Genetic deterioration and repair in pea (Pisum sativum L.) seeds during storage

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GENETIC DETERIORATION AND REPAIR IN PEA (*Pisum sativum* L.) SEEDS DURING STORAGE

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ABSTRACT

The relationship between loss of seed viability and the accumulation of chromosomal aberrations has been investigated in both pea cultivars and wild types. It has been shown here, for the first time with wild type peas, that loss of seed viability is associated with an increase in the frequency of chromosomal aberrations, and that even small losses of viability result in some chromosomal damage in both wild type pea seeds as well as in the seeds of pea cultivars. In addition, for a given loss of viability the frequency of chromosomal aberrations in either low or high moisture content seed lots was the same. However, it has been shown that under identical storage conditions, low moisture content seeds of wild type peas exhibit greater longevity than those of pea cultivars.

The chromosomal aberrations were classified into four categories, i.e. chromatid-type, chromosome-type, mixed and others. After controlled deterioration treatments, chromatid-type aberrations were the most frequent and single-fragment aberrations were predominant in the surviving seeds of both cultivated and wild type peas.

The effects of initial seed moisture content and viability on the susceptibility of seeds to soaking

injury were investigated in pea cv. "Douce Provence". There is a negative correlation between seed viability and susceptibility of seeds to soaking injury, in both high and low moisture content seeds but the damaging effect of soaking was more severe in the low moisture content seed lots.

It has been confirmed in this study that post-storage humidification treatments conducted on pea cultivars "Kelvedon Wonder" and "Douce Provence" avoided imbibition injury. The optimum temperature for humidification and the final moisture content to be reached in pea seeds were determined as 16°C and 16.3 - 18.1%, respectively.

A protocol for priming pea seeds was developed. Post-storage priming treatments at 16°C with PEG-8000 (-0.5, -1.0 and -1.2 MPa), ABA (10^{-3} M) and distilled water for 3, 5 and 7 days ameliorated some of the damage which resulted from ageing. Most of the benefits occurred during the first 3 days with PEG or ABA and during the first 5 days in distilled water. Priming treatments increased the final germination and decreased the mean germination time (MGT) and the frequency of chromosomal aberrations due to the possible repair of some ageing induced damage. The results of the priming experiment suggest that the critical moisture content for

repair of chromosomal damage to occur in pea seeds is likely to be between 32% and 46%.

Possible causes of the damage to chromosomes in aged seeds, repair of ageing induced damage, and the practical significance of the results are discussed in terms of the long-term conservation of seed germplasm.

CONTENTS

CHAPTER 1. INTRODUCTION

With increased knowledge and technology in plant genetics and breeding, the necessity for long term storage of small quantities of various cultivars and wild types becomes apparent, especially in the light of conserving the world's rapidly diminishing genetic resources. An international network of genebanks has been set up under the auspices of the Food and Agriculture Organisation of the United Nations (FAO) and the International Board for Plant Genetic Resources (IBPGR) to conserve genetic stocks. Facilities for storing seed germplasm now exist at Fort Collins, *Colorado - USA*, Tsukuba, *Japan*, Braunschweig, *Germany*, Bari, *Italy*, Izmir, *Turkey*, and many other locations.

In most cases, germplasm is stored as seeds, which provide the easiest, safest and most economical method of long-term conservation of genetic resources. The vast majority of economic crop species (arable and horticultural crops) have seeds which can be successfully dried to low moisture contents and stored at low temperatures for long periods without any loss of viability; these are described as orthodox seeds. However, there are exceptions to these generalizations, in particular those seeds which are known as recalcitrant, which cannot be dried without damage.

Therefore, their survival is typically limited to a few weeks or months. The seeds of many tropical plantation crops, many tropical fruit crops and a number of temperate and tropical species are recalcitrant (Roberts, 1973a).

Recently, Ellis *et al.* (1990a, 1991a,b) have suggested that there is a third category of seed storage behaviour intermediate between the above categories defined by Roberts. Seeds with intermediate storage behaviour, e.g. coffee (*Coffea arabica*) and papaya (*Carica papaya*), can be dried safely to about -90 MPa and sometimes to -150 MPa, but further desiccation reduces viability of the surviving seeds. This range is clearly intermediate between the -2 MPa to -3 MPa at which recalcitrant seeds such as cocoa (*Theobroma cacao*) are killed (Roberts and Ellis, 1989) and the -350 MPa or so which is the limit in orthodox seeds can be safely dried to very low moisture contents (Roberts and Ellis, 1989; Ellis *et al.*, 1989). In species with intermediate-type seeds of tropical origin, reduction in seed storage temperature from 15°C to 0°C and below may also reduce the longevity of the dry seeds (Ellis *et al.*, 1990a, 1991a, b).

Since long-term refrigerated storage is normally the preferred method of genetic conservation for orthodox seeds, and the subject of this research, pea (*Pisum*

sativum), is an orthodox species, the present studies concern the seeds of orthodox type.

A great deal of early work (Abdalla and Roberts, 1968, 1969; Abdul-Baki and Anderson, 1972; Roberts, 1972, 1973b; Villiers, 1973; Ellis and Roberts, 1981; Roos, 1982; Murata *et al.*, 1982, 1984; Dourado and Roberts, 1984a; Sirikwanchai, 1985; Rao *et al.*, 1987a) had shown that there is a close correlation between loss of viability during seed storage and the accumulation of genetic damage in the surviving seeds. In order to examine the amount of genetic damage induced, seeds are aged relatively rapidly by storage under controlled conditions, i.e. at high temperatures and moisture contents. The main advantages of these treatments, compared with collection and storage of seeds from a series of harvests, are decreased time in obtaining samples, the greater genetic uniformity of a single collection of seeds and the elimination of physiological variables such as longevity, viability and depth of dormancy of seeds from successive seasons. In addition, seeds subject to accelerated ageing regimes die through an acceleration of the sequence of events occurring in normal ageing (Berjak and Villiers, 1972). To date this work has concentrated on cultivars. This is the first attempt to look at wild types to see if they conform to previous findings.

To ensure that only high viability seed is stored, the accessions are monitored regularly and once they fall below the regeneration standard (i.e. a pre-determined level of viability loss, 85% in most species), the accession is replaced by regenerating fresher seeds (Ellis *et al.*, 1985). Optimum storage conditions recommended by IBPGR for long term seed conservation are -18°C or less in air-tight containers at a seed moisture content of 5±1% (IBPGR, 1976). It is expected that under such conditions, deterioration occurs albeit very slowly. However, capital investment and running expenses are relatively high for such storage, especially in tropical areas. Therefore, IBPGR has played a pioneering role in research into low-cost technology for long-term seed storage, and has supported research on ultra-dry seed storage (in which seeds would be stored hermetically sealed and below 5% moisture content at ambient temperatures) in China and the UK (IBPGR, 1990, 1991). Ultra-dry seed storage is potentially cheaper and easier to use, particularly for developing countries (IBPGR, 1989).

An IBPGR research project yielding good results has been conducted in the Beijing Botanical Gardens, China. *Brassica* seeds which have been dried to 1-2% moisture content showed no adverse effects on germination, vigour or cell structure, in this project. Furthermore, the

ultra-dry seeds were more tolerant to ageing treatment at 50°C than the control (seeds aged at normal storage moisture content). However, the preliminary results of the other research project on genetic stability of seeds in ultra-dry storage (initiated at the University of Reading, UK in late 1989) indicate that greater chromosome aberrations in root-tip cells accumulate for a given loss in viability in barley (*Hordeum vulgare*) seeds, compared with the control seeds at normal storage moisture content (IBPGR, 1991). Although ultra-dry storage is promising, further experiments investigating physiology and genetic stability have to be carried out before this technology can be recommended for use in genebanks.

Since most of the dry and especially very dry seeds can be susceptible to imbibition damage (due to initial rapid uptake of water) during standard germination test procedures, attention has been given in the last decade to methods of improving seed quality by various post-storage hydration treatments, (Ellis and Roberts, 1982; Rao *et al.*, 1987b; Roberts and Black, 1989; Ellis *et al.*, 1989, 1990b). Both humidification and priming, well known post-storage hydration treatments, are suggested as routine in genebanks not only for germination tests used to monitor viability, but also when sowing seeds to produce plants for regeneration or utilization in

breeding programmes. Ellis *et al.* (1985) recommend that humidification is conducted at 20°C as an all encompassing recommendation. However, the validity of this recommendation should be confirmed in the species under investigation.

There are various ways of assessing the type and extent of chromosomal damage which accumulates in seed storage. An ideal method of evaluating the amount and type of chromosomal aberrations in individual cells (in first mitosis) is examining cells at the metaphase stage. However, the present work was carried out by examining the cells in anaphase due to the need to observe a large number of cell divisions in several different storage treatments, and the relative ease in determination of the frequency of chromosomal aberrations using this technique.

It is important to study the first mitotic divisions in investigating the chromosomal aberrations in non-aged and aged seeds as many of the chromosomal aberrations are eliminated as the root elongates, due to diplontic selection (Gaul, 1961). Furthermore, it has long been known that the occurrence of first mitoses is delayed in aged seeds, e.g. in pea (D'Amato, 1951; Dourado, 1983), barley (Murata *et al.*, 1980; Dourado, 1983), onion (Sirikwanchai, 1985), and lettuce (Rao, 1986). In

order to obtain an accurate estimate of chromosome damage, sampling guidelines produced by Dourado (1983) were followed in this study.

In the first experiment (Chapter 4), controlled deterioration treatments were carried out to investigate the relationship between loss of seed viability and the induction of visible chromosomal damage in pea seeds (*Pisum sativum*) stored at high and low moisture contents. To date, the effect of storage conditions on the accumulation of chromosome damage has concentrated on cultivars. This experiment was conducted to investigate the effect of storage conditions on the accumulation of chromosomal damage in known pea cultivars and wild types. The second objective was to determine the predominant type of chromosomal aberration induced as well as the changes in the frequency of type of chromosomal damage with severity of ageing.

Soaking injury is a common problem in dry seeds due to the rapid uptake of water. The effects of initial seed moisture content and viability on the susceptibility of pea seeds to soaking injury were investigated in the second experiment (Chapter 5).

The effects of humidification treatments to ameliorate any damage caused by either ageing or imbibition damage

during standard germination tests were examined in experiment three (Chapter 6). Optimum conditions (i.e. temperature and moisture content to be achieved) of humidification for pea seeds were determined and the effects of humidification were investigated by cytological examination.

The fourth experiment (Chapter 7) concentrated on post-storage priming treatments. Initially preliminary experiments were carried out to determine the optimum conditions of priming using different osmotic potentials of polyethylene glycol (PEG-8000) for various periods on aged pea seeds. Fungal attack to the seeds was the main problem faced in the preliminary experiments. Then an improved priming method was produced to overcome this problem. In the main priming experiment three osmotic potentials of PEG-8000, one concentration of abscisic acid (ABA) and in addition distilled water were used for varying periods to determine the optimum priming conditions for pea seeds. Finally, the possible effects of post-storage priming treatments on improving viability and repairing ageing induced damage in chromosomes of pea seeds were examined.

Under the General Discussion (Chapter 8); first the quantitative relationship between seed viability and chromosomal aberrations in pea cultivars were compared

with wild types. Secondly, the deleterious effect of rapid uptake of water on seed viability was discussed and thirdly, the beneficial effects of post-storage humidification and priming treatments were elaborated on in terms of seed metabolic activity and DNA repair mechanisms.

CHAPTER 2. LITERATURE REVIEW

2.1. Loss of Seed Viability and the Induction of Genetic Damage in Aged Seeds

The term "genetic damage" is used to designate two types of damage, chromosomal aberrations and gene mutations. Previously, following Kostoff (1935) reviewers have suggested that the history of induction of genetic damage in seeds as they age began with de Vries (1901). He observed a high proportion of mutant phenotypes in five-year old seeds of *Oenothera erythrosepala*, and pointed out that the mutant seeds have greater longevity. However, Priestley's (1985) review has clarified the view that mutants observed by de Vries were not actual gene mutations but manifestations of trisomics, polyploids or other complex genetic segregations peculiar to *Oenothera*. Therefore, the historical pioneers of the discovery that genetic damage accumulates as seeds age are Navashin (1933a, b), working on seeds of *Crepis tectorum*, and Peto (1933), investigating seeds of *Zea mays*. Both researchers found that high frequencies of visible chromosome aberrations occurred in roots produced from old seeds.

It is now well known that there is a correlation between loss of seed viability during storage and the accumulation of chromosome damage. Some of the damage is

microscopically visible and some, like point mutations, is not but is manifest in subsequent generations as heritable phenotypic mutations (Roberts, 1988).

2.1.1. Chromosomal aberrations in aged seeds

Since Peto (1933) and Navashin and Gerassimova (1936), the work on the accumulation of chromosome damage with period of seed storage has been confirmed in a wide range of species. These are briefly described below.

Navashin and Gerassimova (1936) found an increased frequency of chromosome aberrations assessed by cytological observations, in root-tips from old seeds of other species of *Crepis*, i.e. *Crepis dioscoridis* and *Crepis capillaris*.

In work on several varieties of onion (*Allium cepa*), Nichols (1941) pointed out that the frequency of chromosomal aberrations increased with an increase in the age of seed and a decrease in germinability, in root tips at first mitosis.

Increased frequencies of chromosomal aberrations due to seed ageing were reported for *Nothoscordum fragrans* (D'Amato, 1948) and for pea (*Pisum sativum*) (D'Amato, 1951).

Gunthardt *et al.* (1953) found that the frequencies of chromosomal aberration (chromosomal bridges and fragments in mitotic anaphases of root-tip cells) increased with the age of the seeds of common wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare*), rye (*Secale cereale*) and peas.

Harrison and McLeish (1954) and Harrison (1966) confirmed that seed ageing promotes the accumulation of chromosomal aberrations in lettuce (*Lactuca sativa*) seeds stored up to five years. However, they also reported that the frequency of aberrations was poorly correlated with decreasing germinability in six-year old onion seed lots.

Abdalla and Roberts (1968), working with seeds of barley (*Hordeum distichum*), broad bean (*Vicia faba*) and pea, found that the frequency of aberrant cells (anaphase figures with bridges and/or fragments per anaphase cells observed) increased with seed ageing. When the percentage aberrant cells was plotted against seed viability the response curves were essentially identical for each storage condition. Therefore, they concluded that as the percentage viability decreased the percentage of chromosomal aberrations increased. Recently, this conclusion was confirmed by Murata *et al.* (1980, 1982, 1984) in aged barley seeds, and by Dourado and Roberts (1984a) in both aged pea and barley seeds.

Dourado and Roberts (1984a) also emphasized that pea seeds which had already accumulated a few aberrant cells subsequently tend to accumulate more aberration than would be expected by chance. This clumped distribution was marked in seeds of very low viability.

Sirikwanchai (1985) investigated the relationship between percentage of seed viability and the frequency of chromosomal damage in onion seeds. She concluded that there is a negative relationship between seed viability and the frequency of chromosomal aberrations, under dry and moist storage conditions. However, Rao *et al.* (1987a) showed that in lettuce seeds more chromosomal aberrations were found at lower moisture contents (3.3% and 5.5%) than at higher moisture contents (13.0% and 18.0%) for any given decline in percentage germination. Therefore, they suggested that for any given percentage viability, the frequency of aberrations was higher in ultra low moisture content stored seeds. More recently, Roberts *et al.* (1992) indicated that in barley seeds ultra dry storage results in less rapid chromosomal damage than dry seed storage while the frequency of chromosomal aberrations accumulated in the former is greater than that in the latter for a given loss in viability.

Thus, there is now no doubt that the production of chromosomal aberrations during ageing is related to loss

of seed viability but the relationship at ultra low moisture contents needs further work.

2.1.2. Phenotypic mutations in aged seeds

In seed ageing studies, chromosomal aberrations have received the most attention because of their ease of detection. Another advantage is that they are seen immediately after germination, at the first mitotic divisions of the root tips. There are several indications that point mutations, particularly those involving recessive genes, accumulate as seeds age but they can only be detected in the following generations. Besides, large number of progenies have to be observed to detect significant differences between aged and control seeds. Two types of study have been commonly investigated in this situation: pollen abortion analysis and the observation of chlorophyll mutant phenotypes (Roos, 1982; Priestley, 1986; Roberts, 1988).

Pollen viability is one of the best ways for detecting age-induced point mutations. Cartledge and Blakeslee (1933, 1934) were the first to demonstrate that pollen infertility in A_1 plants of jimson weed (*Datura stramonium*) arose because of point mutations as well as chromosomal aberrations. Furthermore, they showed that these defects were promoted by increased temperature and

hydration during storage of the seeds from which the A_1 plants were grown (Cartledge *et al.*, 1936). These conclusions have generally been confirmed by later studies, e.g. in rye-grass (Griffiths and Peglar, 1964), barley, broad beans and peas (Abdalla and Roberts, 1969), pea and wheat (Purkar and Banerjee, 1979, 1983). However, Murata *et al.* (1984) were unable to confirm that pollen infertility was linked to seed ageing in barley.

Recessive chlorophyll mutations induced by seed ageing have been a convenient subject of study because of their distinctive appearance in the A_2 generation (Gunthardt *et al.*, 1953; Floris and Meletti, 1972). However, only a few mutations produce obvious phenotypic changes, in particular those affecting pigmentation or gross morphology. Thus, since only a small range of mutations are easily observed, it is necessary to examine large numbers of plants to detect these. Abdalla and Roberts (1969) have presented extensive analyses of ageing induced chlorophyll mutations, in barley, broad beans and peas. They concluded that under conditions leading to a 50% loss of viability in A_1 seeds, approximately 1 to 3% of the surviving A_1 plants yielded A_2 seeds segregating for chlorophyll mutations. Dourado and Roberts (1984b), working with peas and barley, confirmed and extended these conclusion by examining segregation of A_3 progenies. They found that heritable mutations are

induced by seed ageing, and even small losses of viability of a few percent are also associated with significant increases in the frequency of point mutations. More recently, Rao (1986) examined the relationship between loss of seed viability and induction of phenotypic mutations to establish if especially large amounts of mutations occur in the storage of very dry lettuce seeds. Although increases were observed, they were not statistically significant. Dourado and Roberts (1984b) suggested that it is impossible to avoid the problem entirely when storing seeds for genetic conservation. Even before seeds are stored some deterioration will inevitably have taken place. Such observations support the view that for genetic conservation, seeds should be stored under conditions which minimise loss of viability.

2.2. Factors Which Affect Loss of Viability and the Induction of Chromosomal Aberrations in Stored Seeds

It is now well established that several factors, e.g. seed moisture content, temperature, length of storage and atmospheric gases affect loss of seed viability and the induction of chromosomal aberrations in storage. The effects of these factors are given in detail in the following subsections.

2.2.1. Seed moisture content, temperature and length of storage

A great deal of early work has shown that the main factors affecting longevity are temperature and seed moisture content, and a number of attempts were made to quantify their effect (Roberts, 1988).

The relationship between seed moisture content, temperature and viability was first described quantitatively by Roberts (1960) in viability equations, developed for wheat, which predicted percentage seed viability under a range of temperatures and moisture content after any period of storage. The same predictions were then shown to apply to other species, e.g. rice (*Oryza sativa*) (Roberts, 1961), barley, peas and broad beans (Roberts and Abdalla, 1968).

But, these equations had two faults: they were only accurate within limited ranges of temperature and moisture content and, more important, they failed to take into account the initial quality of the seeds which also has a considerable effect on their longevity. Consequently, the original equations have been modified by Ellis and Roberts (1980a, b), for barley, to produce a single improved viability equation which overcomes these problems, and is applicable over a very wide range of

storage environments: from 3°C to 90°C and 5% to 25% moisture content (f.wt). This equation has, then, been shown to be applicable to all species to which it has been applied, e.g. onion (Ellis and Roberts, 1981), soybean *(Glycine max)*, chickpea (*Cicer arietinum*) and cowpea (*Vigna unguiculata*) (Ellis *et al.*, 1982a) and lettuce (Kraak and Vos, 1987). Recently, Dickie *et al.* (1990) have proved that this viability equation is applicable over a wider range of storage environments: from -20°C to 90°C and 1.8% to 25% (f.wt), in the cereal barley, the grain legumes chickpea, cowpea and soybean, the leaf vegetable lettuce and the timber trees elm (*Ulmus carpinifolia*), mahogany (*Swietenia humilis*) and terb *(Terminalia brassii)*.

The improved viability equation is based on the proposition that in a constant storage environment, the distribution of individual seed life-spans within a population (seed lot) is normal, so that survival curves are cumulative normal distribution of negative slope. Transformation of percentage viability to probits (or normal equivalent deviates) results in linear survival curves and can be described as (Ellis and Roberts, 1980a):

$$v = K_i - p / \sigma \qquad (1)$$

where

v - is probit of percentage viability after storage,

p - is storage period,

σ - is the standard deviation of individual seed life-spans, and

K_i- is the seed lot constant and is the intercept value at zero storage time of the probit survival curve.

The value of K_i indicates the quality and potential longevity of the seed lot and consequently its value varies between seed lots. It depends on both genotype and pre-storage environment. The pre-storage environment includes the conditions experienced around the time of harvest (Roberts, 1983). In addition, the pre-storage environment includes the seed drying process which can also affect the K_i value (Nellist, 1981).

However, the K_i value is not affected by the storage environment which can only affect the slope ($1/\sigma$) of the survival curve: the better the storage conditions, the larger the value of σ. Ellis and Roberts (1980a, b, 1981) showed that σ is affected by seed moisture content and storage temperature as follows:

$$\sigma = 10^{K_E - C_W \log m - C_H t - C_Q t^2} \qquad (2)$$

where

m - is moisture content (percent wet weight basis),

t - is temperature (°C), and

K_E, C_W, C_H and C_Q - are constants within a species but which differ between species.

Substituting the right side of equation (2) for σ in equation (1) gives:

$$v = K_i - p / 10^{K_E - C_W \log m - C_H t - C_Q t^2} \qquad (3)$$

which can be used to predict percentage viability (since v is probit of percentage viability) after any storage period, p, at moisture content, m, and storage temperature, t. K_i has to be determined separately for each seed lot; but within a given species, the four constants (i.e. K_E, C_W, C_H and C_Q) in the exponent of equation 3, which together determined the slope of the survival curve, do not vary. They are not affected by either genotype or pre-storage conditions, i.e. they are not affected by seed quality (Roberts, 1983).

2.2.2. Seed moisture content

We already know that there is a negative logarithmic relationship between seed longevity and moisture content (Ellis and Roberts, 1980b, 1981; Ellis *et al.*, 1982a, 1986, 1988, 1989, 1990b, c; Kraak and Vos, 1987). A lower limit of this relationship has been shown for many diverse species (Ellis *et al.*, 1988; Roberts and Ellis,

1989). The value of this lower limit varies considerably among species, e.g. from 2% in groundnut (*Arachis hypogaea*) (Ellis *et al.*, 1990d) to 6.2% moisture content in pea (Ellis *et al.*, 1989). This lower limit to the otherwise negative logarithmic relations between longevity and moisture thus provides a practical limit to seed desiccation for storage (Ellis, 1991).

However, seed longevity in many crops examined responds identically to differences in equilibrium relative humidity and, thus, water potential. This relationship extends to about 10-11% equilibrium relative humidity, or water potentials close to -350 MPa, below which further desiccation begins to remove the strongly bound water fraction (Ellis *et al.*, 1989, 1990b, c). The partial removal of this fraction has little or no further influence on seed longevity, presumably because it has little chemical potential. It is suggested that most orthodox seeds could be stored at this water potential for long term storage. More importantly, for many oily seeds, it corresponds to values significantly lower than the current recommended seed moisture content of 5% (i.e. between about 2% and 4%, depending on the critical moisture content for the particular species) (Ellis *et al.*, 1990d).

On the other hand, Vertucci and Roos (1990), working with five species (i.e. pea, soybean, peanut, lettuce and sunflower), have proposed that equilibrating seeds between 19% and 27% relative humidity provides the optimum moisture level for maintaining seed longevity during long term storage. Furthermore, they have claimed that the moisture contents between 3% and 7% (depending on the seed) recommended by the IBPGR (1985) may be arbitrary and incorrect, and that the optimal moisture level for storage can be identified more readily by the equilibrium relative humidity (which is almost constant among species) than the seed moisture content. In addition, Vertucci and Roos (1990) have also claimed that the suggestion made by Ellis *et al.* (1989, 1990c, d) that seed moisture contents in equilibrium with about 10 - 11% relative humidity maximize the benefit to longevity from seed desiccation is an underestimate. These different recommendations are discussed in detail in Chapter 8.

There is also an upper limit to the negative logarithmic relations between longevity and moisture. Previously, Villiers (1973, 1975) and Villiers and Edgcumbe (1975) showed in lettuce that seeds stored fully hydrated can survive for long periods without significant loss of viability. However, Ibrahim and Roberts (1983) and Ibrahim *et al.* (1983) concluded that, under anaerobic

conditions the trend of decrease in longevity with increase in hydration continues up to about 27% moisture content, but above this value there is no further change in longevity. In contrast, under aerobic conditions (if oxygen is freely available) longevity increases exponentially up to 44% moisture content, this increase starting at about 15% moisture content at 20°C and at about 24% at 35°C. Therefore, the upper limit occurs whether or not oxygen is available, but there is no improvement to longevity at higher moisture contents without the free availability of oxygen. An upper limit to the negative logarithmic relations between longevity and moisture has also been determined in other species, e.g. in onion (Ward and Powell, 1983), in wheat (Petruzzelli, 1986), in niger (*Guizotia abyssinica*) and tef (*Eragrostis tef*) (Zewdie and Ellis, 1991). According to Roberts (1988) and Zewdie and Ellis (1991), the value of this upper limit (a common minimum water potential) is probably between -14 and -15 MPa.

2.2.3. Temperature

Previously, it was thought that relations between the logarithm of seed viability period and temperature at one moisture content were linear and negative (Roberts, 1961a; Roberts and Abdalla, 1968), and the temperature coefficient, Q_{10} (for change in rate of loss in viability

per 10°C rise in temperature), was not expected to vary with temperature (Roberts, 1972). However, later, it was found in barley seeds that Q_{10} was not stable but increased quite dramatically with increasing temperature. To quantify this variation, the basic semi-logarithmic relationship was modified by the addition of a quadratic term (Ellis and Roberts 1980a, b).

It is now well established that this model describing the relative effect of temperature and the longevity of barley seed between -20°C and 90°C also explains all previous, divergent reports of the value of Q_{10} for a wide range of species, including cereals, legumes, vegetables, grasses and ornamentals (Ellis and Roberts, 1981). Furthermore, this basic model can be applied to other species (Ellis and Roberts, 1981; Ellis *et al.*, 1982a; Kraak and Vos, 1987; Dickie *et al.*, 1990).

2.2.4. Gases

For many years research has been conducted on the effects of a partial vacuum and also gases such as carbon dioxide, oxygen, nitrogen, helium and argon on the longevity of various kinds of seeds (Roberts, 1961b; Bass *et al.*, 1963a, b; Harrison, 1966; Rao and Roberts, 1990). However, the results were complex and some of them were conflicting.

Under most conditions, storage in nitrogen resulted in a longer period of viability than storage in air under similar conditions of temperature and moisture. Increases in the concentration of oxygen during storage decreased the viability period most rapidly (Roberts, 1961; Harrison, 1966). Roberts (1961b) concluded that carbon dioxide was certainly not markedly deleterious to viability. However, Bass *et al.* (1963a, b) indicated little or no special advantage of using a vacuum, air, carbon dioxide, nitrogen, helium or argon in terms of seed viability.

In a further study, Ibrahim *et al.* (1983) proposed that above the critical moisture content, which varies between species, repair mechanisms are activated and are sustained by respiration in the presence of oxygen (see section 2.6). Therefore, seed moisture content is an important factor in a gaseous environment during storage (i.e. at the low moisture content oxygen is deleterious to seed survival but at the high moisture content it has a beneficial effect in improving longevity).

Recently, Rao and Roberts (1990) concluded that the beneficial effect on longevity observed in the nitrogenous atmosphere at low moisture content is only marginal and also, replacing air with nitrogen would not

reduce the amount of genetic damage accumulating for any given loss of seed viability. They also concluded that with the additional technical complications associated with replacing the air in the storage atmosphere, the use of inert gases or a vacuum in controlling genetic deterioration and improving longevity in seed storage seems unwarranted.

2.3. Physiological and Biochemical Changes in Aged Seeds

2.3.1. Physiological changes

Perhaps the most widely accepted and useful index of seed deterioration is reduction in viability, which is often accompanied by reduced seedling growth. Recently, increasing emphasis has been placed on biochemical or physiological changes such as quantitative and qualitative changes in specific enzymes, respiratory metabolism, synthesis of proteins and carbohydrates, leaching of inorganic and organic material, and degradation of storage compounds (Abdul-Baki and Anderson, 1972).

Among the many physiological manifestations of seed deterioration are : changes in seed colour - seed coat and or embryo, delayed radicle emergence and seedling growth, decreased tolerance to suboptimal environmental

conditions during germination and or growth, higher sensitivity to radiation treatments, decreased total germination by a seed population, lower tolerance to adverse storage conditions, increased number of abnormal seedlings, mustiness and increased heat production during storage (Abdul-Baki and Anderson, 1972; Delouche and Baskin, 1973; Copeland, 1976; Bewley and Black, 1982). Of these responses, reduced germinability has been the most widely accepted single criterion of seed deterioration.

2.3.2. Biochemical changes

Seed deterioration begins immediately after maturity regardless of where or how seeds are held. During seed development anabolic processes predominate and bring about a gradual increase in dry matter, including development of an embryo and food reserves. Following maturation biochemical changes continue and eventually catabolic processes predominate and deterioration becomes apparent. Catabolic changes occur more slowly under low temperature and low relative humidity than under high temperature and high relative humidity (Justice and Bass, 1979).

Many biochemical changes have been detected in seeds as they deteriorate. These include changes in respiratory

and synthetic pathways, enzymic activity, food reserves, membranes and barriers, and chromosomes (Abdul-Baki and Anderson, 1972; Delouche and Baskin, 1973; Harrington, 1973; Copeland, 1976; Bewley and Black, 1982).

According to Harrington (1973), the maintenance of seed longevity depends on preventing the destruction of certain compounds essential in triggering the biochemical steps needed to initiate germination. The loss of the ability to produce the plant hormones gibberellic acid, cytokinins, and ethylene necessary for triggering germination is a fundamental ageing process. This loss of ability to produce hormones may be caused by destruction of enzymes by protein denaturation, by activity of free radicles, or by an inability to produce enzymes *de novo* by a breakdown of the DNA-RNA templating mechanism.

Recently, several IBPGR sponsored projects have been carried out to improve the understanding of genetic control of storage characteristics, biochemical basis of good and poor storage, and genetic stability during seed storage. A few of them (which produced interesting results) are as follows:

In a cooperative project (by the Boyce Thompson Institute, Cornell University, USA, the National University of Mexico and CIMMYT, Mexico), experimental

data indicated that the content of polyamine in maize embryos was correlated with seed longevity. Embryos of the C_2 line of maize (which has a long storage life have a significantly higher polyamine content than those of the C_6 line (which has a short storage life). Both polyamine content and good storage characteristics appear to be controlled by nuclear factors. In addition, DNA breakage occurred prior to viability decrease in seeds of the C_2 line (IBPGR, 1989). However, studies involving large numbers of maize lines did not reveal such a correlation (IBPGR, 1991).

Preliminary hybridization studies in peas indicated that a good storage character was controlled by the genes closely associated with the flower colour gene. White-flowered plants produce seeds with a shorter storage life compared with the seeds of coloured-flower plants (IBPGR, 1991).

Genomic alteration after long-term seed storage was studied at Ohio State University, USA. To date, this project has produced the following results (Kamalay, 1992). DNA sequence damage is correlated with seed ageing. DNA repair occurred during seed germination. Standard genomic blotting with single soybean seeds and the method of random amplified polymorphic DNA (RAPD) assay indicated that DNA polymorphism is readily detected

between two cultivars (PI 171451 and Williams). The assessment of DNA sequences and expression of two genes encoding glyoxylate cycle enzyme, isocitrate hyase and malate synthesis is in progress.

In another project (producing interesting results at the Agricultural University, Wageningen, the Netherlands), cauliflower seeds were used to investigate changes in gene expression through storage-induced deterioration and to determine how much damage is repairable during imbibition and germination. The first year's results of this project suggest that the continued synthesis of three particular proteins during early germination is required for rapid germination, and these three proteins are found in the dry seeds (Fujikura and Karssen, 1992).

2.4. Types of Chromosomal Aberrations Found in Aged Seeds

The types of the aberrations observed most frequently in seed ageing studies have involved breakage of the chromosome, which has been compared to that produced from ionizing radiation (Roos, 1982). Evans (1962) described chromosomal aberrations induced by ionizing radiation as being "chromosome" types which involve breakage of both chromatids of a chromosome at identical loci, "chromatid" types where the unit of aberration formation is the

chromatid, and "sub-chromatid" types where the breakages involve a varying number of sub-chromatid strands.

Many authors have attempted to classify ageing-induced aberration in seeds as chromosome or chromatid type. However, opinions have been divided on the question of whether chromosome or chromatid breaks are more significant in aged germinating seeds. Many of the earlier workers suggested that chromosome-type aberrations predominate in aged seeds at anaphase, e.g. Nichols (1941) in onion, D'Amato (1951) in pea, Gunthardt *et al.* (1953) in wheat, rye, barley and peas, Harrison and McLeish (1954) in lettuce and onion, Jackson and Barber (1958) in onion and Abdalla and Roberts (1968) in barley, broad beans and peas. In contrast, there is a tendency for more recent papers to report that most of the damage induced in aged seeds is of the chromatid-type, e.g. in durum wheat (Corsi and Avanzi, 1969; Avanzi *et al.*, 1969; Innocenti and Avanzi, 1971; Floris and Anguillesi, 1974), in barley (Murata *et al.*, 1982; Dourado and Roberts, 1984a), in pea (Dourado and Roberts, 1984a) and in onion (Sirikwanchai, 1985). However, Rao *et al.* (1987b) and Rao and Roberts (1990) have shown in aged lettuce seeds that the frequency of chromosome-type aberrations was predominant to the chromatid-types.

In an extensive study of the first mitotic cycle of aged barley seeds, Murata *et al.* (1982) found that the predominant aberrations were of sub-chromatid and chromatid-types. It is suggested by Kihlman and Hartley (1967) and Kihlman (1970) that the sub-chromatid aberrations are masked chromatid aberrations, because the sub-chromatid aberrations give rise to chromosome type aberrations in the second cell division after chemical treatment. This result leads to the suggestion that the chromosome-type aberrations in aged seeds reported by many workers might be the secondary aberrations resulting from chromatid or sub-chromatid aberrations at first mitosis (Roos, 1982).

Cytogeneticists generally explain the main types of chromosome aberration which are observed on the basis of the particular stage in the cell cycle in which the event leading to the aberration occurred; particularly with respect to the stages before and after DNA replication during interphase (Roberts, 1988). With the important discovery that the incorporation of P^{32} into the DNA of mitotic cells of *Vicia faba* root-tip meristems occurred at some time during interphase (Howard and Pelc, 1953), it soon became apparent that this period of incorporation, or of DNA synthesis, coincided with the time when the transition from chromosome to chromatid-type aberrations occurred (Evans, 1962).

Evans has diagrammatically illustrated the relationship between the types of aberration induced by ionizing radiations in relation to DNA synthesis and stages of development of the mitotic cycle. Adopting the terminology of Howard and Pelc of G_1 for the pre-synthesis period and G_2 for the post-synthetic period, it was evident that irradiation of G_1 cells resulted in chromosome-type breaks, and irradiation of G_2 cells in chromatid-type breaks (Appendix 1).

Ageing-induced aberrations were originally interpreted in terms of the Classical Theory of breakage and reunion proposed by Sax (1940, 1941) and later developed by Lea (1946) from the experiments on X-ray induction of chromosomal aberrations. According to this theory, a sudden energy loss from a quantum of X-radiation (or electromagnetic radiation of shorter wavelength) results immediately in a primary break in the chromatin (DNA duplex) thread, producing a pair of broken ends. Rejoining of two broken ends restores continuity and restitutes the original structure. If two breaks occur close together, the four ends may rejoin in a variety of ways to produce various exchange aberrations. Breaks which do not rejoin are manifest as single fragments.

However, most ageing induced damage is now thought to occur according to the Exchange Theory, originally

proposed by Revell (1959). The Exchange Theory states that the primary event of damage is not a break, unlike as described in the Classical Theory, but an unstable lesion. Evans (1977) states that a lesion may give rise to a break during semi-conservative replication when a break occurs in one of the two duplex DNA threads produced, i.e. in one of the resulting chromatids. Exchanges may also occur providing two sites of potential breaks are in close proximity. Damage resulting from lesions always appears initially as chromatid- type aberrations in the first mitosis following the first replication after the lesion occurs (Roberts, 1988).

Previously, D'Amato and Hoffman-Ostenhof (1956) found that chromosome damage in seeds occurs due to the accumulation of automutagenic substances during storage. In contrast, certain workers who noted predominantly chromatid-type aberrations in aged seed (e.g. Corsi and Avanzi, 1969; Avanzi *et al.*, 1969; Innocenti and Avanzi, 1971; Floris and Anguillesi, 1974; Murata *et al.*, 1982) suggest that no damage is suffered by chromosomes during storage, but it arises during germination following storage. However, these interpretations have been based on the Classical Theory. Later, adopting the ageing induced damage to the Exchange Theory made this issue clearer. Roberts (1988), in his review on "Seed ageing",

suggests that the high proportion of chromatid-type aberrations generally observed does not mean that the primary damage occurs during germination. In fact, the initial lesions occur during seed storage and that the resulting breakages appear during seed hydration at germination when semi-conservative DNA replication occurs.

2.4.1. Factors affecting the accurate estimation of chromosomal damage

As pointed out earlier (section 2.1), the frequency of chromosomal aberrations is correlated with loss of seed viability. However, as the root or shoot elongates, a progressive decrease or elimination of chromosomal aberrations has been observed by several investigators. Navashin (1933b) was probably the first to perceive the reduction in the frequency of chromosomal aberrations with root elongation but did not present any data to support his observations. Peto (1933) noted a reduction in the number of mutated cells in 40 day old root tips of barley compared to one or two day old ones. Nichols (1941) showed that chromosomal aberrations decreased from 10.4% to 1.7% with root growth from 2 to 100 mm in aged onion seeds. Although maximum frequency of chromosomal aberrations ,in 4 year old pea seeds, was observed in roots of 14-18 mm long, no aberrations were found in

roots more than 20-30 mm long (D'Amato, 1951). Kato (1954) also observed a decrease in the frequency of aberrant cells in *Allium odorum*, from 3.9% to 0.1% in seedling of 0.2 - 0.6 cm and >1 cm, respectively.

In other studies, suggestions that the incidence of aberration greatly decreases as the plant develops were confirmed in diverse species, e.g. in peas (Abdalla and Roberts, 1968), onion (Cebrat, 1977), barley (Murata *et al.*, 1984) and lettuce (Rao *et al.*, 1987a). The apparent decrease in aberration frequency is thought to result from the failure of the abnormal cells to compete with those meristem cells containing no aberrations. This has been termed diplontic selection and is a common occurrence in radiation induced mutations (Gaul, 1961).

Moreover, Orlova *et al.* (1975) reported a delay of first mitosis in Welsh onion seeds aged 1.5 - 4 years. In one cultivar, the first mitosis was delayed 5h (from 53h to 58h) with 34% germination in seeds stored 3.5 years, compared to 47% germination of seeds stored 1.5 years. In a different cultivar, the delay was 11h (48-59h) between 2 and 4 year old seeds which germinated at 75% and 29%, respectively. Murata *et al.* (1980) found that a delay in first mitosis was evident in aged pea seeds. In addition, the root length at which first mitosis was observed was longer as germinability declined. Therefore, they

suggested that root length together with germinability might be a more useful indicator of the time of first mitosis in aged seeds than time from start of imbibition.

In order to obtain an accurate estimate of the frequency of chromosomal aberrations in aged root tips the relationship between first mitosis and root length should be examined to ensure minimal under estimation of aberrancies.

2.5. Imbibition Injury

According to Cromarty *et al.* (1982), the use of ultra-dry conditions is important as a potentially inexpensive method of long-term storage of seeds for genetic conservation. Accordingly, IBPGR (1985) suggested that ultra-dry storage of seeds (i.e. storage at 20°C and 2% moisture content, rather than at -20°C and 5% moisture content which was the previous recommendation) was a promising technique and might enable the cost of refrigeration to be reduced or even avoided in some applications. Ellis *et al.* (1986, 1988, 1989) supported this with their work on diverse species.

However, when very dry seeds are imbibed in water, the initial rapid uptake of water can result in a type of damage which has been described as imbibition injury.

This phenomenon was first cited by Kidd and West (1919). They found that the soaking of pea seeds and of bean seeds in excess water for 24-48 hours decreased germination and retarded subsequent growth. Furthermore, it has been confirmed by a number of workers that rapid imbibition has a deleterious effect on very dry leguminous seeds (Klingmüller and Lane, 1960; Pollock and Toole, 1966; Larson, 1968; Orphanos and Heydecker, 1968; Harrison, 1973; Rowland and Gusta, 1977; Powell and Matthews, 1977, 1978; Ellis and Roberts, 1982; Ellis *et al.*, 1982b, 1990d).

Two main factors affect the degree of imbibition injury: (1) the temperature at which imbibition occurs, and (2) the dryness of the seeds. Chilling injury was a term used to describe the observation by Pollock and Toole (1966) that dry seeds of Lima bean (*Phaseolus lunatus*) were injured (either the seeds failed to germinate or the seedlings showed a reduced subsequent growth rate) if they were imbibed at moderately low temperatures (5-15°C) compared to a slightly higher temperature (20°C). Nevertheless, it is now clear that damage, previously attributed to the effects of low temperatures was in fact the result of imbibition injury (Powell and Matthews, 1978). Moreover, seeds of many grain legumes are sensitive to injury during imbibition where moisture is in excess, and this sensitivity is increased by a

lowering of the temperature of imbibition (Ellis *et al.*, 1985).

The dryness of the seeds is also critical to the occurrence of imbibition injury. If seed moisture content is high (18% +) then imbibition injury is usually avoided. In some experimental observations, there appears to be a critical moisture content -below which a constant proportion of seeds fail to germinate as a result of imbibition injury (Ellis and Roberts, 1982; Ellis *et al.*, 1982b). In other observations the proportion of seeds affected increases with decrease in seed moisture content at imbibition (Klingmüller and Lane, 1960; Pollock, 1969; Rowland and Gusta, 1977; Ellis *et al.*, 1982b). In either case it is clear that very dry seeds, as is the case of accessions within a genebank, are particularly susceptible to imbibition injury (Ellis *et al.*, 1985).

Imbibition injury does not only occur in the soaking of very dry seeds but can occur in a standard germination test, even when the germination medium is of low osmotic potential (Ellis *et al.*, 1982b, 1985, 1990b).

Therefore, it is recommended that genebanks which handle very dry seeds adopt humidification to ensure slow initial uptake of water (see chapter 6) prior to testing for germination or field sowing (Ellis *et al.*, 1982b).

2.6. The Concept of Repair in Aged Seeds

It has long been known that the higher the temperature, the moisture content and the oxygen pressure the shorter the viability period (Roberts, 1972). However, it has also been known that fully imbibed seeds may lie in the soil without germinating for very long periods - for decades or even centuries (Villiers and Edgcumbe, 1975; Roberts, 1988). Villiers (1974) pointed out that seedlings grown from dry-stored seeds showed an increase in morphological abnormalities with length of storage, whereas seedlings from imbibed-stored seeds appeared normal, i.e. there was a reversal of the trend of decreasing longevity with increase in moisture content. Moreover, Villiers and Edgcumbe (1975) concluded that loss of viability of seeds in dry storage is caused by the inability of repair and turn over systems to operate in tissues with a low water content. Consequently, damage to macro molecules may accumulate and may only be repaired when the seeds are imbibed for germination, by which time the damage might be too extensive for repair to be effective. In wet-stored seeds, or in seeds in the field, such damage might be repaired, and not begin to accumulate until the cellular maintenance systems themselves become faulty.

Ibrahim and Roberts (1983), and Ibrahim *et al.* (1983) investigated the effects of full imbibition and oxygen for extended longevity in moist storage of lettuce seeds. As a result, they reported that the viability equation would apply up to 15% moisture content in lettuce seeds and below that moisture content oxygen become deleterious to seed longevity. However, above 15% moisture content, oxygen is increasingly beneficial for fully imbibed lettuce seeds. So, the evidence suggests that there is a common moisture content at which there is a transition between oxygen being deleterious to longevity and being beneficial; at which there is a change from increasing moisture content being detrimental to longevity to it being beneficial and, at which seed survival curves change from normal to skewed distribution. The moisture content at which all these changes occur has been called "the critical moisture content" (Ibrahim *et al.* 1983). The value of the critical moisture content varies depending upon species, so that in lettuce it is 15%, in onion it is about 18% (Ibrahim *et al.*, 1983; Ward and Powell, 1983), and in durum wheat it is in the region of 28-30% (Petruzelli, 1986). But it has not yet been defined for peas. According to Roberts (1988), the critical moisture content, which varies between species, may be a reflection of a more fundamental critical water potential which has a similar value in different species, probably somewhere in the region of -14.5 MPa. The main

reason why seeds have different critical moisture contents is probably because of differences in oil content; because, whereas oil contributes to dry weight and therefore affects the moisture content determination, it is hydrophobic and therefore does not contribute to a reduction in water potential.

We already know that repair is not possible below the critical moisture content; but at the critical moisture content there is some repair and possibly turnover, providing oxygen is present to support normal metabolism. As moisture content increases above the critical moisture content, the rate of respiration, repair and turnover increases, i.e. the trend of decreasing longevity with increase in moisture content is reversed (Ibrahim *et al.*, 1983; Roberts, 1988). Consequently, after storing seeds at low moisture contents, DNA repair is possible only when seeds are hydrated during the early stages of germination (Osborne, 1982).

2.7. The Effects of Post-Storage Hydration Treatments on Seed Viability and Chromosomal Aberrations

2.7.1. Humidification

The rapid uptake of water by dry seeds when immersed in water can result in imbibition injury (Pollock and Toole,

1966; Larson, 1968; Harrison, 1973; Powell and Matthews, 1977, 1978; Basu and Pal, 1980). However, the problem can also occur in standard germination tests (Ellis *et al.*, 1982b, 1990b). Cooler temperatures also have a damaging effect on dry seeds during imbibition (Pollock, 1969; Ellis *et al.*, 1982b). Although most experimental work has been concerned with imbibition injury in large seeded legumes, it is now understood that the problem can occur in other species, e.g. forage legumes, cotton and sorghum (Ellis *et al.* 1985). Damage to the seeds (caused by imbibition injury) can be avoided or minimised by humidification (which is a pre-germination treatment based on a slow initial uptake of water to the seeds in a water saturated atmosphere). This concept is now well supported by a number of studies, e.g. in Lima bean (Pollock, 1969), soybean (Obendorf and Hobbs, 1970), French bean (Orphanos and Heydecker, 1968; Pollock *et al.*, 1969), broad bean (Klingmüller, 1961), rice (Basu and Pal, 1980), cowpea (Ellis *et al.*, 1982b), lettuce (Rao *et al.*, 1987b) and pea (Ellis *et al.*, 1990b), and is elaborated further in Chapter 6.

Rao *et al.* (1987b) concluded that humidification of lettuce seeds after storage reversed some of the damage which resulted from ageing. Most of the benefits of humidification occurred during the first 3 days, when seed moisture content rose to 34 percent. Humidification

treatments reduced the frequency of chromosomal aberrations, increased the rate of root growth, and decreased the frequency of morphologically abnormal seedlings.

2.7.2. Priming

As an alternative to humidification treatments, another, and more successful method of physiological advancement involves the initial imbibition of seeds in osmotica which holds the seeds imbibed but at the brink of germination, followed by transfer to water (germination test conditions). This initial imbibition technique has various names, including: advancing, conditioning, osmo-conditioning, priming, osmopriming to name a few. In this study, seeds treated thus will be referred to as "primed" seeds.

Priming can be carried out with a variety of solutes, including solutions of various inorganic salts (such as sodium chloride and potassium nitrate), sugars (particularly mannitol), growth regulators (especially abscisic acid), and polyethylene glycol (PEG). In the last decade, the use of PEG solutions has been the most common way of priming. PEG is a chemically inert, high molecular weight compound that, unlike the lower molecular weight sugars and salts, probably does not

penetrate the cell walls (Khan *et al.*, 1978; Bewley and Black, 1982; Mayer and Poljakoff-Mayber, 1989). Two general rules of priming appear to apply: (1) for any given osmotic potential, a longer priming treatment is required at lower temperatures, and (2) at any given temperature, decreasing the concentration of the osmoticum (up to a certain limit) shortens the priming time. For most seeds the efficacious osmotic potential is in the range of -0.5 to -2.0 MPa, in a temperature range of 10-20°C. Furthermore, the seed must be provided with an ample supply of oxygen, presumably to allow metabolic processes essential for germination to proceed. Also the imbibed seed must be protected against microbial attack or the proliferation of seed-borne pathogens (Bewley and Black, 1985).

It is now well understood that in aged seeds priming promotes faster germination and results in a decrease of mean germination time (Dell' Aquila, 1987; Georghiou *et al.*, 1987; Alvarado and Bradford, 1988; Thanos *et al.*, 1989; Zanzottera and Bray, 1989; Dell'Aquila and Tritto, 1990, 1991) . Priming is also closely linked with the advancement of protein and DNA synthesis rates in germinating embryos of aged seeds. Reinvigoration of aged seeds by priming treatments with PEG (using combination of different concentrations of osmoticum, temperature and duration of treatment) has been shown in a range of

species. Brocklehurst and Dearman (1983), working on carrot, celery and onion, found that germination rate is improved in aged seeds following priming with PEG solutions. It has been shown by Dell'Aquila and his colleagues (Dell'Aquila *et al.*, 1984; Dell'Aquila and Taranto, 1986; Dell'Aquila, 1987; Dell'Aquila and Tritto, 1990, 1991) that a correct application of PEG aqueous solutions may induce a more rapid and uniform germination in aged wheat seeds and also a fast resumption of cell division and DNA-synthesis when the seeds are transferred into distilled water.

Priming with abscisic acid (ABA) can also reversibly block the progress of germination and thereby synchronise and increase the rate of germination of seeds in standard germination tests. This is evident from the recent studies carried out in white mustard (*Sinapis alba*) (Schopfer *et al.*, 1979), celery (*Apium graveolens*) (Biddington *et al.*, 1982), tomato (Liptay and Schopfer, 1983) and carrot (Finch-Savage and McQuistan, 1989). More recently, Finch-Savage and McQuistan (1991) concluded that, ABA-treatment (10^{-4} M solution for 15 days at 15°C) benefited tomato seeds in a similar way to that of priming in PEG solutions at -1.25 MPa for the same duration and at the same temperature. They proposed that ABA can be considered as an alternative to an osmoticum for priming tomato seeds.

A new and potentially important method of priming, known as "non-osmotic priming" or "drum-priming", was developed at the Institute of Horticultural Research (IHR), Wellesbourne, Warwick in the late 1980's by Dr. H. Rowse. The early part of the development history began with laboratory and field trials between 1986 and 1988, and came to fruition in 1990. This highly complex technique utilises a computer and specially developed software to control the amount of water absorbed by each individual seed. Drum-priming has been carried out in carrots, onions, leeks and cauliflowers. The fastest and most uniform germination is observed from seeds after the treatment without being dried, but even with drying back to the original seed moisture content, the speed and uniformity of germination is much better than the untreated seed. The process is likely to be of most value for the priming of large volumes of seed which hitherto have required the use of large quantities of osmotica, usually PEG (Anonymous, 1991). This process has now been commercialised and drum primed seeds are marketed by Sharpes International Seed Company.

As it is well established that during storage various deteriorative processes occur in seeds, the controlled hydration maintained during priming may permit repair processes to take place (Burgass and Powell, 1984). The

repair processes may be of membranes or even of DNA or RNA. Some repair occurs automatically during initial imbibition which is an integral part of the germination process but, in order for repair to chromosomes to be maximized, it may be best if the period for repair could be prolonged before semi-conservative DNA replication during the normal cell cycle consolidates errors beyond the possibility of repair. This approach implies priming treatments involving water potentials below that required for germination, but well above the critical moisture content (see section 2.6) postulated to be the minimum necessary for the repair of cellular components (Rao *et al.*, 1987b).

Instead of trying to enhance longevity in aged seeds after storage, an alternative is to attempt to protect seeds from damage by priming them before storage. Although seed priming increases seed vigour according to criteria such as rates of germination and field emergence (Bradford, 1986; Georghiou *et al.*, 1987; Argerich and Bradford, 1989; Thanos *et al.*, 1989), contradictory results have been obtained with primed seeds and ageing treatments. Dearman *et al.* (1986) showed that priming delayed the loss of viability of onion seeds. However, the same authors found that loss of viability in leek and carrot seeds was dramatically faster in primed seeds compared to control seeds (Dearman *et al.*, 1987). A

reduction in storage life of tomato seeds after priming has also been reported (Alvarado and Bradford, 1988; Argerich *et al.*, 1989).

In summary, the effect of post-storage priming is clear since it is widely accepted as a useful technique to avoid or minimise imbibition injury and to enhance germinability in aged seeds due to its possibly facilitating repair in DNA and chromosomes. However, the effect of pre-storage priming is as yet unclear as the results in a number of studies are conflicting.

CHAPTER 3. GENERAL MATERIALS AND METHODS

3.1. Seed Lots

The pea seeds in this project represented two categories: cultivars and wild types. These two categories were chosen in order to compare their characteristics in terms of genetic deterioration during storage. Seeds of cultivar "Kelvedon Wonder" were purchased from Booker Seeds Ltd., and seeds of cultivar "Douce Provence" from Nutting Seed Company. "Kelvedon Wonder" had an initial moisture content of 11.5%, 99.5% total germination (radicle protrusion) and 95.5% normal germination (morphologically normal seedlings). "Douce Provence" had an initial moisture content of 15.1%, and showed 100.0% total germination with 96.5% normal germination. All seed was stored at 3°C till needed for experimentation.

A very limited amount of germplasm of pea wild types* [JI 750 and JI 959 (originating from Turkey), and JI 181 and JI 1104 (originating from Nepal)] were obtained from the John Innes Institute, Norwich. They were regenerated (bulked up) twice, in 1989 in order to produce sufficient seed for experimentation.

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* Although they were not the actual wild types (but selected lines in primitive cultivation or ecotypes), they were called as wild types in this study.

The first regeneration was carried out in the glasshouses of the University of Bath, situated on campus. Approximately 30-50 seeds of each wild type were sown in pots (Optipot 15F, 6" diameter), containing (Fison's Levington Compost: M2) peat compost, in early February. Irrigating was carried out regularly to maintain sufficient moisture in the compost. The plants were supported with canes and tied up individually, 3 weeks after sowing. The pea plants were harvested in early May. Then seeds of each wild type were separately extracted, cleaned and packaged in hermetically sealed laminated aluminium foil packets. These wild type seed lots were stored in a fridge at 3°C until the next regeneration.

Subsequently, the same wild types were regenerated under polythene tunnels in the Bathampton Field Station of the University of Bath. During the soil preparation, a general fertilizer VITAX GR 112 (manufactured by Vitax Ltd.), which is composed of: 7% N, 7% P_2O_5, 14% K_2O, 5.8% MgO, 0.13% Fe, 540 mg Mn/kg and 100 mg B/kg, was applied and afterwards overhead irrigation was carried out in each polythene tunnel. The soil was near field capacity when the seeds of each wild type were sown, with a combine drill, in late May. Spacing between rows and within rows were 60 cm and 8 cm, respectively. Irrigation, hoeing and weeding were carried out when

necessary. The plants were supported with wired mesh in each row, 2-3 weeks after sowing. In order to protect the plants against aphids, Dimethoate-40 (purchased from Murphy Chemical Ltd.) was applied at a rate of 840 ml in 400-1000 l water/ha ,in late July. The plants of each wild type were harvested separately, between the 10th and 30th August (i.e. JI 181 on 10th, JI 750 on 16th, JI 1104 on 22nd and JI 959 on 30th). Seeds of each wild type were then extracted, cleaned, packed and stored as in the procedure followed after the harvest of the first regeneration. The initial moisture contents [as determined by ISTA (1985a, b)] of JI 750, JI 959, JI 181 and JI 1104 were 11.1%, 11.3%, 10.8% and 10.1%, respectively. All seed lots of the wild types showed 100.0% normal germination.

Low moisture content seeds were required for some of the experiments. These were obtained by drying the seeds at 20°C, in desiccators with regularly regenerated silica gel in a partial vacuum for approximately 6 months in the case of the cultivars and 10 months in the case of the wild types.

Until required for experimentation, all the seed lots were stored either in a cold room or in a fridge both at 3°C, in hermetically sealed laminated aluminum foil packets.

3.2. Phenotypic Descriptions of Pea Cultivars and Wild Types

Phenotypic observations of plant species are carried out for different purposes. They are mostly used to identify certain characteristics of germplasm collections in a gene bank for further breeding work, and for comparison of genetic damage, occurring as phenotypic mutations, related to seed deterioration during seed storage (Abdalla and Roberts, 1969; Floris and Meletti, 1972; Dourado and Roberts, 1984a, b). However, in this research, the observations were carried out to discover if there were any phenotypic differences between the pea cultivars and wild types studied here.

The results of phenotypic observations of pea cultivars and wild types are presented in Tables 3.1 and 3.2. The seed lots were characterised following descriptors and states obtained from the John Innes Institute, Norwich. Cultivated and wild type pea plants are also shown in Photographs 3.1 - 3.

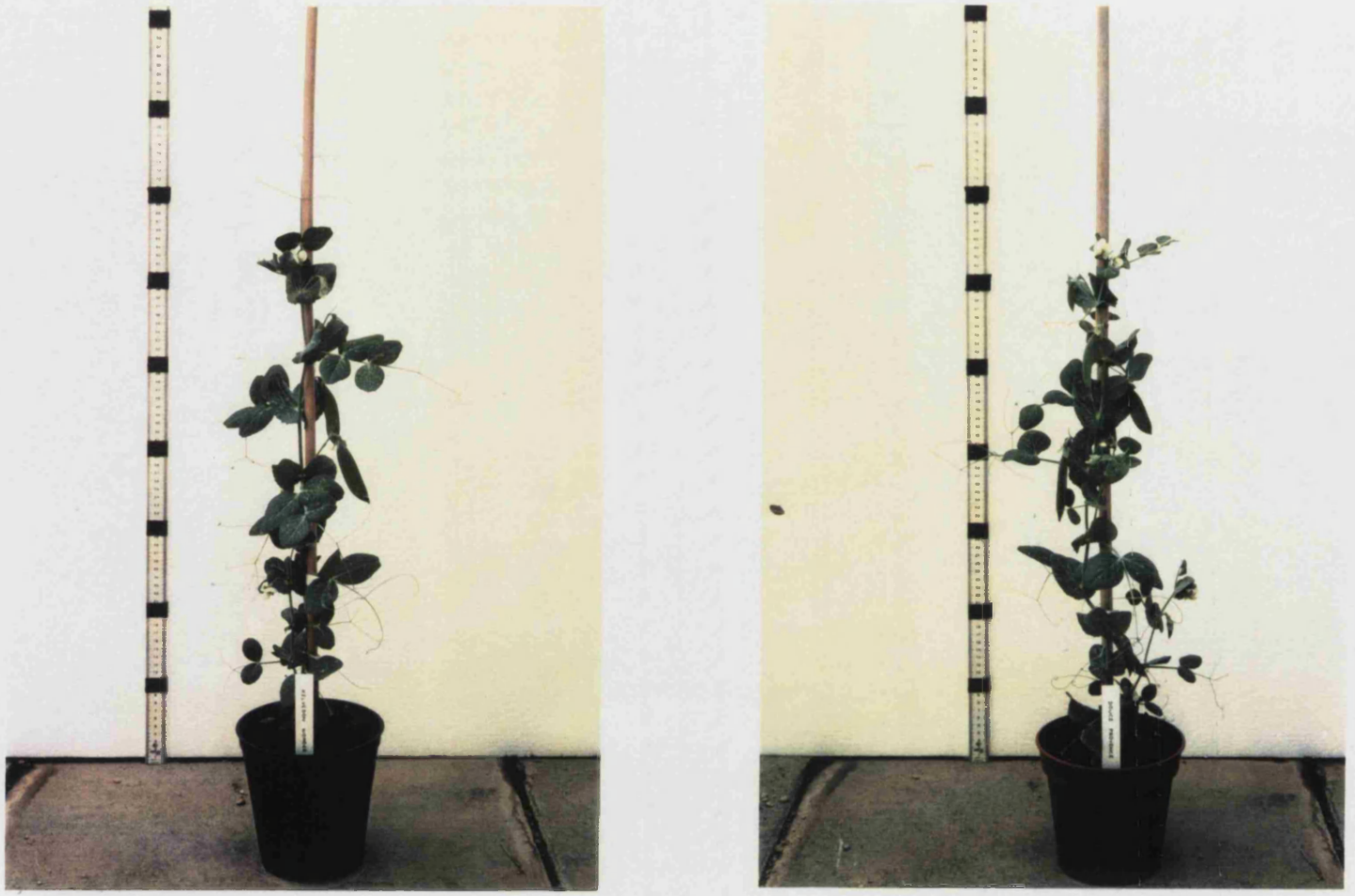
Table 3.1 shows that phenotypic characteristics of pea cultivars (Kelvedon Wonder and Douce Provence) are very similar. However, there are three main differences between these two cultivars, i.e. margins of leaflets, type of seeds and surface of testa.

Table 3.1. Phenotype descriptors and states of pea cultivars.

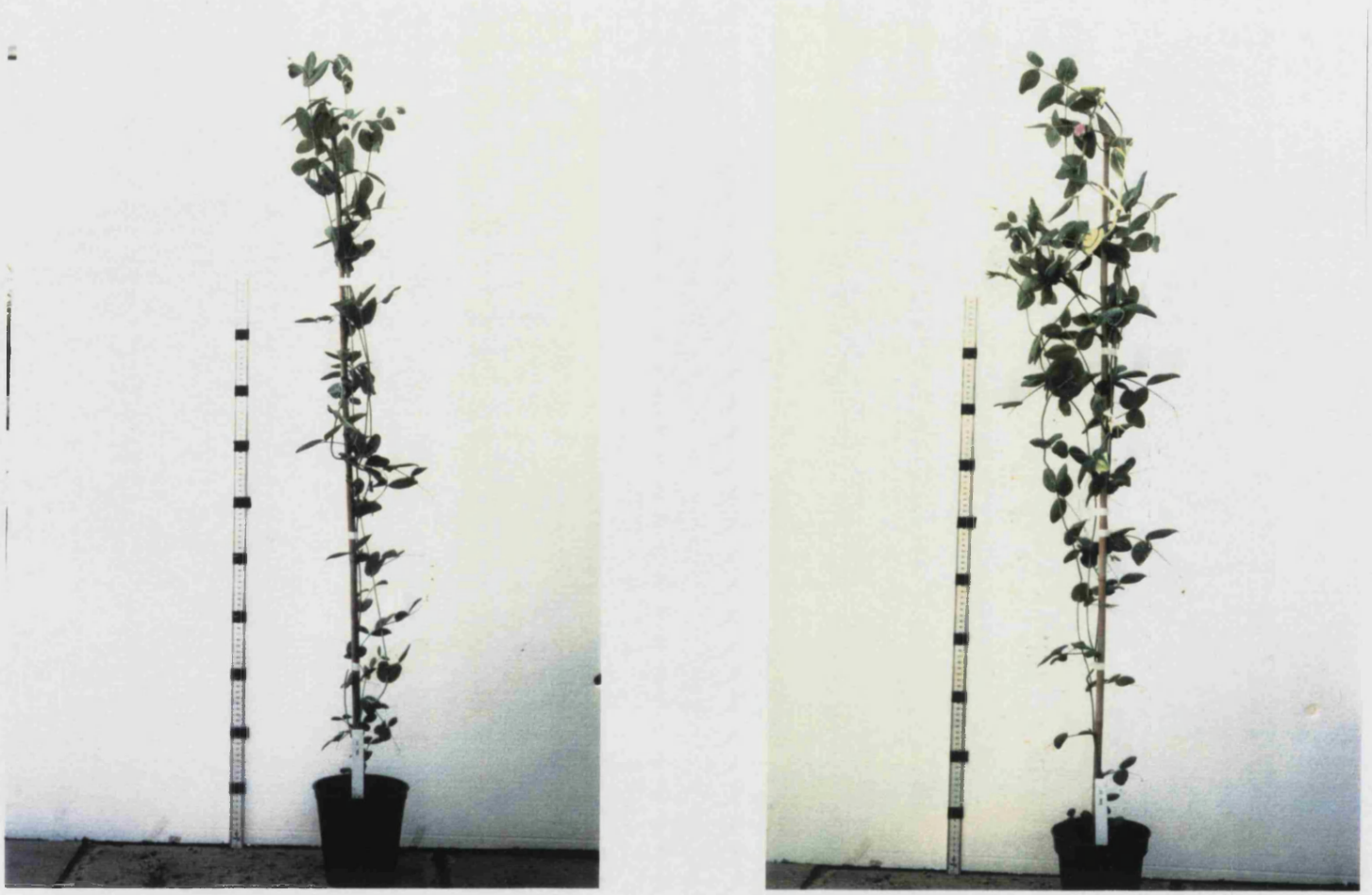
DESCRIPTORS / STATES		KELVEDON WONDER	DOUCE PROVENCE
Recorded			
Date	:	Between 10 July and 30 August 1989	
Habit			
Internode length (mm)	:	67	73
Basal branching	:	0	0
Aerial branching	:	Absent	Absent
Fasciation	:	Absent	Absent
Foliage			
Colour	:	Green	Green
-anthocyanin	:	Absent	Absent
Leaf angle	:	Normal	Normal
Leaflets	:	Normal	Normal
-size	:	Medium	Medium
-angle	:	Normal	Normal
-pulvinii	:	Absent	Absent
-margin	:	Entire	Dentate
-grey spotting	:	Present	Present
-wax upper	:	Present	Present
lower	:	Present	Present
Stipules	:	Normal	Normal
-size	:	Medium	Medium
-grey spotting	:	Present	Present
-wax upper	:	Present	Present
lower	:	Present	Present
-anthocyanin	:	Absent	Absent
Tendrils	:	Normal	Normal
Inflorescence			
Nodes to 1st flower	:	6-8	6-8
Total flowering nodes	:	3-4	3-5
Pedicel	:	Short	Short
Flowers per node	:	1-2	1-2
-size	:	Normal	Normal
-colour	:	White	White
-standard	:	Present	Present
-wings	:	Normal	Normal
-keel	:	Normal	Normal
Pods			
Length	:	Medium	Medium
Width	:	Medium	Medium
Curvature	:	Straight	Straight
Apex	:	Pointed	Pointed
Surface	:	Smooth	Smooth
Wall	:	Thin	Thin
-parchment	:	Present	Present
Colour	:	Green	Green
-anthocyanin	:	Absent	Absent
Pods per node	:	1-2	1-2
Seeds			
Type	:	Wrinkled	Spherical
Sticking	:	No	No
Hilum			
-size	:	Normal	Normal
-colour	:	Cream	Cream
Testa			
-colour	:	Green	Green
-brown marbling	:	Absent	Absent
-anthocyanin	:	Absent	Absent
-surface	:	Dimpled	Smooth
Cotyledon			
-colour	:	Cream	Cream

Table 3.2. Phenotype descriptors and states of John Innes pea wild types.

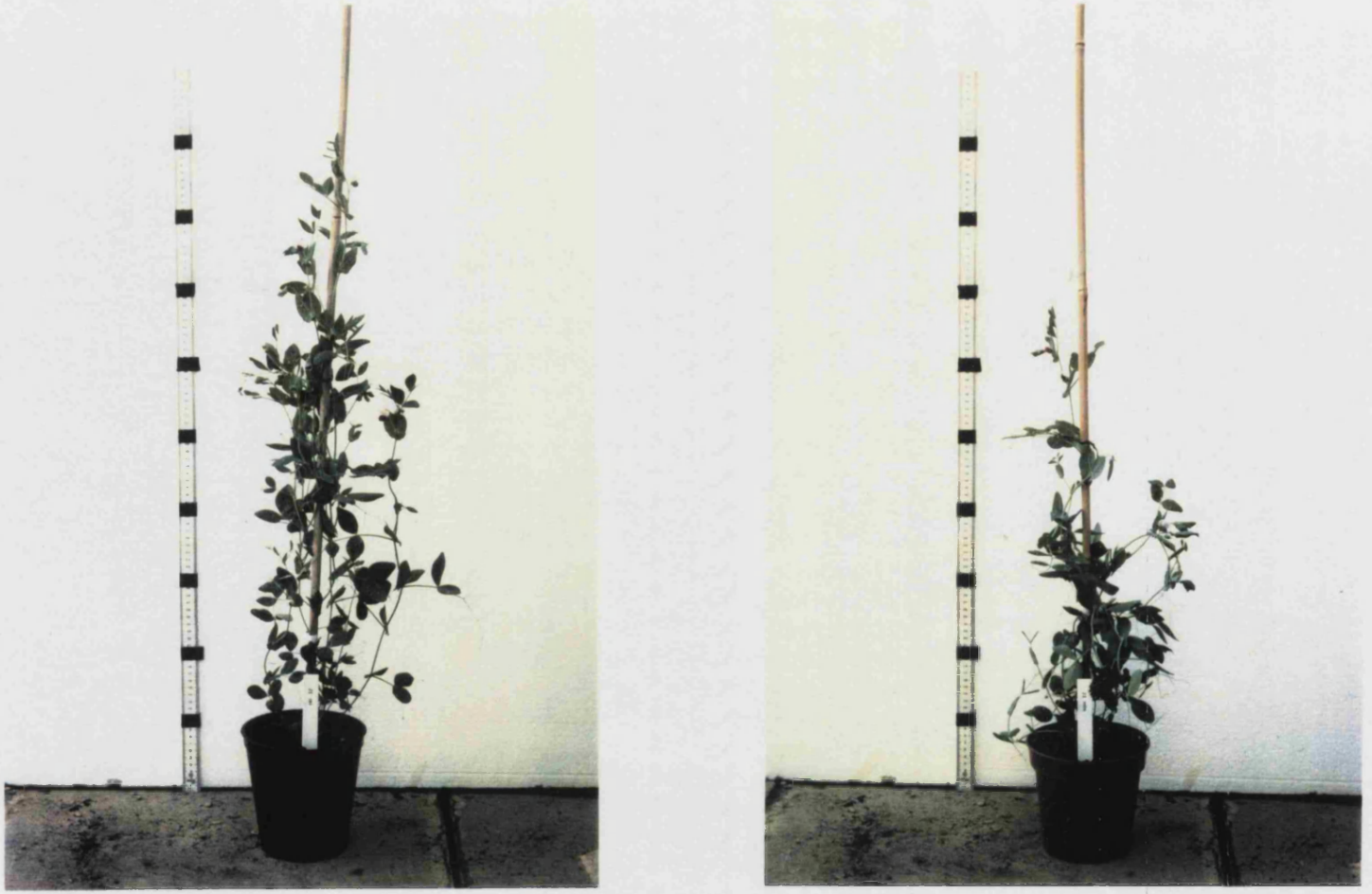
DESCRIPTORS / STATES	JI 181	JI 750	JI 959	JI 1104
Recorded				
Date	: Between 10 July and 30 August 1989			
Habit				
Internode length (mm)	: 80	99	94	77
Basal branching	: 4-5	0	1-3	>5
Aerial branching	: Present	Present	Present	Present
Fasciation	: Present	Present	Present	Present
Foliage				
Colour	: Green	Green	Green	Green
-anthocyanin	: Present	Present	Present	Absent
Leaf angle	: Normal	Normal	Normal	Normal
Leaflets	: Narrow	Normal	Crinkled	Normal
-size	: Small	Medium	Medium	Small
-angle	: Normal	Normal	Normal	Normal
-pulvinii	: Present	Present	Present	Present
-margin	: Entire	Entire	Entire	Entire
-grey spotting	: Present	Present	Present	Present
-wax upper	: Present	Present	Present	Present
lower	: Present	Present	Present	Present
Stipules	: Normal	Normal	Normal	Normal
-size	: Small	Medium	Medium	Small
-grey spotting	: Present	Present	Present	Present
-wax upper	: Present	Present	Present	Present
lower	: Present	Present	Present	Present
-anthocyanin	: Present	Present	Present	Present
Tendrils	: Normal	Normal	Normal	Normal
Inflorescence				
Nodes to 1st flower	: 9-11	14-16	15-17	9-11
Total flowering nodes	: 2-4	3-6	2-4	2-4
Pedicel	: Short	Medium	Medium	Short
Flowers per node	: 1	1-2	2	1
-size	: Small	Large	Large	Small
-colour	: Pink	Pink	Pink	Pink
-standard	: Present	Present	Present	Present
-wings	: Normal	Normal	Normal	Normal
-keel	: Normal	Normal	Normal	Normal
Pods				
Length	: Short	Medium	Medium	Short
Width	: Narrow	Medium	Medium	Narrow
Curvature	: Concave	Straight	Straight	Concave
Apex	: Blunt	Pointed	Pointed	Blunt
Surface	: Smooth	Smooth	Smooth	Smooth
Wall	: Thin	Thin	Thin	Thin
-parchment	: Present	Present	Present	Present
Colour	: Green	Green	Green	Green
-anthocyanin	: Absent	Absent	Absent	Absent
Pods per node	: 1	2	2	1
Seeds				
Type	: Spherical	Spherical	Spherical	Spherical
Sticking	: No	No	No	No
Hilum				
-size	: Small	Normal	Normal	Small
-colour	: Cream	Brown	Brown	Cream
Testa				
-colour	: Green	Cream	Cream	Green
-brown marbling	: Present	Absent	Absent	Present
-anthocyanin	: Absent	Spots	Spots	Absent
-surface	: Smooth	Smooth	Smooth	Smooth
Cotyledon				
-colour	: Cream	Cream	Cream	Cream



Photograph 3.1. Cultivated pea plants : Kelvedon Wonder (left) and Douce Provence (right).



Photograph 3.2. Wild type pea plants : JI 750 (left) and JI 959 (right).



Photograph 3.3. Wild type pea plants : JI 181 (left) and JI 1104 (right).

It is clear from the observed phenotypic chracteristics of wild type peas that JI 750 and JI 959 (originating from Turkey) have some differences compared with JI 181 and JI 1104 (originating from Nepal). These can be summarized by the following descriptors: size of leaflets and stipules, total flowering nodes, size of flowers, length, curvature and apex of pods, size and colour of hilum and colour, brown marbling and anthocyanin of testa (Table 3.2).

Phenotypic characteristics of JI 750 and JI 959 are more similar to the cultivars rather than JI 181 and JI 1104. However, there are a lot of differences between cultivated and wild type peas , e.g. internode length, basal and aerial branching, pulvinii of leaflets, anthocyanin of stipules, nodes to first flower and colour of flowers.

3.3. Seed Moisture Content Determinations

The moisture content of a sample is defined as the loss in weight when it is dried, as a percentage of the original sample.

According to the High Constant Temperature Oven method, in the ISTA Rules (ISTA, 1985a, b), two independently drawn working samples of 4-5 g seeds were weighed to four

decimal places. As pea has large seeds, these were ground before drying as recommended (ISTA, 1985a, b). Petri dishes for the determination were pre-dried in the oven for 15 minutes and left to cool in desiccators for 30 minutes. The container and cover were weighed before and after filling with the ground seed. The container with cover below was then placed quickly in an oven maintained at 130-133°C and dried for 1 hour. The containers and seed were then removed and allowed to cool down in desiccators for 30 minutes before being reweighed.

The moisture content as a percentage by weight was calculated to one decimal place by means of the following formula:

$$(M_2 - M_3) \times 100 / (M_2 - M_1)$$

Where

M_1 - is the weight in grams of the container and its cover,

M_2 - is the weight in grams of the container, its cover and its contents before drying, and

M_3 - is the weight in grams of the container, cover and contents after drying.

The result was taken as the arithmetic mean of the duplicate determinations carried out on a sample if the

difference between the two determinations did not exceed 0.2%. Otherwise, the determination in duplicate, was repeated.

3.4. Germination Tests

All the seed lots were tested for germination percentage on moist rolled paper towels (Scott Fresh Regular, 229 x 330 mm) at 20°C. The percentage viability of the control and aged seeds was determined according to the ISTA Rules (ISTA, 1985a, b) with the modification of eight 25-seed replicates instead of eight replicates of 50 seeds and of an increase in the test duration from 8 days to:

* 11 days in aged cultivars and 14 days in aged wild types, in the case of the high moisture content seed lots,

* 14 days in aged cultivars and 21 days in aged wild types, in the case of the low moisture content seed lots.

Any hard seeds remaining in the tests after 8 and 11 days were counted and then scarified with sandpaper, and the germination tests extended by a further 6 and 10 days in the cultivated and wild type seed lots, respectively.

Normal seedlings comprised : intact seedlings, seedlings with slight defects, and seedlings with secondary infection (i.e. if it was evident that parent seed is not the source of infection and if it was determined that all the essential structures were present). Abnormal seedlings comprised : damaged seedlings, deformed or unbalanced seedlings and decayed seedlings. Seeds in which the radicle had failed to emerge or which were heavily infected were considered dead.

The result of each germination test was considered reliable, if the difference between the highest and the lowest replicate did not exceed the ISTA tolerances (ISTA, 1985b).

3.5. Ageing Treatments

Seed lots of known moisture contents were packaged in hermetically sealed laminated aluminum foil packets from which as much air as possible had been excluded, and labelled individually. The amount of seeds per packet varied according to the anticipated severity of the treatment. However, on average, each packet contained 200 seeds for a germination test plus sufficient viable seed for cytological investigations (50-100 seeds), and in addition 200-300 extra seeds (i.e. a minimum of 450-600 seeds per treatment).

Controlled deterioration of the seeds was carried out in two incubators ; at 50 ± 0.5 °C and 65 ± 0.2 °C, at various seed moisture contents for various time intervals to achieve a range of viabilities. Pea seeds of two cultivars and two wild types were studied (although initially four wild types were regenerated). These treatments are summarised in Tables 4.1 and 4.2 (see Chapter 4).

The improved viability equation (see section 2.2.1) was used to predict the final viability of each seed lot. The values of the viability constants were assumed to be as follows (Ellis, personal communications, 1988):

$K_E = 9.868$

$C_W = 5.389$

$C_H = 0.0328$

$C_Q = 0.000481$

3.6. Pregermination Treatments

A series of experiments were conducted to determine if imbibition damage occurred in these seed lots and to investigate ways of ameliorating such damage.

3.6.1. Soaking

Treatments were carried out by soaking 200 seeds in 200 ml of distilled water for varying periods of time between 0 (control) and 12h, in non-aged and aged seed lots of high and low moisture contents, at 20°C (see Chapter 5).

The seeds were weighed before and after the soaking treatments to determine the final moisture contents (Figure 3.1). The moisture content of the seed samples was determined using the following equation:

$$c = a\ (100 - b)\ /\ (100 - d)$$

where
c - is the final weight of seed,
a - is the initial weight of seed,
d - is the required moisture content (%, f.wt),
b - is the initial moisture content (%, f.wt).

3.6.2. Humidification

The seeds were humidified by placing them over water in a desiccator incubated at varying temperatures (between 15°C and 20°C) for varying periods (between 3 and 7 days) until the desired moisture contents were achieved (see

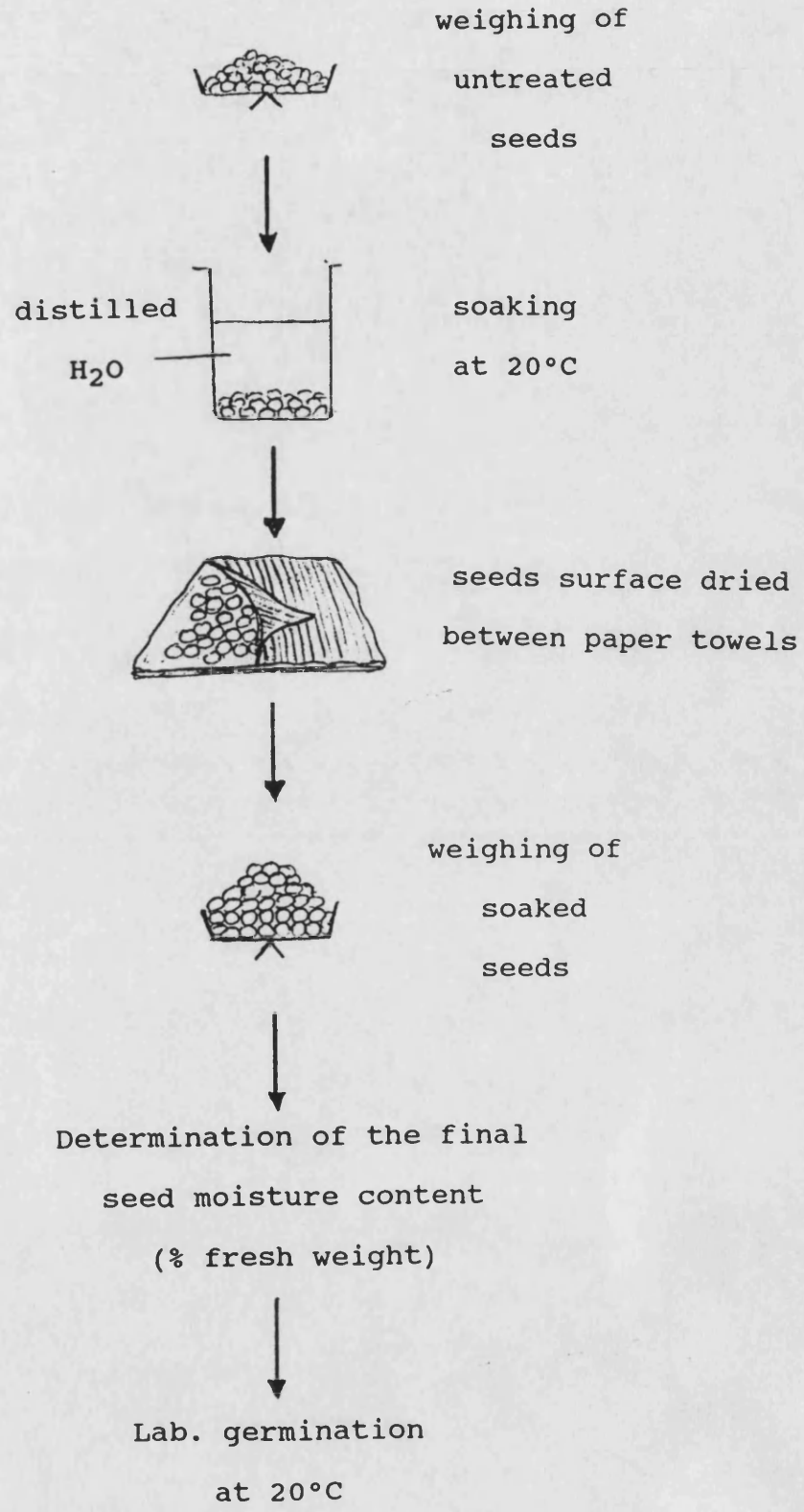


Figure 3.1. General procedure for soaking of pea seeds.

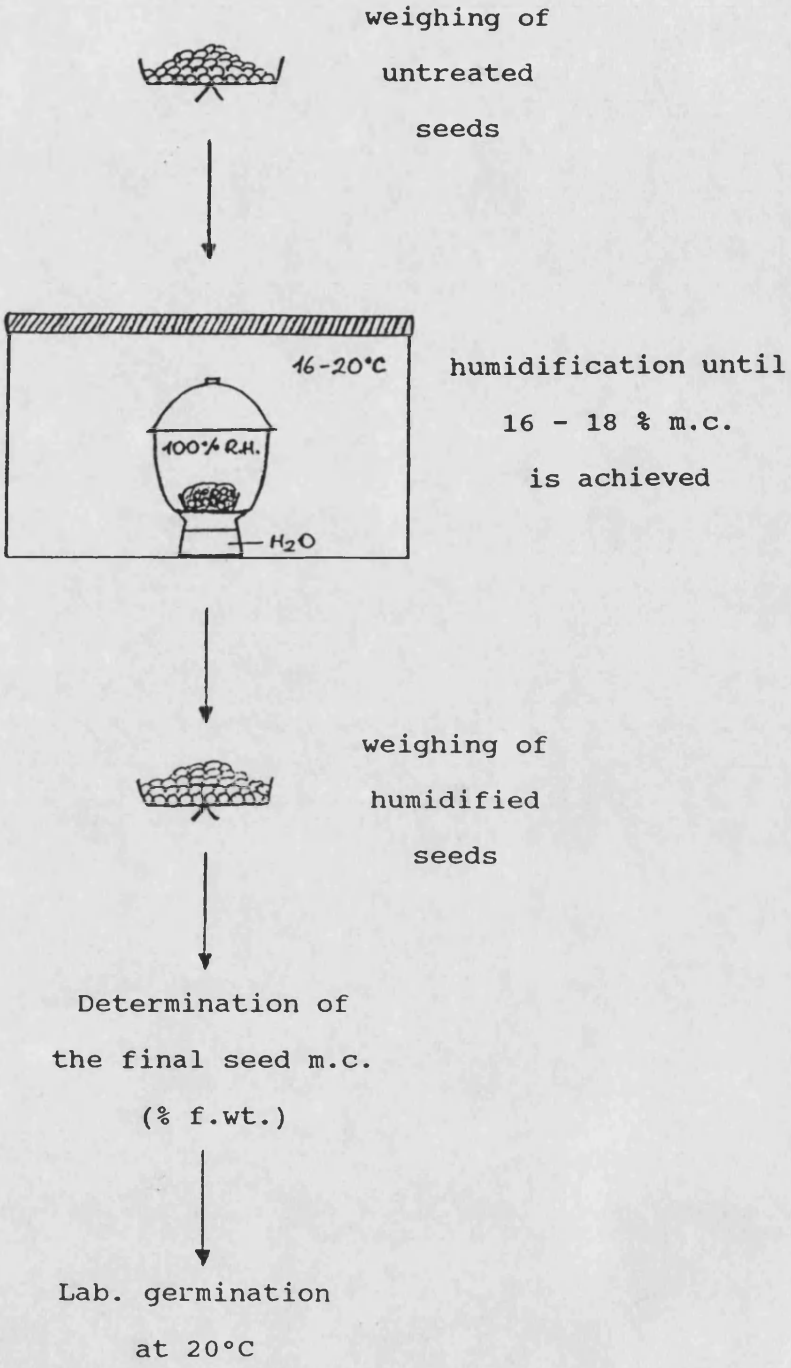


Figure 3.2. General procedure for humidification of pea seeds.

Chapter 7 for details) . The seeds were put in a petri dish above water in order to avoid any possibility of direct water contact (Figure 3.2). The moisture content of the seed samples was determined (from the change in weight during humidification) using the equation in section 3.6.1.

3.6.3. Priming

Priming of the seeds following storage were conducted at 15°C and 25°C in the preliminary experiments and at 16°C in the main experiment (see section 7.2).

Concentrations of PEG-8000 (was formerly PEG-6000, purchased from SIGMA Chemical Company), were determined using the following equation, proposed by Michel and Kaufmann (1973):

$$\psi_S = [\ -(1.18 \times 10^{-2})C - (1.18 \times 10^{-4})C^2 + (2.67 \times 10^{-4})CT + (8.39 \times 10^{-7})C^2T\ ] / 10$$

where

C - is the concentration of PEG-8000 in g/kg H_2O,

T - is the temperature in °C,

ψ_S- is the osmotic potential of the solution in MPa.

The seeds were weighed before treatment with PEG-8000. In the first preliminary experiment, priming was carried

out by immersing paper towels in PEG-8000 solution and in each replicate spreading the seeds evenly on two moist towels and covering with another towel, as in the germination tests (8 replicates of 25 seeds in each treatment were used), based on a modification of the ISTA Rules (ISTA, 1985a, b). The towels were rolled and then arranged in plastic boxes (dimensions 28 x 16 x 9 cm). Following this treatment, the seeds in a beaker were washed in running water for 5 minutes, rinsed in distilled water and then dried at 20°C and 40 ± 5 % r.h. for about 2 hours. Then the seeds were reweighed and the change in weight following priming was determined so that the seed moisture content was estimated from the equation used in section 3.6.1.

The same procedure was followed in the second preliminary priming experiment except with a slight modification in the use of the paper towels immersed in PEG-8000 solution. In this method, 200 seeds were evenly spread on two moist towels which were placed at the bottom of the plastic box and covered with another towel. This was in an attempt to allow greater aeration of the seeds.

In the main priming experiment, the previous methods used were improved so that a more useful and convenient priming method was produced. In this improved method, allowing sufficient aeration of the seeds during priming

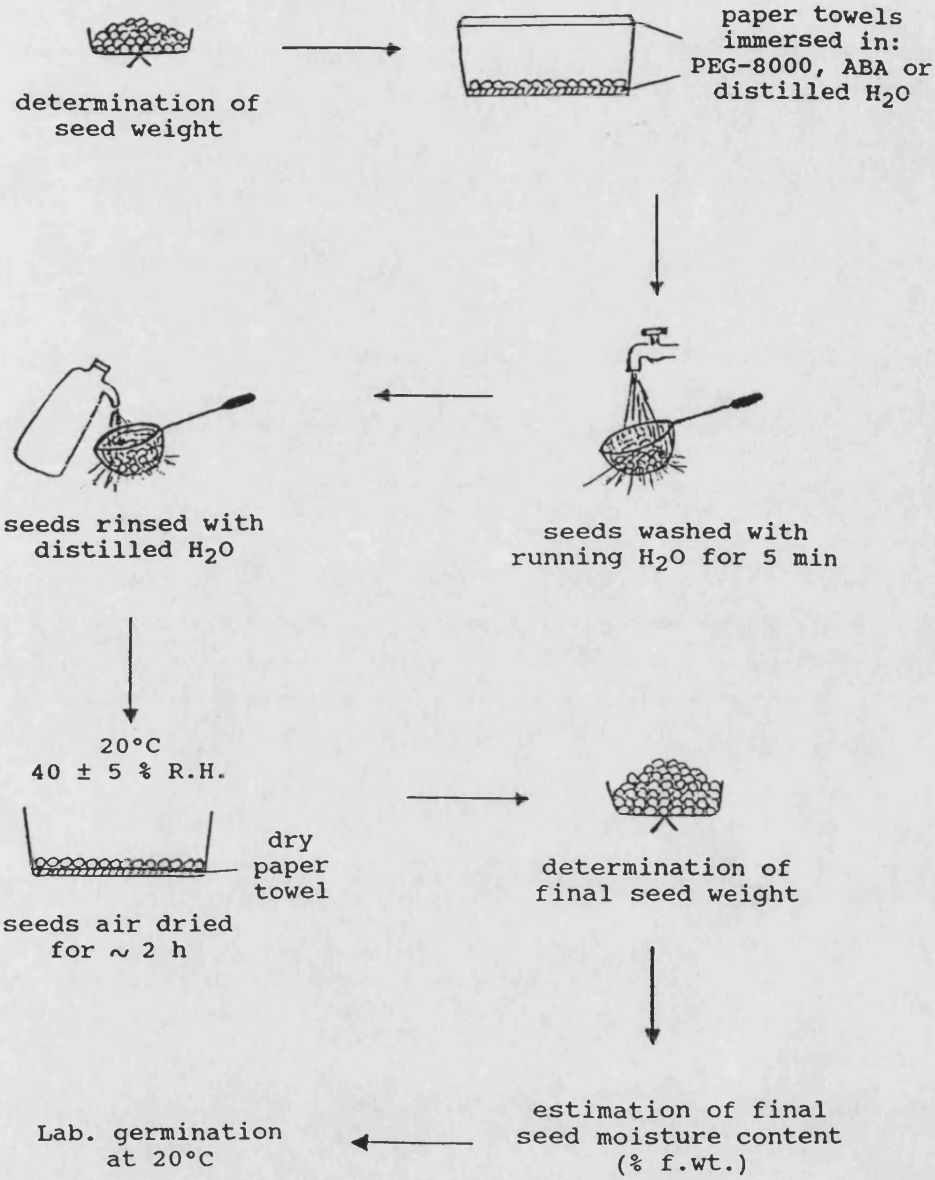


Figure 3.3. General procedure of the improved priming method for pea seeds.

and after priming, washing with tap water and rinsing with distilled water, eliminated the fungal problem. The procedure of the improved priming method was given in detail in section 7.2.

In addition to PEG-8000 solutions, priming with ABA and distilled water were also used in the main experiment (This explained in Chapter 7). The procedure of the improved priming method was followed in treatments with ABA solutions and distilled water, as well (Figure 3.3).

3.7. Cytological Procedures

Radicle-tips for cytological examination were excised over a number of sampling times from the start of germination to ensure that a random selection of fast, medium and slow germinators were selected (Dourado and Roberts, 1984a). Since gross chromosomal aberrations in dividing cells tend to be eliminated through diplontic selection (Gaul, 1961), it is important to sample radicle-tips during the first mitotic divisions in fresh and aged seeds. Therefore, radicles of varying lengths were used depending on the viability of the seed lot. In order to examine the maximum number of cells at first mitosis where normal germination was 80% or more, radicles of 4-8 mm in length were collected. Since the occurrence of the first mitotic division is delayed in

relation to radicle extention in aged seeds (Murata *et al.*, 1980), radicles of 9-13 mm in length were used when viability was between 50 and 80%. With the more severely aged pea treatments (i.e. seeds aged to below 50% viability), radicles were sampled at 14-18 mm following the observations by D'Amato (1951) and Dourado (1983).

The excised radicles were placed in individual glass vials and fixed with glacial acetic acid: absolute alcohol mixture at a ratio of 1:3 for 15 minutes at room temperature. They were then rinsed in distilled water twice and stored in a solution of 70% ethanol at 3°C, in the fridge.

For slide preparation, the radicles were hydrolysed with 1M HCl in a water bath at 60°C for 10 minutes. The acid was then removed and Schiff's Reagent (Feulgen), purchased from BDH Chemicals, added to stain. They were left to stain for 20 minutes in darkness at room temperature. A single radicle was transferred to a glass slide and a 0.8 - 1.0 mm portion excised from the radicle-tip for examination. One drop of 60% lacto-propionic-orcein was added and radicle-tip squashes were made according to Dyer (1979).

Observations were restricted to chromosomal aberrations at late-anaphase, since generally, chromosomal

aberrations are more easily detected at this stage and also because of the relative ease of the procedure when examining large quantities of material. All late-anaphases present in each slide were scored; a minimum of 400 anaphases per treatment were examined. For each treatment between 5 and 12 slides were examined.

3.8. Classification of Chromosomal Aberrations

The total number of aberrations were expressed as a percentage of the total number of anaphase cells observed per treatment. The aberrations were classified as chromatid or chromosome-types, mixed or others (see section 4.2), following Dourado (1983), Sirikwanchai (1985) and Rao (1986).

CHAPTER 4. SEED SURVIVAL CURVES AND THE ACCUMULATION OF CHROMOSOMAL ABERRATIONS IN STORAGE OF CULTIVATED AND WILD TYPE PEA SEEDS

4.1. Introduction

The relationship between loss of viability during seed storage and the accumulation of chromosome aberrations in the surviving seeds has been investigated quantitatively and confirmed in a wide range of species, as reviewed in Chapter 2. However, so far, this relationship has only been examined in cultivated seeds. This experiment is the first attempt to investigate the effect of storage conditions on loss of viability and the accumulation of chromosomal aberrations in wild type peas in comparison with pea cultivars.

Previously, D'Amato (1951) and Abdalla and Roberts (1968) reported mainly chromosome-type aberrations in the root-tips of aged pea seeds. However, recently, Dourado and Roberts (1984a) concluded that the majority of aberrations induced by ageing were of the chromatid-type, and the proportion of these to chromosome-type aberrations remained more or less constant with loss of viability. Therefore, the following experiment was conducted to re-examine the predominant type of chromosomal damage induced in pea seeds under various

storage conditions and also to determine the type and frequency of chromosomal aberrations that accumulate in aged wild type peas. In addition, the changes in the frequency of type of chromosomal damage with severity of ageing were examined both in pea cultivars and wild types.

4.2. Materials and Methods

4.2.1. Survival curves

Pea seeds of two cultivars (Douce Provence and Kelvedon Wonder) and two wild types (JI 181 and JI 1104) were used in these investigations (see section 3.1). The initial seed moisture contents and germination percentages before and after ageing are shown in Tables 4.1 and 4.2. To obtain low moisture content seed lots, the seeds were dried at 20°C over regularly regenerated silica gel in a partial vacuum in desiccators for approximately 6 and 10 months, in the case of cultivars and wild types, respectively. The seed moisture contents of the pea cultivars and wild types were determined by the High Constant Temperature Oven method, i.e. the seeds were dried for 1 hour at 130-133°C (ISTA, 1985a, b). Moisture contents are expressed as percentages of fresh weight and represent means of two determinations.

High and low moisture content seed lots of the cultivars and wild types were aged under different storage conditions as shown in Tables 4.1 and 4.2.

These controlled deterioration treatments were conducted to provide a range of viabilities in each seed lot. The improved viability equation, produced by Ellis and Roberts (1980a, b), and the assumed viability constants for pea seeds (see section 3.5) were used in this study to predict the final viability, after any period of storage, in any air-dry storage environment of known moisture and temperature for any seed lot. Since K_i (the seed lot constant) is equivalent to the probit of percentage viability at the beginning of storage, a single germination test was performed at that time to determine the K_i value of each seed lot. Although that was a crude estimation of initial viability, it was used to provide a general idea of each seed lot.

After storage, non-aged and aged seed lots of pea cultivars and wild types were set to germinate between moist, rolled paper towels at 20°C according to the ISTA Rules (ISTA, 1985a, b) with a modification in seed amount and in test duration (see section 3.4).

Table 4.1. Storage conditions and the resultant viabilities of two pea cultivars.

Cultivars	Storage conditions: Moist. cont. (%,f.wt)	Temp. (°C)	Period (days)	Normal ger. (%)	Abnormal ger. (%)	Total ger. (%)
Kelvedon Wonder	11.5	50	0*	95.5	4.0	99.5
			12	91.5	3.0	94.5
			18	78.0	14.0	92.0
			24	51.0	17.5	68.5
			30	32.0	22.5	54.5
	4.7	65	0*	96.0	1.0	97.0
			17	90.0	6.0	96.0
			39	82.0	7.0	89.0
			50	67.0	5.5	72.5
			70	38.5	13.0	51.5
Douce Provence	15.1	50	0*	96.5	3.5	100.0
			3	90.0	7.0	97.0
			3.5	84.0	6.5	90.5
			4	79.5	7.0	86.5
			6	55.0	13.5	68.5
			7	21.0	11.0	32.0
	4.8	65	0*	94.0	5.0	99.0
			21	88.5	6.0	94.5
			42	73.5	6.5	80.0
			63	66.5	5.0	71.5
			77	51.0	2.0	53.0
			84	44.0	3.0	47.0

* Control (non-aged)

Table 4.2. Storage conditions and the resultant viabilities of two wild type peas.

Wild types	Storage conditions: Moist. cont. (%,f.wt)	Temp. (°C)	Period (days)	Normal ger. (%)	Abnormal ger. (%)	Total ger. (%)
JI 181	10.8	65	0*	100.0	0.0	100.0
			1	100.0	0.0	100.0
			2	100.0	0.0	100.0
			3	99.5	0.0	99.5
			4	98.5	0.5	99.0
			5	97.0	1.5	98.5
			6	75.0	17.5	92.5
			7	36.0	12.5	48.5
	5.1	65	0*	100.0	0.0	100.0
			126	94.0	1.5	95.5
			140	87.5	4.0	91.5
			154	76.0	11.0	87.0
			175	20.0	25.5	45.5
			182	8.5	29.5	38.0
JI 1104	10.1	65	0*	100.0	0.0	100.0
			2	100.0	0.0	100.0
			4	100.0	0.0	100.0
			6	95.0	3.0	98.0
			8	77.0	17.0	94.0
			10	42.0	14.0	56.0
	5.1	65	0*	100.0	0.0	100.0
			112	95.5	0.5	96.0
			126	87.5	6.0	93.5
			140	83.5	7.0	90.5
			154	63.0	11.0	74.0
			168	45.0	16.5	61.5
			189	16.5	22.5	39.0

* Control (non-aged)

Fitted values were obtained by using Generalized Linear Models (GLIM) in a Genstat 5 Computer Programme. Then, the survival curves for the pea cultivars and wild types were produced by plotting storage period against percentages of normal and total germination in comparison with the fitted values (Figures 4.1 - 4.4).

In order to linearize the relationship between normal germination and storage period (Figures 4.5 and 4.6) and between aberrant anaphase cells and normal germination (Figure 4.8), percentages were transformed to probit values using a probit link function in the same computer programme.

Then, the above relationships were analysed by linear regression techniques. Initially, regression analysis was carried out separately for each of the eight storage conditions (see Appendices 2 - 5). Where the individual regression lines fitted to the relationship showed similar intercepts and slopes, a single line was fitted to represent the relationship within cultivars or wild types or the overall relationship. The best fitted lines to the relationships shown in Figure 4.6 (one for the cultivars and the other for the wild types) and Figure 4.8 (a single line for both cultivars and wild types) were also computed using the probit link function in the above computer programme.

4.2.2. Cytology

Chromosomal aberrations induced during storage of pea cultivars and wild types were estimated by examining the first mitotic divisions in the radicle tips of the germinating seeds. A number of sampling times and radicles of varying lengths were used depending on the percentage viability of each seed lot (see section 3.7). Previously, Dourado (1983) produced sampling guidelines for pea cultivars to obtain an accurate estimate of chromosome damage during first mitosis. These sampling procedures were adopted in this study. Once the radicles of pea cultivars and wild types were excised and fixed, radicle-tip squashes were prepared and between 5 and 12 slides were examined in order to score a minimum of 400 anaphases per treatment (see section 3.7).

Chromosomal aberrations observed for each seed lot (at each sampling time, in each storage treatment) were classified into one of the four categories: chromosome or chromatid-types, mixed or others.

Chromatid-type aberrations comprised: single fragments, two or more fragments of unequal size; single bridges, two or more bridges with or without fragments of unequal size. *Chromosome-type* aberrations comprised: one or more double fragments; one or more double bridges with or

without double fragments. Where both chromatid and chromosome-type aberrations were observed within a cell, the damage was classified as *mixed*. A small category, *other* damage including lagging chromosomes with or without any of the other groups were also recorded.

The percentage aberration of each category was calculated for each treatment group.

4.3. Results

4.3.1. The effect of storage on the viability of high and low moisture content seeds

Pea seeds (i.e. two cultivars and two wild types) were aged by storing hermetically for different periods at various temperatures and moisture contents. The ageing treatments decreased the viability of all seed lots of the pea cultivars and wild types with increase in time. The fastest decrease in germination was achieved by storing the seeds at high moisture contents and high temperatures (Tables 4.1 and 4.2.). The estimated percentage viability (both on the basis of normal germination and total germination) under different storage conditions were plotted against time for each cultivar and wild type (Figures 4.1 - 4.4). The solid lines, representing the survival curves described by

negative cumulative normal distributions, were fitted from probit analysis using the Genstat 5 computer programme. It was shown that in high and low moisture content seed lots of each cultivar and wild type, the proposed relationship describing both the criteria (normal germination and total germination with time) closely fit the pattern of negative cumulative normal frequency distribution, supporting the suggestion of Roberts (1972a) that when seeds are stored under constant conditions, the periods of viability of the individual seeds in a population are normally distributed around a mean value.

It is clear from the survival curves shown in Figures 4.1A and 4.2A that the decrease in percentage viability of high moisture content seed lots of pea cv. "Kelvedon Wonder" was slower than those of cv. "Douce Provence". Although similar temperature regimes were applied to each cultivar (Table 4.1), the initial seed moisture content of the former was lower than the latter, i.e. 11.5% and 15.1%, respectively. However, after desiccation of the seeds of both cultivars, the low moisture content seeds of cv. "Kelvedon Wonder" (4.7%) showed a faster decline in viability compared with the 4.8% moisture content seed lot of cv. "Douce Provence" (Figures 4.1 and 4.2).

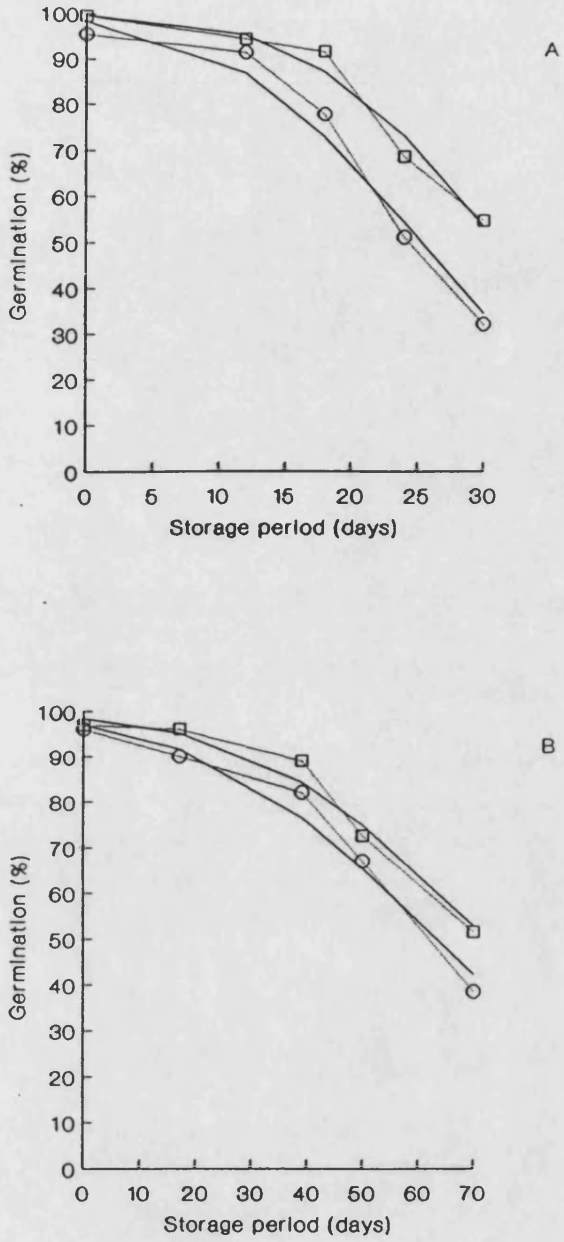


Figure 4.1. The survival curves ofpea cv."Kelvedon Wonder", stored hermetically for various periods at the following temperatures and moisture contents: (A) 50°C, 11.5% m.c.; (B) 65°C, 4.7% m.c. The percentage viability was plotted as normal germination (····⊙····) and total germination (····□····). Solid curves (———) representing the relationship as a negative cumulative normal distribution were fitted by probit analysis.

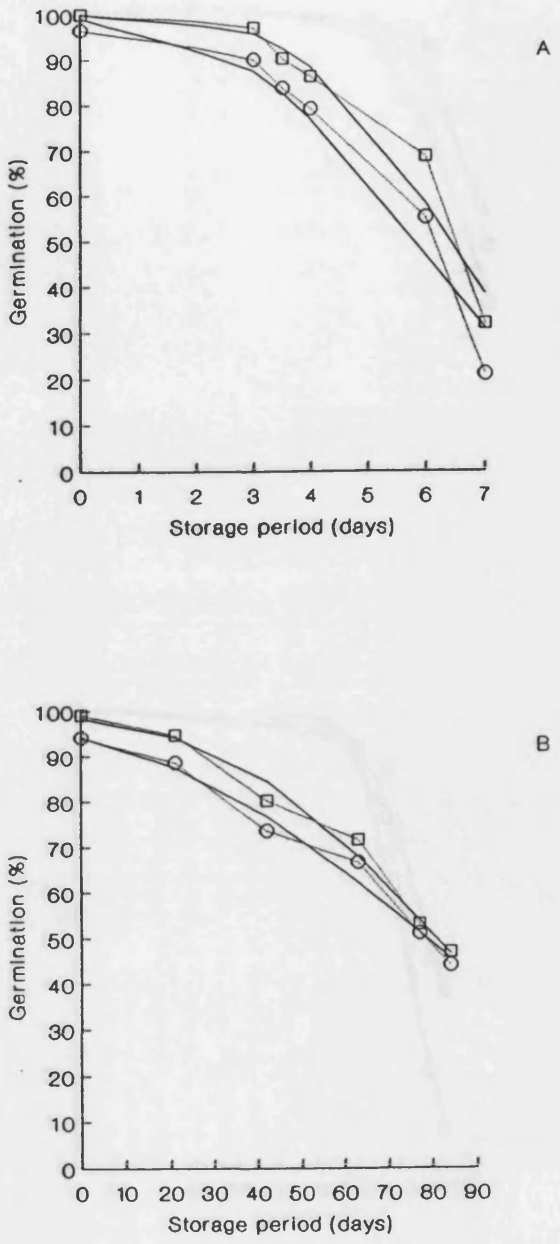


Figure 4.2. The survival curves of pea cv. "Douce Provence", stored hermetically for various periods at the following temperatures and moisture contents: (A) 50°C, 15.1% m.c.; (B) 65°C, 4.8% m.c. The percentage viability was plotted as normal germination (···o···) and total germination (···□···). Solid curves (———) representing the relationship as a negative cumulative normal distribution were fitted by probit analysis.

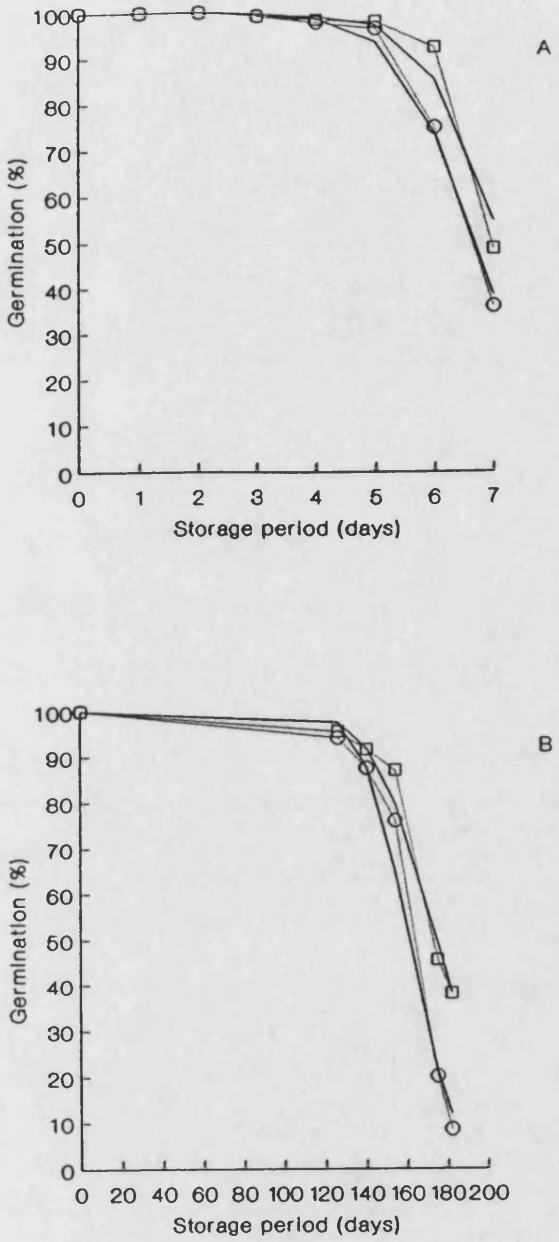


Figure 4.3. The survival curves of pea wild type JI 181, stored hermetically for various periods at the following temperature and moisture contents: (A) 65°C, 10.8% m.c.; (B) 65°C, 5.1% m.c. The percentage viability was plotted as normal germination (···o···) and total germination (··□··). Solid curves (———) representing the relationship as a negative cumulative normal distribution were fitted by probit analysis.

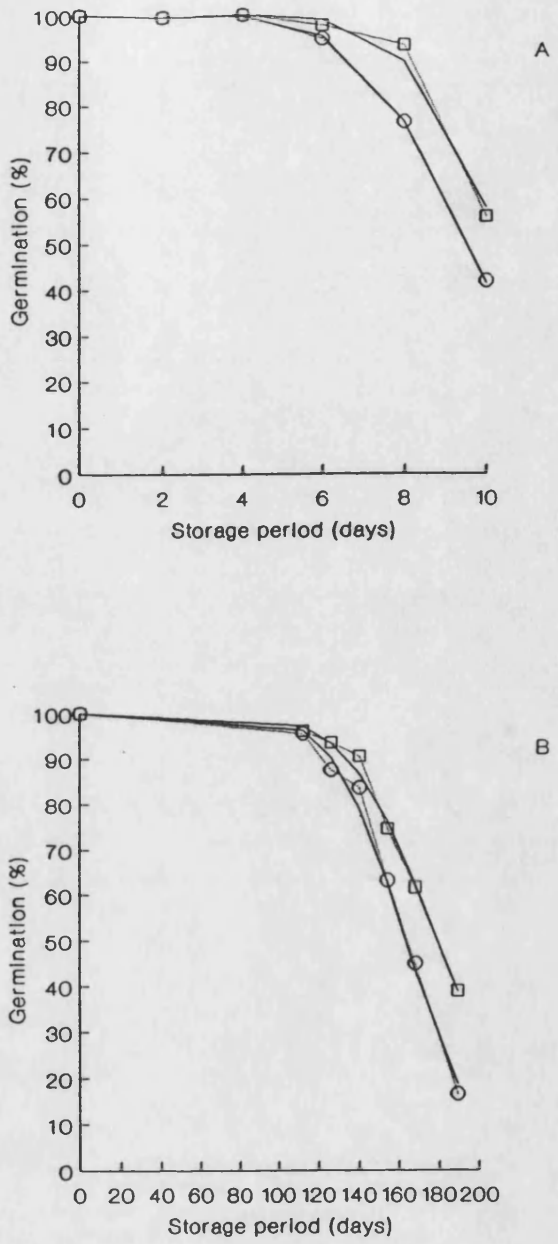


Figure 4.4. The survival curves of pea wild type JI 1104, stored hermetically for various periods at the following temperature and moisture contents: (A) 65°C, 10.1% m.c.; (B) 65°C, 5.1% m.c. The percentage viability was plotted as normal germination (····⊙····) and total germination (··⊡··). Solid curves (———) representing the relationship as a negative cumulative normal distribution were fitted by probit analysis.

Both high and low moisture content seed lots of wild type peas, JI 181 (10.8% and 5.1%, respectively) and JI 1104 (10.1% and 5.1%, respectively) were aged at 65°C. The viability curves of the wild type peas (Figures 4.3 and 4.4), under the above storage conditions, also showed that loss of seed viability is related to an increase in seed moisture content, storage temperature and duration, i.e. increasing each of these factors caused a decrease in seed viability.

In addition, the percentage of normal germination of each seed lot was plotted on a probit scale against storage period in order to clearly define the relationship between seed viability and storage period under different storage conditions, in cultivated and wild type peas. Figures 4.5 and 4.6 show that there is a linear relationship between the probit of percentage normal germination and storage period under all the storage conditions.

It is shown in Figure 4.5 that in the high moisture content seed lots of cultivars and wild types an increase in either seed moisture content or temperature causes a steeper slope in each seed lot and therefore an increase in the rate of loss of seed viability. For example, in the pea cultivars (both stored at 50°C), the slope of the 11.5% moisture content seed lot is shallower than that of

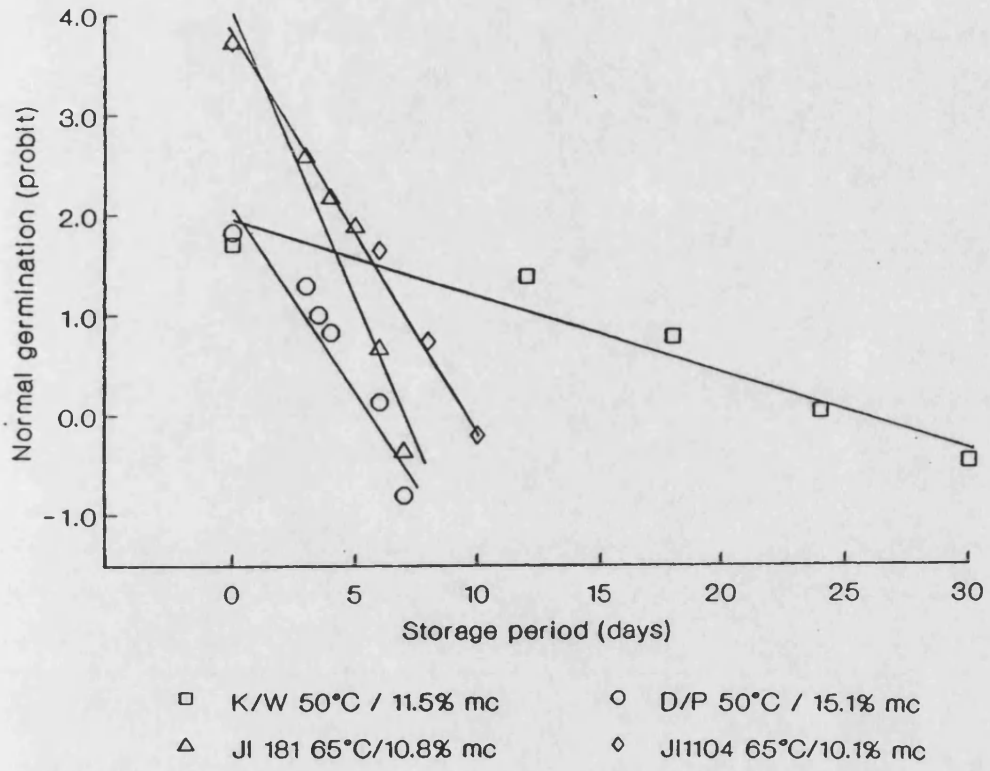


Figure 4.5. The relationship between storage period and normal germination (probit values) in the high moisture content seeds of pea cultivars and wild types stored at two different temperature regimes.

the 15.1% moisture content seed lot (-0.075 and -0.359, respectively). However, in the wild type peas, the higher storage temperature (65°C) results in a steeper slope in each seed lot compared with the pea cultivars. In addition, even an apparently small increase in the seed moisture content also results in a steeper slope in wild type peas, i.e. the slope of JI 181 (-0.554) is steeper than that of JI 1104 (-0.387) and their moisture contents are 10.8% and 10.1%, respectively (see Appendix 6).

Furthermore, Figure 4.6 is convincing evidence that in low moisture content pea seeds, loss of probit viability in cultivars show similar trends to wild types under similar storage conditions (at 65°C and ca. 5% seed moisture content) since there is no significant difference between the slopes of the cultivars and wild types ($P > 0.05$). The slope of the best fitted regression line for the cultivars (-0.023) is almost parallel to that of the wild types (-0.025). However, the intercept of the line for pea wild types (4.096) is higher than that of the cultivated peas (1.659). This suggests that the storage life of pea wild types is longer than that of pea cultivars when stored under similar conditions (see Appendix 7).

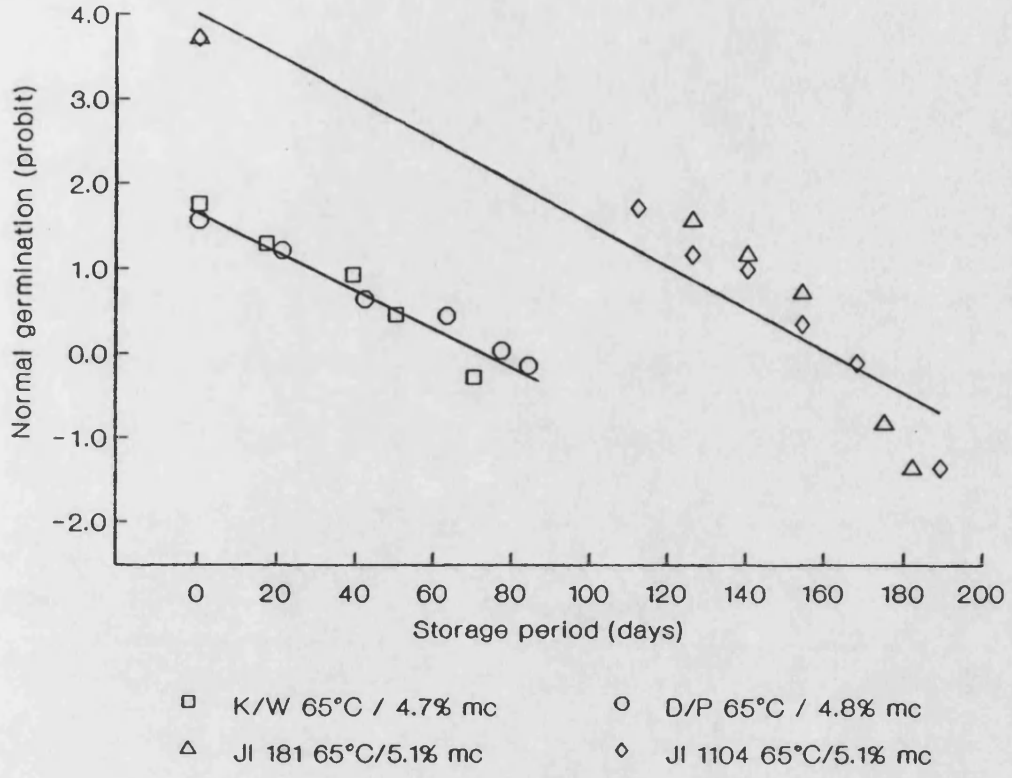


Figure 4.6. The relationship between storage period and normal germination (probit values) in the low moisture content seeds of pea cultivars and wild types stored at 65°C.

4.3.2. The effects of temperature, moisture content and time on the relationship between loss of seed viability and the accumulation of chromosomal aberrations

The effect of storage conditions on the accumulation of chromosomal aberrations in pea cultivars and wild types are detailed in Appendices 2 - 5. The frequency of visible chromosome damage (as percentage aberrant anaphase cells at first mitosis) in the radicle tips of the surviving seeds were plotted against storage period (Figure 4.7). It is clear, that in both cultivars and wild types, the frequency of aberrant cells increased with increase in storage period. Figure 4.7A shows that at high seed moisture contents the rate of accumulation of chromosomal aberrations in pea cultivars stored at 50°C and also in pea wild types stored at 65°C increased. However, in the low moisture content seed lots (Figure 4.7B), pea cultivars showed a faster increase in the frequency of aberrant anaphase cells relative to pea wild types although they were stored at the same temperature, i.e. 65°C. In addition, the moisture contents of pea cultivars, Kelvedon Wonder (4.7%) and Douce Provence (4.8%), were also similar to those of the wild type peas, JI 181 and JI 1104 (5.1%).

The percentage of normal germination and the total frequency of aberrant anaphase cells in each seed lot

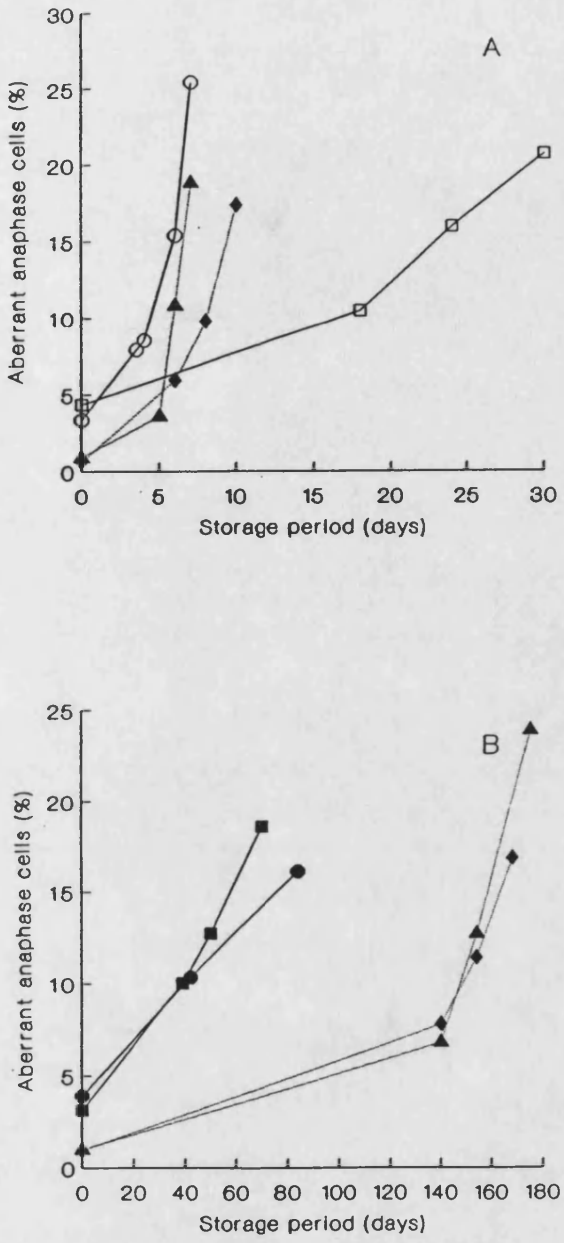


Figure 4.7. The relationship between storage period and the frequency of aberrant anaphase cells in surviving seeds of pea cultivars and wild types stored under the following conditions:

A (high moisture contents)

(□) Kel. Won., 50°C/11.5%
(○) Dou. Pro., 50°C/15.1%
(▲) JI 181 , 65°C/10.8%
(◆) JI 1104 , 65°C/10.1%

B (low moisture contents)

(■) Kel. Won., 65°C/4.7%
(●) Dou. Pro., 65°C/4.8%
(▲) JI 181 , 65°C/5.1%
(◆) JI 1104 , 65°C/5.1%

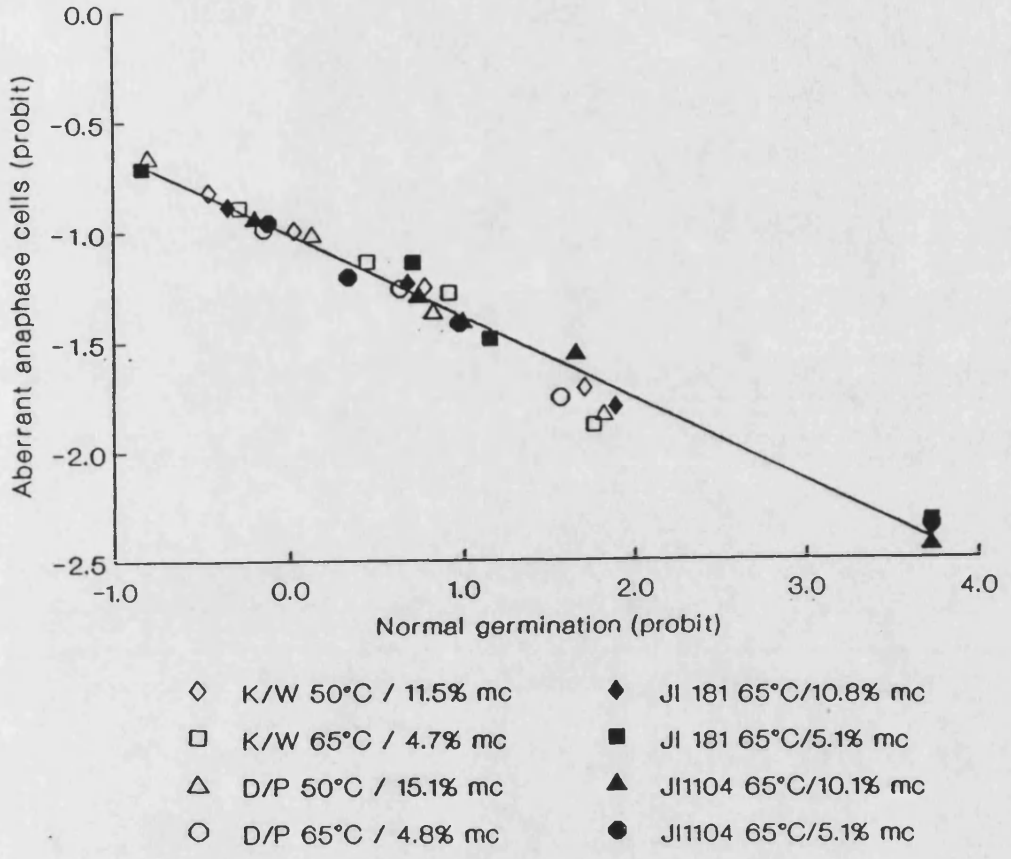


Figure 4.8. The relationship between percentage normal germination and the total frequency of aberrant anaphase cells in surviving seeds, plotted on probit scale, in pea cultivars and wild types under different storage conditions (see Appendices 2 - 5).

(irrespective of type) from the various storage conditions were plotted on probit scales in order to define the relationship between seed viability and the frequency of aberrant anaphase cells under the various storage conditions (Figure 4.8). It can be seen that there is a negative relationship between normal germinating seedlings and the frequencies of aberrant anaphase cells under different storage conditions, and probit transformation of both variables linearized this relationship in pea cultivars as well as in wild type peas. As the intercept and slope of the regression line for each of the eight storage conditions are not significantly different ($P > 0.05$), a single line fitted to the overall relationship clearly shows that in pea cultivars and wild types the frequency of aberrant anaphase cells for any given loss of viability does not increase with decrease in seed moisture content.

4.3.3. Types and frequencies of chromosomal aberrations

Some of the common types of chromosomal aberrations observed in the anaphase cells of the first mitotic divisions in cultivated and wild type pea seeds after storage are shown in Plates 1 - 3.

Figures 4.9 - 4.12 illustrate the relationship between storage period and the frequency and type of chromosome

damage that accumulate. There is a general increase in all types of aberrations with decrease in percentage germination of each cultivar (Figures 4.9 and 4.10) and wild type (Figures 4.11 and 4.12). However, the relative frequency of the various types of aberration with loss of viability varied in the different storage treatments although chromatid-type aberrations were predominant to the other categories under all the storage conditions in each cultivar and wild type.

It is clear from Figure 4.9A that chromatid-type aberrations appear predominant but the proportion of the category "others" is also high in the seeds of cv. "Kelvedon Wonder" stored at 50°C and 11.5% m.c. A similar trend can be seen in the seeds stored at 65°C and 4.7% m.c. but a high proportion of the category "others" (2.19%) only appears in the 70 day aged seed lot, which has 38.5% normal germination (Figure 4.9B). The seeds of pea cv. "Douce Provence" also show similar trends in storage at 50°C and 15.1% m.c.(Figure 4.10A) as well as at 65°C and 4.8% m.c. (Figure 4.10B).

In the low moisture content seed lots of JI 181 (Figure 4.11B) and JI 1104 (Figure 4.12B), the proportion of each aberration category, at any similar viability level, was almost identical to the cultivars with the exception that in the cultivars, storage at high seed moisture contents

resulted in a higher frequencies of the category "others". In addition, Figures 4.11 and 4.12 show that in wild type peas, chromosomal aberrations (i.e. chromatid-type aberrations) are present even at 100% seed viability.

In addition, mixed-type aberrations only occurred below 80% normal germination in all the seed lots of both cultivars and wild types (Figures 4.9 - 4.12).

The data from all the treatments within each seed moisture content of each cultivar and wild type were pooled separately to illustrate the overall frequency of different types of aberration (as percentage of the total number of anaphase cells) accumulated during storage (Appendices 9 - 12). The pooled results confirmed that chromatid-type aberrations were predominant at each moisture content level of each cultivar and wild type (i.e. the proportions of chromatid-type aberration to the total frequency of aberrations varied between 73% and 89%). The most common type of chromatid aberration were single fragments (1F), followed by two fragments of unequal size (2F) and single bridges (1B).

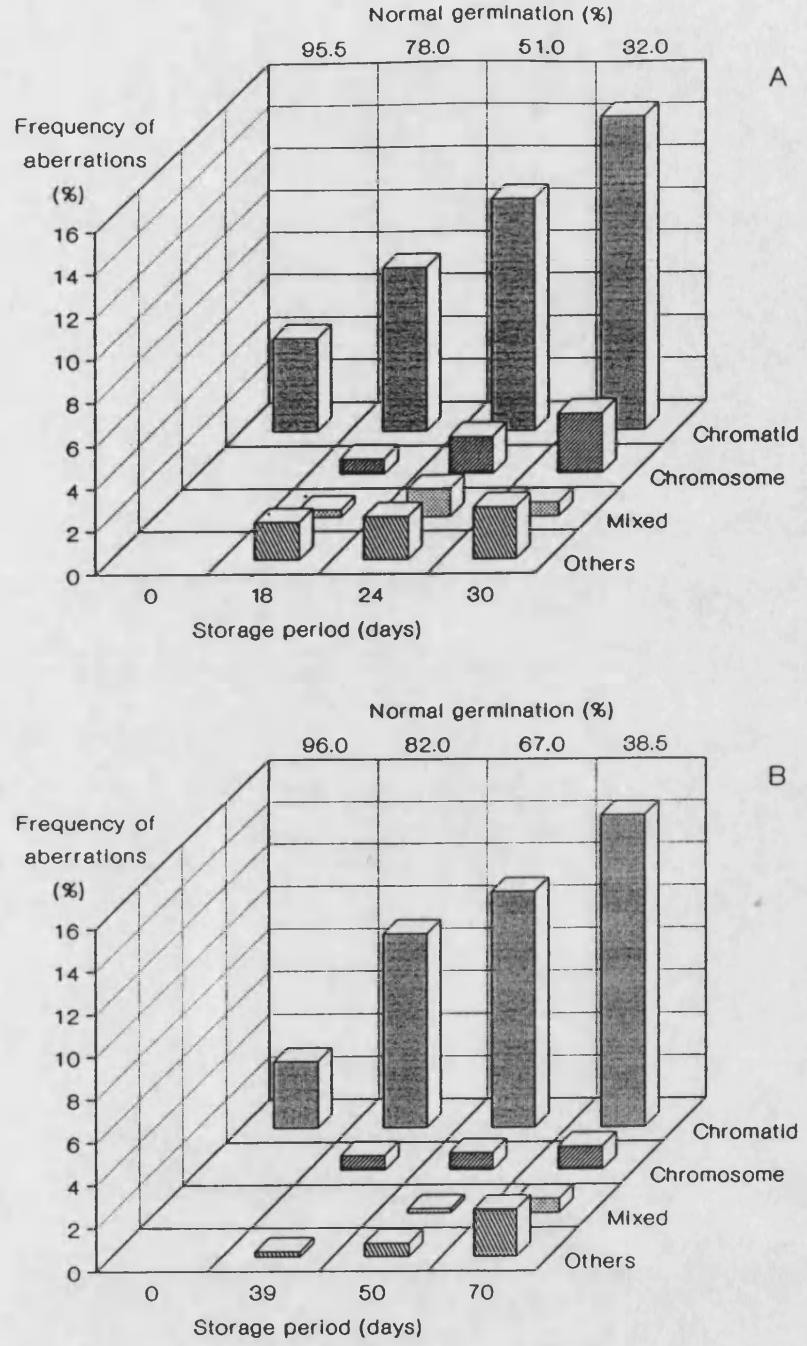


Figure 4.9. The relationship between storage period / percentage normal germination and the frequency of cells containing various types of chromosomal aberrations in pea cv. "Kelvedon Wonder", under different storage conditions (A, 50°C / 11.5% m.c.; B, 65°C / 4.7% m.c.).

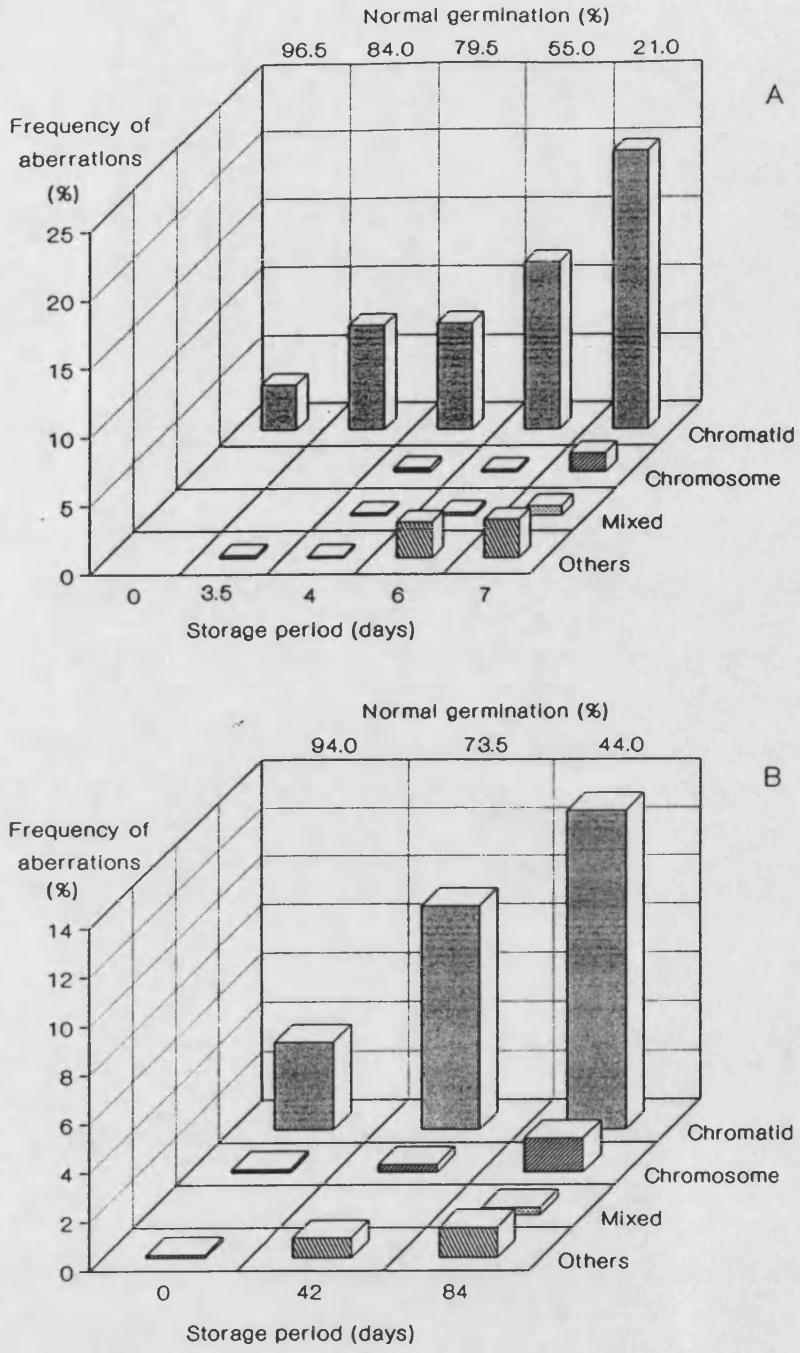


Figure 4.10. The relationship between storage period / percentage normal germination and the frequency of cells containing various types of chromosomal aberrations in pea cv. "Douce Provence", under different storage conditions (A, 50°C / 15.1% m.c.; B, 65°C / 4.8% m.c.).

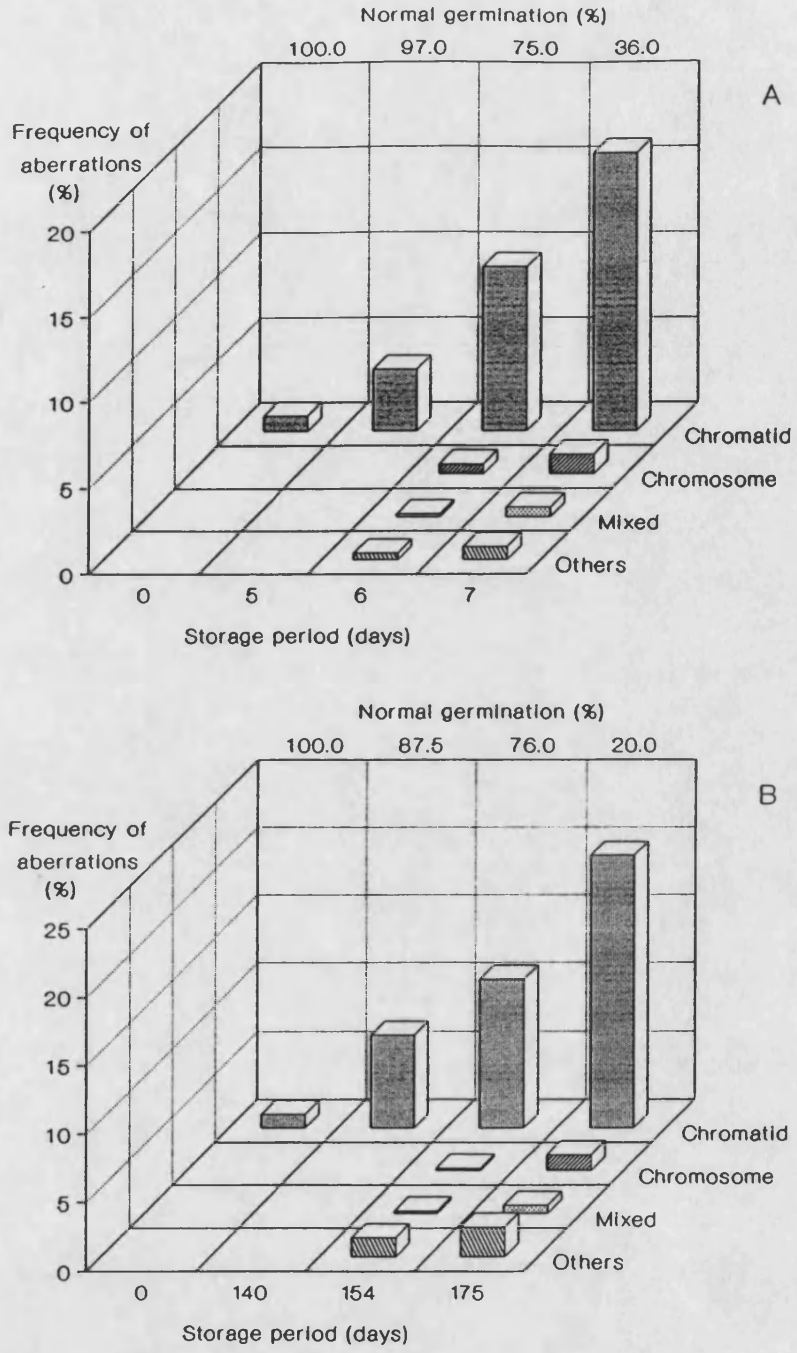


Figure 4.11. The relationship between storage period / percentage normal germination and the frequency of cells containing various types of chromosomal aberrations in pea wild type "JI 181" , under different storage conditions (A, 65°C / 10.8% m.c.; B, 65°C / 5.1% m.c.).

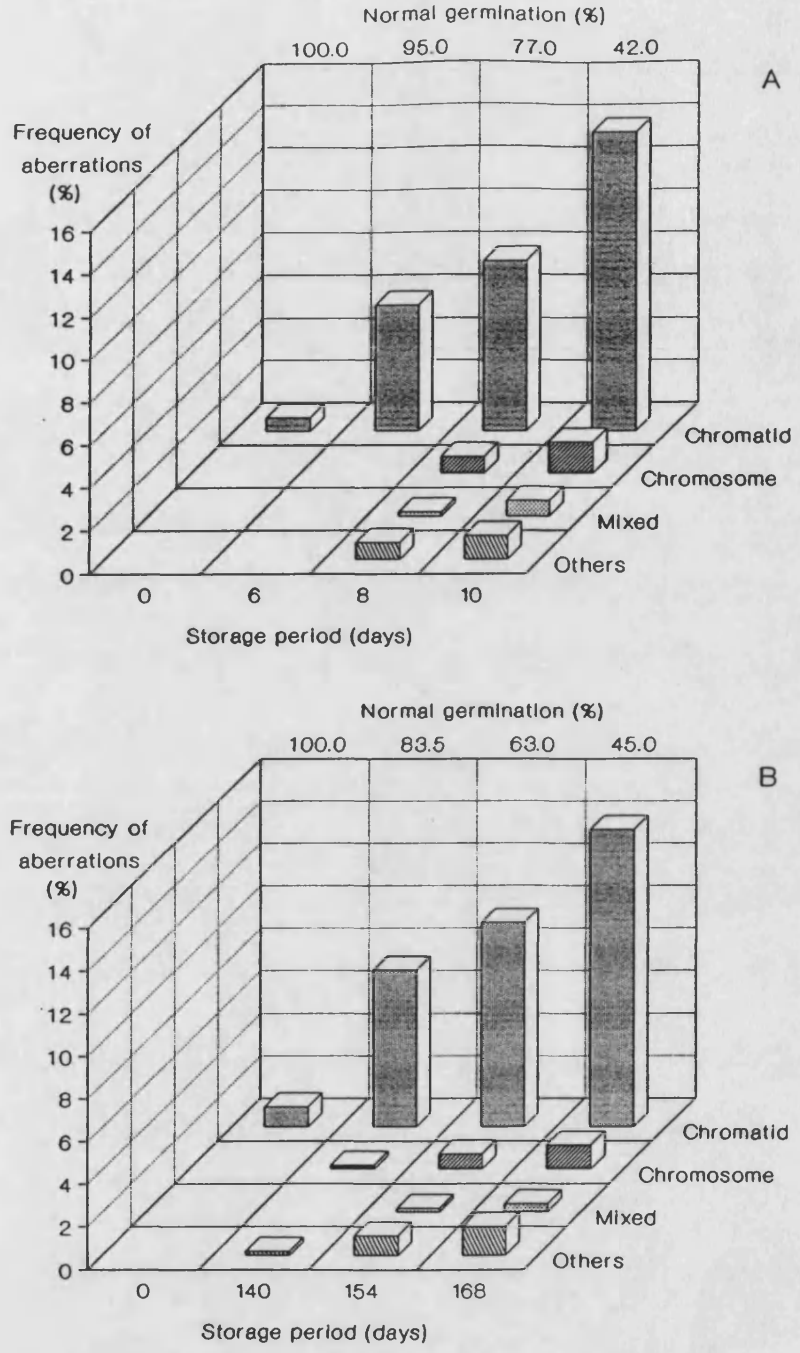


Figure 4.12. The relationship between storage period / percentage normal germination and the frequency of cells containing various types of chromosomal aberrations in pea wild type "JI 1104" , under different storage conditions (A, 65°C / 10.1% m.c.; B, 65°C / 5.1% m.c.).

4.4. Discussion

Roberts (1972a) proposed that life spans of the individual seeds in the population are normally distributed around a mean value. The present results from the ageing treatments in the two cultivars and two wild types confirmed this suggestion in seed lots of both high and low seed moisture contents. Figures 4.1 - 4.4 are convincing evidence that the survival curves (of both normal and total germinations) were very close fits to the pattern of negative cumulative normal distributions in the high and low moisture content seed lots of the pea cultivars and wild types. Figures 4.5 and 4.6 also support this showing the negative linear relationship between the probit of percentage normal germination and storage period under various temperature and seed moisture content regimes.

In the present study, data were presented to show that the longevity of cultivated and wild type pea seeds was affected by temperature, seed moisture content and time, i.e. increasing any of these factors decreased viability (Figures 4.1 - 4.6). Each seed lot of the wild types had 100.0% initial viability, and loss of seed viability was slower in the wild type peas compared with the pea cultivars under similar storage conditions (Figure 4.6).

Previously, a number of researchers concluded that there is a negative relationship between loss of seed viability and the accumulation of chromosomal aberrations in diverse species (see section 2.1.1). In peas, this relationship was confirmed by Abdalla and Roberts (1968) and Dourado and Roberts (1984a). The present results are consistent with the previous findings. In addition, this relationship is also confirmed here in the wild type pea seeds (JI 181 and JI 1104).

Moreover, Abdalla and Roberts (1968), working on barley, broad beans and peas, concluded that the relationship between loss of seed viability and accumulation of aberrant cells during first mitosis were asymptotic, i.e. there was an initial rapid rise in aberrant cells with decrease in seed viability but that as viability decreased below about 50% further increases in the frequency of aberrant cells were hardly detectable. However, in this study, it was shown that in pea cultivars and wild types there was a further considerable increase in the frequency of aberrant cells below 50% viability. Villiers (1975), working on lettuce, Murata *et al*. (1979, 1982), working on barley and Dourado and Roberts (1984a), working on barley and peas support the present findings that the relationship is typically sigmoid. Since the percentage normal germination were

transformed to probit, this relationship is shown as linear in Figure 4.8.

Both in cultivated and wild type pea seeds, loss of viability and the induction of chromosomal aberrations occurred more rapidly in seeds of high moisture content than low moisture content. From the previous work on pea it was concluded that even small losses of seed viability were associated with some damage (Dourado and Roberts, 1984a). These results confirm this conclusion in the wild type pea seeds as well as in the seeds of pea cultivars. It is shown that small losses of seed viability, e.g. from 100% normal germination (control) to 97% in JI 181 and from 100% (control) to 95% in JI 1104, resulted in some chromosomal damage (i.e. from 0.875% to 3.593% in JI 181 and from 0.626% to 5.925% in JI 1104; see Appendices 4 and 5). In addition, these results also suggest that in wild type peas chromosomal damage is present even at 100% seed viability. However, previously, Abdalla and Roberts (1968) showed that in pea cv. Meteor there was no chromosome abnormalities at 100% seed viability.

Previously, Harrison (1966) found that lettuce seeds stored at 18°C and 6% moisture content had a much higher frequency of chromosomal aberrations for a given decline in viability compared with seeds stored at 10% moisture

content. Recently, Rao (1986) showed that in lettuce seeds more chromosomal aberrations were induced at low moisture content (5.5%) compared to a similar viability resulting from ageing at a high moisture content (13.0%). This is in contrast to the work presented here on peas. However, Abdalla and Roberts (1968), who investigated the effects of temperature and seed moisture content on the induction of chromosome damage in seeds of barley, broad beans and peas during storage, concluded that in each species a standard amount of chromosome damage accumulates for a given loss of viability under different storage conditions. The findings of Abdalla and Roberts are in close agreement with the results of this study which suggest that in cultivated and wild type pea seeds there is no difference between the effects of various storage conditions in terms of the accumulation of aberrant anaphase cells for a given loss of viability (Figure 4.8). However, in the present study, the frequency of aberrant anaphase cells observed for a given loss of viability was more than was found in the work of Abdalla and Roberts. They found that the frequency of aberrant cells, e.g. for ca. 50% viability was between 5% and 6% (estimated values from Figure 3 of Abdalla and Roberts, 1968) under different storage conditions. But it was found here that the frequency of aberrant cells for the above conditions varied between 15% and 16% (Appendices 2 - 5). Apart from the above differences, the

relative effect of different storage conditions on the frequency of chromosomal aberrations for a given loss of viability is similar in both studies carried out in pea seeds.

There is some debate on which type of chromosomal aberration is predominant in aged seeds. Although Abdalla and Roberts (1968) have reported the preponderance of chromosome-type aberrations over chromatid-types in aged seeds of barley, pea and broad bean, more recently, it has been concluded that aberrations in aged seeds were predominantly of the chromatid-type, e.g. in wheat (Innocenti and Avanzi, 1971), in barley (Murata *et al.*, 1982, 1984; Dourado and Roberts, 1984a) and in pea (Dourado and Roberts, 1984a). Recently, Rao *et al.* (1987b) and Rao and Roberts (1990) have shown that in aged lettuce seeds the frequency of chromatid-type aberrations was predominant at high moisture contents whilst the frequency of chromosome-type aberrations was predominant at low moisture contents.

It is confirmed here that chromatid-type aberrations are predominant in the first mitotic divisions of high and low moisture content aged pea seeds. The proportion of chromatid-type aberrations to the total frequency of aberrations was over 84% (with the exception of 72% in

the 11.5% m.c. seed lot of cv. Kelvedon Wonder) in each cultivated and wild type pea seed lot.

Certain workers believe that the visible chromosome damage observed in anaphase cells of radicle tips arises during germination following ageing treatment rather than during ageing itself (Corsi and Avanzi, 1969; Innocenti and Avanzi, 1971; Floris and Anguillesi, 1974; Murata *et al.*, 1982). However, according to the Exchange Theory of chromosome damage, the high proportion of chromatid-type aberrations generally observed does not mean that the primary damage occurs during germination. It is argued that the initial lesions actually occur during seed ageing and that the resulting breakages appear during seed hydration at germination when semi-conservative DNA replication occurs (Roberts, 1988). As the majority of aberrations observed in this study were chromatid-type aberrations, it would seem that damage to the DNA occurs as seeds age during storage. This damage is only manifest during seed germination after DNA replication and results in only chromatid-type aberrations in the first mitosis. However, if the aberrant cell goes through a further mitosis, the abnormality may become a chromosome-type aberration (Roberts, 1988). Therefore, in this study, any chromosome-type aberrations observed in any of the seed

lots probably represent second mitosis (see Appendices 2 - 5).

In summary, this study shows that in pea cultivars and wild types loss of seed viability is a function of storage temperature, seed moisture content and storage period; and there is also a negative relationship between loss of seed viability and accumulation of chromosomal aberrations, i.e. even small losses of viability result in some chromosomal damage. This study also suggests that under different storage conditions, the same frequencies of chromosomal aberrations accumulate at an identical viability level, in both high and low moisture content seeds of pea cultivars and wild types. However, it appears that under identical storage conditions, low moisture content wild type peas exhibit greater longevity than the two cultivars investigated. Therefore, it is concluded here that the current recommendation of IBPGR (1976) for long term genetic conservation, i.e. storage of orthodox seeds at 5 ± 1% moisture content and -18°C, is still valid. However, the Vertucci and Roos (1990) controversy with regard this recommendation (see section 2.2.2) is discussed further in Chapter 8.

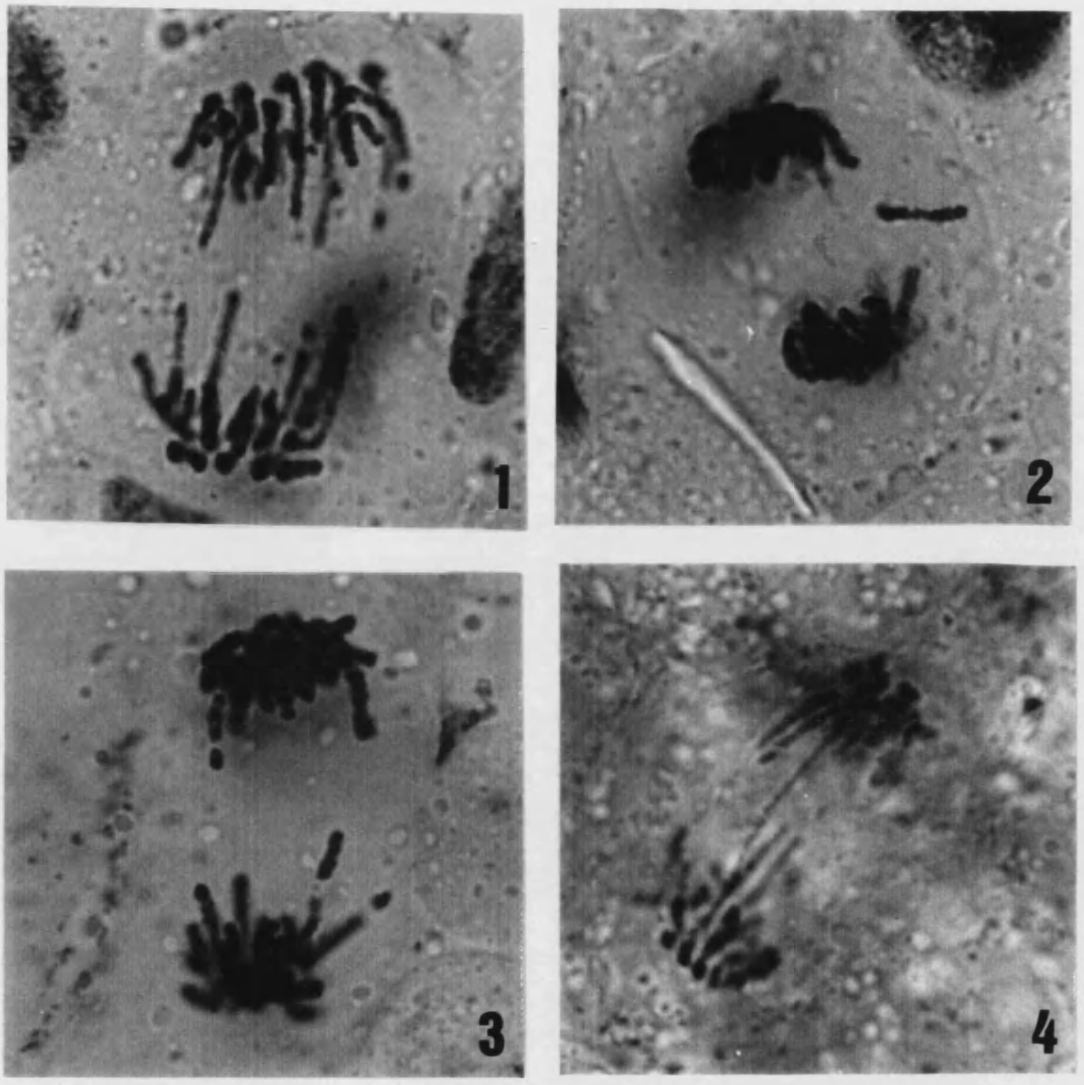
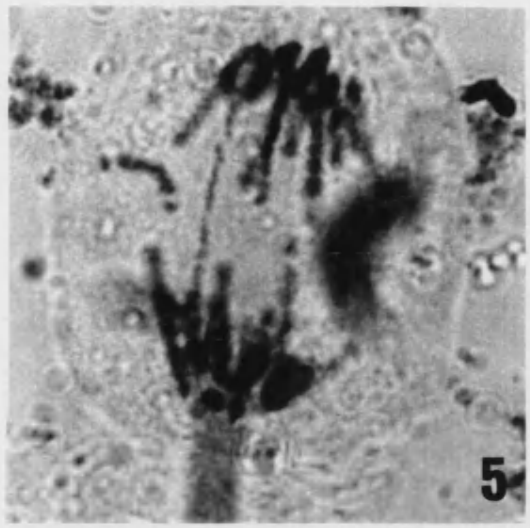


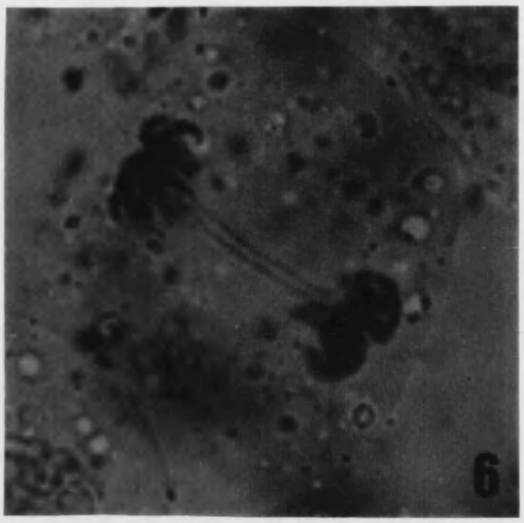
Plate 1. Chromosomal aberrations observed in the anaphase cells of the first mitotic divisions in radicle-tips of aged pea seeds (Magnification x 1850).

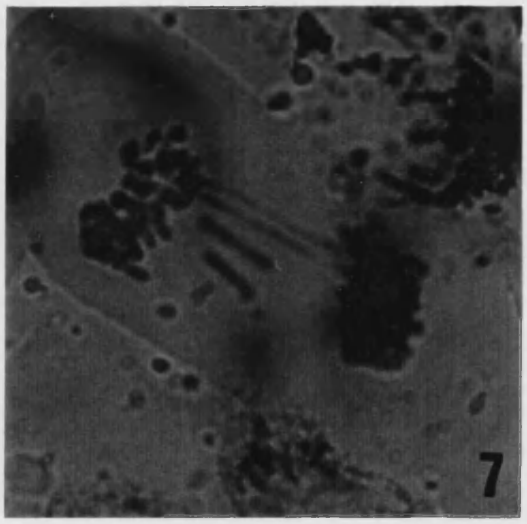
1. Normal anaphase

2-3. Single fragment

4. Single bridge







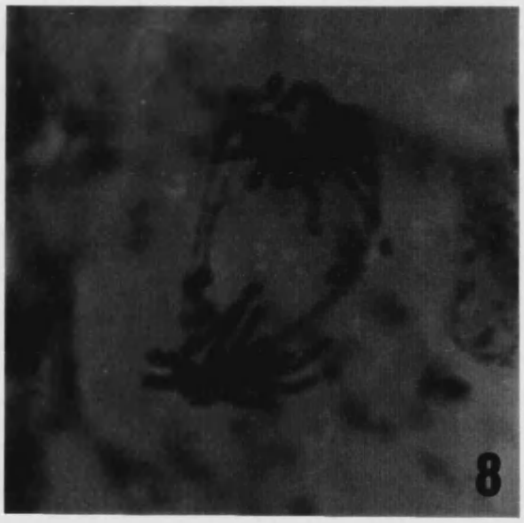
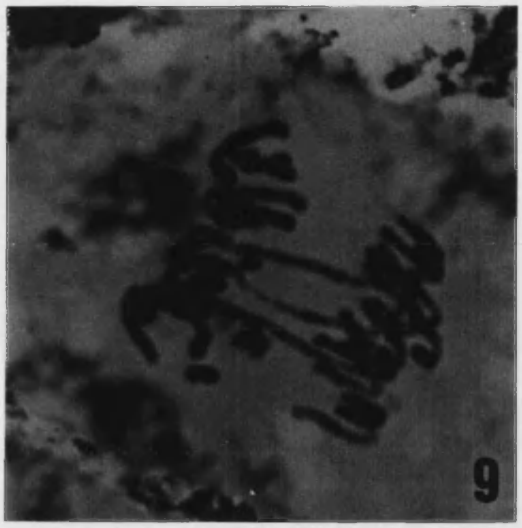
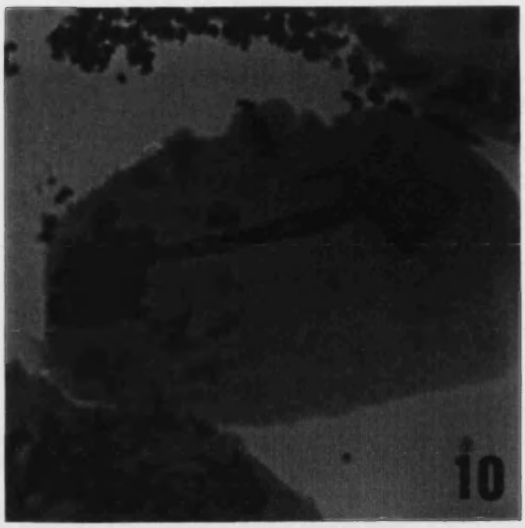
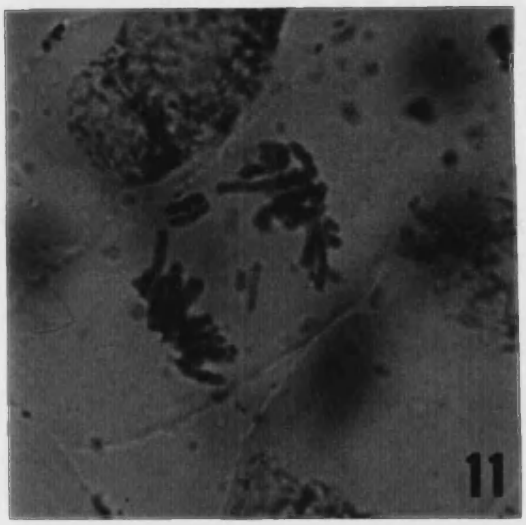


Plate 2. Chromosomal aberrations observed in the anaphase cells of the first mitotic divisions in radicle-tips of aged pea seeds (Magnification x 1850).

5. Single bridge with single fragment and two dot fragments
6. Two bridges
7. Two bridges with two unequal fragments
8. Multiple bridges







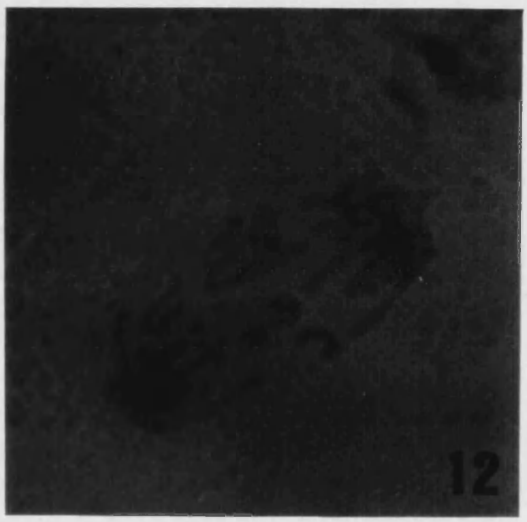


Plate 3. Chromosomal aberrations observed in the anaphase cells of the first mitotic divisions in radicle-tips of aged pea seeds (Magnification x 1850).

9. Multiple bridges with multiple fragments
10. Double bridge
11. Double fragments with two unequal fragments
12. Lagging chromosome with multiple fragments

CHAPTER 5. THE EFFECT OF SEED MOISTURE CONTENT AND VIABILITY ON THE SUSCEPTIBILITY OF PEA SEEDS TO SOAKING INJURY

5.1. Introduction

Previously, Kidd and West (1919), Klingmüller and Lane (1960), Pollock and Toole (1966), Larson (1968), Orphanos and Heydecker (1968), Harrison (1973), Rowland and Gusta (1977), Powell and Matthews (1977, 1978) and Ellis and Roberts (1982) concluded that rapid imbibition had a deleterious effect on leguminous seeds which had been dried to very low moisture contents. It is now well established that when very dry seeds, such as those stored for long term genetic conservation, are tested for germination, the rapid uptake of water which ensues on contact with water can lead to imbibition injury and decreased germination (Ellis *et al.*, 1982b, 1990b).

Seed physiologists recognize three main stages during imbibition (Ellis *et al.*, 1985; Gray, 1989):

* Stage 1, the initial uptake of water which is usually complete in 6-24 h depending on the species. All seeds, even dead ones, take up water rapidly during this stage.

* Stage 2, the initiation of nucleic acid and protein

synthesis in preparation for the emergence of the radicle. This stage may last two or three times as long as stage 1.

* Stage 3, a short stage characterized by the rapid uptake of water, generally less rapid than stage 1, cell expansion and the penetration of the radicle through the seed covering structures.

During the initial water uptake in dry pea seeds soaking injury could result from a lack of oxygen, the harmful effect of water on imbibing tissue, a rapid leakage into the surrounding medium of solutes such as sugars, organic acids, ions, amino acids and proteins or any combination of these (Larson, 1968; Bewley and Black, 1985). It is widely recognised that rapid early leakage during imbibition of dry pea embryos (with seed coats removed) result from the death of cells caused by the physical disruption of membranes. Consequently, imbibition injury results in reduced respiration and germination, a decline in the rate of food reserve transfer from the cotyledons to the growing axis, and a lower growth rate in the seedlings produce. However, intact pea seeds do not leak so copiously into the surrounding medium during imbibition compared to the dry pea embryos (Larson, 1968; Simon and Raja Harun, 1972; Powell and Matthews, 1978; Bewley and Black, 1985). Furthermore, the percentage

normal germination following rapid imbibition of intact pea seeds with a low initial seed moisture content (7.5%) was lower as compared to seeds with a moisture content of 13.5% or higher (Rowland and Gusta, 1977).

This study investigates the effect of soaking non-aged and aged pea seeds, each with high and low moisture contents, in water for different periods of time in order to determine:

* the effect of initial seed moisture content on loss of viability of intact seeds during the soaking treatment,
* the effect of initial seed viability on susceptibility to soaking injury,
* the joint effects of initial seed moisture content and initial seed viability on soaking injury.

5.2. Materials and Methods

Seeds of pea "cv. Douce Provence" purchased from Nutting Seed Company were used in this study. These seeds had an initial moisture content of 15.1%, and showed 100.0% total germination and 96.5% normal germination. Low moisture content seeds were obtained by drying the seeds over regularly regenerated silica gel in a partial vacuum at 20 °C for 6 months. Desiccation from 15.1% to 4.8% moisture content resulted in a slight decrease in viability (i.e. the dried seed lot showed 99.0% total

germination and 94% normal germination). Seed moisture contents were determined on ground samples by the High Constant Temperature Oven method (ISTA, 1985a,b). Low viability samples of high and low moisture content seed lots were obtained by ageing the seed for 4 days at 50°C and for 42 days at 65°C, respectively.

High and low viability seed samples of high and low moisture content seed lots were then subjected to soaking treatments, for varied periods of time, in order to determine the final viabilities and the effect of soaking on non-aged and aged pea seeds. The treatments were carried out by soaking 200 seeds in 200 ml of distilled water for 0 (control), 1, 2, 4, 8 and 12 h in low moisture content (4.8%) seeds, and in high moisture content (15.1%) seeds at 20°C.

Following these treatments, the seeds were surface dried between paper towels. They were weighed to four decimal places before and after the soaking treatments to determine the final moisture contents, using the equation in section 3.6.1.

All the seed lots were then tested for germination between moist rolled paper towels at 20°C according to the International Seed Testing Association Rules (ISTA, 1985a, b), except for a reduction in the number of seeds

tested from 400 to 200 and an increase in the test duration from 8 to 14 days. The hard seeds remaining in test after 8 days were counted and then scarified by rubbing the testa with sandpaper and the germination test extended by 6 days. Seedlings were evaluated and counted according to the criterion of normal germination (ISTA, 1985a,b). The results (seed moisture content vs. normal germination) were subjected to probit analysis using GLIM in a GENSTAT 5 Computer Programme.

5.3. Results

The proportions of abnormal seedlings and dead seeds were affected by soaking, in all treatment groups. Normal germination (i.e. proportion of seeds capable of producing morphologically normal seedlings) was much more sensitive than total germination (i.e. the proportion of seeds including morphologically normal and abnormal radicles) to soaking. These results are presented in Figures 5.1 - 5.4. Therefore, the base line of the soaking experiment is the percentage of normal germinating seedlings.

Figure 5.1 shows the effect of soaking on a high viability, high moisture content seed lot. Before the experiment normal germination of the seed lot was 96.5% and after 12 h soaking it was 94.0%. Although the loss

of viability was 2.5% after 12 h soaking, the difference was significant ($P<0.01$). However, the effect of soaking was more striking in the rest of the treatment groups (Figures 5.2-5.4).

In the lower viability, high moisture content seed lot (Figure 5.2), the percentage of normal germination was 79.5 before any soaking treatment. After 12 h soaking normal germination of the seed lot decreased to 3.0%. Therefore, the effect of soaking on seed viability was highly significant ($P<0.001$).

Figure 5.3 shows the effect of soaking on a high viability, low moisture content seed lot. Normal germination of the seed lot was 94.0% before the experiment. There was a sharp decrease in normal germination within the first hour of soaking. However, seed viability remained stable within the second hour and was then followed by another sharp decrease. After 12 h soaking normal germination of the seed lot decreased to 56.0%. The effect of soaking on decreasing seed viability was also significant ($P<0.001$) in this seed lot.

In addition, Figure 5.4 (which represents the effect of soaking on a lower viability, low moisture content seed lot) shows a very similar trend to Figure 5.3 with regard to loss of seed viability during soaking. Initial normal

germination of this seed lot was 73.5% and after 12 h soaking it was 16.5%. Statistical analysis of the data supports that the difference was highly significant ($P<0.001$).

Accumulated analysis of deviance confirmed that the effect of change in seed moisture content, the effect of initial seed viability were significant ($P<0.001$) in high moisture content seed lots (Figures 5.1 and 5.2) as well as low moisture content seed lots (Figures 5.3 and 5.4). Although the effect of the interaction between change in seed moisture content and initial seed viability was highly significant ($P<0.001$) in the high moisture content seed lots, it was significant at $P<0.05$ in the lower moisture content seed lots. This interaction in the latter seed lots occurred, most probably, due to their similar trends to imbibition injury. It is clear in high and low moisture content pea seeds that the lower the seed viability the more susceptible the seeds to imbibition injury.

No more than a 2.5% reduction in normal germination was observed in the 15.1% moisture content non-aged seeds following 12 hour soaking compared to the control (Figure 5.1). Nevertheless, the equivalent treatments decreased the normal germination from 94% (control) to 56% in 4.8% moisture content non-aged seeds (Figure 5.3). Although

the final moisture content (55.6%) of the high moisture content seed lot was higher than the lower moisture content lot (47.9%), the decrease in normal germination in the latter seed lot was approximately 15-fold compared to the former. According to the accumulated analysis of deviance of these two non-aged seed lots; the effect of change in seed moisture content, the effect of initial seed moisture content, and the interaction between change in seed moisture content and initial seed moisture content were significant ($P<0.001$). In other words, low moisture content seeds are more susceptible to soaking injury than high moisture content seeds. Also, the longer the time of soaking the greater the amount of injury.

In aged seed lots of high and low moisture contents (Figures 5.2 and 5.4), accumulated analysis of deviance showed that the effect of initial seed moisture content was significant ($P<0.01$). In addition, both change in seed moisture content and the interaction between change in seed moisture content and initial seed moisture content were significant ($P<0.001$). During soaking treatments of aged seed lots, imbibition injury occurred in lower seed moisture contents in the 4.8% m.c. seed lot compared with the 15.1% m.c. seed lot. Evidence for this is shown in Figures 5.2 and 5.4 that, e.g. seed moisture content reached for normal germination to fall to 50%, was ca. 33% in high moisture content aged seed lot

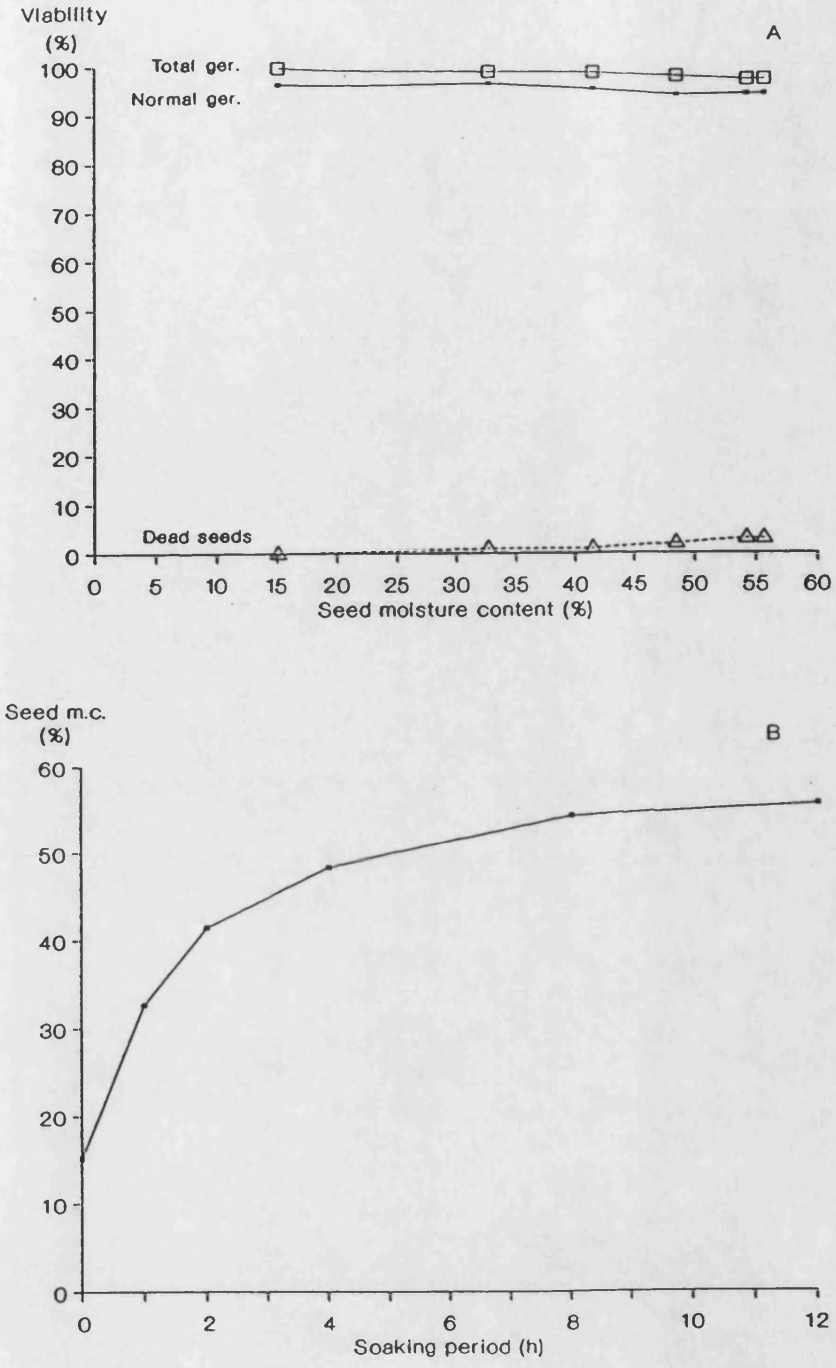


Figure 5.1. The relationship between (A) percentage viability and seed moisture content and (B) change in seed moisture content over time, in high viability (96.5%), high moisture content (15.1% m.c.) pea seeds after soaking in distilled water at 20°C.

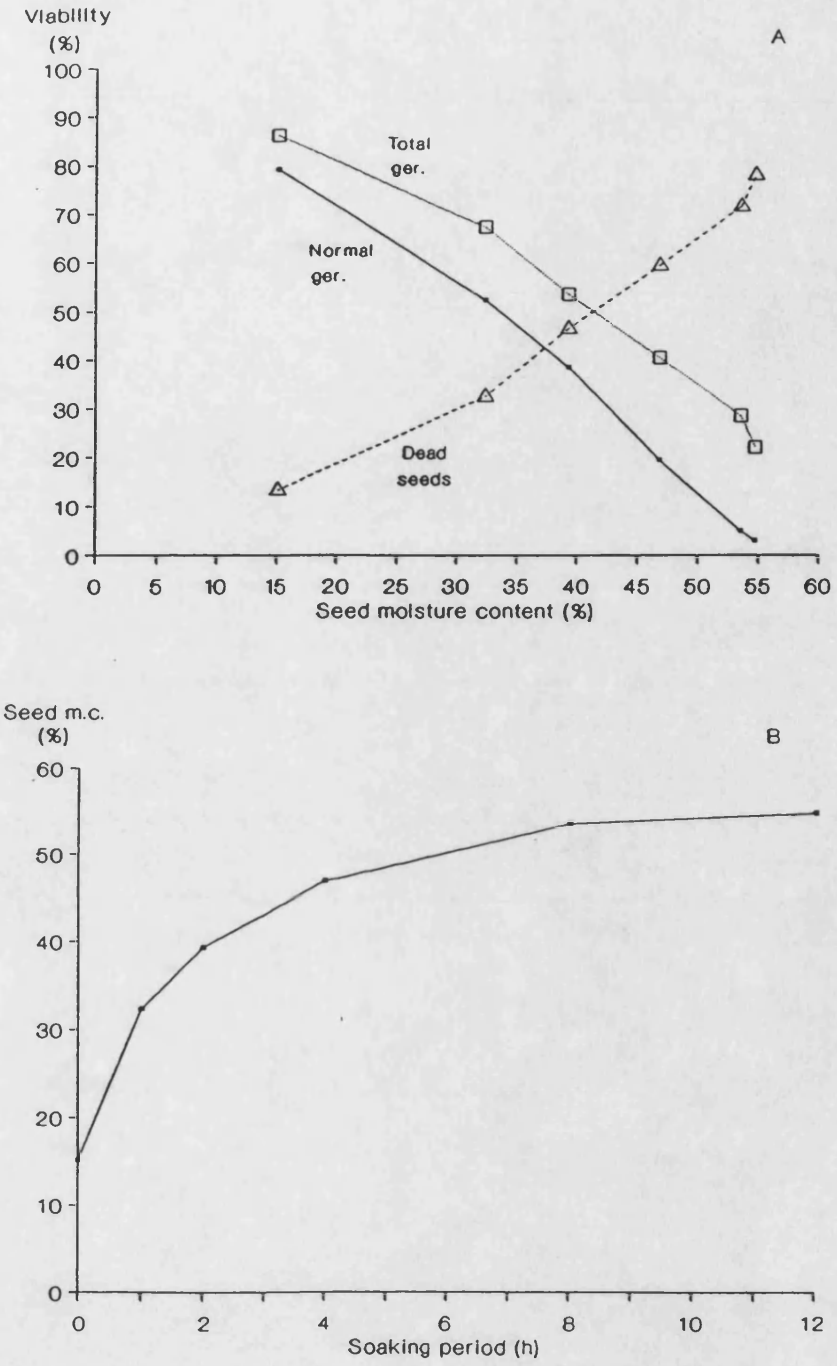


Figure 5.2. The relationship between (A) percentage viability and seed moisture content and (B) change in seed moisture content over time in seeds aged at 50°C/ 15.1% m.c. for 4 days (with a resultant viability of 79.5%) after soaking in distilled water at 20°C.

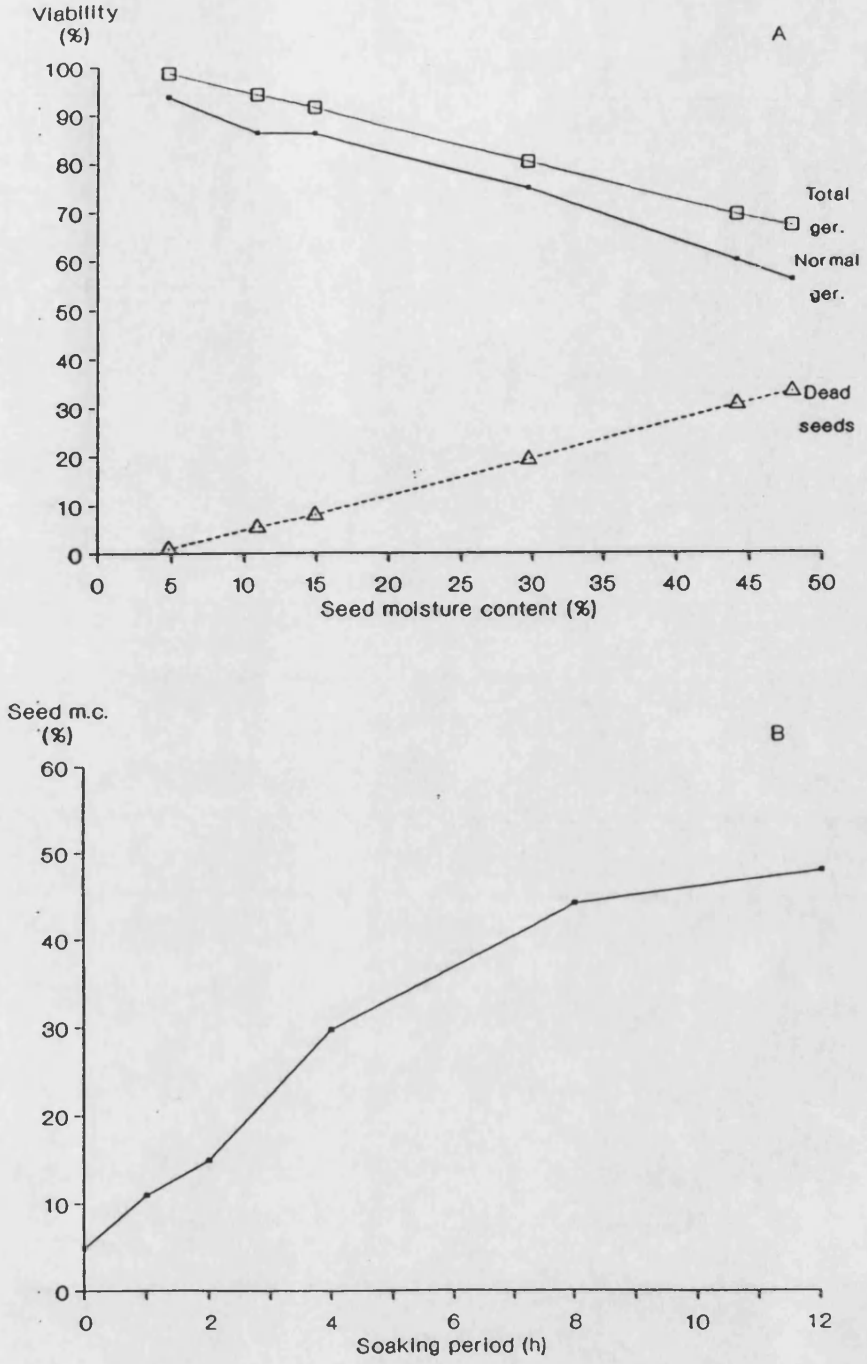


Figure 5.3. The relationship between (A) percentage viability and seed moisture content and (B) change in seed moisture content over time, in high viability (94.0%), low moisture content (4.8% m.c.) pea seeds after soaking in distilled water at 20°C.

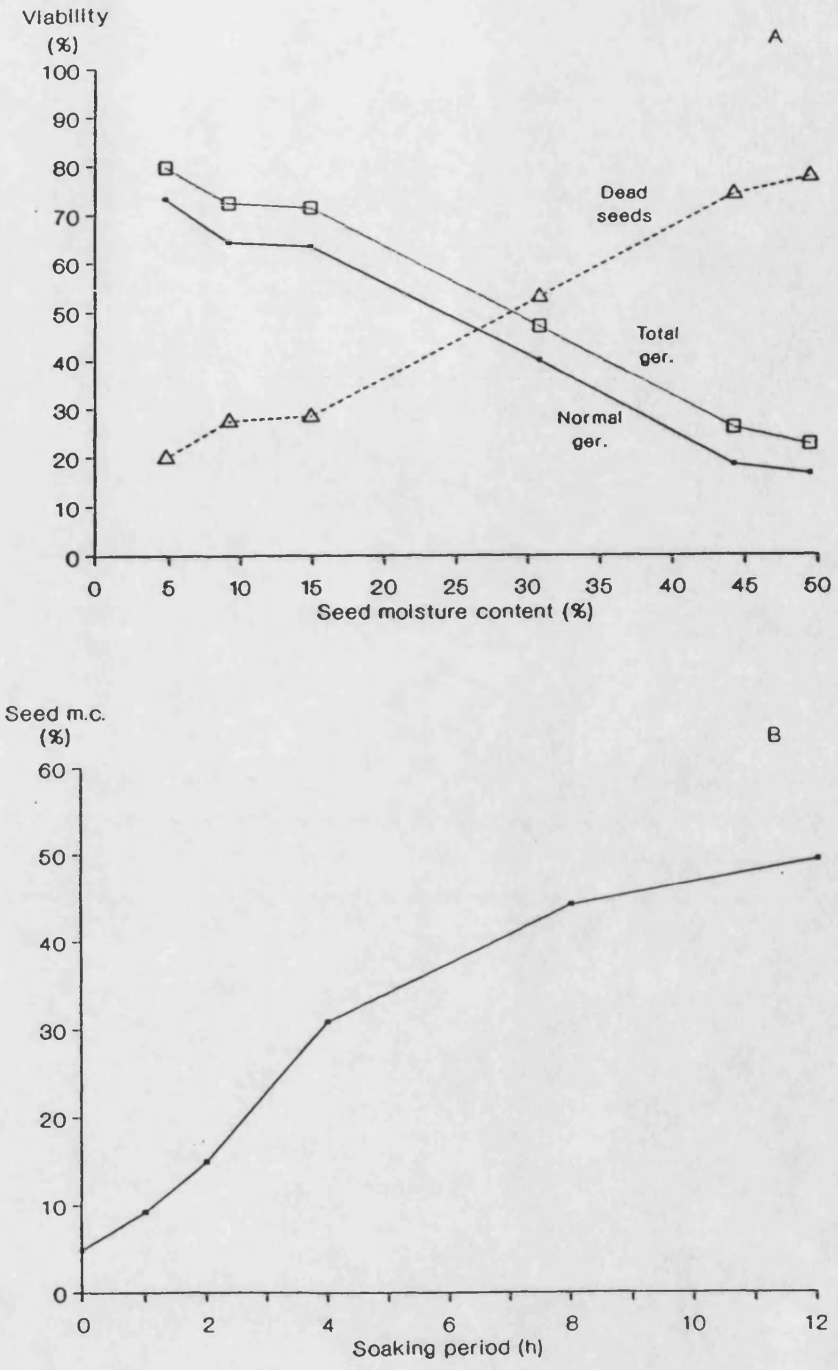


Figure 5.4. The relationship between (A) percentage viability and seed moisture content and (B) change in seed moisture content over time in seeds aged at 65°C/ 4.8% m.c. for 42 days (with a resultant viability of 73.5%) after soaking in distilled water at 20°C.

while it was ca. 24% in low moisture content aged seed lot. However, the time taken to reach the above mentioned seed moisture content was ca. 1 hour in the former and was ca. 2.5 hours in the latter seed lot.

Consequently, low moisture content aged seeds were more susceptible to imbibition injury than high moisture content aged seeds during the soaking treatment.

5.4. Discussion

Dry and especially very dry pea seeds can be susceptible to imbibition injury during standard germination test procedures (Ellis *et al.*, 1990b). However, there was only a slight reduction in the proportion of pea seeds germinating normally (i.e. from 96.5% to 94%) after desiccation from 15.1% to 4.8% seed moisture content, in this experiment. This contrasts with the reduction from 92% to 65% normal germination for pea seeds dried from 14.8% to 4.8% moisture content reported by Ellis *et al.* (1990b). The discrepancy may be due to the lower vigour of pea seeds used in the previous study. In other words, the K_i value [which is the seed lot constant in the improved viability equation proposed by Ellis and Roberts (1980a)] of pea seeds in the previous study was ca. 1.4 (which was equivalent to 92% initial normal germination). However, in the present study, the seeds had a K_i value

of ca. 1.9 with 96.5% initial normal germination. In an earlier study, Ellis and Roberts (1982) reported that desiccation from 16.8% initial seed moisture content to low moisture contents (at least down to 7%) was not deleterious to pea seed germination, which was initially 98%. Subsequently, Ellis *et al.* (1990b) showed that desiccation from 14.8% initial seed moisture content to 6.9% moisture content resulted with the decrease in normal germination from 92% to 79%. Thus, it appears that the initial viability of pea seeds is an important factor affecting imbibition injury during standard germination tests after desiccation to low moisture contents.

It is clear in the present results that when pea seeds are soaked in water, loss of viability which occurs as a result of rapid imbibition is inevitable, in low moisture content seeds. The percentage normal germination after the soaking treatments was impressively, and significantly, lower at 4.8% moisture content than 15.1% moisture content, in the case of non-aged seed lots (Figures 5.1 and 5.3). This is consistent with previous findings in peas (Kidd and West, 1919; Pollock and Toole, 1966; Larson, 1968; Harrison, 1973; Rowland and Gusta, 1977; Powell and Matthews, 1978; Ellis and Roberts, 1982 and Ellis *et al.*, 1990b).

Since, hitherto, the effect of initial viability of pea seeds on imbibition injury has not been observed, soaking of high and low moisture content aged seed lots were involved in this study. The present results provide clear evidence that in pea seeds there is a negative correlation between seed viability and susceptibility of seeds to imbibition injury, in high moisture content seeds (Figures 5.1 and 5.2) as well as low moisture content seeds (Figures 5.3 and 5.4). Furthermore, the effect of ageing on imbibition injury was more severe in low seed moisture content than in high seed moisture content, during the soaking treatment (Figures 5.2 and 5.4).

In order to prevent imbibition injury in laboratory conditions, pea seeds should be rehydrated following desiccation. This will be evaluated in Chapter 6.

CHAPTER 6. THE EFFECTS OF HUMIDIFICATION TREATMENTS ON VIABILITY AND THE ACCUMULATION OF CHROMOSOMAL ABERRATIONS IN HIGH AND LOW MOISTURE CONTENT PEA SEEDS

6.1. Introduction

It is now clear that when dry seeds are imbibed in water, the initial rapid uptake of water can result in imbibition injury (see section 2.5 and Chapter 5). Humidification is a pre-germination treatment, providing a slow initial water uptake to the seeds in a saturated atmosphere (100% R.H.), to avoid or minimise imbibition injury.

A number of researchers have reported the beneficial effects of this pre-germination technique in a range of species. Basu and Pal (1980) concluded that in 12 month-old rice seeds, the injury caused to the seed by rapid water uptake during imbibition could be avoided by slow hydration (pre-humidification) accomplished by seed moisture equilibration with a saturated atmosphere (100% R.H. at 25°C) for 24 - 72 hours. However, prolonged seed moisture equilibration at a high temperature should be avoided because of its ageing effect (Heydecker and Coolbear, 1977). The combined effects of time, temperature and moisture could result in loss of seed viability (Ellis *et al.*, 1990b). However, if the period

of humidification is insufficient to raise the seed moisture content to a sufficiently high level, imbibition injury can not be avoided following the humidification treatment (Ellis *et al.*, 1982b). Thus, Ellis *et al.* (1985) proposed that the moisture content of dry seeds should be increased slowly to between 16% and 18% by absorption of water vapour in a saturated atmosphere instead of rapid imbibition of liquid water at 20°C. Recently, Ellis *et al.* (1990b) recommend that the moisture content of dry and very dry pea seeds should be raised to between about 18% and 20% by humidification treatments at 20°C before beginning germination tests.

Furthermore, Rao *et al.* (1987b) showed that in lettuce seeds humidification increased the rate of seedling growth and decreased the number of morphological abnormalities which occur during seedling development. They also showed that a humidification treatment increased the percentage of normal germination and decreased the frequency of cells showing chromosomal aberrations in aged lettuce seeds during first mitotic divisions due to the repair of DNA damage induced during storage.

Experiments presented in this chapter were carried out to determine the optimum conditions of humidification for pea seeds. Therefore, the effects of humidification

treatments on viability and the accumulation of chromosomal aberrations were investigated in low moisture content pea seeds (cv. Kelvedon Wonder) in Experiment 1 as well as in the high moisture content ones in Experiment 2. In order to maximise the possible benefits of humidification, further experiments were carried out in terms of the optimum temperature (Experiment 3) and moisture content to be reached (Experiment 4) during the humidification period. Having established these, as a continuation of the soaking injury experiment (see Chapter 5), the effects of humidification treatments on avoiding or minimising imbibition damage and possibly on recovering any damage which resulted from ageing were examined in high and low moisture content seed lots of pea cv. "Douce Provence".

6.2. Materials and Methods

High and low moisture content seed lots of two cultivars (Kelvedon Wonder and Douce Provence) used in the following experiments were obtained from the storage treatments described in Chapter 4. Both non-aged and aged seed lots of these cultivars (Tables 6.1 and 6.3) were stored in hermetically sealed aluminium foil packets, in a fridge at 3°C until required for experimentation.

The seeds were weighed to four decimal places and placed in a petri-dish. Then, the petri-dish and seeds were placed on a metal gauze, above water, in a sealed desiccator to avoid any possibility of direct water contact. The seeds were humidified in this water saturated atmosphere (Tables 6.1 and 6.3) at varying temperatures (between 15°C and 20°C) for varying periods (between 3 and 7 days). After each humidification treatment, the seeds were re-weighed and the change in weight was determined so that the seed moisture content of the samples could be determined using the equation in section 3.6.1.

Germination tests of non-humidified and humidified seed lots were conducted by placing the seed between moist, rolled paper towels at 20°C as described in the ISTA Rules (ISTA, 1985a, b), except with a reduction in seed amount and an increase in the test durations of aged seeds and low moisture content seeds (see section 3.4).

The amount of improvement in seed viability after humidification was so small that it would not be found significant by a probit analysis, i.e. there was not enough evidence using this approach to prove that humidification was causing an improvement. However, in every case considered humidification caused an improvement in seed viability (Tables 6.1 and 6.3).

Therefore, these data were analysed using the SIGN Test (Siegel and Castellan, 1988). The combination of 6 experiments in cv. "Kelvedon Wonder" and 4 experiments in cv. "Douce Provence", i.e. total 10 pairs/conditions (non-humidified and humidified) were subjected to a one-sided test.

The cytological methods used were as described previously in section 3.7. Chromosomal aberrations observed within each cell were classified as chromatid or chromosome-types, mixed or others (see section 4.2 for details).

6.3. Results

6.3.1. The effects of temperature, moisture content and time on the response of pea seeds (cv. Kelvedon Wonder) to humidification treatments

(i) Experiment 1

As low moisture content seeds are more susceptible to soaking injury than high moisture content seeds (see Chapter 5), post-storage humidification was initially applied to 4.7% moisture content non-aged seed lots of pea cv. Kelvedon Wonder (Table 6.1). Holding the seeds in a saturated atmosphere at 20°C for 5 days (until the seed moisture content was raised to between 13.0 and 13.4%)

led to an increase in normal germination and decreased the percentage of abnormal seedlings although there was no alteration in total germination. In the non-aged (control) 4.7% m.c. treatment, no abnormal seedlings were recorded after humidification. Similarly, the frequency of anaphase cells which showed chromosomal aberrations at the first mitotic divisions decreased with an increase in seed viability in each seed lot. The reduction in the proportion of aberrant anaphase cells was 1.06% in the control, 5.61% in seeds aged for 39 days and 7.37% in seeds aged for 50 days at 65°C, 4.7% m.c. (Table 6.1).

In general, there was a clear reduction in all categories of chromosomal aberrations (particularly in chromatid-type aberrations) in both fresh and aged seed lots of 4.7% moisture content seeds after the humidification treatments (Figure 6.1). The frequency of chromatid-type aberrations, following the humidification treatments, decreased from 3.11, 9.07 and 11.04% to 1.60, 3.50 and 4.59% in 0 (control), 39 and 50 day aged seed lots, respectively. However, small differences in the distributions of chromosomal aberration types were noted, i.e. chromosome and mixed-type aberrations slightly increased in the humidified control seeds, and a small increase in mixed-type aberrations was observed after humidification in 39 day aged seeds. The most common aberration type was the chromatid-type.

Table 6.1. The influence of post-storage humidification of pea seeds (cv. Kelvedon Wonder) on viability and the frequency of aberrant anaphase cells at the first mitotic divisions.

Storage conditions			Humidification		Subsequent			Aberrant
Initial moist.cont. (%, f.wt)	Temp. (°C)	Period (days)	Temp. (°C)	Period (days)	moisture content (%, f.wt)	Normal germination (%, mean ± s.e)	Abnormal germination (%, mean ± s.e)	anaphase cells (%)
4.7	65	0	-	-	(4.7)	96.0 (± 1.69)	2.0 (± 0.66)	3.11
			20	5	13.4	98.0 (± 2.00)	0.0 (± 0.00)	2.05
		39	-	-	(4.7)	82.0 (± 2.62)	7.0 (± 1.81)	10.00
			20	5	13.2	85.5 (± 1.50)	3.5 (± 1.59)	4.39
		50	-	-	(4.7)	67.0 (± 3.91)	5.0 (± 1.50)	12.68
			20	5	13.0	72.0 (± 6.93)	3.0 (± 1.63)	5.31
11.5	50	0	-	-	(11.5)	95.5 (± 0.91)	4.0 (± 1.07)	4.33
			20	5	18.1	97.5 (± 0.66)	2.0 (± 0.50)	2.08
		18	-	-	(11.5)	78.0 (± 3.02)	14.0 (± 2.27)	10.48
			20	5	17.3	84.0	9.0	9.10
			18	5	17.0	87.0	7.0	6.37
			16	5	16.6	87.0 (± 3.09)	5.0 (± 1.50)	5.98
		24	-	-	(11.5)	51.0 (± 3.36)	17.5 (± 3.20)	15.94
			20	3	16.3	39.5	32.0	*
			16	5	16.3	57.5 (± 5.04)	15.0 (± 2.36)	12.15
			15	7	16.6	39.0	25.0	*

- Non-humidified

* Not examined

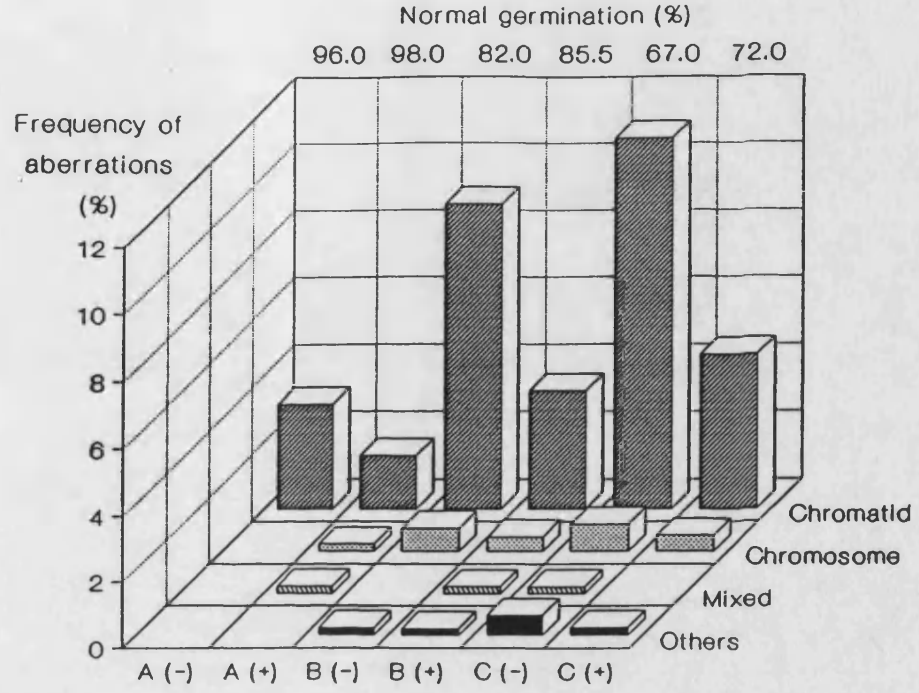


Figure 6.1. A comparison of the frequencies of the major types of chromosomal aberrations for pea seeds (cv. Kelvedon Wonder) non-humidified (-) or humidified in a saturated atmosphere at 20°C (+) prior to the germination test following ageing at 65°C and 4.7 percent moisture content for 0d/Control (A), 39d (B) or 50d (C).

(ii) Experiment 2

It was shown in Chapter 5 that even high moisture content pea seeds (especially aged seeds) are susceptible to soaking injury. Therefore, another attempt has been made to examine the response of 11.5% moisture content non-aged and aged lots of pea cv."Kelvedon Wonder" to humidification treatments.

Initially, non-aged (control) seeds of the 11.5% moisture content sample were humidified for 5 days at 20°C, as this treatment worked well in the low moisture content (4.7%) seed lots. After humidification, when the seed moisture content was raised to 18.1%, the percentage of normal germination slightly increased and, in addition, the percentage of abnormal seedlings and the frequency of aberrant anaphase cells at the first mitotic divisions decreased (Table 6.1).

However, humidification at 20°C in a saturated atmosphere did not work in the 24 day aged samples of the 11.5% moisture content seed lot. When the seed moisture content was raised to 16.3%, after 3 day humidification, the percentage of normal germination decreased (from 51.0 to 39.5) and abnormal germination increased (from 17.5 to 32.0) contrary to the previous findings. Thus, it was decided to lower the temperature in order to slow down

the initial rate of water uptake of the seeds. Unfortunately, humidification at 15°C for 7 days, i.e. until a similar seed moisture content (16.6%) was reached, gave similar results with humidification at 20°C in terms of a decrease in normal germination (Table 6.1). Eventually, the same seed lot (50°C / 11.5% m.c. - 24 day aged) was subjected to humidification at 16°C for 5 days. This final treatment gave promising results, viz. increasing the seed moisture content, improving the percentage of normally germinating seedlings, decreasing the percentage of abnormal germination and the proportion of chromosomal aberrations occurring during first mitosis (Table 6.1 and Figure 6.2). Therefore, it is thought that in severely aged pea seeds (i.e. about 50% viability) humidification at 16°C (which is a critical temperature just above the chilling injury zone for pea seeds) is suitable in terms of increasing normal germination while decreasing the frequency of chromosomal aberrations. It appears that in this seed lot humidification at either 20°C or 15°C was deleterious.

(iii) Experiment 3

In a separate experiment (with 50°C / 11.5% m.c. - 18 day aged seed lot), seeds were subjected to humidification treatments for 5 days at 16, 18 and 20°C and seed moisture content of each sample raised to 16.6, 17.0

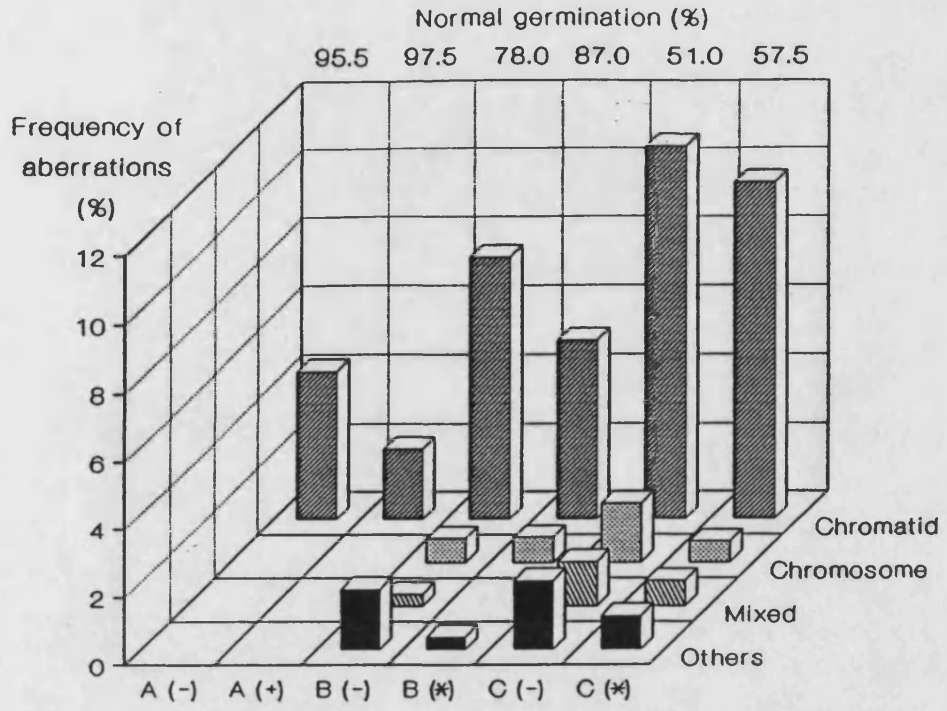


Figure 6.2. A comparison of the frequencies of the major types of chromosomal aberrations for pea seeds (cv. Kelvedon Wonder) non-humidified (-) or humidified in saturated atmospheres at 20°C (+) and 16°C (*) prior to the germination test following ageing at 50°C and 11.5 percent moisture content for 0d/Control (A), 18d (B) or 24d (C).

and 17.3%, respectively (Table 6.1). This experiment was carried out to determine the optimum temperature during humidification. Following the humidification treatments under the conditions mentioned above in non-aged and aged seed lots of 11.5% moisture content seed, the percentage of normal germination increased between 6.0% and 9.0% and the reduction in the frequency of aberrant anaphase cells which showed chromosomal aberrations was between 1.38% and 4.5% (Table 6.1). Furthermore, all the categories of chromosomal aberrations decreased after humidification treatments and the greatest relative reduction was observed in the category "others" (Figure 6.3). These results suggest that humidification at any temperature was beneficial to pea seeds of 78.0% viability. However, humidification at 16°C and 18°C gave better results than 20°C with regard to an increase in normal germination and a decrease in the proportion of chromosomal aberrations. As the water uptake of the seeds is slower at 16°C than 18°C, it was concluded that 16°C is the optimum temperature for humidification of aged pea seeds although either temperature improved the percentage normal germination and decreased the percentage abnormal germination and the frequency of aberrant anaphase cells to an identical level (Table 6.1 and Figure 6.3).

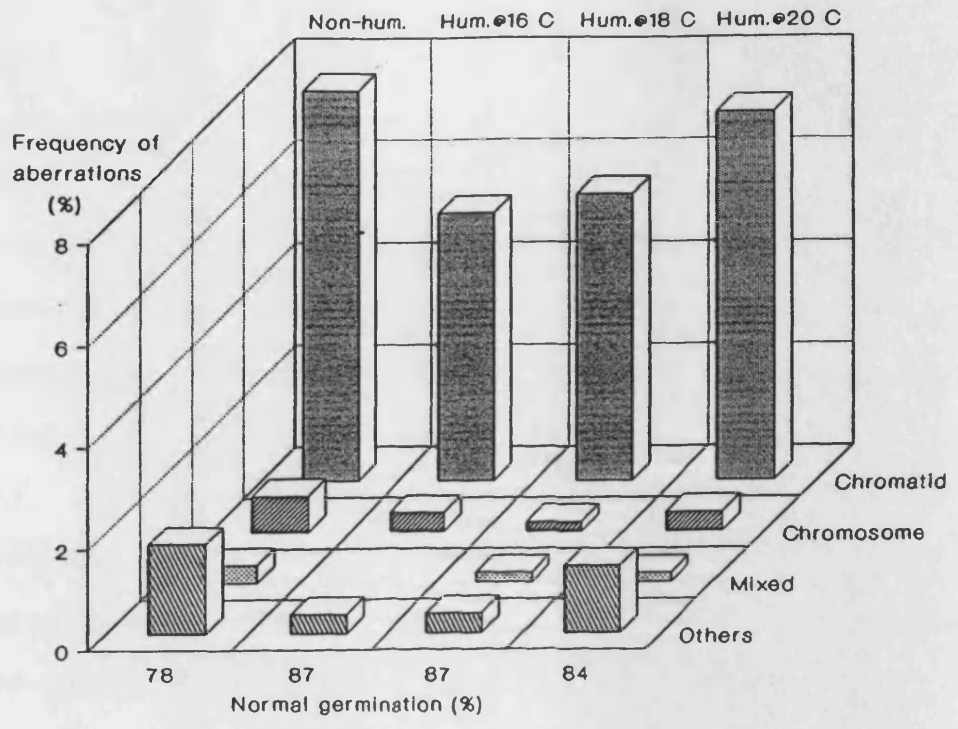


Figure 6.3. The influence of humidification at different temperatures on seed viability and the frequencies of the major types of chromosomal aberrations in a 50°C / 11.5% m.c. - 18d aged seed lot (resultant viability = 78.0%) of pea cv. "Kelvedon Wonder".

(iv) Experiment 4

In order to determine the optimum moisture content to be reached, a seed lot of 50°C / 11.5% moisture content stored for 18 days was held in a saturated atmosphere at 16°C for varying periods, to increase the seed moisture content from 11.5% to between 14.1% and 21.6%. The percentages of normal and abnormal germination were analysed following five humidification treatments compared with non-humidified (control) seeds (Table 6.2). As can be seen, increasing the seed moisture content between 14.1% and 20.1% results in an increase in normal germination and a decrease in abnormal germination. However, within the limits of 16.3% and 18.1%, normal germination increased to a maximum and abnormal germination decreased to a minimum level. Therefore, it was concluded that the optimum seed moisture content to be reached is between 16.3% and 18.1%.

The overall results in this section confirmed that in humidification treatments a decrease in the frequency of chromosomal aberrations and an increase in the viability of non-aged and aged pea seeds (cv. Kelvedon Wonder) of high and low moisture contents was observed (Figure 6.4).

Table 6.2. The relationship between normal and abnormal germination in seeds stored at 50°C / 11.5% m.c. for 18 days and then equilibrated to a range of seed moisture contents.

Humidification Temp. (°C)	Period (days)	Final seed m.c. (%, f.wt)	Normal germination (%,mean ± s.e)	Abnormal germination (%,mean ± s.e)
-	0	11.5	78.0 (± 3.02)	14.0 (± 2.27)
16	2	14.1	83.5 (± 2.56)	9.0 (± 1.65)
	4	16.3	85.0 (± 3.09)	6.5 (± 1.50)
	7	18.1	85.5 (± 1.68)	7.5 (± 1.59)
	9	20.1	85.0 (± 1.25)	9.5 (± 1.50)
	16	21.6	70.5 (± 4.34)	16.0 (± 4.66)

- Non-humidified (Control)

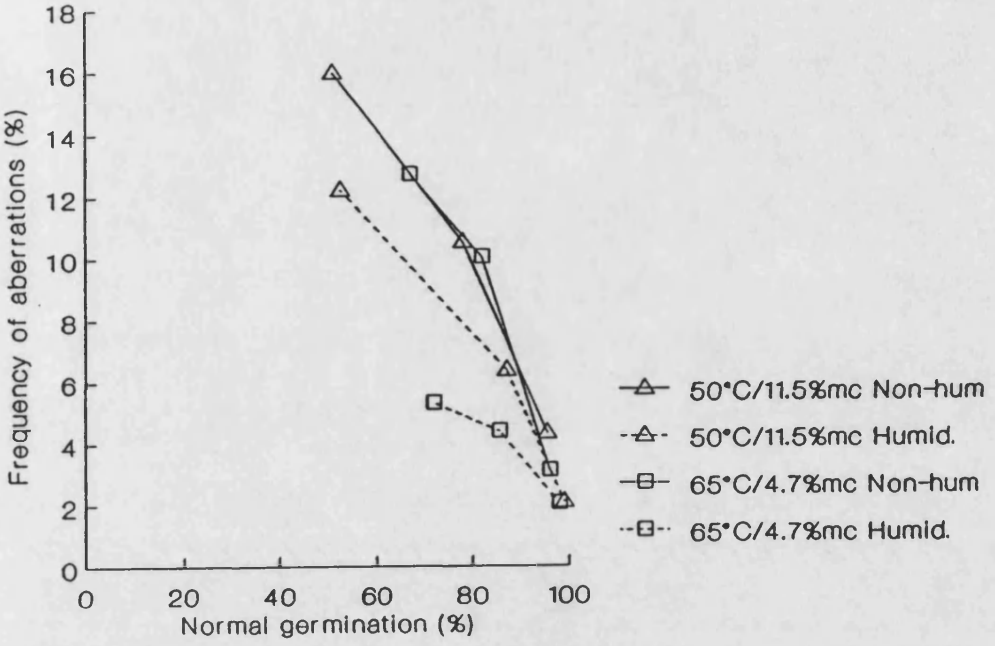


Figure 6.4. The effect of humidification treatments on the viability of high and low moisture content pea seeds (cv. Kelvedon Wonder) and the frequency of aberrant anaphase cells occurring during first mitotic divisions.

6.3.2. The effects of humidification treatments on the viability and the accumulation of chromosomal aberrations in aged pea seeds (cv. Douce Provence)

Previously, in Chapter 5, it was shown that in pea seeds (cv. Douce Provence) there is a negative correlation between seed viability and susceptibility of seeds to imbibition injury, in high moisture content seeds (15.1%) as well as low moisture content seeds (4.8%). In this particular section, humidification treatments were applied to the same seed lots used in Chapter 5 in order to examine the effects of this pre-germination treatment. As the optimum seed moisture content to be reached in pea seeds during any humidification treatment had been determined in section 6.3.1, high and low moisture content non-aged and aged seed lots were humidified until their moisture contents reached between 16.3% and 18.1%. All the seed lots were held at 16°C in a water saturated atmosphere (Table 6.3).

The results obtained from the humidification treatments, in pea seeds cv. "Douce Provence", also confirmed that imbibition injury can be removed by using this technique prior to a standard germination test. As in cv. "Kelvedon Wonder", humidification treatments led to an increase in percentage normal germination and decreased the percentage abnormal germination. In particular, the

Table 6.3. The influence of post-storage humidification of pea seeds (cv.Douce Provence) on viability and the frequency of aberrant anaphase cells at the first mitotic divisions.

Storage conditions			Humidification		Subsequent			Aberrant
Initial moist.cont. (%, f.wt)	Temp. (°C)	Period (days)	Temp. (°C)	Period (days)	moisture content (%, f.wt)	Normal germination (%, mean ± s.e)	Abnormal germination (%, mean ± s.e)	anaphase cells (%)
4.8	65	0	-	-	(4.8)	94.0 (± 1.85)	5.0 (± 1.96)	3.90
			16	6	16.5	96.5 (± 0.91)	2.5 (± 0.73)	2.05
		42	-	-	(4.8)	73.5 (± 2.72)	6.5 (± 1.99)	10.33
			16	7	16.4	86.0 (± 3.02)	1.0 (± 0.66)	6.43
15.1	50	0	-	-	(15.1)	96.5 (± 0.91)	3.5 (± 0.91)	3.32
			16	3	17.7	99.5 (± 0.50)	0.5 (± 0.50)	1.77
		4	-	-	(15.1)	79.5 (± 3.16)	7.0 (± 1.46)	8.53
			16	4	18.1	85.0 (± 1.96)	4.5 (± 1.40)	5.84

- Non-humidified

maximum benefit of humidification was observed in the 65°C / 4.8% m.c. - 42 day aged seed lot (resultant viability = 73.5%).

Furthermore, humidification treatments decreased the frequency of anaphase cells which showed chromosomal aberrations by between 1.85% and 3.90% in the low moisture content seeds and between 1.55% and 2.69% in the high moisture content seed lots (Table 6.3). Each type of chromosomal aberrations was also decreased in all seed lots after humidification treatments (Figure 6.5). Similar to the findings in section 6.3.1, the greatest relative reduction was observed in the category "others".

The data obtained from high and low seed moisture contents were separately pooled (each into non-humidified and humidified groups) to illustrate the overall frequency of the different types of aberration (Appendices 13 and 14). It is clear from the pooled results that chromatid-type aberrations were the most common at each moisture content level, in both non-humidified and humidified seed groups. The frequency of each aberration type decreased with humidification. In particular, the greatest reduction was noted in chromatid-type aberrations (Figure 6.5). However, no chromosome-type aberration was observed after humidification. Furthermore, the frequency of "others"

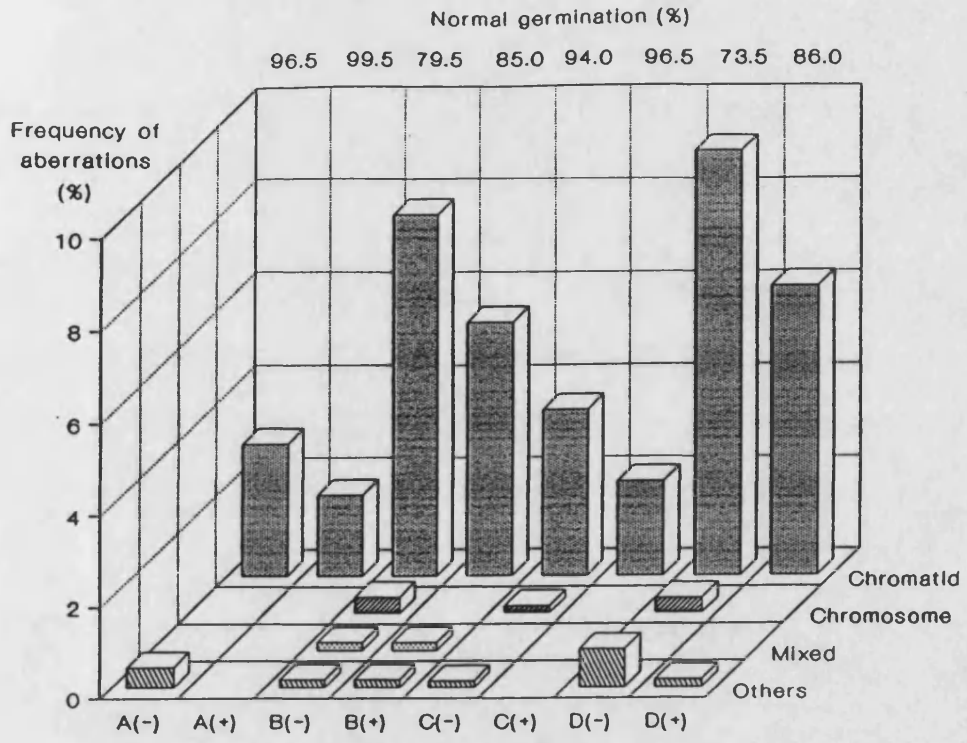


Figure 6.5. A comparison of the frequencies of the major types of chromosomal aberrations for pea seeds (cv. Douce Provence) non-humidified (-) or humidified in a saturated atmosphere at 16°C (+) prior to the germination test following ageing at 50°C and 15.1 percent moisture content for 0d/Control (A), 4d (B); and at 65°C and 4.8 percent moisture content for 0d/Control (C) and 42d (D).

(the second most common aberration type following chromatid-type aberrations) showed a marked decrease.

6.4. Discussion

Humidification treatments have been recommended to slowly increase the seed moisture content by absorption of water vapour instead of rapid imbibition of liquid water. The aim has been to avoid imbibition injury which otherwise might occur when seeds are set to germinate (Ellis *et al.*, 1985). As humidification also raises moisture contents above the critical values for repair in oily seeds (e.g. lettuce), it provides an increased opportunity before germination, for the repair of the ageing-induced sub-cellular damage which is an inevitable consequence of storage (Rao *et al.*, 1987b). However, the critical moisture content has as yet not been determined for pea seeds. Since peas are non-oily seeds, Roberts (personal communication, 1989) suggests that the critical moisture content might be in the region of 30-40%. It is clear from the present results that increasing the seed moisture content even up to 30% would take weeks (e.g. it took 16 days to increase the seed moisture content from 11.5% to 21.6%; see Table 6.2). As prolonged humidification treatments have damaging effects to seeds rather than beneficial effects (Heydecker and Coolbear, 1977), it is probably impossible for non-oily seeds (like

peas) to facilitate repair of ageing-induced DNA damage by the use of humidification treatments. However, the frequency of anaphase cells which showed chromosomal aberrations at the first mitotic divisions decreased in all the seed lots (i.e. high and low moisture content fresh and aged seed lots) of pea cultivars "Kelvedon Wonder" and "Douce Provence" (Table 6.1 and 6.3) although the seed moisture contents, reached in the humidification treatments, do not seem to be sufficient to induce enzyme activity and possible repair and turnover. Also, there was generally a considerable decrease in each type of the chromosomal aberrations after humidification treatments (Figures 6.1, 6.2 and 6.5). Furthermore, the greatest reduction was observed in the chromatid-type aberrations.

In the present study, the moisture content of the pea seeds after any humidification treatment was determined on a wet weight basis, and this was the overall moisture content of a particular seed lot (i.e. the moisture content of each seed within the seed lot may well have been different). In addition, there is a possibility that within each seed the embryo was in fact at a different moisture content to the cotyledons, i.e. from the hypothesis that since rapid initial water uptake during imbibition causes a sharp increase in moisture content of pea embryos relative to cotyledons (Powell and Matthews,

1978), the moisture content of the embryo is expected to be higher after humidification treatment. This suggests that the overall embryo moisture content of a particular seed lot might have been at a higher moisture content which could have facilitated repair. Therefore, this might have been the cause of a decrease in the frequency of chromosomal aberrations in pea seeds after humidification treatments. However, without any further investigation of the current hypothesis, it remains as a speculation.

It is evident from the results shown in Tables 6.1 and 6.3 that the humidification treatments which were carried out in the pea cultivars "Kelvedon Wonder" and "Douce Provence" avoided imbibition injury and improved seed viability in every case considered ($P<0.001$). Two separate but connected factors may be concerned with the humidification of seeds: a) temperature and b) final moisture content to be reached over a period of time. Therefore, it is concluded here that the nearer the temperature is to just above the limit of chilling injury, the slower the initial water uptake from the saturated atmosphere and the more benefit is gained from the humidification treatment. The results of this study suggest that the maximum benefit of humidification can be obtained when non-aged and aged pea seeds are humidified at 16°C. In addition, the optimum seed

moisture content to be reached in a humidification treatment was found to be between 16.3% and 18.1%. These conclusions are acceptable in both high and low moisture content seed lots of cv. "Kelvedon Wonder" (11.5% and 4.7%, respectively) and cv. "Douce Provence" (15.1% and 4.8%, respectively). This beneficial effect of a humidification treatment to dry seeds is compatible with the findings of Ellis *et al.* (1985). However, they suggested humidification at 20°C. In the present study it is concluded that humidification at 16°C gives better results in aged pea seeds, and 20°C should only be used for fresh (non-aged) seeds. It is also concluded here that as aged seeds were more susceptible to damage caused by rapid water uptake (see Chapter 5), they require a slower rate of initial water uptake than non-aged seeds in humidification treatments.

In summary, this study supports the previous suggestions that the injury caused to the seed by rapid water uptake during soaking or standard germination tests could be avoided by pre-humidification treatments (Basu and Pal, 1980; Ellis *et al.*, 1982b, 1985, 1990b; Rao *et al.*, 1987b).

CHAPTER 7. THE EFFECT OF PRIMING TREATMENTS ON THE VIABILITY AND ACCUMULATION OF CHROMOSOMAL DAMAGE IN AGED PEA SEEDS

7.1. Introduction

The technique of seed priming, in which seeds are held before germination in an osmoticum, which is high enough to permit almost complete imbibition but just enough to prevent germination, for a prolonged period (Heydecker *et al.*, 1975), has been carried out in aged seeds by a number of researchers (Brocklehurst and Dearman, 1983; Dell'Aquila *et al.*, 1984; Coolbear *et al.*, 1984; Dell'Aquila and Taranto, 1986; Rao *et al.*, 1987b; Georghiou *et al.*, 1987; Thanos *et al.*, 1989; Dell'Aquila and Tritto, 1990, 1991). It was shown in section 2.7.2 that the correct application of post-storage priming treatments induced a more rapid and uniform germination and results in a decrease of mean germination time when seeds are transferred into germination conditions. Mean germination time may be considered an appropriate manifestation of seed vigour, its variation being closely associated with changes of protein and DNA synthesis in deteriorated and primed and surface-dried seeds (Dell'Aquila, 1987).

Considerable attention has been given to the possibility

that the effects of ageing and genetic deterioration could be reversed or ameliorated if seeds were held close to full imbibition. For example, recently Rao *et al.*, (1987b) concluded that post-storage priming treatments in lettuce seeds reduced the frequency of chromosomal aberrations, increased the rate of root growth, and decreased the frequency of morphologically abnormal seedlings. Furthermore, Khan *et al.*, (1978) showed that priming of lettuce seeds increased the activity of such enzymes as acid phosphatase and esterase. They also showed that priming reduced the time of imbibition required for the onset of RNA and protein synthesis and polyribosome formation and increased the total amount of RNA and protein synthesized in lettuce seeds. Therefore, Rao (1986) proposed that extention of the lag period between imbibition and DNA replication in aged seeds should permit more complete repair of DNA lesions induced during storage. Since priming provides seeds a prolonged pregermination period, the incidence of chromosomal aberrations at first mitosis is reduced due to the stimulation of DNA repair mechanisms.

The experiments presented in this chapter were carried out to investigate the possible effect of post-storage priming treatments on viability and ageing induced chromosomal aberrations in pea seeds possibly due to repair of the nuclear damage. Previously, priming of pea

seeds was attempted by Khan *et al.* (1978). Over 8 days of priming, they used 25, 35 and 50% (-0.85, -1.56 and -3.0 MPa, respectively) PEG at 15°C. They concluded that priming with PEG not only shortened the time of germination but invigorated the seeds as evidenced by increased percentage germination and faster rate of root and shoot growth. Apart from this work, there is scant information on priming methods and optimum conditions for successful priming in pea seeds. Preliminary experiments were conducted in order to establish the optimum conditions (i.e. temperature, concentration of PEG-8000 solution and period of the treatment) and a convenient procedure for priming pea seeds.

Furthermore, exogenous ABA can arrest the progress of germination prior to radicle protrusion, e.g. in mustard (Schopfer *et al.*, 1979), in celery (Biddington *et al.*, 1982), in tomato (Liptay and Schopfer, 1983; Finch-Savage and McQuistan, 1991) and in carrot (Finch-Savage and McQuistan, 1989). Therefore, it might be possible to prime aged pea seeds in ABA in order to extend the lag period between imbibition and germination (DNA replication) and to permit an extended period for repair of the subcellular damage.

In the main priming experiment, three different concentrations of PEG-8000, one concentration of ABA and

also distilled water were used for different periods to compare the effect of different osmotica on the viability and accumulation of chromosomal damage in aged pea seeds. The effect of priming on mean germination time was also examined (in addition to final viability and frequency of chromosomal aberrations) in pea seeds after storage.

7.2. Materials and Methods

Preliminary experiments were carried out to determine the effects of different temperatures and concentrations of PEG-8000 for different periods on the viability of aged pea seeds.

In the first preliminary experiment, PEG-8000 solution of -0.5 MPa was applied (as described in section 3.6.3) to the 11.5% moisture content seeds of pea cv. "Kelvedon Wonder", aged for 18 days at 50°C. Priming of the seeds was conducted at 15°C and 25°C for 0 (non-primed / control) , 7 and 14 days (Table 7.1).

The seed lot used in the second preliminary experiment was the same as in the soaking injury experiment (i.e. 15.1% moisture content seeds of pea cv. "Douce Provence", aged for 4 days at 50°C). Priming was carried out with the use of PEG-8000 solution of -1.0, -1.2, -1.4 and -1.6 MPa for 0 (non-primed/control), 7 and 14 days at

Table 7.1. The effect of different temperatures and osmotica on the final moisture content of pea seeds (cv. Kelvedon Wonder and Douce Provence).

Cultivars	Priming temp. (°C)	Conc. of PEG-8000 solution (g/kgH_2O)	Osm.Pot. (MPa)	Initial seed m.c.(%)	Final seed m.c. after 7d (%)	Final seed m.c. after 14d (%)
Kelvedon	15	184	-0.5	11.5	55.4	60.9
Wonder	25	202	-0.5	11.5	56.6	56.7
Douce	15	273	-1.0	15.1	41.2	42.6
Provence	15	303	-1.2	15.1	38.7	39.2
	15	329	-1.4	15.1	36.6	35.7
	15	354	-1.6	15.1	34.8	35.2

15°C (Table 7.1).

The methods of priming in both preliminary experiments were similar except with the use of the paper towels immersed in PEG-8000 solutions. The seeds were spread on two moist towels, covered with another towel and then rolled and arranged in plastic boxes (dimensions : 28 x 16 x 9 cm) in the first experiment. However, in the second experiment two moist towels were placed at the bottom of the plastic box and pea seeds were spread on them; another moist towel was then placed on top of the seeds to allow greater aeration.

After priming, washing and drying (see section 3.6.3), the change in seed weight was determined and the final seed moisture content was estimated (Table 7.1) from the equation given in section 3.6.1. Germination tests were carried out according to the ISTA Rules (ISTA, 1985a, b) with the modifications cited in previous chapters.

A large sample of pea seeds (cv. Douce Provence) of initial moisture content 15.1% and aged at 50°C for 3.5 days were used in the main experiment. This seed lot had 84.0% normal germination (which was just below the regeneration standard (85%) of accessions in genebanks), and 7.914% of the root tip cells in the first mitotic division at anaphase showed chromosomal aberrations. This

seed lot was then divided into two batches; one for experimentation (4500 seeds) and the other as a reserve (1500 seeds).

The priming procedure used in the preliminary experiments were modified and a useful priming method was established for pea seeds, in the main experiment. The priming procedure was slightly changed in each experiment in order to establish a more convenient priming method for pea seeds. A new improved method, which avoided exposure to fungal attack, was followed in the main experiment. In this improved method, 4 paper towels were immersed in 50 ml PEG-8000 solution. Then the seeds were spread on two moist towels as a single layer as in the second preliminary experiment. However, the other two towels were placed on the lid of the plastic box in order to allow maximum aeration of the seeds during priming.

Priming was carried out with three different concentrations of PEG-8000 (-0.5, -1.0 and -1.2 MPa), one concentration of ABA (10^{-3} M) and distilled water against the control (non-primed) for 3, 5 and 7 days in each treatment. Thus, 15 priming treatments and one unprimed (control) were used in the main experiment.

Previously, in Chapter 6, the optimum humidification temperature for pea seeds was determined as 16°C. Since

both humidification and priming are hydration treatments, priming was conducted at 16°C in the main experiment.

In each treatment, 250 seeds (i.e. 200 seeds for germination test and 50 seeds for cytology work) were placed as a single layer onto the priming medium. In order to remove the seeds of the 15 different treatments on the same day, priming was set up at day 0, day 2 and day 4 for 7, 5 and 3 day treatments, respectively.

Once all the treatment groups were removed from the 16°C incubator at day 7, the seeds of each treatment were put in a wire mesh strainer and washed with tap water for 5 minutes and then rinsed with distilled water. Following this, the seeds of each treatment were dried at 20°C and 40 ± 5 % R.H. for about 2 hours. The change in weight following priming was determined and the final seed moisture content of each treatment group was estimated from the equation used in section 3.6.1.

Germination tests were set up using a Completely Randomized Block Design and the percentage viability of the control and primed seeds were determined according to the ISTA Rules (ISTA, 1985a, b) except for a reduction in the number of seeds tested from 400 to 200 in each treatment. In the mean time, the remaining 50 seeds in

each treatment were set to germinate for cytological examination.

Four incubators running at 20°C were used as four different blocks of the Randomized Block Design during the germination tests. Each incubator comprised 16 treatment groups (i.e. 15 different priming groups and 1 unprimed-control) and each treatment group comprised 50 seeds (two replicates of 25 seed each). In each incubator (block), germination boxes (treatment groups) were placed randomly.

Germination test results were recorded daily until day 8. Mean germination time was calculated according to the equation of Ellis and Roberts (1981):

$$MGT = \Sigma(Dn) \; / \; \Sigma n$$

where

n - is the number of seeds which germinate on day d, and

D - is the number of days counted from beginning of the germination test.

As an alternative, the coefficient of velocity of germination was also calculated according to the equation suggested by Kotowski (1926):

$$CVG = \Sigma n \times 100 \; / \; \Sigma Dn$$

However, MGT is equivalent to (1 / CVG) x 100. Therefore, as the use of MGT values is more common, they were presented in the results section and CVG values in Appendices 17 a - c.

The final germination percentages of normal seedlings were analysed by analysis of variance (Randomized Blocks ANOVA) after arcsin transformation. MGT and CVG values were also analysed separately according to the Randomized Blocks ANOVA.

Radicle-tips for cytological examination were excised at regular intervals from the start of germination for each treatment group. Cytological procedures given in section 3.7 were followed and the total number of aberrations were expressed as a percentage of the total number of anaphase cells observed per treatment. Chromosomal aberrations in the first mitotic divisions were classified as chromatid or chromosome-types, mixed or others (see section 4.2).

7.3. Results

7.3.1. Preliminary Priming Experiments

In the first preliminary experiment, the final moisture contents of pea seeds (cv. Kelvedon Wonder) primed with

-0.5 MPa PEG-8000 solution at 15°C were 55.4% and 60.9% for 7 day and 14 day treatments, respectively; and at 25°C they were 56.6% for 7 day and 56.7% for 14 day treatments. As the final seed moisture content reached was greater than 50%, seeds which had germinated were observed in each treatment group (Table 7.2). However, the aim of priming was to hold the seeds imbibed just below the moisture contents for germination for a longer period.

Although this first attempt seemed a failure, priming at 15°C gave promising results with regard to the final viability, compared with 25°C treatments. In particular, 7 day priming at 15°C increased the proportion of normal germination from 78.0% to 88.5%. However, the viability of pea seeds decreased with the increase in priming period (i.e. in 14 day treatment) due to fungal attack of the seeds.

The results presented in Table 7.2 show that priming at 25°C for 7 and 14 days caused a decrease in percentage normal germination and an increase in dead seeds. Since the seeds were severely infected by *Fusarium spp.* in 7 and 14 day treatments at 25°C, it was decided to use 15°C in a further experiment on post-storage priming of pea seeds.

Table 7.2. The effect of post-storage priming treatments with -0.5 MPa PEG-8000 on the final viability of pea seeds (cv. Kelvedon Wonder).

Priming temp. (°C)	Duration of priming (days)	Subsequent seed m.c. (%, f.wt)	Normal ger. (%)	Total ger. (%)	Dead seeds (%)
Control	-	11.5	78.0	92.0	8.0
15	7	55.4[a]	88.5	96.0	4.0
	14	60.9[b]	52.0	63.5	36.5
25	7	56.6[c]	34.5	46.0	54.0
	14	56.7[d]	22.5	32.0	68.0

The proportion of seeds which germinated during priming:
[a]3% [b]46% [c]18% [d]20%

In order to avoid germination during priming, 15.1% moisture content pea seeds (cv. Douce Provence), aged at 50°C for 4 days, were primed with PEG-8000 solutions which had lower osmotic potentials, in the second preliminary experiment.

Table 7.3 shows the effect of post-storage priming treatments at 15°C with four different osmotica of PEG-8000 on the final viability of pea seeds. It is clear from the results that decreasing the osmotic potentials reduced the final seed moisture content reached, in both 7 and 14 day priming treatments. In addition, the viability of pea seeds, decreased with increase in priming period and decrease in osmotic potential values due to fungal infection.

In each treatment, 14 day priming had a damaging effect on seed viability. Also, 7 day treatments of -1.4 and -1.6 MPa osmotic potentials resulted in a decrease in final viability of the aged pea seeds. Although, in general, priming had a negative effect on seed viability, 7 day treatments of -1.0 and -1.2 MPa osmotic potentials did not reduce the viability of pea seeds compared with the control. Therefore, these results suggest that seed viability is lost with a decrease in osmotic potential of PEG-8000 solution and an increase in the priming period. The results also suggest that fungal problems, during

Table 7.3. The effect of post-storage priming treatments at 15°C with four different osmotica of PEG-8000 on the final viability of pea seeds (cv. Douce Provence).

Osmotic pot. (MPa)	Duration of priming (days)	Subsequent seed m.c. (%, f.wt)	Normal ger. (%)	Total ger. (%)	Dead seeds (%)
Control	-	15.1	79.5	86.5	13.5
-1.0	7	41.2	79.5	88.0	12.0
	14	42.6	34.5	58.0	42.0
-1.2	7	38.7	79.0	87.0	13.0
	14	39.2	50.0	68.5	31.5
-1.4	7	36.6	60.5	75.0	25.0
	14	35.7	10.5	27.0	73.0
-1.6	7	34.8	49.0	61.5	38.5
	14	35.2	21.0	40.5	59.5

priming, should be remedied either by allowing more aeration of the seeds or by treating the seeds with a fungicide (e.g. Thiram) before priming treatments.

7.3.2. The Main Priming Experiment

The moisture content of pea seeds (cv. Douce Provence), previously stored at 50°C , 15.1% moisture content for 3.5 days, rose to between 30-50% within 3, 5 and 7 days of priming at 16°C with PEG-8000 (-0.5, -1.0 and -1.2 MPa), ABA (10^{-3} M) solutions and distilled water. The final seed moisture content varied between 30% and 40% in the case of the PEG solutions, and between 40% and 50% in the ABA solution or distilled water during priming for 7 days (Figure 7.1). No seeds germinated in PEG or ABA treated samples over a period of 7 days. Although 16% of the seeds germinated after 7 days treatment with distilled water, seeds primed up to 5 days in distilled water showed no rupture of the testa.

Figure 7.2 shows that the highest increase in percentage germination was noted in seeds primed for 3 and 5 days in distilled water, followed by 3 day treatments of 10^{-3} M concentration of ABA and -1.0 MPa osmotic potential of PEG. Least Significant Difference (LSD) test results after a Randomized Blocks ANOVA with the arcsin transformed values of percentage normal germination

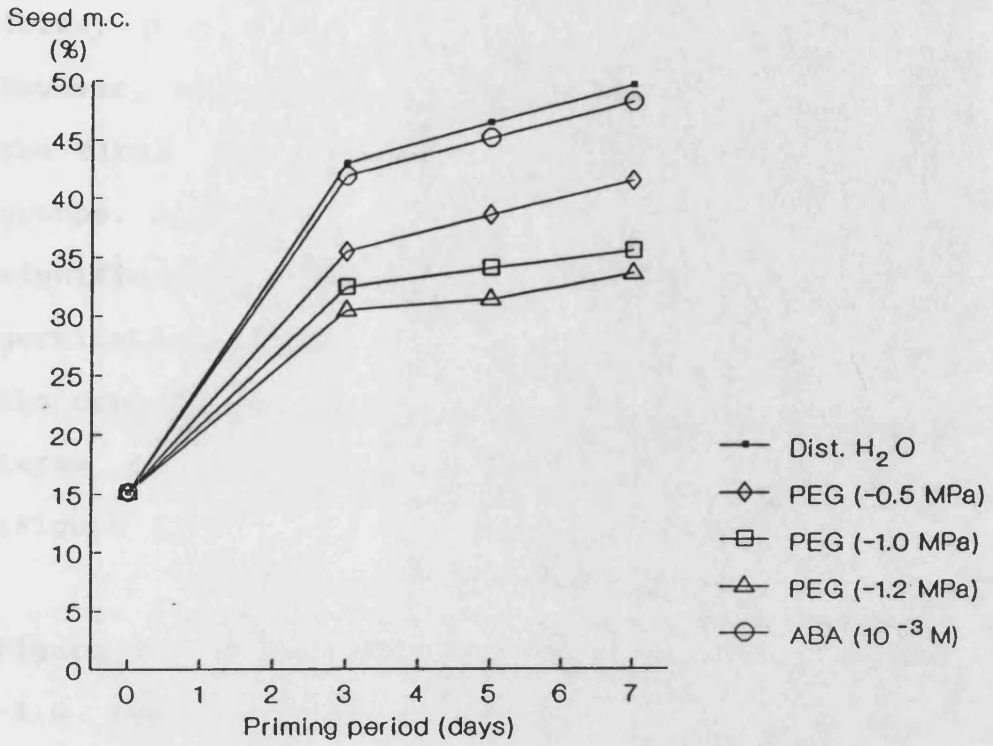


Figure 7.1. The effect of post-storage priming treatments on the final moisture content of pea seeds (cv. Douce Provence).

(see Appendices 15 b and c) confirmed that the above treatment groups were significantly different (LSD = 4.264, $p < 0.05$) from the control (non-primed) seeds. However, no significant differences were evident between the final normal germination values of these treatment groups. Although 7 day priming with ABA and -1.0 MPa PEG significantly decreased the final percentage of normal germination, there was no significant difference between the control and each of the remaining treatment groups in terms of the percentage of final normal germination (Figure 7.2).

Figure 7.3 shows that priming for 3 days with PEG (-0.5, -1.0 and -1.2 MPa) and ABA (10^{-3} M) solutions and distilled water reduced the mean germination time (MGT) of the seeds, when they were transferred to standard germination test regimes. The fastest rate of germination was observed in the 7 day treatment in distilled water. An LSD test, following a Randomized Blocks ANOVA (see Appendices 16 a and b) was conducted on this data and showed that the MGT value of 7 day treatment with distilled water was significantly different (LSD = 0.278, $P < 0.05$) compared with the other treatment groups. However, 16% of the seeds in this treatment germinated during priming. Thus, 5 day priming with distilled water (which was the second best response) was considered the most effective treatment. Although

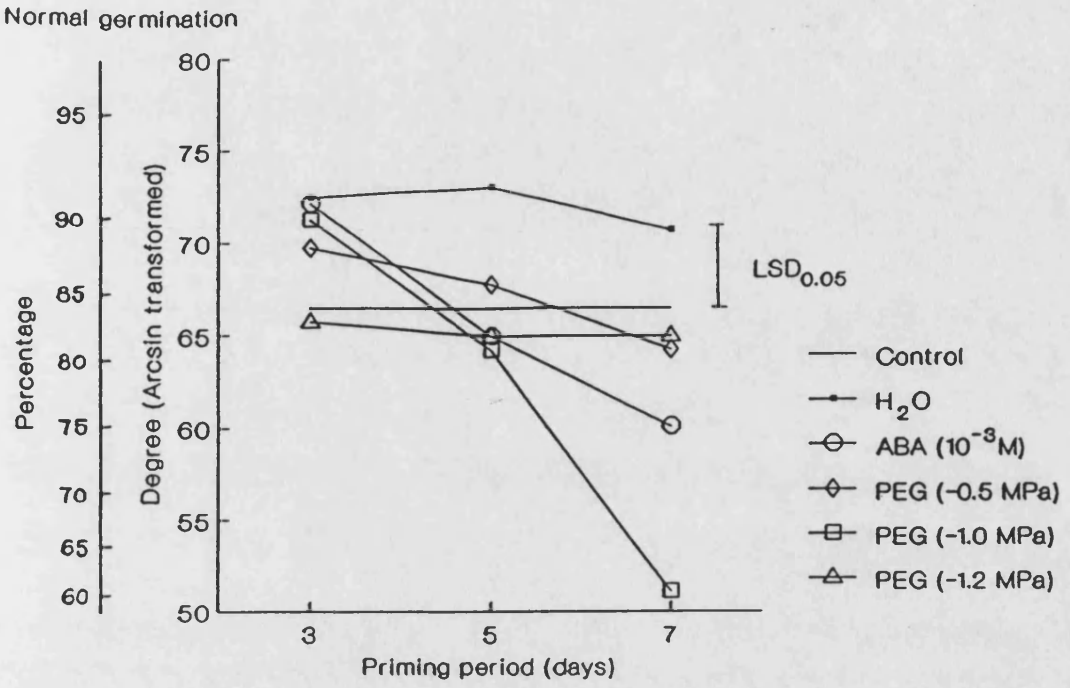


Figure 7.2. The influence of post-storage priming treatments on the subsequent percentage normal germination (arcsin transformed values) of pea seeds cv. Douce Provence which had 84% initial normal germination (Vertical bar, LSD = 4.264; $P<0.05$).

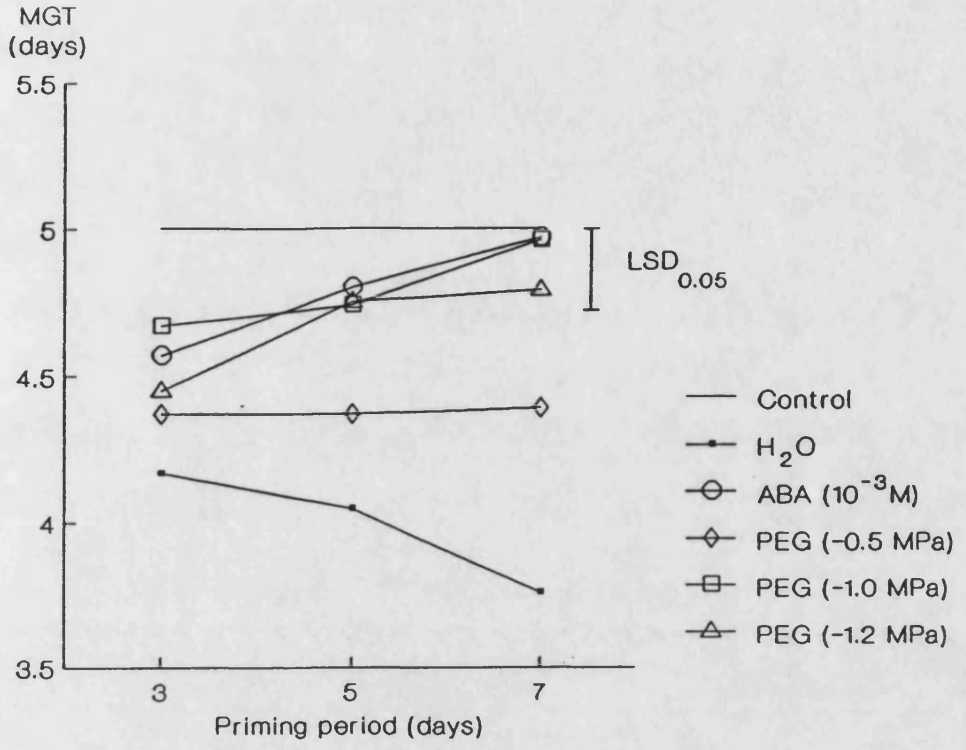


Figure 7.3. The influence of post-storage priming treatments on the mean germination time (MGT) of pea seeds (cv. Douce Provence) which had 84% initial normal germination (Vertical bar, LSD = 0.278; $P < 0.05$).

there was no significant difference between MGT values after 3 and 5 day treatments in distilled water, the MGT value of the 5 day treatment was significantly higher than all the other treatment groups. The 3 day treatment in distilled water resulted in an intermediate category in terms of the MGT value. Therefore, the effect of this treatment was not significantly different from 3, 5 or 7 day treatments of PEG solution with -0.5 MPa osmotic potential. It is clear from the results that there was no significant difference between the MGT value of control (non-primed seeds) and 5 and 7 day treatment groups of ABA and the remaining PEG solutions (i.e. -1.0 MPa and -1.2 MPa). Identical results were obtained from the ANOVA of CVG values (see Appendices 17 a - c).

The cumulative normal germination curves of the distilled water primed seeds (irrespective of the priming period) showed that germination occurred earlier and that the final percentages of normal germination of these seeds were higher relative to the control seeds (Figure 7.4.A). However, after priming with ABA (Figure 7.4.B) and PEG solutions (Figure 7.4.C-E) only 3 day treatments produce earlier curves in terms of cumulative normal germination, relative to the control seeds. The percentage of final normal germination was also higher than the control seeds in these treatments except with the PEG solution of -1.2 MPa osmotic potential.

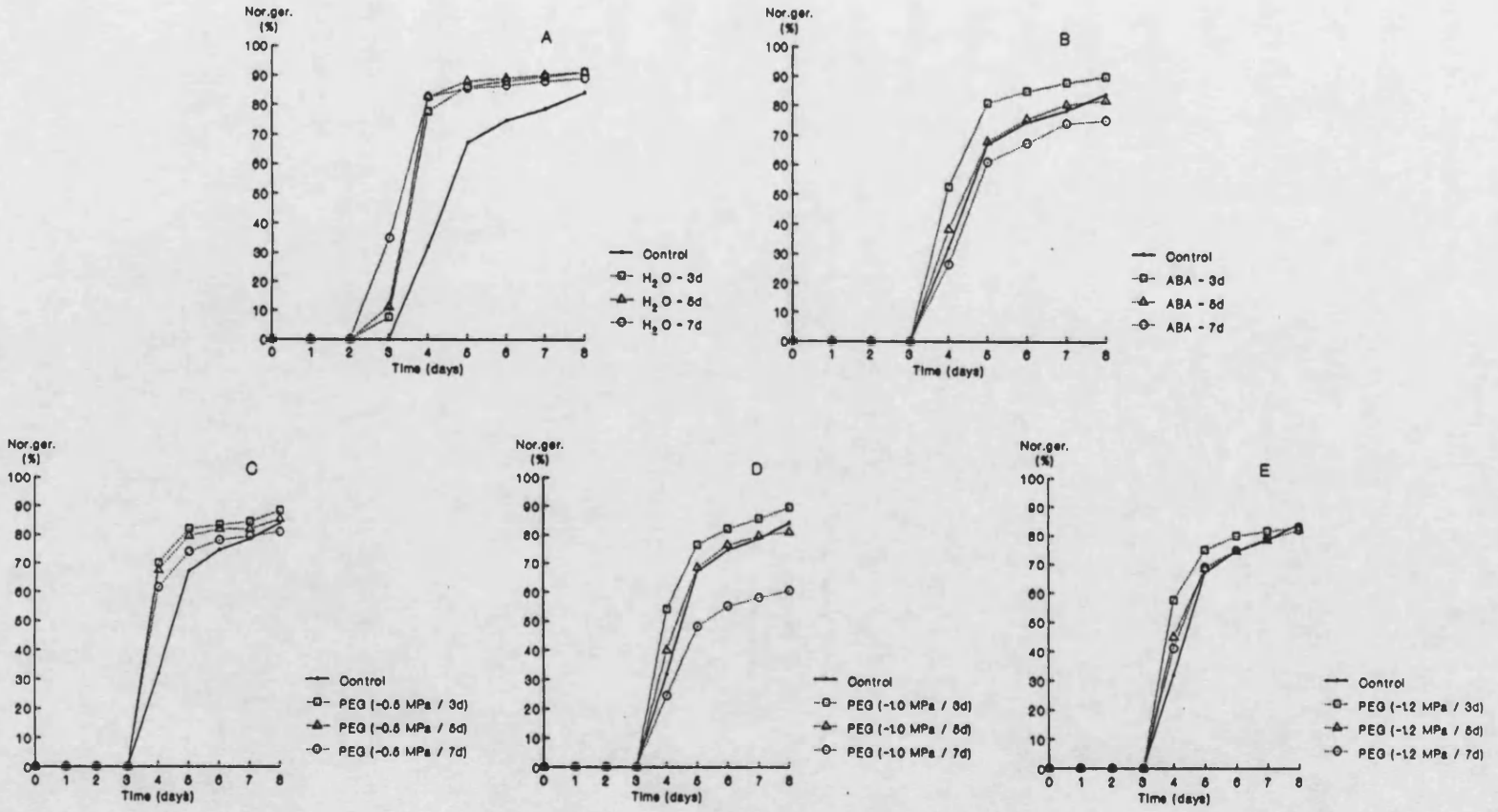


Figure 7.4. Normal germination time course of pea seeds (cv. Douce Provence) following post-storage priming treatments at 16°C with distilled H_2O (A); 10^{-3} M ABA (B); -0.5 MPa PEG (C); -1.0 MPa PEG (D); -1.2 MPa PEG (E) for 3, 5 and 7 days.

However, 5 and 7 day treatments of ABA and PEG solutions gave conflicting results (Figure 7.4.B and D). For example, 5 day treatments of ABA and -1.0 MPa osmoticum of PEG did not alter the germination rate of the seeds although 7 day treatments slowed the rate of germination and decreased the percentage of final normal germination, compared with the non-primed control seeds. The cumulative normal germination curves of the seeds treated with -0.5 MPa PEG for 5 and 7 days also occurred earlier than the curve of the control seeds. However, the percentage of final normal germination was similar to control in either treatment (Figure 7.4.C). Finally, the response of seeds to 5 and 7 day treatments with -1.2 MPa PEG were also similar to the control, in terms of the rate of germination and the percentage of final normal germination (Figure 7.4.E).

The results presented in Figure 7.5 show the effect of selected priming treatments on percentage normal germination and the frequency of chromosomal aberrations during the first mitotic divisions in pea seeds (cv. Douce Provence) aged at 50°C, 15.1% moisture content, for 3.5 days.

Non-primed (control) seeds had 84.0% normal germination, and 7.914% of the root-tip cells in the first mitotic division at anaphase showed chromosomal aberrations.

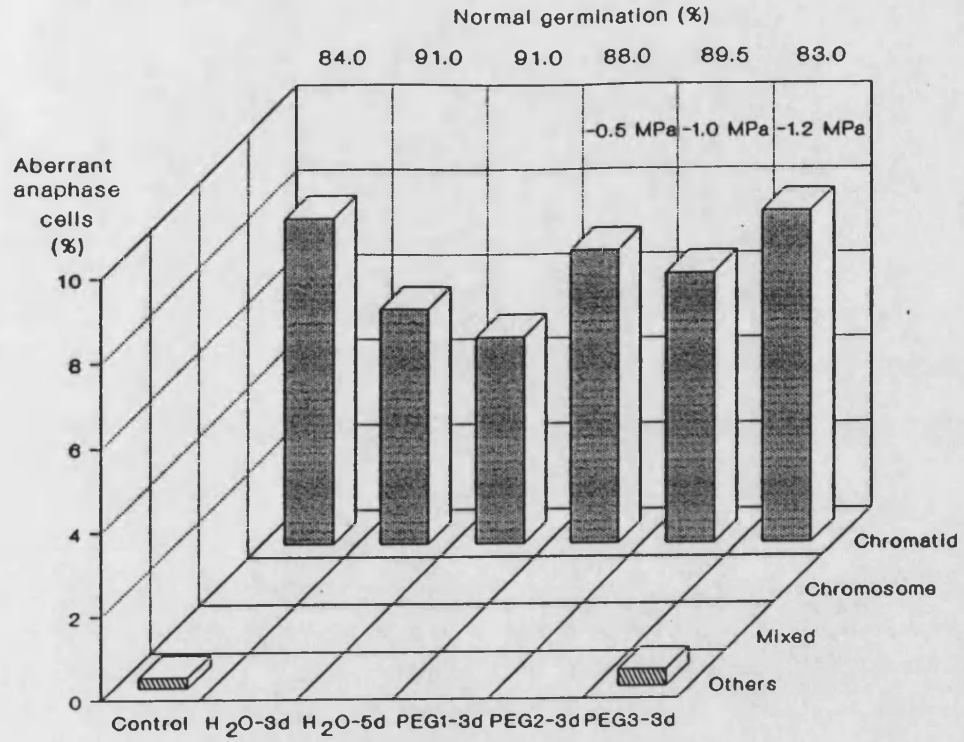


Figure 7.5. Comparison of the frequencies of chromosomal aberrations for pea seeds (cv. Douce Provence) non-primed (control) or primed in different osmotica at 16°C prior to the germination test following storage at 50°C with 15.1% moisture content for 3.5 days.

Since priming at 16°C improved the germination rate and the final germination of aged pea seeds, particularly priming for 3 days with ABA or PEG solutions and for 3 and 5 days with distilled water, cytological examinations were carried out on the seed samples of these treatments. However, after priming for 3 days with ABA, the cells of 4-8 mm root-tips (excised from the seeds between 48 and 64 hours after initiation of imbibition) showed no mitotic division. Since the ABA treatment caused a mitotic delay, thus no anaphase cells were observed under the conditions cited above, the effect of priming with ABA on the frequency of chromosomal aberrations in aged pea seeds could not be presented here.

As would be expected, priming with distilled water for 3 and 5 days decreased the chromosomal aberrations of the seeds to the lowest level among the seed lots examined for cytology. Within the water primed seed samples, 3 day treatments decreased the frequency of chromosomal aberrations to 5.544% whilst 5 day treatment resulted in a further slight decrease in chromosomal damage (i.e. 4.863%). However, the final percentage of normal germination of the seeds was 91.0%, in both cases. Therefore, the maximum benefit of priming was evident when the seeds were imbibed with distilled water at 16°C and the highest osmotic potential (0 MPa) for 5 days. This is probably because this treatment increased the

seed moisture content to 46.4% which is very close to full hydration and thus allows repair to ageing induced damage.

Priming with PEG solutions also reversed some of the ageing induced damage to chromosomes although they were not as effective as distilled water priming. The reason was probably due to their lower osmotic potentials. 3 day treatments with PEG solutions of -0.5, -1.0 and -1.2 MPa osmotic potentials increased the moisture content of the seeds to 35.4, 32.4 and 30.4%, respectively. Although the effect of priming on the decrease in visible chromosomal aberrations during first mitotic divisions was almost similar in PEG treated seeds with either -0.5 MPa or -1.0 MPa osmoticum (6.897% and 6.357%, respectively), the seeds treated with -1.2 MPa osmoticum responded like control seeds (8.200% and 7.914%, respectively).

Two different types of chromosomal aberrations (i.e. chromatid-type and others) were observed in almost similar frequencies in control seeds and in the seeds treated with -1.2 MPa osmoticum of PEG. Neither chromosome-type nor mixed-type aberrations were observed in these seed lots. Furthermore, the chromosomal aberration category "others" practically disappeared in the distilled water primed seeds as well as the other two treatments of PEG (Figure 7.5).

7.4. Discussion

The results presented in the preliminary experiments are convincing evidence that holding aged seeds at high moisture contents (at the brink of germination) by an osmoticum does not always favour recovery from ageing induced damage nor improve germination unless the correct conditions for priming apply. The main problem in the preliminary experiments was fungal contamination of the aged seeds during priming. Loss in seed viability also increased with a decrease in the osmoticum of the PEG solution and an increase in the priming period. Also, it was realised that in the preliminary experiments the seeds had insufficient aeration, through priming between rolled paper towels in the first preliminary experiment or by just covering in the second. When seeds were allowed sufficient aeration (with the use of the improved priming method), no fungal contamination occurred in the main experiment. Since priming was successful in the main experiment, the results of the main experiment only are considered in this section.

It is clear from the results obtained in priming with distilled water that increasing the seed moisture content just below full hydration provided the maximum benefits of priming. For example, when the seeds were primed with distilled water for 3 and 5 days (the final moisture

content reached 42.9% and 46.9%, respectively) the percentage of final normal germination increased to 91.0%, which was the highest viability obtained (Figures 7.1 and 7.2). However, 3 and 5 day priming with ABA (10^{-3} M) gave conflicting results. Although 3 day ABA treated seeds showed 90.0% normal germination which was significantly different from the control ($P < 0.05$), the 5 day treatment considerably decreased the normal germination to 82.0%, i.e. below the control seeds (84.0%). However, ABA treated seeds reached 41.8% m.c. after 3 days and to 45.1% after 5 days, a similar trend with the distilled water treated seeds. These results suggest that in aged pea seeds prolonged priming treatments with ABA result in loss of viability compared with the control seeds.

A significant reduction in the mean germination time (MGT) was observed ($P < 0.05$) following 3 day priming with each of the osmotica. Although 5 and 7 day treatments of ABA and two PEG solutions (-1.0 MPa and -1.2 MPa) did not significantly ($P > 0.05$) differ in the MGT compared with the control, the seeds treated with distilled water and -0.5 MPa osmoticum of PEG significantly reduced ($P < 0.05$) the MGT over a 7 day period. Not surprisingly, once again, 5 day priming with distilled water was the best treatment for aged pea seeds (Figure 7.3). Cumulative normal germination curves of the

primed seeds also suggested that distilled water treatments provided the maximum benefit regarding earliness of germination (Figure 7.4.A-E).

Very little work has been carried out on the priming of aged seeds and related effects on seed viability and rate of germination. Previously, Simak (1976), using 14-year-old Scots pine seeds primed in PEG, noted not only faster germination but also a small increase in germinability. Brocklehurst and Dearman (1983) also found that post-storage priming with PEG solutions increased the rate of germination compared to that of unprimed (control) seeds in carrot, celery and onion. Later, Dell'Aquila and his colleagues (Dell'Aquila *et al.*, 1984; Dell'Aquila and Taranto, 1986; Dell'Aquila, 1987; Dell'Aquila and Tritto, 1990, 1991) showed that in aged wheat seeds priming with PEG did not affect the final germination, but the mean germination time (reciprocal of germination rate) was reduced.

Furthermore, in pepper seeds, post-storage priming with mannitol also gave conflicting results in terms of the final germination. Pepper seeds which were primed in 0.4 M mannitol solution (ψs of osmoticum -0.991 MPa) for 4 days (at 25°C, in darkness) after storage at 35°C for up to 6 months, did not have any effect on the final seed viability (Georghiou *et al.*, 1987). Later, Thanos *et*

al. (1989) stored pepper seeds at 25°C for over a 3-year period and then primed with mannitol as described in Georghiou *et al.* (1987). However, they showed that there was a significantly higher germinability (assessed at 15°C) in comparison with the corresponding unprimed seeds. On the other hand, both Georghiou *et al.* (1987) and Thanos *et al.* (1989) concluded that post-storage priming with mannitol enhanced the germination rate of pepper seeds.

Therefore, this study has confirmed the findings of Simak (1976) and Thanos *et al.* (1989) that post-storage priming treatments at 16°C with distilled water (for 3-5 days) and with PEG-8000 (-0.5, -1.0, -1.2 MPa) and ABA (10^{-3}M) solutions (for 3 days) increased the final germination and the rate of germination, i.e. decreased the MGT as also pointed out by Dell'Aquila and his colleagues.

Biochemical studies reported by Khan *et al.* (1978) have concluded that RNA and protein metabolism are enhanced by priming suggesting that this treatment makes available to seed precursors utilized for macromolecular synthesis. The changes in activities of such enzymes as esterase, phosphotase and 3-phospho-glyceraldehyde dehydrogenase suggest that mobilization of seed storage materials as carbohydrates, fats and

proteins may underlie the increased germination and vigour induced by priming.

Cytological examination after priming showed that in aged seeds the frequency of visible chromosomal aberrations decreased whilst the percentage of normal germination increased. It is clear from the results presented in Figure 7.5 that the maximum benefit of priming occurred when the seeds were treated with distilled water for 5 days although 3 day treatments of distilled water and PEG solutions with -0.5 MPa and -1.0 MPa osmotica also reversed some of the ageing induced chromosomal aberrations. Although priming with ABA increased the percentage of normal germination in aged pea seeds, the frequency of chromosomal aberrations could not have been examined due to a mitotic delay which possibly induced by the ABA solution. Mayer and Poljakoff-Mayber (1989) suggest that the inhibitory effect of exogenously applied ABA on germination gradually disappears on removal of the seeds from the ABA solution and by washing them. However, following a post-storage priming treatment with ABA, what causes a mitotic delay in pea seeds during germination is as yet unclear.

In conclusion, the results of the present study suggest that priming at 16°C with distilled water for 5 days is the optimum treatment for aged pea seeds to increase the

percentage normal germination and decrease the mean germination time and the frequency of chromosomal aberrations. The results also suggest that priming of aged seeds in high osmotica would have artificially prolonged the lag phase between imbibition and germination (DNA replication) and hence led to a possible repair of the induced damage. In addition, this work is the first evidence showing that the critical moisture content for repair of chromosomal damage to occur in pea seeds is between 32% and 46%.

CHAPTER 8. GENERAL DISCUSSION

8.1. Loss of Seed Viability and the Accumulation of Chromosomal Aberrations in Pea Cultivars and Wild Types during Storage

It has long been known that there is a close relationship between loss of seed viability and the accumulation of chromosomal aberrations in a range of species. The main factors affecting this relationship are seed moisture content, temperature and storage period, i.e. increasing either results in an increase in loss of seed viability and an increase in the frequency of chromosomal aberrations. This study has confirmed the previous findings in pea seeds (Gunthardt *et al.*, 1953; Roberts *et al.*, 1967; Abdalla and Roberts, 1968; Dourado and Roberts, 1984a) with regard to the above relationship. Furthermore, this study is the first attempt to investigate the validity of this relationship in wild type pea seeds. The results in Chapter 4 show that there is also a negative relationship between loss of viability and accumulation of chromosome damage in wild type pea seeds. This relationship was examined within the range of 96.5% and 21% normal germination in cultivars, and 100% and 20% normal germination in wild types. In addition, these results are also compatible with Dourado and Roberts (1984a), showing that significant increases in

the percentage of aberrant cells (during the first mitotic divisions) can occur in association with very small losses of viability.

However, the seeds of wild type peas showed greater longevity compared with the cultivated pea seeds. This might be due to the higher initial seed viability (i.e. greater K_i value) and or different genotypic characteristics (which tolerate adverse storage conditions) of wild types. The results of the IBPGR sponsored project (by the Boyce Thompson Institute, Cornell University, USA, the National University of Mexico and CIMMYT, Mexico) on genetic control of storage characteristics in pea suggest that the storage life of the seeds produced from white flowered plants is shorter than those of coloured-flower plants (IBPGR, 1991). Similarly, phenotypic observations carried out in this research show that cultivated pea plants produce white flowers whilst wild type pea plants produce pink flowers (see section 3.2).

Previously, Rao (1986) concluded that in lettuce, the frequency of chromosomal aberrations in the surviving seeds in relation to a given loss of seed viability, increased with decrease in the seed moisture content (within the range 5.5-13.0%). However, this study has shown that there is no significant difference between

high and low moisture content pea seeds in terms of the accumulation of chromosomal aberrations at an identical viability level, i.e. low moisture content seeds do not exhibit a greater frequency of aberrations than high moisture content seeds of equivalent viability. Wild type pea seeds showed the same trend to that of pea cultivars. Rao (1986) also concluded that lipid peroxidation-mediated-free radical injury might be mainly responsible for the increased chromosome damage at low moisture contents. However, since pea seeds are high in starch (52%) and low in lipid (6%) content (Bewley and Black, 1985), unlike lettuce seeds, it is probable that lipid peroxidation-mediated-free radical injury in pea seeds is not as effective as in lettuce seeds. Therefore, this might be a reason why the negative relationship between seed moisture content and the amount of chromosome damage for a given loss of viability found in lettuce seeds did not occur in pea seeds.

The present findings are compatible with those of Abdalla and Roberts (1968), working with barley, broad beans and peas, in that for a given percentage survival of seed, the mean frequency of aberrant cells in the survivors is the same, irrespective of how rapidly the loss of viability occurred, or what combination of environmental conditions led to it. They showed this relationship in pea seeds under different storage conditions, i.e.

storage temperatures between 25 and 45°C, and seed moisture contents between 13.0 and 18.3%. The present study suggests that in cultivated and wild type pea seeds this relationship also applies to storage temperatures varying between 50 and 65°C, and seed moisture contents between 4.7 and 15.1%.

Since the accumulation of genetic damage is a symptom of loss of seed viability, desiccation of seeds to low moisture contents (5 ± 1%) and storage between -18 and -20°C are recommended to increase the storage life of orthodox seeds (IBPGR, 1976) and reduce the frequency of genetic damage. However, the relationship between seed longevity and moisture content is subject to a low moisture content limit below which there is no further improvement in longevity and the value of which can vary among species (see section 2.2.2). Ellis *et al.* (1989, 1990c, d) expressed this limit as an equilibrium relative humidity (i.e. around 10-11%) since this is a common value among species. However, Vertucci and Roos (1990) found that in seeds of five species (pea, soybean, peanut, lettuce and sunflower) when these seeds were stored below a critical moisture level of about 19% relative humidity (RH), all five seed lots showed reduced viability within 4 months. They have thus concluded that the low moisture content limit is between 19% and 27% RH. Thus, they criticized the suggestion made by Ellis

et al. (1989, 1990c, d), that the low moisture content limit is between 10% and 11% RH, as an underestimate, citing also problems associated with interpreting ageing experiments at 65°C and insensitive germination tests.

However, it has long been known that there is no interaction between the effects of temperature and moisture on seed longevity when these relations are quantified by the seed viability equation proposed by Ellis and Roberts (1980a, b); and that there is a continuous relation between seed longevity and temperature between -20°C and 90°C (see sections 2.2.1 and 2.2.2). Therefore, there is no evidence that the relative effect of moisture content on longevity varies with the experimental temperature of 65°C used in the work of Ellis and his colleagues. Moreover, they reported longevity estimated from entire survival curves rather than a single germination test result. It is well known that when entire survival curves are examined, this can be a sensitive method of investigation.

Furthermore, Bass and Stanwood (1978) reported the results of 16 years of storage of sorghum at temperatures between -12°C and 32°C in which storage at 4.8% moisture content was shown to result in less loss in viability than storage at 7.0% moisture content. In sorghum, 4.8% moisture content is close to the value in equilibrium

with the 10-11% RH recommended by Ellis and his colleagues, while 7.0% moisture content is in equilibrium with a value close to the lower end of the range of 19-27% RH recommended by Vertucci and Roos (see Justice and Bass, 1979). Similarly, this present study suggests that in the seeds of pea cultivars and wild types, low moisture content (4.7-5.1%) seed lots [even below the low moisture content limit of pea seeds, i.e. 6.2% (Ellis *et al.*, 1989)] display greater longevity and are not unduly affected by drying (Chapter 4).

Therefore, there is a considerable amount of evidence to suggest that the proposal that moisture contents in equilibrium with 19-27% RH are optimal for seed longevity (Vertucci and Roos, 1990) is, in fact, an overestimate.

However, these findings do suggest that different species may behave in contrasting ways in terms of the relationship between seed moisture content and the accumulation of chromosomal aberrations with loss of viability, and this may be a function of oil content; a hypothesis which should be further investigated.

8.1.1. Types of Chromosomal Aberrations in Aged Seeds

Previously, it was proposed by Sax (1940, 1941) that ageing-induced aberrations were related to the Classical

Theory of breakage and reunion whereby aberrations were produced as a direct result of breaks in the chromatin due to ionizing radiations. This suggests that chromosome-type aberrations result when breakages occur in cells in G_1 (gap before DNA replication), while chromatid-type aberrations occur as a result of breakages in S (DNA replication stage) and G_2 (gap after DNA replication). However, later, Revell (1959) proposed the Exchange Theory, as an alternative to the Classical Theory. The Exchange Theory states that the primary event of damage is not a break but an unstable lesion that only develops into aberrations when the cell passes through the DNA replication stage. Most of the work on chemical mutagens and ultra-violet light showed that the damage resulting from lesions should always appears as chromatid-type aberrations in the first mitosis following the first cycle of DNA replication. However, if the aberrant cell goes through a further mitosis, the abnormality may become a chromosome-type aberration (Kihlman, 1966; Bender *et al.*, 1974; Evans, 1977; Roberts, 1988). Thus, recent reports on ageing-induced aberrations have adopted the Exchange Theory since the mechanism of chromosome aberration production in aged seeds, e.g. barley and pea (Dourado 1983) onion (Sirikwanchai, 1985) and lettuce (Rao 1986), behaves similarly to that of chemical mutagens or ultra-violet light.

This present study suggests that in aged seeds of pea cultivars and wild types, chromatid-type aberrations are predominant, both in high moisture content (between 73% and 89%) and low moisture content (between 85% and 87%) seed lots. Therefore, adopting the ageing-induced damage Exchange Theory, where chromosome-type aberrations are observed in this study this indicates that the cells were scored at second mitosis. These results are compatible with those of Dourado (1983), who found that in aged pea seeds the proportion of chromatid-type aberrations was ca. 93% (based on the present classification of chromosomal aberrations, see section 4.2).

Previously, Abdalla and Roberts (1968), concluded that in aged seeds of barley, broad beans and peas, chromosome-type aberrations were predominant. They proposed that the embryo cells in stored seeds were in early interphase (i.e. in presynthetic stage, G_1) and that the aberrations brought about by seed ageing were chromosome-type. However, it is now known that ageing has a similar effect to chemical mutagens or ultra-violet light on producing chromosomal aberrations and that ionizing radiations are the only physical agents which produce chromosome-type aberrations by immediate breakage in interphase (Roberts, 1988).

Recently, Rao (1986) found that in lettuce the frequency of chromosome-type aberrations was predominant at low moisture content (i.e. between 3.3% and 5.5%) aged seeds. He also found that chromatid-type aberrations were predominant in lettuce seeds aged between 8.1% and 18.1% moisture contents. In the case of the low moisture content aged seeds, Rao claimed that the chromosome-type aberrations were the true aberrations and not duplicated chromatid-type aberrations in the second mitotic divisions since, for example, there had been a number of instances where radicles showing very few mitotic cells often contained all chromosome-type aberrations. Therefore, he suggested that the latent lesions in DNA were converted into potential breaks even before DNA replication.

8.1.2. Possible Causes of the Damage to Chromosomes in Aged Seeds

Although a number of hypotheses have been proposed to explain the cause of the damage to chromosomes as seeds age, what exactly affects this phenomenon is as yet unclear. These hypotheses include: the presence of background irradiation, accumulation of automutagenic substances, lipid autoxidation, to name a few. Roberts (1972), Abdul-Baki and Anderson (1972), Heydecker (1973), Bewley and Black (1985) and Priestley (1986) have

reviewed these in details. Hence, only some of the important points are discussed here. The discovery of the mutagenicity of x-rays and other radioactive sources led to the hypothesis that a gradual accumulation of chromosome damage in seeds stored under normal conditions resulted from normal background radiation. However, Giles (1940) pointed out that the cause of spontaneous chromosome aberration in *Tradescantia* could not be explained by background radiation. Similarly, Gunthardt *et al.* (1953) concluded that the dosage of natural radiation, including cosmic radiation, received by seeds in storage was insufficient to account for the frequencies of cytogenetic changes observed in the aged seeds.

Later, another hypothesis which attempted to explain the spontaneous mutability of aged seeds on a metabolic basis gained popularity. The idea that chromosome damage is a consequence of the accumulation of automutagenic substances resulting from normal metabolism of seeds during ageing received a lot of attention. Many investigators have applied aqueous or lipophilic extracts from aged seeds to healthy tissue in the expection of identifying mutagenic ageing products. Although apparent success has been reported by some workers (e.g. Gisquet *et al.*, 1951; Keck and Hoffman-Ostenhof, 1952; Kato, 1954; Jackson, 1959), others have raised serious doubts

concerning the means by which the purported mutagens have been assayed in many of the studies (Marquardt, 1949a, b; Gori, 1953; D'Amato, 1953; Abdalla and Roberts, 1968). Therefore, the attempts of explaining the possible cause of chromosomal aberrations in aged seeds by the use of automutagenic substances were obscured.

Several attempts to explain the increase in chromosomal aberrations in aged seeds that focused on lipid autoxidation have engendered much speculation. The production of free radicals and hydroperoxides is an autocalytic autoxidation reaction because each break of a lipid double bond produces two free radicals each of which can in turn induce a break at another double-bond. The progressive inactivation of enzymes, denaturation of other proteins, and disruption of DNA and RNA slowly destroys the functioning of a cell (Harrington, 1973; Bewley and Black, 1985).

Lipid autoxidation is accelerated by high temperature and inhibited by the exclusion of oxygen (Schultz *et al.*, 1962). The one anomalous factor is seed moisture. Below moisture levels at which fungi destroy viability, the drier the seed the greater the amount of lipid autoxidation. Harrington (1973) states that seed longevity increases as seed moisture is lowered from 12-14% to 4-6%. Below 4-6% seed moisture, longevity

decreases in line with increased lipid autoxidation. Therefore, lipid autoxidation only becomes serious below 4-6% moisture and other factors must be more important above this level. Moreover, the lipid contents of the seed might be an important factor. As discussed earlier (in section 8.1), only dry seeds that are rich in storage lipids could have been exposed to chromosome damage as a consequence of lipid peroxidation-mediated-free radical injury. From the standpoint of the above hypothesis, it is perhaps unfortunate that evidence in favour of the free radical hypothesis is somewhat weak, and there is no direct evidence for the buildup of free radicals within aged seeds.

Current thought is that loss of viability occurs due to loss of DNA integrity. The first evidence for the presence of naturally occurring breaks in DNA with loss of viability was provided by Cheah and Osborne (1978). They showed the reduction in molecular weight of DNA with loss of viability by first isolating nuclei and then either lysing them directly on alkaline sucrose density gradients or subjecting the extracted DNA to electrophoretic separations on polyacrylamide or agarose gels. This loss of DNA integrity could be the source of chromosomal aberrations and impaired transcription observed during germination of low viability seeds. Further evidence comes from the work of Elder *et al.*

(1987), who showed that fragmentation of nuclear DNA and loss of DNA integrity, occur progressively in the embryos of aged rye seeds. In addition, Dandoy *et al.* (1987) concluded that the number of apurinic or apyrimidinic (AP) sites found in DNA of radicle cells of maize embryos decreased after 2 years of storage at 20°C. Therefore, they suggested that the damage which has accumulated in DNA during storage was related to a decrease in the number of AP sites.

Recently, some biochemical and molecular biological parameters, related to damage to chromosomes as seeds age, have been investigated by several researchers. Guy *et al.* (1991) used restriction fragment-length polymorphism (RFLP) techniques to detect damage to DNA. They detected ageing-induced chromosome changes by the use of molecular probes in wheat seeds, and these changes were first detectable after 12 hours of ageing at 45°C. In another experiment carried out by Kraak *et al.* (1992), DNA was isolated from dry tomato seeds, from seeds at several stages of imbibition, from seedlings and from leaves of plants grown from control and aged seeds. To date, 7 restriction enzymes in combination with 15 probes have been examined. Probes for single copy and repetitive DNA were used. However, they have not yet succeeded in detecting changes in DNA and they have, therefore, concluded that RFLP techniques appear not suitable to

detect chromosome breakage and other changes in DNA due to ageing of tomato seeds.

8.2. The Effects of Humidification Treatments on Minimizing Imbibition Injury in Dry Seeds

Since the rapid uptake of water results in imbibition injury in dry seeds, post storage humidification (in a water saturated atmosphere) is a useful pre-germination treatment to avoid or minimise damage to the seeds. This is now well supported by a number of workers, e.g. Klingmüller (1961), Pollock (1969), Basu and Pal (1980), Ellis *et al.* (1982b, 1985, 1990b) and Rao *et al.* (1987b), to name a few. The results of this study confirm the previous findings in high and low moisture content non-aged and aged pea seeds. In addition, Ellis *et al.* (1982b) suggest that the seed moisture content should be raised to a sufficiently high level to make the most of the humidification treatment. However, it is also important for the seeds not to be humidified for long periods because of its ageing effect (Heydecker and Coolbear, 1977). In other words, there should be an optimum temperature and seed moisture content range to be reached during any humidification treatment in order to obtain the maximum benefit to the seed.

Therefore, Ellis *et al.* (1985, 1990b) suggest humidification treatments at 20°C before beginning germination tests. However, the suggestions for optimum seed moisture content to be reached in a humidification treatment vary between 16% and 18% (Ellis *et al.*, 1985), and between 18% and 20% (Ellis *et al.*, 1990b). This study suggests that humidification at 20°C should only be used for non-aged (high viability) pea seeds. However, as has been discussed in Chapter 5, aged pea seeds (both high and low moisture contents) are more susceptible to rapid uptake of water so that they require slower initial water uptake in a humidification treatment than non-aged seeds. Therefore, the results of this study also suggest that 16°C is the optimum temperature for the humidification of aged pea seeds since it provides a slower initial water uptake to the seeds. In addition, the optimum moisture content to be reached is between 16.3% and 18.1% in aged pea seeds.

Recently, Rao *et al.* (1987b) showed that in aged lettuce seeds humidification treatments increased the percentage of normal germination and reduced the frequency of cells showing chromosomal aberration due to some repair of ageing-induced sub-cellular damage since humidification raised the seed moisture content above the critical value (i.e. 15% moisture content for lettuce seeds). Although it seems unlikely that pea seeds can facilitate repair of

ageing-induced damage by humidification treatments as the expected critical moisture content is above 30% (Roberts, personal communication, 1989); on the basis of the hypothesis posed in Chapter 6, the pea embryos in this study might have reached a higher moisture content than the surrounding cotyledons and this could have facilitated repair during humidification. This study shows that humidification treatments caused a decrease in the frequency of chromosomal aberrations in pea seeds (see Tables 6.1 and 6.3). Thus, this hypothesis should be investigated as a possible answer to this phenomenon of a reduced frequency of chromosomal aberrations following humidification.

The results presented in Chapter 6 are convincing evidence that humidification treatments avoid imbibition injury which otherwise occur during initial rapid uptake of water, and cause an improvement in seed viability.

8.3. The Effects of Post-Storage Priming Treatments on the Repair of Ageing-Induced Damage in Seeds

It was shown in Chapter 7, that damage to the DNA and other sub-cellular systems, during storage could be repaired to some extent by holding the seeds imbibed but at the brink of germination, by post-storage priming treatments. These experiments indicated that post-storage

priming treatments increased the percentage final germination and decreased the mean germination time (MGT) and these results are compatible with that of previous workers in a range of species (Brocklehurst and Dearman, 1983; Dell'Aquila, 1987; Dell'Aquila and Tritto,1990, 1991; Georghiou *et al.*, 1987; Alvarado and Bradford, 1988; Thanos *et al.*, 1989; Gray *et al.* 1990, 1991). The results also confirmed that the frequency of chromosomal aberrations during first mitotic divisions is decreased due to the stimulation of DNA repair mechanisms (Rao, 1986). However, many of the chromosomal aberrations observed at the first mitosis are eliminated as the root elongates due to diplontic selection (Gaul, 1961). Therefore, the effect of post-storage priming treatments on heritable mutations (which has not been studied here) is worth investigating in further work.

Villiers (1973) proposed that deterioration in dry stored seed results from the lack of operable systems to repair and replace organelles and that the full consequences of damage accumulating during storage are not evident until imbibition. Furthermore, it is probable that for most seeds, the first hours of imbibition are marked by the activation of cellular repair processes that gradually eliminate the lesions acquired in dry storage (Berjak and Villiers, 1972; Osborne, 1982). Presumably, it is the existence of such repair metabolism in the

extended lag period before DNA replication (which is attained by holding the seeds in solutions of high osmotic potential) that permits the repair of some of the ageing induced damage. Although a limited number of investigations into the biochemical changes occurring in primed and subsequently germinated seeds have noted improvements in RNA, protein and DNA synthesis and the activity of certain enzymes (Khan *et al.*, 1978; Coolbear and Grierson, 1979; Blowers *et al.*, 1980; Dell'Aquila and Taranto, 1986; Fu *et al.*, 1988; Bray *et al.*, 1989; Dell'Aquila and Bewley, 1989), firm evidence concerning the molecular basis of repair in aged seeds during priming treatments is still lacking.

It has been demonstrated that in leek, protein synthesis was induced in both embryo and endosperm tissues during priming at 15°C (Bray *et al.*, 1989; Zanzottera and Bray, 1989; Davison and Bray, 1991). Fujikura and Karssen (1992) also concluded that in cauliflower seeds, the expression of certain proteins was enhanced during priming at 20°C. However, Dell'Aquila and Bewley (1989) concluded that protein synthesis decreased in the embryonic axes of pea seeds during priming at 25°C but increased upon their return to water, though not to the level exhibited by the axes germinated on water. These conflicting results in the above studies might have occurred due to different conditions (i.e. not only

temperatures but osmotica and priming periods, as well) used in the priming treatments.

Previously, Coolbear and Grierson (1979) showed that priming did not affect the DNA content of tomato seeds. However, recently, it has been shown that, DNA synthesis was detectable in leek embryos during the priming period in the absence of any cell division (Zanzottera and Bray, 1989; Bray *et al.*, 1989), but no DNA synthesis was detectable in leek endosperm tissue (Zanzottera and Bray, 1989). During priming, rRNA levels also increased in the embryos of tomato (Coolbear and Grierson, 1979), leek (Zanzottera and Bray, 1989; Clarke and James, 1991; Davison *et al.*, 1991) and peanut (Fu *et al.*, 1988). Bray *et al.* (1989) showed that the RNA content of leek embryos doubled during the priming period.

The above studies suggest that many metabolic processes become activated during priming but identification of those factors induce repair during priming still awaits conclusive substantive evidence.

Since repair of ageing induced damage is only possible at a critical moisture content which varies depending upon species (Ibrahim *et al.*, 1983; Ward and Powell, 1983; Petruzelli, 1986), when conducting post-storage priming treatments, it is essential that seeds are held above the

critical moisture content for considerably long periods of time and are properly ventilated (Bewley and Black, 1985). The critical seed moisture contents has been determined in some species, i.e. in lettuce, 15% (Ibrahim *et al.*, 1983), in onion, 18% (Ward and Powell, 1983), and in durum wheat, between 28 and 30% (Petruzelli, 1986). The critical moisture content varies between species due to oil content of the seed, i.e. in oily seeds it is lower relative to seeds low in lipid content. Since the oil content of pea seeds is very low, the critical moisture content should be very high compared to oily seeds (e.g. lettuce and onion). This study confirms Roberts' suggestion (personal communication, 1989) that the critical moisture content for pea seeds might be in the region of 30-40%. However, more work is needed to narrow the range of critical moisture content for pea seeds, which was identified as between 32% and 46% in this research.

The priming temperature also affects the rate of repair (see section 2.7.2). Although this study suggests that 16°C is the optimum temperature for the post-storage priming treatments of pea seeds, this needs to be investigated further before any categorical statement is made.

In summary, the results of the post-storage priming treatments in pea seeds confirmed the previous suggestion made by Rao (1986), working in lettuce, that priming treatments before sowing the seeds for regenerating accessions should be recommended as a general practice in gene banks (especially when the seeds have undergone considerable deterioration). Seed priming is not only useful for regeneration purposes but also reduces the incidence of heritable genetic damage which is manifested as phenotypic mutations.

8.4. Conclusions

For long term genetic conservation of orthodox seeds, the conditions recommended by the International Board for Plant Genetic Resources are that of dry storage at a moisture content of 5 ± 1% and a storage temperature of -18°C or less (IBPGR, 1976). Under the recommended conditions, even though loss of viability will be very slow; it is assumed that the accumulation of genetic damage with such losses of viability will nevertheless occur. This, together with the possibility of genetic selection in genetically heterogeneous accessions (because there is evidence that seed viability is, in part, genetically controlled) has led to recommendations by IBPGR, that accessions should not be allowed to fall below 85% viability before they are regenerated, i.e. a

fresh stock of seed is grown to replace the accession in gene banks (Roberts and Ellis, 1984).
This present study confirms that the accumulation of chromosomal aberrations is associated with even small losses in viability in both wild type and cultivated peas and it is therefore essential that the regeneration standard be kept high (Roberts and Ellis, 1984).

Results from these experiments on pea cultivars and wild types indicate that for a given loss of viability, there is no significant difference between high (10.1-15.1%) and low (4.7-5.1%) moisture content seed lots in terms of the mean frequency of chromosomal aberrations observed during first mitosis. However, these results conflict with previous findings in onion (Sirikwanchai, 1985) and lettuce (Rao, 1986) which showed more chromosomal aberrations in low moisture content seeds than those of high moisture content at an identical viability level. But, the present results are strong evidence against Vertucci and Roos (1990) that desiccation of pea seeds close to the low moisture content limit (i.e. 6.2%) results in an increase in their storage life.

Although cytological examination of chromosomal aberrations is indicative of genetic damage, it can not be used on its own to assess the problem. However, it is useful if conducted in parallel with investigations on

the induction of gene mutations. In view of the current interest in the use of ultra-dry seed storage, it is suggested that the effect of storage with low moisture contents should be studied in relation to loss of seed viability and the accumulation of genetic damage (both chromosomal aberrations and phenotypic mutations) in peas and a range of other species.

This study has shown that post-storage humidification treatments prevent imbibition injury, which is a common problem in very low moisture content seeds. However, the possible benefits of humidification should be further investigated over a range of seed moisture contents and ageing regimes in both pea cultivars and wild types.

Finally, this study shows that priming is an effective means of restoring viability and repairing age induced chromosomal damage in pea seeds.

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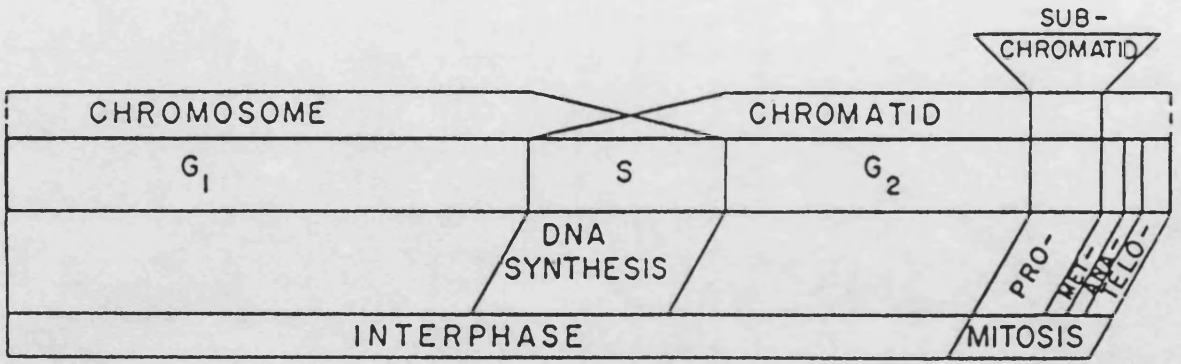
APPENDICES



Appendix 1. Diagrammatic representation illustrating the relationship between the types of aberration induced by ionizing radiations in relation to DNA synthesis and stage in development of mitotic cycle (from Evans, 1962).

Appendix 2. The relationship between viability and accumulation of chromosomal aberrations in pea seeds (cv. Kelvedon Wonder).

Stor. cond. (°C/%mc)	Stor. period (days)	Normal germ. (%)	Total no. of anaph. examined	Chromosomal Aberrations				Total no. of aber. anaph.	Total aber. cells (%)
				Chro-matid	Chro-mosome	Mixed	Others		
50/11.5	0	95.5	439	4.33				19	4.33
	18	78.0	563	7.64	0.71	0.36	1.78	59	10.49
	24	51.0	690	10.87	1.74	1.30	2.03	110	15.94
	30	32.0	611	14.73	2.78	0.66	2.46	126	20.63
65/4.7	0	96.0	482	3.11				15	3.11
	39	82.0	430	9.07	0.70		0.23	43	10.00
	50	67.0	489	11.04	0.82	0.21	0.62	62	12.69
	70	38.5	457	14.66	1.09	0.66	2.19	85	18.60

Appendix 3. The relationship between viability and accumulation of chromosomal aberrations in pea seeds (cv. Douce Provence).

Stor. cond. (°C/%mc)	Stor. period (days)	Normal germ. (%)	Total no. of anaph. examined	Chromosomal Aberrations				Total no. of aber. anaph.	Total aber. cells (%)
				Chro-matid	Chro-mosome	Mixed	Others		
50/15.1	0	96.5	662	2.87			0.45	22	3.32
	3.5	84.0	417	7.67			0.24	33	7.91
	4	79.5	563	7.81	0.36	0.18	0.18	48	8.53
	6	55.0	416	12.26	0.24	0.24	2.64	64	15.38
	7	21.0	422	20.38	1.42	0.71	2.84	107	25.36
65/4.8	0	94.0	667	3.60	0.15		0.15	26	3.90
	42	73.5	600	9.17	0.33		0.83	62	10.33
	84	44.0	640	13.13	1.41	0.31	1.25	103	16.10

Appendix 4. The relationship between viability and accumulation of chromosomal aberrations in wild type pea seeds (JI 181).

Stor. cond. (°C/%mc)	Stor. period (days)	Normal germ. (%)	Total no. of anaph. examined	Chromosomal Aberrations				Total no. of aber. anaph.	Total aber. cells (%)
				Chro-matid	Chro-mosome	Mixed	Others		
65/10.8	0	100.0	686	0.88				6	0.88
	5	97.0	501	3.59				18	3.59
	6	75.0	509	9.63	0.59	0.20	0.39	55	10.81
	7	36.0	516	16.28	1.16	0.58	0.78	97	18.80
65/5.1	0	100.0	709	0.99				7	0.99
	140	87.5	502	6.77				34	6.77
	154	76.0	505	10.89	0.20	0.20	1.39	64	12.68
	175	20.0	511	19.96	1.17	0.59	2.15	122	23.88

Appendix 5. The relationship between viability and accumulation of chromosomal aberrations in wild type pea seeds (JI 1104).

Stor. cond. (°C/%mc)	Stor. period (days)	Normal germ. (%)	Total no. of anaph. examined	Chromosomal Aberrations				Total no. of aber. anaph.	Total aber. cells (%)
				Chro-matid	Chro-mosome	Mixed	Others		
65/10.1	0	100.0	639	0.63				4	0.63
	6	95.0	557	5.93				33	5.93
	8	77.0	501	7.98	0.80	0.20	0.80	49	9.78
	10	42.0	542	14.02	1.48	0.74	1.11	94	17.35
65/5.1	0	100.0	743	0.94				7	0.94
	140	83.5	503	7.36	0.29		0.20	39	7.76
	154	63.0	554	9.57	0.72	0.18	0.90	63	11.37
	168	45.0	529	13.99	1.13	0.38	1.32	89	16.82

Appendix 6. The intercepts and slopes of the survival curves of high moisture content seed lots of pea cultivars and wild types aged under different storage conditions.

Storage Conditions	Intercepts	Slopes
K/W[a] 50°C/11.5% m.c.	1.945	-0.075
D/P[b] 50°C/15.1% m.c.	2.111	-0.359
JI 181 65°C/10.8% m.c.	4.084	-0.554
JI 1104 65°/10.1% m.c.	3.794	-0.387

[a] cv. "Kelvedon Wonder"

[b] cv. "Douce Provence"

Appendix 7. The intercepts and slopes of the survival curves of low moisture content seed lots of pea cultivars and wild types stored at 65°C (Bold values represent the best fitted lines for cultivars and wild types).

Storage Conditions	Intercepts	Slopes
K/W[a] 65°C/4.7% m.c.	1.813	-0.028
D/P[b] 65°C/4.8% m.c.	1.570	-0.020
	1.659	**-0.023**
JI 181 65°C/5.1% m.c.	4.115	-0.025
JI 1104 65°C/5.1% m.c.	4.074	-0.025
	4.096	**-0.025**

[a] cv. "Kelvedon Wonder"

[b] cv. "Douce Provence"

Appendix 8. The intercepts and slopes of the regression lines fitted for the relationship between the probit percentage normal germination and the probit total frequency of aberrant anaphase cells in cultivated and wild type pea seeds aged under different storage conditions (Bold values represent the best fitted line for cultivars and wild types).

Storage Conditions	Intercepts	Slopes
K/W[a] 50°C/11.5% m.c.	-0.988	-0.412
K/W 65°C/4.7% m.c.	-0.963	-0.480
D/P[b] 50°C/15.1% m.c.	-0.996	-0.449
D/P 65°C/4.8% m.c.	-1.030	-0.455
JI 181 65°C/10.8% m.c.	-1.025	-0.370
JI 181 65°C/5.1% m.c.	-0.994	-0.360
JI 1104 65°C/10.1% m.c.	-0.997	-0.381
JI 1104 65°C/5.1% m.c.	-1.056	-0.350
	-1.019	**-0.379**

[a] cv. "Kelvedon Wonder"

[b] cv. "Douce Provence"

Appendix 9. Types and frequencies of chromosomal aberrations observed in seeds of pea cv "Kelvedon Wonder" during storage under the conditions shown in Table 4.1.

Type of aberrations(*)	No of aberrations (%) at 4.7 % m.c.	11.5 % m.c.
Chromatid-type		
1F	79 (38.537)	81 (25.736)
2F	24 (11.707)	40 (12.739)
MF	27 (13.171)	16 (5.096)
1B	31 (15.122)	53 (16.879)
2B	6 (2.927)	3 (0.955)
MB	3 (1.463)	2 (0.637)
1B+1F	3 (1.463)	13 (4.140)
1B+2F	0 (0.000)	6 (1.911)
1B+MF	2 (0.976)	7 (2.229)
2B+1F	1 (0.488)	0 (0.000)
2B+2F	0 (0.000)	4 (1.274)
MB+2F	0 (0.000)	1 (0.318)
MB+MF	0 (0.000)	2 (0.637)
	176 (85.854)	227 (72.611)
Chromosometype		
DF	11 (5.366)	16 (5.096)
DB	1 (0.488)	15 (4.777)
2DB	0 (0.000)	1 (0.318)
DF+DB	0 (0.000)	1 (0.318)
	12 (5.854)	33 (10.509)
Mixed-type		
1F+DF	0 (0.000)	1 (0.318)
2F+DF	1 (0.488)	1 (0.318)
1F+DB	0 (0.000)	2 (0.637)
2F+DB	0 (0.000)	4 (1.274)
MF+DB	0 (0.000)	2 (0.637)
1B+DF	1 (0.488)	2 (0.637)
2B+DF	1 (0.488)	0 (0.000)
1B+DB	1 (0.488)	1 (0.318)
	4 (1.952)	13 (4.139)
Others		
LC	6 (2.927)	14 (4.459)
LC+1F	1 (0.488)	6 (1.911)
LC+2F	2 (0.976)	1 (0.318)
LC+MF	0 (0.000)	1 (0.318)
LC+DF	0 (0.000)	1 (0.318)
2LC+1F	1 (0.488)	0 (0.000)
2LC+2F	0 (0.000)	1 (0.318)
2LC+MF	2 (0.976)	1 (0.318)
2LC+DF	0 (0.000)	1 (0.318)
LC+1B	0 (0.000)	5 (1.592)
LC+MB	0 (0.000)	1 (0.318)
LC+DB	0 (0.000)	1 (0.318)
2LC+1B	1 (0.488)	0 (0.000)
LC+1B+1F	0 (0.000)	2 (0.637)
LC+2B+2F	0 (0.000)	2 (0.637)
LC+2B+MF	0 (0.000)	1 (0.318)
2LC+1B+MF+DF	0 (0.000)	1 (0.318)
1RC+1F	0 (0.000)	1 (0.318)
	13 (6.343)	40 (12.734)
Total	205 (100.003)	313 (99.993)

(*) Abbreviations:

F : Fragment D : Double LC : Lagging chromosome
B : Bridge M : Multiple RC : Ring chromosome

Appendix 10. Types and frequencies of chromosomal aberrations observed in seeds of pea cv "Douce Provence" during storage under the conditions shown in Table 4.1.

Type of aberrations	No. of aberrations (%) at 4.8 % m.c.	15.1 % m.c.
Chromatid-type		
1F	78 (40.838)	105 (38.321)
2F	33 (17.277)	47 (17.153)
MF	23 (12.042)	24 (8.759)
1B	17 (8.901)	23 (8.394)
2B	2 (1.047)	7 (2.555)
MB	0 (0.000)	4 (1.460)
1B+1F	6 (3.141)	12 (4.380)
1B+2F	3 (1.571)	4 (1.460)
1B+MF	1 (0.524)	4 (1.460)
MB+MF	0 (0.000)	1 (0.365)
	163 (85.341)	231 (84.307)
Chromosome-type		
DF	7 (3.665)	6 (2.190)
DB	5 (2.618)	3 (1.095)
	12 (6.283)	9 (3.285)
Mixed-type		
2F+DF	0 (0.000)	1 (0.365)
MF+DF	0 (0.000)	1 (0.365)
1B+DF	1 (0.524)	1 (0.365)
2B+DF	0 (0.000)	2 (0.730)
1B+DB	1 (0.524)	0 (0.000)
	2 (1.048)	5 (1.825)
Others		
LC	9 (4.712)	19 (6.934)
LC+1F	1 (0.524)	2 (0.730)
LC+2F	2 (1.047)	3 (1.095)
LC+MF	1 (0.524)	2 (0.730)
LC+1B	1 (0.524)	1 (0.365)
LC+1B+MF	0 (0.000)	1 (0.365)
MLC+1B	0 (0.000)	1 (0.365)
	14 (7.331)	29 (10.584)
Total	191 (100.003)	274 (100.001)

Appendix 11. Types and frequencies of chromosomal aberrations observed in seeds of pea wild type (JI 181) during storage under the conditions shown in Table 4.2.

Type of aberrations	No. of aberrations (%) at 5.1 % m.c.	No. of aberrations (%) at 10.8 % m.c.
Chromatid-type		
1F	108 (47.577)	87 (49.432)
2F	45 (19.824)	21 (11.932)
MF	19 (8.370)	8 (4.545)
1B	15 (6.608)	21 (11.932)
2B	5 (2.203)	6 (3.410)
MB	2 (0.881)	3 (1.705)
1B+1F	3 (1.324)	7 (3.977)
1B+2F	0 (0.000)	2 (1.136)
2B+1F	0 (0.000)	1 (0.568)
2B+2F	1 (0.441)	0 (0.000)
2B+MF	0 (0.000)	1 (0.568)
	198 (87.228)	157 (89.205)
Chromosome-type		
DF	4 (1.762)	2 (1.136)
DB	3 (1.324)	7 (3.977)
	7 (3.086)	9 (5.113)
Mixed-type		
1F+DF	3 (1.322)	0 (0.000)
2F+DF	1 (0.441)	0 (0.000)
2F+DB	0 (0.000)	2 (1.136)
MF+DF	0 (0.000)	1 (0.568)
2B+DB	0 (0.000)	1 (0.568)
	4 (1.763)	4 (2.272)
Others		
LC	10 (4.405)	3 (1.705)
LC+1F	1 (0.441)	0 (0.000)
LC+2F	0 (0.000)	1 (0.568)
LC+MF	5 (2.203)	1 (0.568)
2LC	1 (0.441)	0 (0.000)
2LC+2F	0 (0.000)	1 (0.568)
LC+1B	1 (0.441)	0 (0.000)
	18 (7.931)	6 (3.409)
Total	227 (100.008)	176 (99.999)

Appendix 12. Types and frequencies of chromosomal aberrations observed in seeds of pea wild type (JI 1104) during storage under the conditions shown in Table 4.2.

Type of aberrations	No. of aberrations (%) at 5.1 % m.c.	No. of aberrations (%) at 10.1 % m.c.
Chromatid-type		
1F	79 (39.899)	74 (41.111)
2F	33 (16.667)	20 (11.111)
MF	22 (11.111)	9 (5.000)
1B	21 (10.606)	33 (18.333)
2B	7 (3.535)	5 (2.778)
MB	0 (0.000)	2 (1.111)
1B+1F	3 (1.515)	2 (1.111)
1B+2F	0 (0.000)	4 (2.222)
1B+MF	3 (1.515)	1 (0.556)
2B+1F	1 (0.505)	2 (1.111)
2B+2F	1 (0.505)	1 (0.556)
	170 (85.858)	153 (85.000)
Chromosome-type		
DF	5 (2.525)	6 (3.333)
DB	7 (3.535)	6 (3.333)
	12 (6.060)	12 (6.666)
Mixed-type		
1F+DF	0 (0.000)	2 (1.111)
2F+DF	0 (0.000)	1 (0.556)
MF+DF	1 (0.505)	0 (0.000)
1F+DB	0 (0.000)	1 (0.556)
1B+DF	2 (1.010)	0 (0.000)
2B+DF	0 (0.000)	1 (0.556)
	3 (1.515)	5 (2.779)
Others		
LC	6 (3.030)	3 (1.667)
LC+1F	3 (1.515)	2 (1.111)
LC+2F	0 (0.000)	1 (0.556)
LC+MF	3 (1.515)	1 (0.556)
LC+1B	1 (0.505)	2 (1.111)
LC+1B+1F	0 (0.000)	1 (0.556)
	13 (6.565)	10 (5.557)
Total	198 (99.998)	180 (100.002)

Appendix 13. The effect of humidification in a saturated atmosphere at 16°C on the changes in the frequency of chromosomal aberrations in pea seeds (cv. Douce Provence) with an initial moisture content of 4.8 per cent.

Type of aberrations	No. of aberrations (%) at 4.8% m.c.	
	Non-humidified	Humidified at 16°C
Chromatid-type		
1F	44 (3.472)	24 (1.932)
2F	14 (1.105)	9 (0.724)
MF	11 (0.868)	0 (0.000)
1B	4 (0.316)	10 (0.805)
2B	1 (0.079)	0 (0.000)
1B+1F	4 (0.316)	4 (0.322)
1B+2F	1 (0.079)	1 (0.081)
1B+MF	0 (0.000)	1 (0.081)
	79 (6.235)	49 (3.945)
Chromosome-type		
DF	3 (0.237)	0 (0.000)
DB	1 (0.237)	0 (0.000)
	4 (0.316)	0 (0.000)
Others		
LC	2 (0.158)	0 (0.000)
LC+MF	1 (0.079)	0 (0.000)
LC+1B	1 (0.079)	0 (0.000)
LC+2B	1 (0.079)	1 (0.081)
	5 (0.395)	1 (0.081)
Total no. of aberrant cells	88 (6.946)	50 (4.026)
Total no. of anaphases analysed	1267 (100.0)	1242 (100.0)

Appendix 14. The effect of humidification in a saturated atmosphere at 16°C on the changes in the frequency of chromosomal aberrations in pea seeds (cv. Douce Provence) with an initial moisture content of 15.1 per cent.

Type of aberrations	No. of aberrations (%) at 15.1 % m.c.	
	Non-humidified	Humidified at 16°C
Chromatid-type		
1F	28 (2.286)	14 (1.126)
2F	11 (0.898)	5 (0.402)
MF	7 (0.571)	5 (0.402)
1B	5 (0.408)	9 (0.724)
2B	4 (0.327)	1 (0.080)
MB	1 (0.082)	0 (0.000)
1B+1F	7 (0.571)	0 (0.000)
1B+2F	0 (0.000)	6 (0.483)
1B+MF	0 (0.000)	2 (0.161)
MB+MF	0 (0.000)	1 (0.080)
	63 (5.143)	43 (3.458)
Chromosome-type		
DF	2 (0.163)	0 (0.000)
	2 (0.163)	0 (0.000)
Mixed		
DF+2F	1 (0.082)	0 (0.000)
DF+1B+2F	0 (0.000)	1 (0.080)
	1 (0.082)	1 (0.080)
Others		
1LC	3 (0.245)	1 (0.080)
1LC+MF	1 (0.082)	0 (0.000)
	4 (0.327)	1 (0.080)
Total no. of aberrant cells	70 (5.714)	45 (3.618)
Total no. of anaphases analysed	1225 (100.0)	1243 (100.0)

Appendix 15a. Germination percentages of aged pea seeds primed at 16°C in different osmotica for varying periods compared with non-primed (control) seeds.

	R E P L I C A T E S				
TREATMENTS	1	2	3	4	MEANS
CONTROL	84	86	86	80	84.0
H_2O 3d	92	94	86	91	91.0
H_2O 5d	90	84	96	94	91.0
H_2O 7d	90	86	88	92	89.0
$PEG_{0.5}$ 3d	88	86	90	88	88.0
$PEG_{0.5}$ 5d	86	86	90	80	88.5
$PEG_{0.5}$ 7d	82	84	82	76	81.0
$PEG_{1.0}$ 3d	88	90	94	86	89.5
$PEG_{1.0}$ 5d	82	78	80	84	81.0
$PEG_{1.0}$ 7d	48	62	64	68	60.5
$PEG_{1.2}$ 3d	86	86	80	80	83.0
$PEG_{1.2}$ 5d	78	86	84	80	82.0
$PEG_{1.2}$ 7d	78	84	86	80	82.0
ABA 3d	86	94	90	90	90.0
ABA 5d	80	86	84	78	82.0
ABA 7d	70	80	76	74	75.0

Appendix 15b. Arcsin transformed values of the percentage normal germination of aged pea seeds primed at 16°C in different osmotica for varying periods compared with non-primed (control) seeds.

	R E P L I C A T E S				
TREATMENTS	1	2	3	4	MEANS
CONTROL	66.4	68.0	68.0	63.4	66.4
H_2O 3d	73.6	75.8	68.0	72.5	72.5
H_2O 5d	71.6	66.4	78.5	75.8	72.5
H_2O 7d	71.6	68.0	69.7	73.6	70.6
$PEG_{0.5}$ 3d	69.7	68.0	71.6	69.7	69.7
$PEG_{0.5}$ 5d	68.0	68.0	71.6	63.4	67.6
$PEG_{0.5}$ 7d	64.9	66.4	64.9	60.7	64.2
$PEG_{1.0}$ 3d	69.7	71.6	75.8	68.0	71.1
$PEG_{1.0}$ 5d	64.9	62.0	63.4	66.4	64.2
$PEG_{1.0}$ 7d	43.9	51.9	53.1	55.6	51.1
$PEG_{1.2}$ 3d	68.0	68.0	63.4	63.4	65.7
$PEG_{1.2}$ 5d	62.0	68.0	66.4	63.4	64.9
$PEG_{1.2}$ 7d	62.0	66.4	68.0	63.4	64.9
ABA 3d	68.0	75.8	71.6	71.6	71.6
ABA 5d	63.4	68.0	66.4	62.0	64.9
ABA 7d	56.8	63.4	60.7	59.3	60.0

$$LSD_{0.05} = t_{0.05} \sqrt{2\ MSE\ /\ n}$$
$$= 2.014 \times 2.117$$
$$= 4.264$$

Appendix 15c. The results of Completely Randomized Blocks ANOVA and the LSD value at 5% significance level for the arcsin transformed values of percentage normal germination.

---------- ANALYSIS OF VARIANCE ----------

HEADER DATA FOR: C:PRIMING1 LABEL: NORMAL GERM. (ARCSIN)
NUMBER OF CASES: 4 NUMBER OF VARIABLES: 16

RANDOMIZED BLOCKS ANOVA

GERMINATION TEST RESULTS OF PRIMED PEA SEEDS

TREATMENT	MEAN (%)	MEAN (TRANSF.)		N
CONTROL	84.0	66.450	cd	4
H_2O - 3D	91.0	72.475	a	4
- 5D	91.0	73.075	a	4
- 7D	89.0	70.725	ab	4
PEG_1 - 3D	88.0	69.750	abc	4
- 5D	85.5	67.750	bcd	4
- 7D	81.0	64.225	de	4
PEG_2 - 3D	89.5	71.275	ab	4
- 5D	81.0	64.175	de	4
- 7D	60.5	51.125	f	4
PEG_3 - 3D	83.0	65.700	cd	4
- 5D	82.0	64.950	d	4
- 7D	82.0	64.950	d	4
ABA - 3D	90.0	72.175	a	4
- 5D	82.0	64.950	d	4
- 7D	75.0	60.050	e	4

BLOCK	MEAN	N
1	65.388	16
2	67.231	16
3	67.569	16
4	65.763	16
GRAND MEAN	66.488	64

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F RATIO	PROB.
TREATMENT	1841.340	15	122.756	13.695	4.4E-12
BLOCK	55.326	3	18.442	2.057	0.119
ERROR	403.364	45	8.964		
TOTAL	2300.030	63			

$$LSD_{0.05} = t_{0.05} \sqrt{2\ MSE\ /\ N}$$
$$= 2.014 \times 2.117$$
$$= 4.264$$

$\therefore$ Values not associated with the same letter are significantly different ($P < 0.05$).

Appendix 16a. MGT values of aged pea seeds primed at 16°C in different osmotica for varying periods compared with non-primed (control) seeds.

	R E P L I C A T E S				
TREATMENTS	1	2	3	4	MEANS
CONTROL	4.93	4.88	5.00	5.20	5.00
H_2O 3d	4.37	4.13	4.00	4.17	4.17
H_2O 5d	4.36	4.17	3.79	3.87	4.05
H_2O 7d	3.98	3.70	3.54	3.80	3.76
$PEG_{0.5}$ 3d	4.41	4.33	4.33	4.39	4.37
$PEG_{0.5}$ 5d	4.33	4.21	4.38	4.55	4.37
$PEG_{0.5}$ 7d	4.24	4.36	4.36	4.58	4.39
$PEG_{1.0}$ 3d	4.68	4.64	4.57	4.79	4.67
$PEG_{1.0}$ 5d	5.03	4.72	4.52	4.67	4.74
$PEG_{1.0}$ 7d	5.25	4.81	5.06	4.71	4.96
$PEG_{1.2}$ 3d	4.49	4.72	4.22	4.37	4.45
$PEG_{1.2}$ 5d	4.82	4.74	4.86	4.57	4.75
$PEG_{1.2}$ 7d	5.31	4.92	4.44	4.47	4.79
ABA 3d	4.64	4.47	4.60	4.58	4.57
ABA 5d	5.10	4.60	4.81	4.69	4.80
ABA 7d	5.49	4.63	4.55	5.19	4.97

$$LSD_{0.05} = t_{0.05} \sqrt{2\ MSE\ /\ n}$$
$$= 2.014 \times 0.138$$
$$= 0.278$$

Appendix 16b. The results of Completely Randomized Blocks ANOVA and the LSD value at 5% significance level for the MGT values.

---------- ANALYSIS OF VARIANCE ----------

HEADER DATA FOR: C:PRIMING2 LABEL: MGT VALUES
NUMBER OF CASES: 4 NUMBER OF VARIABLES: 16

RANDOMIZED BLOCKS ANOVA

MGT VALUES OF PRIMED PEA SEEDS

TREATMENT	MEAN	N
CONTROL	5.003 g	4
H_2O - 3D	4.168 bc	4
- 5D	4.055 b	4
- 7D	3.755 a	4
PEG_1- 3D	4.365 cd	4
- 5D	4.368 cd	4
- 7D	4.385 cd	4
PEG_2- 3D	4.670 ef	4
- 5D	4.735 fg	4
- 7D	4.958 g	4
PEG_3- 3D	4.450 de	4
- 5D	4.748 fg	4
- 7D	4.785 fg	4
ABA - 3D	4.573 def	4
- 5D	4.800 fg	4
- 7D	4.965 g	4

BLOCK	MEAN	N
1	4.716	16
2	4.502	16
3	4.439	16
4	4.538	16
GRAND MEAN	4.549	64

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F RATIO	PROB.
TREATMENT	7.508	15	0.501	13.130	9.0E-12
BLOCK	0.677	3	0.226	5.924	1.7E-03
ERROR	1.715	45	0.038		
TOTAL	9.901	63			

$$LSD_{0.05} = t_{0.05} \sqrt{2\ MSE\ /\ N}$$
$$= 2.014 \times 0.138$$
$$= 0.278$$

$\therefore$ Values not associated with the same letter are significantly different ($P < 0.05$).

Appendix 17a. CVG values of aged pea seeds primed at 16°C in different osmotica for varying periods compared with non-primed (control) seeds.

	R E P L I C A T E S				
TREATMENTS	1	2	3	4	MEANS
CONTROL	20.29	20.48	20.00	19.23	20.00
H_2O 3d	22.89	24.23	25.00	23.96	24.02
H_2O 5d	22.96	24.00	26.37	25.82	24.79
H_2O 7d	25.14	27.04	28.21	26.29	26.67
$PEG_{0.5}$ 3d	22.68	23.12	23.08	22.80	22.92
$PEG_{0.5}$ 5d	23.12	23.76	22.84	21.98	22.93
$PEG_{0.5}$ 7d	23.56	22.95	22.91	22.84	22.82
$PEG_{1.0}$ 3d	21.36	21.53	21.86	20.87	21.41
$PEG_{1.0}$ 5d	19.90	21.20	22.10	21.43	21.16
$PEG_{1.0}$ 7d	19.05	20.81	19.75	21.25	20.22
$PEG_{1.2}$ 3d	22.28	21.18	23.67	22.86	22.50
$PEG_{1.2}$ 5d	20.74	21.08	20.59	21.86	21.07
$PEG_{1.2}$ 7d	18.84	20.34	22.51	22.35	21.01
ABA 3d	21.57	22.38	21.74	21.84	21.88
ABA 5d	19.61	21.72	20.79	21.31	20.86
ABA 7d	18.23	21.62	21.97	19.27	20.27

$$LSD_{0.05} = t_{0.05} \sqrt{2\ MSE\ /\ n}$$
$$= 2.014 \times 0.636$$
$$= 1.281$$

Appendix 17b. The results of Completely Randomized Blocks ANOVA and the LSD value at 5% significance level for the CVG values.

---------- ANALYSIS OF VARIANCE ----------

HEADER DATA FOR: C:PRIMING3 LABEL: CVG VALUES
NUMBER OF CASES: 4 NUMBER OF VARIABLES: 16

RANDOMIZED BLOCKS ANOVA

CVG VALUES OF PRIMED PEA SEEDS

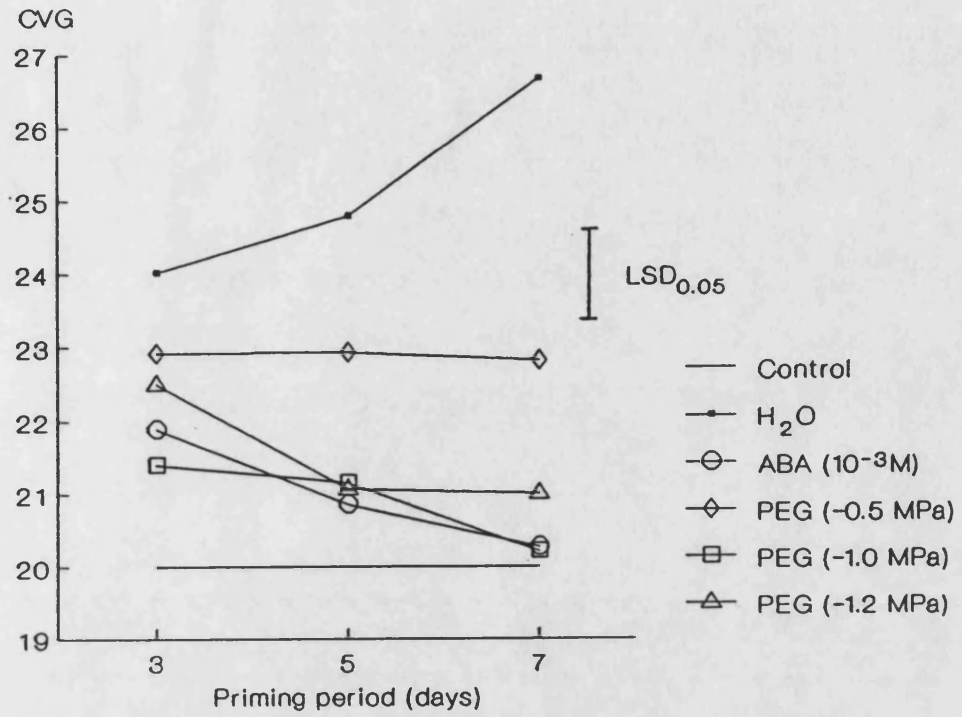
TREATMENT	MEAN	N
CONTROL	20.000 h	4
H_2O - 3D	24.020 bc	4
- 5D	24.788 b	4
- 7D	26.670 a	4
PEG_1- 3D	22.920 cd	4
- 5D	22.925 cd	4
- 7D	22.815 cd	4
PEG_2- 3D	21.405 efg	4
- 5D	21.158 fgh	4
- 7D	20.215 gh	4
PEG_3- 3D	22.498 de	4
- 5D	21.068 fgh	4
- 7D	21.010 fgh	4
ABA - 3D	21.883 def	4
- 5D	20.858 fgh	4
- 7D	20.273 gh	4

BLOCK	MEAN	N
1	21.389	16
2	22.340	16
3	22.712	16
4	22.185	16
GRAND MEAN	22.156	64

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F RATIO	PROB.
TREATMENT	201.140	15	13.409	16.593	1.4E-13
BLOCK	14.918	3	4.973	6.153	1.3E-03
ERROR	36.367	45	0.808		
TOTAL	252.424	63			

$$LSD_{0.05} = t_{0.05} \sqrt{2\ MSE\ /\ N}$$
$$= 2.014 \times 0.636$$
$$= 1.281$$

$\therefore$ Values not associated with the same letter are significantly different ($P < 0.05$).



Appendix 17c. The influence of post-storage priming treatments on the coefficient of velocity of germination (CVG) of pea seeds (cv. Douce Provence) which had 84% initial normal germination (Vertical bar, LSD = 1.281; $P < 0.05$).