aleurone tissues.

This study is limited by the fact that there is no information on the uptake efficiency or stability of  $GA_3$  in the aleurone cells of differently aged or treated wheat seeds. Also aleurone responses were measured after a 42 h incubation period and thus changes in initial responses are not known. Despite these limitations, this study suggests the possibility of changes in tissue sensitivity to plant growth regulators during ageing of wheat seeds.

## CHAPTER 8 GENERAL DISCUSSION

### 8.1 EVALUATION OF KEY FINDINGS AND LIMITATIONS OF THIS STUDY

#### 8.1.1 Pre-Sowing Treatments for Protection or Invigoration of Wheat Seeds

The results of this study clarify some of the basic confusion in the literature about the utility of different pre-sowing hydration treatments for improving the germination performance of stored wheat seeds. The short and longer hydration treatments were found to have clearly different roles: i.e. protection of germination capacity (Sec. 5.2.2.2) and invigoration (Sec. 5.2.2.3) respectively for stored seeds. Use of short hydration treatments after storage or longer hydration before storage either proved to be of little value or caused severe damage to viability and germination performance.

The invigoration of unaged seeds by longer hydration treatments confirms the earlier work of Hanson (1973). The earlier Indian work (Basu's group) with respect to protection of germination capacity by pre-storage short soaking treatments was also confirmed during this study (Sec. 5.2.2.2). However, the deleterious effects of longer hydration treatments prior to storage, observed during this study (cf. preliminary study of Lush *et al.*, 1981), clearly disagrees with the findings of Rudrapal and Basu (1982) and also with the recent report of Dell'Aquila and Tritto (1990).

As yet there is no clear evidence for the mechanisms involved in the protection of germination capacity by short soaking treatments. Although this study showed that leaching of germination inhibiting substances can occur during these

short soaking periods, this may not be directly related to protective effects in storage. This is because, similar protection of stored seeds can also be obtained by simple exposure of seeds to saturated atmosphere (see Rudrapal and Basu, 1982) which apparently does not allow leaching of any metabolites from seeds although it may be possible that, under these conditions, toxic substances are removed from active sites of damage within the seed.

Short soaking periods (post-storage) appear to be insufficient to allow physiological repair of ageing damage because, in the present study, they were unable to produce appreciable decreases in times to 50% radicle emergence (T50's) of aged seeds. This clearly contrasts with the suggestions of Goldsworthy *et al.* (1982), that effective repair processes are possible in the shorter time scales of 5 to 30 minutes. This group used higher imbibition temperatures than those described as optimal for germination of wheat seeds (ISTA, 1985). In this study a soak temperature of 35°C, suggested as optimal by Goldsworthy *et al.* (1982), proved deleterious for low vigour seeds (Sec. 4.2.2 and 4.2.3), while substantial invigoration of aged seeds (measured as T50's) was gained from longer hydration treatments using temperatures nearer the optimum for germination (Sec. 5.2.2.3). This study also showed that the invigoration effects on aged seeds can be retained upon drying of seeds to approximately original moisture levels. Earlier work on aged seeds by Dell'Aquila's group (Dell'Aquila *et al.*, 1984; Dell'Aquila, 1987), did not attempt to dry the seeds and instead germinated them immediately after longer hydration treatments. Some possible mechanisms of invigoration of aged seeds, using a longer hydration treatment, are discussed in Section 8.1.2.

The protection of viability by short hydration treatments without influencing seed vigour during storage, coupled with rapid germination losses despite substantial invigoration by

longer hydration treatments prior to storage, suggest that mechanisms of damage to vigour may not be the same as those causing viability losses in wheat seeds. As vigour losses generally precede losses in seed viability during storage, several workers (e.g. Ellis and Roberts, 1981; Dearman *et al.*, 1986) had earlier proposed the hypothesis that progressive accumulation of ageing damage leading to vigour losses may finally culminate in seed death. However, the results of this study agree with those of Coolbear *et al.* (1984) on tomatoes, who found that seed viability and vigour are not necessarily expressions of the same deteriorative sequence.

#### 8.1.2

#### **Relationship Between $\alpha$ -amylase or Protease Activity and Germination Performance in Wheat Seeds**

Although invigoration due to hydration treatments in different crop species has been shown to be accompanied by several types of physiological and biochemical changes (e.g. Hanson, 1973; Coolbear and Grierson, 1979; Khan *et al.*, 1980; Dell'Aquila *et al.*, 1984), very few attempts have been made (for e.g. Coolbear and Grierson, 1979; Khan *et al.*, 1980) to understand their relationship to changes in germination performance of seeds. In wheat, systematic studies have not been made to understand the relationship between hydrolytic enzyme activities and germination performance. In the following sections this aspect will be discussed from the results of a most effective invigorative pre-sowing treatment (PEG treatment: 20 h hydration in PEG solution at 20°C followed by drying back. Details in Sec. 3.3.2.2).

#### 8.1.2.1

#### *Relationship between $\alpha$ -amylase activity and germination performance*

The present study showed a build up of  $\alpha$ -amylase activity during PEG treatment of unaged seeds (Sec. 7.2.1.1), a finding which was not detected in the work of Hanson (1973). In

addition to this, rapid emergence of radicles (measured as T50's) (Tab. 7.1) accompanied by rapid development of  $\alpha$ -amylase activity (cf. Hanson, 1973) during subsequent germination of treated seeds suggest possible advancement, during hydration, of some of the desiccation tolerant steps involved in these early germination processes. Further, exposure of unaged-untreated seeds to ageing conditions was found to increase the lag periods of these two events, whereas PEG treatment after ageing counteracts both the ageing effects causing early radicle emergence as well as rapid development of  $\alpha$ -amylase activity during germination (Sec. 7.2.2.1). These results suggest that, in addition to completion of desiccation tolerant processes of germination, longer hydration periods allowed for repair of age induced damage to early germinative events.

However,  $\alpha$ -amylase activity appears to have no direct relationship with radicle emergence rate. Evidence for this includes the observation that after treatment, unaged and aged seeds have similar T50's (Tab. 7.3) despite having different levels of  $\alpha$ -amylase activity (Fig. 7.4). Also treated seeds exposed to rapid ageing conditions, contain higher levels of  $\alpha$ -amylase activity (Fig. 7.9) but much delayed radicle emergence compared to similarly aged untreated seeds (Tab. 7.6). This clearly disagrees with a hasty conclusion (based on correlative evidence) made by Petruzzelli and Taranto (1990) that  $\alpha$ -amylase production is always linked to changes in germination rates in wheat seeds.

However increased seedling dry weights in unaged or aged seeds subjected to PEG treatment (Sec. 5.2.2.3) may be partly due to rapid mobilization of starch reserves mediated by rapid development of  $\alpha$ -amylase in them during germination.

In germinating seeds, the time lag for  $\alpha$ -amylase production can be influenced mainly by the lag period for gibberellin

synthesis in the embryo and/or the lag period for aleurone enzyme production. In the present study, ageing did not seem to affect the lag period for aleurone enzyme production when tested in vitro (Fig. 7.10), which suggests that delayed gibberellin synthesis in the embryo might be the likely cause of delayed  $\alpha$ -amylase production in germinating seeds. However, more direct evidence is needed on this aspect of ageing damage.

In the literature there is no clear explanation of ageing effects on aleurone tissue with respect to its ability to produce  $\alpha$ -amylase enzyme. Earlier suggestions on the occurrence of ageing damage to aleurone tissue with respect to production of  $\alpha$ -amylase (Preliminary studies by Aspinall and Paleg, 1971) were contradicted by more recent work of Artsruni and Panosyan (1984), although none of these appeared to be well planned attempts to provide clear answers on this aspect (see Sec. 7.3.1.3). In vitro studies during the present investigation showed a significant reduction in the ability of aged aleurone to produce  $\alpha$ -amylase, in response to exogenously applied gibberellic acid, compared to unaged tissue (Sec. 7.2.4.1). The effects of such aleurone deficiency on in vivo  $\alpha$ -amylase production during germination of aged seeds, however, needs further investigation.

The reversal of age induced damage to aleurone enzyme production, in response to  $GA_3$  in vitro, as a result of PEG treatment of aged seeds is a particularly interesting result (Sec. 7.2.4.2). This finding not only provides additional support for the hypothesis of operation of repair processes during longer hydration of seeds, but also makes the wheat aleurone system look attractive for studying the mechanisms of changes in the sensitivity of plant tissues to plant growth regulators (cf. Gibson and Paleg, 1982).

### 8.1.2.2 *Relationship between protease activity and germination performance*

There appears to be no work relating protease activity to germination performance in wheat seeds. In general, protease activity (azocoll substrate, pH 6.8) was not significantly influenced either by ageing or by PEG treatment (Sec. 6.2.4). An exception to this was 60 d controlled deterioration (which results in complete loss of viability) where there was a significant drop in enzyme activity of untreated seeds. This loss was prevented by PEG treatment of seeds prior to storage. As observed with  $\alpha$ -amylase, the base levels of protease activity (Tab. 6.1) were not related to changes in germination rates (T50's in Tab. 7.3) observed after ageing of untreated seeds or after PEG treatment of either unaged or aged seeds. Changes in germinative protease activity were, however, not assessed during this study. Despite some apparent evidence of qualitative changes to matrix proteins of the starchy endosperm due to PEG treatment (Sec. 6.2.1), or to high molecular weight proteins ( $\approx 270$ kD) during subsequent storage of treated seeds (Sec. 6.2.2), there was no quantitative change in storage proteins (Sec. 6.2.3). Further studies are needed to find out if there is any change in the pattern of storage protein degradation during germination, as a result of PEG treatment.

Also separate studies on proteins and proteolytic enzyme activity changes in the embryo appear necessary as they might be more closely related to vigour or viability losses than changes in whole seeds.

### 8.1.2.3 *Comparison of the effects of PEG treatment with those of sprouting damage*

An additional implication of this study was that PEG treatment appeared, in some ways, to mimic sprouting damage occurring during maturation of wheat seeds under wet harvest conditions.

For example similar to the effects of PEG treatment on unaged seeds (Sec. 7.2.1.1 and 6.2.1), sprouting damaged wheat seeds have been reported to contain higher levels of  $\alpha$ -amylase activity (Sargeant, 1979) and show some clearance of matrix proteins in the starchy endosperm (MacGregor and Matsuo, 1982). Surprisingly however, treated seeds showed neither evidence of damage to stored starch in situ, nor evidence that the increased  $\alpha$ -amylase might affect bread quality during baking (see Appendix 5). These results clearly contrast with sprouting damage, in which the increased  $\alpha$ -amylase levels are known to cause damage to starch both within the seeds (e.g. MacGregor and Matsuo, 1982) as well as subsequently during baking (see Haverson and Zeleny, 1988). Some evidence in the literature suggests that different groups of  $\alpha$ -amylase isoenzymes of wheat may have different abilities to degrade granular starch (see Kruger and Lineback, 1987). However, these are in vitro studies and may not explain the starch degrading abilities of  $\alpha$ -amylase isoenzymes in vivo.

### 8.1.3

#### **Effects of High vs Lower Moisture Ageing in Wheat Seeds**

Some evidence in soybean (Priestley and Leopold, 1983 and also see Bewley, 1986) and tomato (Francis and Coolbear, 1987) seeds suggest that deteriorative events occurring under high and lower moisture seed ageing may be different. However no such attempts have been made in wheat seeds, and higher moisture rapid ageing techniques are still being used (e.g. Ram and Wiesner, 1988) for studying the metabolism of seed deterioration. The present studies clearly demonstrate that the physiological events occurring during lower moisture storage are different from those at higher seed moisture regimes. For example membrane permeability increased during lower moisture controlled deterioration (15% SMC), but did not change during accelerated ageing at saturated humidity ( $\sim$  25% SMC) although losses in germination capacity were of a similar magnitude under both storage regimes (Sec.

5.2.1). Similarly plumule growth was differently affected by the two ageing methods. Thus despite their convenience, rapid ageing techniques involving higher seed moisture regimes may not be useful for studying the metabolism of deterioration in wheat seeds.

#### 8.1.4

#### **Limitations of this Study**

The following are some of the important limitations of this research which need further clarification in order to understand some of the observed ageing or treatment effects more clearly.

- 1) The present study did not investigate the influence of microflora during seed deterioration. There were no symptoms of microbial damage, like mould growth or rotting, during ageing or treatment of seeds and during their subsequent germination. Accordingly the use of disinfectant chemicals on whole seeds was avoided as they might introduce additional variables affecting the germination responses (e.g. Peck et al., 1976).
- 2) A major part of this research was conducted on a single seed lot of wheat as it was intended to characterise the ageing and treatment effects in a single system in as much detail as possible. However, variation in responses between seed lots must always be expected. For example, in Ram and Wiesner's (1988) work different cultivars and seed lots of wheat responded similarly to accelerated ageing conditions although changes in physiological and biochemical parameters of seed quality varied significantly.
- 3) Initial studies (Sec. 4.2) revealed that the effects of ageing or pre-sowing treatments evaluated

by germination trials at 10 and 20°C were basically similar. Hence, in order to emphasise the ageing or treatment effects, most of the subsequent studies relating to evaluation of germination performance were conducted at 10°C. However, this study provides no information on the germination performance of differently aged or treated seeds in other types of stress conditions which might be encountered in the field (for example, under water stress).

- 4) Changes in membrane permeability were examined only by indirect methods; i.e. by measuring leakage of electrolytes from seeds. However, biochemical changes to membrane structure were not studied.
- 5) There was some indirect evidence that small decreases in T50's due to short soaking treatments could be due to leaching of germination inhibiting substances from wheat seeds. However, no attempt was made to identify the nature of the germination inhibiting compounds.
- 6) The major part of enzyme studies were conducted using seed extracts. However, it must be realised that, enzyme activity measured this way may not always truly represent the activity in situ, due to losses of compartmentalization between enzymes and their inhibitory compounds and/or proteolytic activity during seed extraction. Also enzyme extraction efficiency itself can be variable. Although the extraction procedure and assay for  $\alpha$ -amylase was validated as far as possible (Sec. 3.9.1), this was not done for the protease studies.

- 7) Some studies (Sec. 2.4.2) suggest differences in the ability of  $\alpha$ -amylase isoenzymes to degrade granular starch in vitro. However in the present case,  $\alpha$ -amylase assays were restricted to the measurement of gross enzyme activities. Accordingly no information was obtained on any changes in isoenzyme composition within the seeds. Similarly no attempt was made to differentiate different types of proteolytic enzymes within the seeds.
- 8) Owing to limited resources, scanning electron microscope examination of the starchy endosperm was confined to the scutellum-crease region. Thus, the extent of protein matrix degradation (Sec. 6.2.1) in other parts of endosperm as a result of pre-sowing treatment of wheat seeds is not known.
- 9) Although the lag phase for aleurone  $\alpha$ -amylase production in vitro did not seem to change due to either ageing or PEG treatment, this needs further confirmation by measuring enzyme activity changes at more frequent intervals than those used in this study. Aleurone responses (in vitro) to exogenously applied GA<sub>3</sub> changed significantly due to both ageing and pre-sowing treatment, however the mechanisms of these changes were not investigated. Also the impact of such changes on aleurone responsiveness to hormones in vivo is not known.

## 8.2

### SCOPE FOR FUTURE WORK

Many areas for future study are suggested by both the limitations of this work and some questions of major importance

which it raises. These will be discussed briefly in the following sections:

### 8.2.1

#### **Metabolism of Deterioration in Wheat Seeds**

As vigour and viability losses appear not to be the results of the same deteriorative sequence (Sec. 8.1.1), it raises key question about what cellular events are critical for these two different deteriorative processes. In this regard, the pre- and post- storage hydration treatments reported in this study appear to have the potential to be useful investigative tools.

They appear to provide a good opportunity for the examination of the relationship between membrane damage and vigour or viability losses, because membrane permeability was affected in a variety of ways by changes in storage and treatment conditions. Suggested mechanisms of membrane damage include both oxidative and hydrolytic damage (Priestley, 1986). By examining losses in differently unsaturated fatty acids and by-products of lipid peroxidation, it may be possible to determine under what conditions lipid peroxidation is a major cause of damage. On the other hand the significance of hydrolytic damage could be determined by studying changes in the activity of phospholipases, proteases and the resulting hydrolysis products. Assessment of in situ enzyme activities seems very important and in this regard, the usefulness of histochemical blotting procedures (Okamoto et al., 1980) and application of immunoassay principles need to be explored. A special attention should also be given to the mechanisms of enzyme activity changes, for example by studying changes in the levels of enzyme activity inhibitors or damage to lysosomal membranes.

Another important question is, how far does the evidence from wheat and soybean that the metabolism of higher and lower

moisture storage being different, applies to other crop species. Until clarification is obtained for other crop species it is advisable to avoid use of higher seed moisture regimes for studying the metabolism of seed deterioration.

### 8.2.2 **The Role of Plant Growth Regulators and Tissue Sensitivity in Seed Ageing**

The role of plant growth regulators (PGR's) in seed ageing is not at all clear (Priestly, 1986). Recent work of Petruzzelli and Taranto (1990) in wheat do not provide any evidence as to whether gibberellins are limiting  $\alpha$ -amylase production in aged seeds. In the present study there was some indication that gibberellin synthesis during germination may be delayed as a result of ageing of wheat seeds. It is, however, not known if there are any quantitative deficiencies in gibberellin production due to ageing. Recently there has been considerable improvement in immunoassay techniques and development of monoclonal antibodies that may help in the detection and quantification of gibberellins in plant tissues (Eberle *et al.*, 1986). Time-course studies on gibberellin synthesis during germination may provide some answers regarding the effects of ageing on gibberellin production in wheat seeds. There is also a need for the investigation of the influence of PGR's on germination processes in non-dormant embryos other than their key role in endosperm mobilization. There seems to be little information on this (e.g. see Bryant, 1985).

The results of in vitro studies on aleurone tissue also raises another key question about the importance of tissue sensitivity to PGR's in seed ageing. Losses in aleurone responsiveness to GA<sub>3</sub> with respect to  $\alpha$ -amylase production as a result of ageing was, at least in part, reversed by PEG treatment of aged wheat seeds. Accordingly, this treatment appears to be a very useful tool for studying changes in aleurone sensitivity to PGR's. Before proceeding with this work, however,

there is a need to confirm the present observation that aleurone lag phase times are not altered due to ageing or PEG treatment. Subsequently detailed studies on the pattern of aleurone responses over a range of GA<sub>3</sub> concentrations using differently aged or treated seeds may help in understanding possible mechanisms of changes in aleurone sensitivity, in terms of receptivity, affinity to PGR molecules or cellular response capacity (Firn, 1986), to gibberellic acid.

#### 8.2.2.1 *Stability of gibberellins and $\alpha$ -amylase mRNA in wheat seeds*

It has been reported that the structural properties or cellular events that influence mRNA stability in germinating cereals are not very clear (see Fincher, 1989). In the present study, quick resumption of  $\alpha$ -amylase production by treated seeds suggests that some of the steps involved in the enzyme production may be tolerant of desiccation. The survival of active gibberellins and  $\alpha$ -amylase mRNA's in treated seeds needs verification. Storage of treated seeds caused damage to  $\alpha$ -amylase metabolism. Using different storage and treatment conditions it maybe possible to study the cellular events, that influence the stability of  $\alpha$ -amylase mRNA in the aleurone and scutellum of wheat.

#### 8.2.2.2 *Physiological studies on sprouting damage in seeds*

In some ways, treatment-induced increases in  $\alpha$ -amylase activity appears similar to those in sprouting damaged wheat seeds. However, the enzymes of treated seeds neither damaged starch in situ, nor seemed to affect the baking quality of the flour (Sec. 8.1.3). Accordingly identification of  $\alpha$ -amylase isoenzymes in treated seeds would be crucial additional information, and the relationship of these isoenzymes to in vivo starch degradation should be explored.

Other crop species have also been reported to suffer weathering damage similar to wheat (e.g. Murthy et al., 1984 in sorghum;

Woodstock *et al.*, 1985 in cotton). Pre-sowing treatments, similar to those used in this study might be developed to suit wheat and other crop species in order to study the effects of weathering damage and associated problems with seed quality, either for planting or for milling purposes.

### 8.2.3

#### **Commercial Utilization of Pre-sowing Treatments**

The high costs of handling in relation to the advantages gained, are likely to render these Pre-sowing treatments uneconomical for general use. However, the usefulness of these treatments in over-coming different types of environmental stress conditions, for example poor soil moisture availability, increased soil salinity or low soil temperature, needs to be explored. Secondly, as aged seeds respond much better than unaged seeds (Fig. 5.8) these treatments may be useful under some special circumstances such as the multiplication of germplasm or breeder seed stock stored for longer periods, or in cases where the seed crop has failed and the quality of carry-over seed needs to be maximised.

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Appendix 1 Scanning electron microscopic studies of the starchy endosperm.

Dry wheat seeds (12.5 to 13.5% seed moisture) were bisected longitudinally using a clean razor blade exposing both the embryo and endosperm in a middle plane. One half of each seed, with its cut surface facing upwards, was glued to aluminium 'scanning electron microscope' specimen slab using a conductive silver paint. The glued half seeds were then sputter coated with 200 Å gold. The cut surface of these seeds was examined using a 'Cambridge 250 MKIII' scanning electron microscope. The examination was localized to the region near scutellum-crease junction. Special attention was paid to find if there were any apparent changes to matrix proteins and starch grains in aged and/or treated seeds compared to unaged-untreated seeds. Randomly selected samples of 10 seeds were examined for each treatment.

Appendix 2 Method for HPLC analysis of seed proteins.

Albumins and globulins were extracted from 100 mg flour using 2 ml of 2% saline solution. After centrifugation, gliadins were extracted from the pellet using 2 ml 70% ethanol. The residue obtained following another centrifugation was used for extracting glutenins. The residue was extracted for 16 to 20 hours with 2 ml of 0.1 M Tris (hydroxymethyl) methylamine (pH 6.8) containing 2% (w/v) sodium dodecyl sulphate and 1% (w/v) dithiothreitol. The long extraction time was found to be necessary to ensure complete and reproducible extraction of glutenins. The reaction mixture was then centrifuged (48,000 g for 20 minutes) and the clear solution used for HPLC. The HPLC instrumentation and analysis of data are as described by Sutton et al. (1989). A Waters 600 solvent delivery/control system with a Waters WISP 712 automatic sample injection and a Waters 418 variable-wavelength ultraviolet detector were used. The column was a 220 x 4.6 mm Brownlee Aquapore RP-300 fitted with an 18 x 3.5 mm Brownlee Aquapore RP-18 Guard column. Eluted components were detected at 210 nm and chromatographic traces were recorded using the Waters 810 Baseline software operating on a personal computer. For details see Sutton et al. (1989).

Appendix 3 Quantitative changes (relative estimates) in four HMW glutenin subunits as affected by ageing and/or pre-sowing treatment of wheat seeds. The data represent peak areas (mV. sec) recorded during HPLC of the HMW glutenin fraction in a single replicate assay. Figures in parenthesis represent percentage losses in protein compared to unaged, untreated seeds.

	HMW Glutenin Subunits* - peak areas (mV. sec)			
	2	7	8	12
Unaged, untreated	11,310	16,995	4,346	6,818
unaged, treated	9,664 (14.6%)	13,755 (19.1%)	3,599 (17.2%)	5,578 (18.2%)
40 d aged, untreated	10,028 (11.3%)	15,073 (11.3%)	3,670 (15.6%)	6,035 (11.5%)
40 d aged, treated after ageing	10,845 ( 4.1%)	15,590 ( 8.3%)	4,026 ( 7.4%)	6,594 ( 3.3%)
40 d aged, treated before ageing	8,610 (23.9%)	12,741 (25.0%)	3,230 (25.7%)	5,093 (25.3%)

Note: Assays conducted by 'Wheat Research Institute', Christchurch. As no standards have been used, the above figures are only relative estimates and specific to the instrumentation and assay conditions used.

Abbreviations: mv.sec = millivolts per second; HPLC = high performance liquid chromatography; HMW = high molecular weight

\* HMW Glutenin subunit nomenclature after Payne and Lawrence (1983).

Appendix 4 Quantitative changes (relative estimates) in different seed protein fractions due to storage of treated wheat seeds.  
 The data represent peak areas (mV. sec) recorded during HPLC of seed proteins in a second replicate assay.

	Peak areas (mV. Sec)			
	2% Saline Solubles	Gliadins	HMW Glutenins	LMW Glutenins
Unaged, untreated	128,400	130,740	15,210	63,160
40 d aged - treated before ageing	118,400	114,300	15,040	61,450

Note: Assays conducted by 'Wheat Research Institute', Christchurch. As no standards have been used, the above figures are only relative estimates and specific to the instrumentation and assay conditions used.

Abbreviations: mV.sec = millivolts per second; HPLC = high performance liquid chromatography; HMW = high molecular weight.

Appendix 5 Effect of pre-sowing treatment (20 h hydration in -0.37 MPa PEG solution at 20°C followed by drying back) of wheat seeds, cv. Karamu, prior to storage (CD at 15% seed moisture and 35°C) on the baking performance of flour.

Baking Results					
Ageing/Treatment	Work input (Wh/Kg)	Water absorption (%)	Load volume score	Texture score	Bake score total
Unaged - untreated	4.5	62.5	10.5	8.5	19.0
40 d aged - treated before ageing	4.7	62.0	12.5	8.0	20.5

Abbreviations: PEG = polyethylene glycol; CD = controlled deterioration; Wh/Kg = watt hours/kilogram.

Note: Work input is energy required to bring dough to maximum viscosity. The loaf volume score is a figure derived from actual loaf volume and texture score is a subjective assessment of crumb texture on a numerical scale of 0 to 14. Bake score is the sum of numerical values of texture and loaf volume scores. Higher bake score is indicative of higher baking quality of flour. The above data do not show a clear difference in the baking performance between the two samples tested. For details of loaf evaluation methods see Swallow and Baruch (1986).