Physiological and anatomical studies in seed coat regulation of water uptake in soybeans (*Glycine max* L. Merril)

DEMOSTHENIS HAHALIS

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Abstract

This study examined i) the imbibition behaviour of a wide range of genotypes with different seed coat characteristics; ii) the use of a polymer to regulate the rate of water uptake and iii) the mechanism of regulation of water uptake by the seed coat in soybeans (*Glycine max* L. Merril.). Seed coat structure was studied by using light, fluorescence and scanning electron microscopy. The effects of different methanol and chloroform pre-treatments on seed coat permeability to water were also assessed.

In this study, imbibition damage due to rapid water uptake was well documented in a wide range of soybean genotypes. In addition a line (VLS-1) was identified that possessed a delayed-permeability seed coat characteristic that offered protection against imbibition damage. This characteristic was likely to be due to a lack of pits in the abaxial region of the seed. In contrast, genotypes with a high proportion of deep and wide open pits in the abaxial region of the seed offered minimal protection against imbibition damage.

Coating seeds (24 mg per seed) with a polymer containing vinyl acetate, vinyl chloride, ethylene and acrylate regulated the rate of water uptake, and offered protection against imbibition damage. Seedling emergence from polymer coated seeds was also improved.

Deposits and pits occurred in the surface of the seed coat in most genotypes. Deposits were shown to be composed of hydrophilic polysaccharide material, since staining with calcofluor was observed. Water permeability mapping indicated that pits were the sites of the initial water penetration. However, in hard seeds, pits appeared to function in a different way to soft seeds and this is fully discussed within the thesis.

Prolonged methanol pre-treatments were highly effective in increasing the water uptake when seeds were imbibed immediately after the pre-treatments. However, drying of seeds after the organic solvent pre-treatments restored permeability to water to untreated control levels. Results from the absorption spectrum of the methanol and chloroform supernatants, indicated that the effect of the pre-treatments were not due to the extraction of UV-absorbed material from the seed coat. In hard seeds, the location of the water impermeability barrier was near the outermost part of the palisade cell layer. The nature of the barrier was not identified by comparative anatomical and histochemical studies between hard and soft seeds.

A mechanism for the water uptake regulation by the soybean seed coat was proposed. The proposed mechanism involved: i) a diminished role of the cuticle and its components (epicuticular and intracuticular waxes), ii) a key role for pits as initial sites of water penetration, and iii) swelling or collapse of the cellulosic and/or pectic material in the subcuticular and palisade cell layer that could regulate water penetration through the seed coat. The common behaviour of a wide range of genotypes tested indicated that the above mechanism of regulation of water uptake by the seed coat is likely to be universal in soybeans.

Declaration

I hereby declare that this thesis is of my own composition, and that all assistance has been duly acknowledged. The results presented herein have not previously been submitted for any other degree or qualification.

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CHAPTER 1

General Introduction

1.1. Importance of soybeans

Soybean (*Glycine max* L. Merril.) is a member of the family Leguminosae, sub-family Papilionideae and the genus Glycine (Hinson and Hartwing, 1982). It probably originated from *Glycine soja* which grows wild in the northern and north-eastern provinces of China, adjacent areas of Russia, Korea and Japan (Hinson and Hartwing, 1982). Flowers are borne on short racemes originating in the leaf axis; each raceme bears 3 to 15 small, purple, pink or white flowers. Self pollination is the rule with only about 1% of cross pollination (Weiss, 1983). Soybean seeds are borne in pods, each pod contains about 4 seeds. Seed shape varies from spherical to flattened and elongated. Seed coat varies from yellow to black but most cultivated genotypes are yellow seeded. Seed weight in cultivated genotypes varies from 100mg to 200 mg per seed (Hinson and Hartwig, 1982).

Soybean seeds sent from China by missionaries were planted at the Royal Botanic Garden Kew, London, in 1790 (Morse, 1950). Soybean was introduced into the United States in 1804 but in the early stages, it was mostly grown as a forage crop (Hammond *et al.*, 1951). It was around 1920's when the Americans realised its food value and started harvesting the crop for grain (Probst and Judd, 1973). As a result, there has been a continuous increase in the cropped area which resulted in doubled world production between 1969 and 1982, mainly because of increases in output from the USA and Brazil (Hume *et. al.*, 1985). Four countries, namely USA, Brazil, China and Argentina, account for about 90 to 95% of the world soybean production (Smith and Huyser, 1987).

The grain is mainly composed of proteins, lipids, carbohydrates and minerals with proteins and oil being the most important constituents. Most of the improved varieties contain about 40% protein, about 20% oil, about 34% carbohydrate and 5% ash on a dry weight basis (Orthoefer, 1978). The grain composition makes soybean an excellent source of protein and oil, in contrast with cereals which contain almost no oil and about 8 to 20% protein on a dry weight basis (Hardy and Havelka, 1975).

Soybean meal and oil are the most traded and utilised meal and vegetable oil products in the world: soybean oil accounts for about 20% of total world fats and oil production and about 30% of total edible vegetable oil production, more than any other single vegetable or animal source (Smith and Huyser, 1987). Its oil is made up of both saturated (palmitic, stearic) and unsaturated fatty acids (linoleic, oleic and linolenic) (Hinson and Hartwig, 1982). In contrast, a high percent of the meal remaining after the extraction of oil is used as a protein supplement in livestock and poultry feeds worldwide. Soy-protein in common with other legumes is low in methionine and cysteine but high in tryptophane (Hinson and Hartwig, 1982). Soybean seeds are the cheapest source of good quality edible plant protein (Cambell 1979). Additionally, soybean meal is used to a smaller extent as a food either in the Orient which is part of the local diary or elsewhere in the world as a meat substitute or protein enrichment.

Although demand for soybean meal is mainly in developed countries, several tropical and subtropical countries have been trying to introduce developed varieties or modify existing management systems for a large-scale soybean production. In the tropics, soybean meal could be used as a protein supplement to increase the protein content of traditional dishes such as maize or porridge (IITA, 1987). Soybean could also play an important role in bridging protein malunitrion. In West Africa, for example, great efforts have been made to grow soybean on a large scale in the Ivory Coast, Ghana, Nigeria and Cameroon (Nangju *et al.*, 1980). However, soybean introduction to the lowland tropics has met with little success (Singh and Rachie, 1987). In Pakistan, poor storage environment and lack of expertise during planting has acted as a disincentive to more extensive soybean cultivation, although the country is faced with a serious shortage of edible oil and the crop is considered to be suitable for about 1.5 million hectares (Ehsanullah, 1993).

1.2. Seed quality

Seed quality is a multi-component concept namely analytical purity, seed health, germination and vigour. Several laboratory tests are needed to assess seed quality with analytical purity, seed health and germination being determined in routine tests (Perry, 1980). A farmer perceives seed quality as a component required in order to produce vigorous seedlings, and then an established plant and ultimately an economic yield (Carver, 1980). Crop failures caused by poor seed quality have been well

documented over many years. In soybeans, the most severe quality problems relate to low germination and vigour and the single most recognised and accepted index of seed quality is the ability of seeds to germinate (Copeland and MacDonald, 1985).

1.2.1. Seed viability

Seed viability is the ability of seeds to germinate. However, there is no common measurement of seed germination. To the seed physiologist, a viable seed would be one that a clear radicle protrusion through the seed coat is manifested (Copeland and McDonald, 1985). To the seed analyst the definition of germination is "the emergence and development from the seed embryo of those essential structures which are indicative of the ability to produce a normal plant under favourable conditions" (Copeland and MacDonald, 1985). In other words, the criterion of viability is the production of normal seedlings rather than the germination itself.

The main aim of the standard germination test is to determine the practical value of a seed lot under controlled and standardised conditions (Wellington, 1965). However, under adverse field conditions, the results from laboratory tests over-predict field emergence by varying percentages depending on the relative adversity of soil conditions (Athow and Caldwell, 1956; TeKrony and Egli, 1977). Another problem with standard germination tests is that differences in the magnitude of 5% between lots are often not statistically different, although could reflect great differences in other performance characteristics (Ellis and Roberts, 1980). Finally, the loss of capacity to germinate is almost the last event that happens as seed deteriorates and ultimately dies (Priestley, 1986). Therefore, the standard germination test does not take into account the very substantial loss in performance potential that occurs before germination capacity is lost.

1.2.2. Seed vigour

In general, seed vigour is the potential for growth and the ability to sustain life. Over the last two decades, seed vigour has become recognised as an important quality constituent. Although the term seed vigour has been used for a long time, particularly during the 1970's, there was no agreed meaning before the International Seed Testing Association (ISTA) adopted the following definition: "the sum total of all those

properties of seed which determine the level of activity and performance of the seed lot during germination and seedling emergence" (Perry, 1978). McDonald (1980) defined vigour as those properties that determine the potential for rapid and uniform seedling emergence over a range of field conditions.

Despite differences of opinion on its definition there is a general agreement that vigour measurements can be used for predicting the potential performance in the field of soybeans (Pollock and Roos, 1972; Yaklich and Kulik, 1979; Oliveira *et al.*, 1984; Loeffler *et al.*, 1988). Heydecker (1972) reported that soybean seed vigour may show its effects at survival in storage, emergence in the field, establishment of mature plants and production of full yield at the end of the plants life cycle. However, Egli and TeKrony (1979) observed that high vigour seed lot resulted in improved crop stands but not necessarily high yield. Over the years, the identification of the vigour level of seeds has been a prime target of research, and therefore several tests have been developed. McDonald (1980) suggested that any vigour test should be inexpensive, rapid, uncomplicated, reproducible and correlated with field performance. Vigour tests could be broadly grouped in three main categories: physical, physiological and biochemical.

Physical tests

Within this category are those that measure the effect of seed size and weight upon the rate of seedling emergence (Woodstock, 1969; Perry, 1969; McDonald, 1975).

Physiological tests

Physiological tests may be carried out under favourable or unfavourable conditions. Under favourable conditions, the most common test is the measurement of speed of germination or the seedling growth (Perry, 1980). Under unfavourable conditions, the cold test has been widely used in maize (McDonald, 1975) and soybeans (Johnson and Wax, 1978; Perry, 1980). Other stress tests, involve measurement of germination after exposure of seeds to ageing conditions. Accelerating ageing is the test where seeds are rapidly aged by exposing them to hot and humid environment (e.g. 41°C and 100% relative humidity). In soybeans, the accelerating ageing test has been used to estimate longevity in storage (Delouche and Baskin, 1973), seed vigour during seed production (Rasyad *et al.*, 1990) and to predict field emergence (TeKrony and Egli, 1977). Controlled deterioration test involves the ageing under stable seed moisture and temperature conditions

(Matthews, 1980). Controlled deterioration has been widely used by Ellis and colleagues to develop the viability equation for predicting seed deterioration (Ellis and Roberts, 1980).

Biochemical tests

Vigour tests within this group are based on the measurement of chemical reactions or processes related to seed germination. These include vital staining reactions, enzyme activity, processes involved in biosynthesis and measurement of membrane integrity (Woodstock, 1973). The tetrazolium test and the electrical conductivity test are the most widely used biochemical tests.

The tetrazolium test is based on a vital staining reaction which occurs when the reduction of the colourless 2,3,5 tri-phenyl tetrazolium chloride by the redox dehydrogeneases of living seed tissue results to a formation of red formazan (Cottrel, 1948). The test is considered as a powerful diagnostic tool in the detection of low viability due to ageing or other causes such as mechanical or heating damage to seeds (Woodstock, 1973). In addition, the staining with tetrazolium chloride was used as a seed vigour indicator to illustrate that seeds with injured cotyledons were susceptible to adverse conditions in the field leading to poor emergence (Moore, 1973). It was later, that a close association between the poor staining of the cotyledons and the high solute leakage in low vigour pea seeds was observed (Matthews and Rogerson, 1976). Staining of the cotyledons with tetrazolium chloride has been widely used to reveal imbibition damage in many grain legumes, as proposed by Powell and Matthews (1978).

The conductivity test is based on the measurement of the electrical conductivity of the water in which seeds have been soaked (Perry, 1969). Matthews and Bradnock (1967, 1968) first used this test was as a routine vigour test for peas. Seed lots with high conductivity readings were found to have poor field emergence and vigour whereas those with low conductivity measurements had a high vigour and seedling emergence (Matthews and Bradnock, 1967). Over the years, the conductivity test has been widely used to stress the negative relationship between leakage of electrolytes and low vigour in many grain legumes, including peas (Perry, 1970; Bedford, 1974; Rowland, 1981), soybeans (Yaklich *et al.*, 1979; Oliveira *et al.*, 1984), chickpeas (Legesse and Powell, 1992), French beans (Powell *et al.*, 1986a) and faba beans (Kantar *et al.*, 1996).

1.2.3. Seed characteristics related to viability and vigour

Seed size is frequently adopted as a quality characteristic in soybeans although the implications and practical significance of seed size to seed quality are poorly understood. There is some contradiction in the literature in relation to the effect of seed size on vigour, germination, seedling growth and establishment.

According to Heydecker (1972), larger seeds with more "initial capital" often have at least an initial advantage over smaller seeds. However, different soybean seed sizes within cultivars had little or no effect on germination, field emergence and initial plant stand (Singh *et al.*, 1972; Johnson and Lueders, 1974; Smith and Camper, 1975). Edward and Hartwig (1971) used isogenic soybean lines differing in seed size and found that the small (95 mg per seed) and medium (135 mg per seed) gave more rapid emergence and greater root development than the larger seeds (225 mg per seed). This result was consistent at different soil moisture contents at which germination occurred. In agreement, Green *et al.* (1965) reported that genotypes with small seeds were generally associated with larger germinability and seedling emergence than genotypes with large seeds in fields in Missouri, USA. A possible explanation for the poor emergence of large seeds may be that the large cotyledons provide excessive resistance in medium and heavy soils, and are more susceptible to mechanical damage during handling in comparison to the smaller seeds (Shibles *et al.*, 1975). Hoy and Gamble (1985) studied the seed performance of different sizes in 18 different lots of three different cultivars. They reported that the largest seeds had the lowest percentage of normal seedlings and highest leakage conductivity values.

Egli *et al.*, (1990) compared the effect of seed size on the seedling growth within similar range of vigour levels (as measured by using the accelerating ageing test). They reported that larger seeds produced heavier seedlings than the small seeds but seed size had no effect on seedling growth rate. Burris (1973) reported that the largest seeds within seed lots exhibited superior seedling emergence and greater leaf area than small seeds. Fontes and Ohlrogge (1972) reported that in field conditions larger seeds within seed lots produced fewer barren plants compared to small seeds. Hopper *et al.*, (1979) reported that although the smallest seeds within a seed lot germinated more slowly than the larger seeds, in sand media the smaller emerged faster than the largest seeds.

1.3. The role of ageing in seed quality

Earlier reports suggested that some of the important manifestations of seed ageing were changes in seed colour, delay in germination, susceptibility to adverse storage conditions, higher sensitivity to radiation treatments, reduced growth of seedlings and increased number of abnormal seedlings (Toole *et al.*, 1948).

In general, as seeds age, they maintain germinability for some time; then enter a period decline during which some fail to germinate while others germinate and grow normally (Abdul-Baki and Anderson, 1972). The time which marked the first detectable decline in germinability did not coincided with the actual beginning of deterioration; a great decline in synthesis of carbohydrates and proteins occurred while the germinability still remained unchanged (Abdul-Baki, 1969). Although no physiological interpretation of the characteristic germinability-decay curve has been firmly established, one attractive hypothesis has been advanced by Roberts *et al.* (1967). According to this hypothesis, the germination of a seed is dependent on the proper functioning of a relatively large number of key cells in the embryo. The hypothesis assumes that some of these key cells are rendered non-functional during deterioration by some unspecified factor. The interaction of this debilitating process with the cellular population is describable in terms of a Poisson distribution.

Ageing causes an irreversible degenerative change in the quality of seeds after it attains its maximum quality level (Coopeland and McDonald, 1985). According to Roberts (1972) the membrane damage is the earliest biochemical indication of the onset of ageing which is followed by a sequence of events such as impaired biosynthesis which caused slower growth and a greater susceptibility to environmental stress resulting in poor emergence, morphological aberrations and finally the lost ability of seed to germinate. Ageing is recongnised as a major cause of differences in the vigour. Soybean seeds could suffer considerable deterioration before harvest in the mother plant depending on the climatic conditions (Green *et al.*, 1965; TeKrony *et al.*, 1980; Ellis *et al.*, 1987), during and immediately after harvest (Green *et al.*, 1966; Lueders and Burris, 1979; TeKrony *et al.*, 1987), and during the storage (Roberts, 1986).

1.4. The role of imbibition in seed quality

Germination and seedling growth require energy and molecular building blocks (substrates) for synthesis of the new tissues. Energy and substrates are obtained by enzyme-catalysed metabolic processes in the tissues of the germinating seeds (Woodstock, 1988). Water is essential for cellular metabolism for at least three reasons: a) for enzyme activity, b) solubilization and transport of reactants and c) as a reactant (Priestley, 1986). According to Woodstock (1988), although necessary imbibition is a period of peril. The ability of the seed to traverse this period successfully depends on the inherent soundness and vigour of the seed (Woodstock, 1988). Vertucci (1989) suggested that the rate of water penetration was critical to the success of subsequent germination. If water uptake is too slow, germination is reduced perhaps because of fungal infection or accelerated deterioration. If water uptake is too rapid, seeds are subject to imbibition damage (Vertucci, 1989).

Larson (1968) investigated the effect of the rate of water uptake during imbibition on subsequent seed performance. He reported that when pea embryos (seeds without the coats) were imbibed in water showed an increased solute leakage and reduced seedling growth compared to seeds which were imbibed with the seed coat present. However, it was much later that the phenomenon was properly recognised when pea embryos (seeds without the seed coat) soaked into water by Powell and Matthews (1978). They reported that high leakage of electrolytes coupled with poor staining of the cotyledons with tetrazolium chloride was observed. This phenomenon was called imbibition damage (Powell and Matthews, 1978). Initially, it was supported that the rapid water uptake resulted in the death of the outer layers of cells of the cotyledons which occurred within minutes of the start of imbibition (Powell and Matthews, 1978). However, later it was shown by the same authors that the failure to stain actually resulted from the loss of the substrate for the dehydrogenase enzymes that reduce tetrazolium chloride to the red formazan (Powell and Matthews, 1981). Imbibition damage was, therefore, caused by impaired cell function on the surface of the cotyledons which allowed a high loss of solutes and resulted in a failure to stain with tetrazolium chloride. Further support to the existence of imbibition damage in soybeans, navy beans, peas and groundnuts was given by Duke and Kakefuda (1981) using another methodology. They used Evan's blue, a non-permeable to the seed coat stain; when embryos imbibed in an aqueous stain solution, the outermost cells of the cotyledons were stained whereas no staining in the intact seeds was observed. The consequences of imbibition damage may have an

effect at a later stage of imbibition and germination. Imbibition damage resulted in reduced respiration, germination, a decline in the rate of food reserve transfer from the cotyledons to the growing axis and a lower seedling growth (Powell and Matthews, 1979).

Powell *et al.*, (1984) proposed that imbibition damage as revealed by an increased leakage of electrolytes and low percentage of cotyledons fully stained with tetrazolium chloride was a common situation in all grain legumes. Indeed, using the above methodology, imbibition damage has been observed in soybeans (Semple, 1981; Oliveira *et al.*, 1984), dwarf French beans (Powell *et al.*, 1986b), chickpeas (Knights and Mailer, 1989; Legesse and Powell, 1992), long beans (Abdullah *et al.*, 1992), faba beans (Rowland, 1981; Kantar *et al.*, 1996). Imbibition damage may not only result in a physiological damage of the cells on the surface of the cotyledons but also in an increased predisposition of seeds to the infection by soil borne fungi (Powell and Matthews, 1980). There have been proposed two explanations for the previous situation; either the increased predisposition may result because the tissue acts an initial site of fungal infection and/or the solutes leakage into the soil causes an increase in the inoculum potential (Matthews, 1971).

The possibility of a direct relationship between the imbibition damage and the low seedling emergence under soil condition conditions could be explained of the previous basis. In several studies, the soil condition has been involved in the seedling emergence and growth. Earlier studies, in peas, have shown that high soil moisture was closely correlated with low seedling emergence and growth (Hull, 1937; Baylis, 1943). The infection of soil-borne fungi of *Pythium* species has been reported as a cause of a poor field emergence in soybeans (Hill and West, 1982; Peske, 1983), chickpeas (Legesse and Powell, 1992), peas (Matthews and Whitbread, 1968; Matthews, 1971). Further evidence of the involvement of the soil-borne fungi in the low seedling emergence is the fact that seed fungicide treatment results in improvement of germination. The improvement in seedling emergence due to fungicide treatments has been reported in several grain legumes including soybeans (Gupta and Chatrath, 1983; Wall *et al.*, 1983; Ferriss *et al.*, 1987), peas (Jacks, 1963; Matthews and Bradock, 1967) and chickpeas (Kaiser and Hannan, 1983).

1.5. Regulation of water uptake during imbibition

In general, imbibition is affected by the seed properties as well as the environment in which seeds germinate. A water gradient between the seed and the environment is the driving force for water uptake but permeability of seed to the medium is more important in determining the rate (Bewley and Black, 1985). Seed permeability is a complex function of seed morphology, structure, composition, moisture and temperature (Vertucci, 1989). Practically, regulation of rate of water uptake during imbibition could be achieved by use of osmo-conditioners as imbibing media, use of polymer coating, and finally by the seed coat itself.

1.5.1. Use of osmo-conditioners

Polyethylene glycol (PEG), a non-toxic high-molecular weight compound has been widely used as an osmoconditioner to reduce the rate of water uptake during imbibition. Woodstock and Tao (1981) reported results in the effect of slow imbibition in PEG in the subsequent growth of the excised embryonic axis of soybeans. They concluded that seed deterioration decreased the ability of seed axis to tolerate rapid water uptake at the start of the imbibition. Slow imbibition in PEG of low vigour seeds resulted in an improvement of the axis growth at levels comparable to the unaged control seeds (Woodstock and Tao, 1981). These results were further supported by the findings of Woodstock and Taylorson (1981). They reported that slow imbibition in PEG could play a vital role in avoiding the rapid water uptake in soybean seeds. Tilden and West (1985) reported a reversal of the effect of ageing by slowly imbibing seeds. This treatment reduced electrolyte leakage and increased seed vigour, providing evidence for a metabolic repair of plasma membranes and other sub-cellular components when seeds imbibed at a slow rate.

1.5.2. Polymer coating

Reduced rate of water uptake could be achieved by the application of relatively hydrophobic polymers. The use of polymers is widespread in seeds of particular crops like vegetables or sugarbeet. These type of polymers are highly hydrophilic, and are added to seeds together with insecticides and fungicides to offer better resistance to pathogen attacks (Powell and Matthews, 1988). However, use of polymers to regulate the rate of water uptake requires polymers with hydrophobic qualities.

Preliminary results by applying a thin layer of lanolin to seeds were open the possibility of the use of polymers to regulate the water uptake (Priestley and Leopold, 1986). West *et al.* (1985) reported results about the control of only moisture uptake but not water uptake by using two polymers. More recently, Hwang and Sung (1991), applied ethyl cellulose as a hydrophobic layer and observed that water uptake was not stopped completely but it was slowed down.

1.5.3. Seed coat

The soybean seed coat has often been regarded as the only, or at least primary, regulator in the exchange of water, nutrients and gases between the seed and outside atmosphere (Woodstock, 1988). The functional significance of the seed coat in soybeans lies in its protective roles for the underlying embryonic tissues. These roles include: mechanical protection from injury due to striking or abrasion, physically holding seed parts together and shielding the embryo, retention of water and protection against desiccation following imbibition, and avoidance of imbibition injury from excessively rapid water uptake (Woodstock, 1988).

According to Delouche (1974), the soybean seed is poorly designed to resist mechanical damage due to the fact that the embryonic axis lies against the basal margins of the cotyledons, and this position together with the thin seed coat make the seed vulnerable to mechanical injury. Many studies have shown that the presence of the soybean seed coat during imbibition resulted in a lower rate of water uptake than embryos and hence in protection against imbibition damage (Duke *et al.*, 1983; Duke *et al.*, 1986; Duke and Kakefuda, 1981; Tully *et al.*, 1981). To carry out this role, the seed coat should be also intact. Powell and Matthews (1979) showed that pea seeds with scarified seed coats had a higher rate of water uptake and higher level of imbibition damage than seeds with intact coats. In addition, Powell and Matthews (1979, 1980) suggested that the presence of pea seeds with cracked coats within seed lots was associated with the vigour level of the seed lot. Similar association between the presence of seeds with cracked coats and low vigour has been reported in commercial soybean seed lots (Oliveira *et al.*, 1984) and faba beans (Kantar *et al.*, 1996).

In grain legumes, several reports have shown the existence of genotypic differences in seed coat characteristics that could be related to different rates of water uptake. One of the most widely referred seed coat characteristic is the pigmented

colour of the seed coat. In soybeans, seeds with black colour seed coat had a lower rate of water uptake than those with yellow colour seed coats (Tully *et al.*, 1981; Kuo, 1989). Powell *et al.* (1986b) in dwarf French beans reported that seeds with black seed coats had a lower rate of water uptake, a lower leakage, a higher percentage of cotyledons fully stained and a higher field emergence than seeds with white seed coat. Similarly, the previous relationship between seed coat colour and imbibition behaviour has been reported in chickpeas (Kaiser and Hannan, 1983; Legesse and Powell, 1992).

Seed coat in soybeans (botanically known as testa) is marked with a hilum (seed scar) that varies in shape from linear to oval. At the one end of the hilum is the micropyle, a tiny hole formed by the integuments during seed development but frequently covered by a cuticle at maturity. At the other end of hilum is the raphe, a small groove extending to the chalaza where the integuments were attached to the ovule proper (Williams, 1950). The seed coat proper has three distinct layers: i) epidermis, ii) hypodermis, and iii) inner parenchyma layer (Thorne, 1981). The epidermal layer consists of closely packed, thick-walled palisade cells (macrosclereids). A cuticle is present on the outer wall of the macrosclereids. The hypodermis consists of a single layer of sclerified cells (hourglass cells) variously elongated and separated from each other. The unevenly thickened cell walls are thin at the end of the cell and very thick at the central, constricted portion of the cell. These cells thus form a strong supporting layer with considerable intercellular space (Wilcox, 1987). The inner parenchyma tissue consists of six to eight layers of thin-walled, flattened cells that lack contents. The parenchyma tissue is essentially uniform throughout the entire seed coat except the hilum where it forms three distinct layers (Dzikowski, 1936).

According to Ragus (1987), the significance of hilum to water uptake was far lesser to that of seed coat itself. The micropyle served important functions, being the route by which pollen tubes entered the ovule and the passageway for movement of gases and moisture to and from the developing embryo (Carlson, 1973). It was also the route for passive invasion by pathogens into the seed (Vaughan *et. al.*, 1985). In the literature there is some contradiction on the role of micropyle in relation to water uptake. Saio (1976), observed that the micropyle acted as a major route for water to enter the seed at germination. He also added that hardseedness (a situation in which water uptake was inhibited) was associated with a closed micropyle. However, Yaklich *et. al.*, (1986) through a series of SEM studies observed hard seeds with an

open micropyle. Phillips (1968), tested the hypothesis if there was any preferential water uptake through the micropyle but no difference between the uncovered and epoxy glue micropyle-covered seeds was observed. A possible explanation for hardseedness in soybeans with an open micropyle may be that a blockage or closure occurs farther down in the micropylar channel (Vaughan *et. al.*, 1987).

McDonald *et al.* (1988), observed that after 8 hours soaking, the seed coat of the cultivar "Williams 82" either had no effect or a slightly promotive effect on water uptake into the seed. However, at longer than 8h imbibition, seeds with the coat present had a higher amount of water absorbed than embryos (McDonald *et al.*, 1988). This might be due to the large water holding capacity of the seed coat. Indeed, Vertucci and Leopold (1983) observed that under conditions of excess water, the seed coat absorbed 3.0 times its dry weight after 4 hours compared to a soybean cotyledon which absorbed about 1.2 times its dry weight. Also, the seed coat in the cultivar "Williams 82" found to aid in tangential and radial movement of water around the seed that both cotyledons hydrate evenly (McDonald *et al.*, 1988). Such mechanism of water transport (if proved to be universal in soybeans) could be very important in a soil environment where pockets of water exists as the seed to soil contact is poor. The thickness of the seed coat in soybeans differed considerably from one part of the seed to the other with the thick part to be the hilar region and the thin part to be the dorsal region (Hwang and Suang, 1991). Some reports have concluded that a thicker seed coat was generally found on a smaller seed (Calero *et. al.*, 1981; Yaklich *et al.*, 1986). In contrast, Hwang and Suang (1991) showed that large seeds had thicker seed coats than seeds of medium and small size. Comparison was done on the thickness at the hilum (thick part) and the distal area (thin part) of the seed coat (Hwang and Suang, 1991).

It is generally believed that thicker seed coats offered more resistance to water penetration, although there were only weak negative correlation between the ratio of seed coat dry weight to embryo dry weight and the rate of water uptake (Mugnisjah *et. al.*, 1987; Calero *et. al.*, 1981; Yaklich *et. al.*, 1986). McDonald *et. al.* (1988) suggested that water firstly penetrates the dorsal region and then the ventral region of the seed. Pereira and Andrews (1985), showed that the seed coat decreased in thickness away from hilum; this was attributed to the presence of hourglass cells that gradually decreased in size from the hilum until they disappeared in the region distal to hilum. Hourglass cells were ascribed a cushioning role by absorbing excess water and preventing the seed coat wrinkling (Pereira and Andrews, 1985). McDonald *et.*

al., (1988) showed that regardless of the hydration environment, the portion of the seed coat nearer to hilum (larger hourglass cells) absorbed more water than that of distal to the hilum. According to McDonald *et al.* (1988), hourglass cells were ideally tailored as moisture reservoirs and this proposed function had obvious advantages to seeds planted in soils with fluctuating moisture levels.

Structural features (pits, deposits) are present in the surface of the soybean seed coat. Pitting of the seed coat surface was observed in several previous reports with surveys of large number of soybean genotypes (Wolf and Baker, 1972; Newell and Hymowitz, 1978; Calero *et. al.*, 1981; Wolf *et. al.*, 1981). The number of pits varied from 21 to 6 per 75 nm^2 but that number was a poor indicator of permeability to water (Calero *et. al.*, 1981). The pits frequently appeared to be either closed slots or open passageways which penetrated to various degrees the thickness of the palisade layer. Below the pits, frequently there was an oval-shaped cavity (Wolf *et. al.*, 1981). Although, the presence of deposits in the surface of the soybean seed coat is well documented, some reports have suggested that deposits were cutins which hinder the water uptake (Calero *et al.*, 1981; Ragus, 1987) whereas other reports have suggested that deposits were residues to the endocarp (Wolf *et al.*, 1981; Yaklich *et al.*, 1986) which may play little role in the water uptake during imbibition. The function of pits and deposits is still intriguing without a clear evidence of their involvement in the water uptake during imbibition. According to Bedi and Basra (1993) an understanding of seed coat characteristics in relation to regulation of the water uptake is imperative so that plant-breeders could exploit this knowledge for cultivar improvement to avoid the detrimental effects of the rapid water uptake during imbibition in seed performance.

1.6. Aims and objectives

The present study aimed: (a) To identify genotypes that were resistant to imbibition damage and then if such genotypes are demonstrated to determine the seed coat characteristics responsible for any observed improvement in imbibition behaviour. (b) To investigate the applicability of the use of a polymer to avoid imbibition damage and to improve seedling emergence and growth. (c) To investigate the mechanism of regulation of water uptake by the soybean seed coat.

The objective of experiments in Chapter 3 was to determine whether particular seed coat characteristics were correlated with high or low levels of imbibition damage. The imbibition behaviour of a wide range of soybean genotypes with different seed size, physical seed coat characteristics and seed coat colour were examined. The importance of the hilum region in water uptake compared with the dorsal or abaxial region of the seed coat was also examined. Finally, the importance of the seed coat adherence to the embryo was assessed.

The objective of experiments in Chapter 4 was to investigate whether the condition (hard or split) of the seed coat could influence seed performance during ageing. The importance of the occurrence of seeds with a high proportion of hard or split seed coats within a genotype was assessed. Comparative studies between five genotypes in the seed performance during ageing were made. The genotypes were grown in the field under similar conditions, were of medium seed size and required similar time to flowering and maturity. However, the condition of the seed coat was different. In particular, in cv. Douglas seeds had intact or split seed coat and in cv. Pioneer-9581 seeds had normal or hard seed coat.

The objective of experiments in Chapter 5 was to investigate whether polymer coating could regulate the rate of water uptake, prevent imbibition damage and improve germination, seedling emergence and growth. The beneficial role of a polymer was studied in seeds of cv. Douglas that was prone to imbibition damage due to a high proportion of seeds with seed coat splits. In addition, the importance of the initial seed quality was assessed by using seeds of two vigour levels (high or low vigour). Seeds of low vigour level were produced after 2 days of accelerated ageing. Finally, the performance of polymer coated seeds was assessed under natural soil conditions or temporary flooding soil conditions.

The objective of experiments in Chapter 6 was to investigate the role of surface structure of the seed coat (e.g. deposits and pits) in regulation of water uptake. Six genotypes with differences in the amount of deposit material present and in the structure and density of pits were examined. Caclofluor was used as a water-soluble fluorescent stain. Finally, changes in the seed coat structure due to organic solvents pre-treatments were associated with changes in the water permeability of the seed coat.

The objective of experiments in Chapter 7 was to localise and identify the nature of the water impermeability barrier in the soybean seed coat. Localisation of the barrier was made in hard seeds by using calcofluor as a water-soluble fluorescent stain. Identification of the nature of the barrier was attempted by comparative anatomical and histochemical studies between hard and soft seeds. Finally, changes in the hard seed coat structure due to organic solvents pre-treatments were associated with changes in the water permeability of the hard seed coat.

The final outcome of the proposed research may help to further understand the mechanism of regulation of water uptake by the seed coat, and this should help: 1) to provide solutions in order to maintain high seed quality, and 2) to increase the fundamental knowledge in seed coat regulation of the water uptake which could subsequently be used in breeding programmes for selection of genotypes with desirable seed coat characteristics.

CHAPTER 2

General Materials and Methods

2.1. Seed Material

Soybean cultivars and lines were kindly supplied around the end of 1993 beginning of 1994 from the following sources: i) Ten genotypes (2 cultivars, 8 lines) from the Germplasm Centre of the Asian Vegetable Research and Development Centre (AVRDC), Tainan, Taiwan; ii) Ten lines from the seed company KWS, Einbeck, Germany; iii) Ten cultivars from the seed company Rustica Semences, Mondonville, France; iv) Ten lines from the National Bureau for Plant Genetic Resources (NBPGR), India and v) Ten cultivars from Agricultural Research Station, Mingora, Pakistan. Seed samples were posted to the researcher as air-mail small parcels. Immediately after reception, seeds were tested for moisture content and germinability (Table 2.1). Seeds were stored in a cold store at 5°C in sealed plastic bags until needed. From the 50 genotypes originally obtained, a selection of genotypes was made in order to cover a wide range of seed size and seed coat colour (Table 2.1).

2.2. Seed characteristics

The seed moisture content of soybean seeds was determined by the hot oven method at 103 ± 2°C for 17h (ISTA, 1985). Four samples each of 2 g were used to determine the seed moisture content. Each sample was ground and placed in a pre-weighed and pre-dried glass beaker. At the end of the drying period, the beakers were placed in a desiccator with blue silica in the bottom to cool for about 30 minutes. After cooling, the beakers were weighed and the percentage moisture content was calculated on fresh weight basis. Hundred seed weight was based on average of three replications of 100 seeds each per cultivar. Seed coat colour and hilum colour were observed with the naked eye. Seed surface area was calculated by the equation describing a prolate spheroid: Area= $2pab\ [(\sin^{-1}e)/e] + 2pb^2$, where a is half the length of the longest axis, b is half the average of width and thickness, and e is the eccentricity of seeds which is calculated by the equation: $e= (a^2-b^2)^{0.5}/a$ (Kuo, 1989).

Table 2.1. Characteristics of the soybean genotypes used in this study.

Genotype	seed coat colour	production year	moisture content (%)	normal seedlings (%)
Source: AVRDC				
GC 84128-17-2-1	yellow	1991	8.6	86
AGS 292	yellow	1992	8.4	85
G 2120	green	1990	8.1	88
Suwan-155	black	1992	7.9	87
Suwan-156	black	1992	8.2	84
CC 84051-32-1	green	1992	8.3	92
GC 88037-38-2-2	brown	1992	8.8	89
SS 87040-2-1	green	1992	8.9	93
Source: KWS				
KWS-2	partially black*	1992	8.9	87
KWS-3	yellow	1992	7.8	88
KWS-5	green	1992	9.1	90
KWS-A	partially black*	1992	8.8	86
KWS-C	partially brown*	1992	8.5	85
KWS-E	yellow	1992	8.8	91
Source: Rustica				
Picador	yellow	1992	9.1	91
Toreador	yellow	1992	9.6	89
Essor	partially brown*	1992	8.8	91
Sapporo	yellow	1992	9.4	94
Source: India				
Pusa-16	yellow	Unknown	7.9	88
Pusa-40	yellow	Unknown	8.1	85
JS 7980	yellow	Unknown	8.2	91
VLS-1	black	Unknown	8.6	91
Source: Pakistan				
Forrest	yellow	1993	9.6	94
Douglas	yellow	1993	9.1	91
HSC-591	yellow	1993	8.1	96
HSC-401	yellow	1993	8.2	92
Pioneer-9581	yellow	1993	8.4	88

* the rest of the seed was yellow.

Table 2.3. List of chemicals used.

Name	Formula	Supplier	Code
Polyethylene glycol 8000	-	Sigma	P-2139
2,3,5-triphenyl tetrazolium chloride	$C_{18}H_{12}N_6$	Fisons	T/3703/43
Potassium phosphate	K_2HPO_4	Sigma	P-5379
Potassium phosphate	Na_2HPO_4	Sigma	S-7907
Sodium phosphate	NaH_2HPO_4	Sigma	S-8282
Sodium-hypochlorite	NaOCl	Anderson	UN1791
Dithane-945	-	Rohm & Hass	2085
Hydrochloric acid	HCl	BDH	UN1789
Phloroglucinol	$C_6H_6O_3$	Sigma	P-3502
Glutaraldehyde	$C_5H_8O_2$	Sigma	G-5882
Ethanol	C_2H_5OH	Fisons	F/0400/08
Calcium Sulfate	$CaSO_4$	Sigma	C-3771
LR White resin	-	London Resin	R/1281
Calcofluor white M2R	$C_{40}H_{42}N_{12}S_2Na_2$	Sigma	C-0647
Glycerol	$C_3H_8O_3$	Fisons	G/0650/17
Chloroform	$CHCl_3$	Fisons	C/4920/PB08
Toluidine Blue O	$C_{15}H_{16}N_3SCl$	Sigma	T-3260
Vanillin	$C_8H_8O_3$	Sigma	V-2375
Ruthenium red	-	Sigma	R-2751
Fluorol yellow	$C_{22}H_{16}O$	Sigma	F-5520
Aniline blue	-	Fisons	A/7300/46

Seed coat dry weight was determined by removing the coat and comparing the seed weight before and after the seed coat removal. Seed coat dry weight per unit area was the quotient of seed coat dry weight and seed surface area.

Hard seeds were defined as those with no obvious sign of imbibition (wrinkling of the seed coat) after 10h soaking in distilled water at room temperature as proposed by Abdullah *et al.* (1992). Soft seeds were those with obvious sign of imbibition after 30 minutes of soaking in distilled water. Hardseedness was determined as the percentage by weight of hard seeds in the seed sample; each mean represents the average of four replications of approximately 100 g of seeds.

2.3. Germination test

The germination of the different soybean genotypes was carried out using the standard rolled paper towel method according to recommended International Seed Testing Association conditions (ISTA, 1985).

Seeds were dipped in 1% sodium hypochlorite for 10 seconds and then rinsed thoroughly with distilled water before being placed for germination. Seeds were placed 2 cm apart in a single row on the lower half of a double sheet of Kimberley Clarke Hi-Dri 7390 rolled paper towel (220 x 380 mm) wetted with distilled water, and covered with a third moistened paper towel. The paper towels were fully saturated, and then placed in a photographic tray and allowed to drain freely before use. The bottom edge was folded up, the towels rolled up and held in shape by elastic bands. The rolls were placed upright in a basket, covered but not sealed with a polythene bag to reduce evaporation of water and placed into an incubator at 25°C in the dark. After 8 days of incubation, germination assessment was carried out according to the rules of International Seed Testing Association (ISTA, 1985). Seedlings were classified as normal, abnormal seedlings or dead seeds. Seedlings were evaluated in accordance with the ISTA Handbook of Seedling Evaluation (Bekendam and Grob, 1979). Normal seedling is described as an embryonic plant that must consist of a complete root and shoot axis that has the capacity of normal growth under favourable conditions (Bekendam and Grob, 1979).

2.4. Seedling emergence and growth from compost

2.4.1. Determination of the field capacity

Seedling emergence and growth was determined in seeds that were sown in a loam based John Innes Compost No 2 that had a pH 6.5. The moisture content was determined following the high constant temperature oven method (ISTA, 1985). The field capacity of the compost was determined as follows: Random samples of the compost were used to fill three pre-weighed 20 cm diameter pots. Pots plus compost were weighed and placed in a photographic tray containing a reservoir of 5cm of water. The pots were watered from the top in order that capillary water movement could be established. After 24h, the pots were allowed to drain for about 6h until capillary movement had virtually stopped before being weighed. The difference between the dry weight of the compost and the weight of the compost after drainage represents the 100% field capacity of the compost.

2.4.2. Seedling emergence and growth

Pots 20 cm diameter were filled with compost up to 5cm from the top. Twenty five seeds were spread over the surface of the compost in such a way that seeds were clearly separated. The seeds were gently firmed into position before being covered with another 4cm of compost which was gently firmed. To achieve the desired percentage of field capacity, water was added to each pot. Subsequently, pots were placed for incubation and the weight of the pots was checked daily.

2.5. Water uptake

2.5.1. Measurements from a bulk of seeds

Twenty weighed seeds were placed in a 200 ml beaker and were covered with 50ml of distilled water. At different intervals, the seeds were removed from water, blotted dry on a paper towel to remove excess of water and weighed. Afterwards, the seeds were quickly returned to the water and the procedure repeated later as a function of time. Changes in weight due to water uptake were expressed as percentage weight increase of the initial weight of the seeds. Each mean represents the average of three replications with 20 seeds each.

2.5.2. Measurements from individual seeds

The water uptake was measured from ten individually weighed seeds each one placed inside a square compartment of a compartmentalised square Petri dish. Each compartment was fully covered with approximately 4ml distilled water at the beginning of the experiment. At the end of the experiment, the water left was enough to completely cover the seeds. The seeds were held at room temperature and at pre-determined intervals, each seed was removed from water, blotted dry, weighed and returned to water for the subsequent measurement. The weight of water absorbed per seed was calculated, and each mean represents the average of ten seeds.

2.6. Leachate conductivity

Four replications of 25 weighed seeds were placed into 350ml clean beakers, and then 200 ml distilled water was added. Control beakers with distilled water were also set up. The beakers were covered with aluminum foil and incubated at 25°C for 24 hours. After this period of soaking the conductivity of the soak water was measured using a GP 383 Conductivity meter (1 cm platinum cell), EDT Instruments. The conductivity of distilled water used as a control was then subtracted from the leachate conductivity of the samples. The electrode of the conductivity meter was rinsed in distilled water before transferring between solutions. The conductivity of the soak solution was expressed per gram of seeds for each replication (mS/g/cm). Each value was a mean of 4 replications.

2.7. Assessment of living tissue with tetrazolium chloride

The tetrazolium chloride was prepared according to ISTA (1985). The percentage of living tissue on the cotyledons was determined by carefully removing the seed coat from fully imbibed seeds. The cotyledons of each seed were separated and placed in a 1% (w/v) solution of 2,3,5-triphenyl tetrazolium chloride (TTC) for 3 hours at 25°C kept in the dark. The staining on the abaxial surface of the cotyledons was assessed as proposed by Powell and Mathews (1978). The results were expressed as a percentage of cotyledons fully stained with tetrazolium chloride, as proposed by Powell and Matthews (1978). Each mean represents the average of four replications

with 20 seeds each. From each seed only one cotyledon was assessed whereas the other cotyledon was discarded.

2.8. Accelerated ageing test

Accelerated ageing test subjects seeds to a combination of high temperature (41°C) and relative humidity (around 100%) for short periods (3 to 4 days); the seeds then removed from stress conditions and placed under optimum germination conditions (Copeland and McDonald, 1985).

Seeds were treated with Dithane M-45 (1.5 g per Kg of seed) before the ageing test in order to suppress the growth of saprophytic fungi during the accelerated ageing test. Seeds were spread in a single layer on a muslin cloth that was firmly attached over a circular metallic sieve with a rubber band. The metallic sieve was placed inside a desiccator that was placed in a water bath running at 41°C (±1 °C). Seeds were situated about 3 cm above the surface of the water inside the desiccator. Seeds were protected against condensation by wrapping the lid of the desiccator with a double sheet of muslin cloth. The desiccator lid was sealed to the base with vaseline. The surface of the water in the water bath was cover with plastic balls to reduce the evaporation of the water. The water bath with the desiccator were covered with a single aluminum foil to reduce heat losses. The temperature of the water in the water bath was constantly measured. Additionally, changes in the moisture content of the seeds during the ageing test were daily measured, as described in section 2.2. Preliminary experiments with seeds from different genotypes showed that this set-up for the accelerated ageing test had no fluctuations in temperature or in the level of seed moisture content during the ageing test. After the ageing process, the seeds were spread out to dry at room temperature for 4 days before being tested for germinability as described in section 2.3.

2.9. Seed coat anatomy and histochemistry

The sequence of the procedures for the anatomical and histochemical investigation of the seed coat, outlined by O'Brien and McCully (1981), was as follows:

1. Preparation of the tissue

Dry soybean seeds were soaked in distilled water for 2hours. From each seed, segments of the seed coat approximately 2 mm x 3 mm were taken from either the ventral side or the dorsal side of the seed.

2. Fixation

The cut segments of the seed coat were placed in 2.5% w/v glutaraldehyde in 0.1M NaH_2PO_4/Na_2HPO_4 buffer, pH 7.2 for 2h at room temperature. Fixation was ended by replacing the fixative and then washing 3 times in the buffer (15 min each wash) at room temperature.

3. Dehydration

The gradual removal of the water from the tissue was accomplished by replacing the buffer in successively higher concentrations (30%, 50%, 70%, 90% and 100%) of ethanol. The duration of each dehydration treatment was 15min at room temperature. The dehydration process was finished by overnight soaking in 100% ethanol which had been dried over $CaSO_4$.

4. Infiltration and embedding

Infiltration was achieved by adding up to 50% of the initial volume the LR White Resin drop by drop over a 30 min period. The vials were placed on a rotary motor for continuous rotation. Subsequently, the resin/ethanol mixture was replaced twice with pure resin; once for 8h and then for 16h with continuous rotation. Embedding was achieved by placing the material in gelatin capsules and then the capsules inside a vacuum oven for 16h at 60 °C.

5. Sectioning

The finished block with the tissue was trimmed and then placed in the microtome where sections measuring 1.5 mm thickness for light and fluorescence microscopy were prepared using a glass knives. Sections were hooted on drops of water on a slide and then dried on a slide warmer.

2.10. Use of calcofluor as a water-soluble fluorescent stain

2.10.1. Water penetration sites in the surface of the seed coat

In short periods, calcofluor enters cuticular discontinuities and binds to the immediately-underlying polysaccharide wall, providing an unambiguous map of the sites of water entry in stigma surfaces (Heslop-Harrison and Heslop-Harrison, 1982).

Whole seeds were briefly soaked in 0.1%, w/v aqueous solution of calcoflour white M2R. Seeds were washed carefully with distilled water to remove any stain excess from the surface of seed coat and then blotted dry on a paper towel. From each seed, around 2 mm x 3 mm segments of the seed coat were taken from ventral, dorsal or abaxial side of the seed. The segments were mounted in glycerol and the surface was observed as quickly as possible with a Leitz Ortholux II epifluorescence microscope, using filter set II (exciter filter, maximum transmission 340-360; barrier filter, maximum transmission >490). Controls of the untreated material were also studied.

2.10.2. Depth of calcofluor presence within the seed coat

Whole seeds were soaked in 0.1% w/v aqueous solution of calcoflour white M2R. Seeds were washed carefully with distilled water to remove any stain excess from the surface of seed coat and then blotted dry on a paper towel. From each seed, around 2 mm x 3 mm segments of the seed coat were taken from the ventral, dorsal or abaxial side of the seed. In order to avoid further penetration or diffusion of the stain due to contact with water, fixation was omitted. The segments were dehydrated in absolute ethanol for 3 times with 15min each. Infiltration, embedding and sectioning was done as described in Section 2.9. Hand cut sections of fresh material were also prepared. Epifluorescence from the sections was observed as described in section 2.10.1.

2.11. Scanning Electron Microscopy (SEM)

Seeds were placed for air-drying in muslin bags and then in an air-incubator for air-drying set at 30 °C for 4 days. Subsequently, they were kept inside a desiccator with blue silica gel in the bottom until required. Specimens were mounted on

aluminum stubs, gold coated and examined in a Cambridge S250 at an accelerating voltage of 7.5 Kv. Micrographs were taken using a Kodak TMX (100 ASA) film.

2.12. Organic solvent pre-treatments

Twenty weighed seeds were placed in 50 ml of different combinations of methanol or chloroform solvents inside a 250 ml conical flask, at room temperature. The flask was tightly covered with aluminum foil held in place with plastic rubber. They were then placed on a shaker for continuous shaking at 200 rpm for the required period of time. At the end of the treatment, seeds were removed from the organic solvent and blotted dry.

2.13. Absorbance measurements

The organic solvent was decanted of the seeds at the end of the pre-treatment period and then centrifuged at 10,000 rpm for 5 min. The absorbance spectrum of the supernatant from 215 to 350 nm was measured using a Beckman DU 65 spectrophotometer. The pure organic solvents were used as blanks.

2.14. Statistical Analysis

Most experiments were conducted in completely randomised design. Experiments in Chapter 5 were conducted, analysed as a two factor (genotype and ageing) or three factor (genotype, ageing, water regime) experiments. In most cases, the analysis was carried out, or treatments were applied, in four replications. However, results from the individual seeds were based in ten replications.

ANOVA were performed in all data, using STATGRAPHICS Release 6.0 (Statistical Graphics Corp., Manugistics Inc.), testing for normal distribution of data sets prior to analysis. All of the data were found either to fit normal distribution or be with acceptable levels of skewness therefore no transformations were applied. Results from the ANOVA tests are presented in the Appendixes section. The mean comparison was based on the two tailed paired t-tests.

All graphs were created using the graphic programme EXCEL Release 5.0. In all figures the standard error of the means (s.e.m.) are displayed.

CHAPTER 3

Imbibition behaviour of soybean (*Glycine max* L. Merril) genotypes with different seed coat characteristics

3.1. Introduction

The presence of the seed coat offers a significant protection to the embryo against the damage of the embryo cells during imbibition. In peas (*Pisum sativum*), seeds without the testa failed to stain with tetrazolium chloride following imbibition in water (Powell and Matthews, 1978). In soybeans, several reports have shown that the intact seed coat retards the rapid water uptake and, hence, protects against imbibition damage as revealed by solute leakage and damage of the cells in the embryo (Duke and Kakefuda, 1981; Tully *et al.*, 1981; Duke *et al.*, 1983). To carry out the important function of protecting the embryo from imbibition damage, the seed coat needs to be intact. In peas, artificially scarified seeds showed a rapid water uptake which resulted in high solute leakage and low vital staining of the cotyledons (Powell and Matthews, 1979). In soybeans, Oliveira *et al.*, (1984) reported that split seed coats in many low vigour seed lots resulted in rapid water uptake causing damage to the cells in the cotyledons and high levels of leakage of electrolytes. The detrimental effect during imbibition of artificially induced or naturally occurring seed coat cracks or splits has been reported in several grain legumes including dwarf French beans (Powell *et al.*, 1986a, 1986b), faba beans (Kantar *et al.*, 1996) and cowpeas (Legesse and Powell, 1992).

High levels of leakage have been shown to result from imbibition damage due to the rapid water uptake causing death of the cells on the surface of the cotyledons in a number of grain legumes including peas (Powell, 1985), dwarf French beans (*Phaseolus vulgaris*; Powell *et al.*, 1986a, 1986b), faba beans (*Vicia faba;* Rowland, 1981; Kantar *et al.*, 1996), chickpeas (*Cicer arietinum*; Knights and Mailer, 1989; Legesse and Powell, 1992), cowpeas (*Vigna unguiculata*; Beigley and Hoper, 1981), long beans (*Vigna sesquipedalis*; Abdullah *et al.*, 1992).

From the seed coat characteristics, seed coat colour and seed coat adherence to embryo have been reported as important factors regulating the rate of water uptake.

In soybeans, seeds with dark coloured coats have been associated with low rate of water uptake (Tully *et al.*, 1981; Kuo, 1989). Similar close association between pigmented seed coat and a low rate of water uptake has been reported in several grain legumes including snap beans (*Phaseolus vulgaris;* Deakin, 1974; Wyatt, 1977), long beans (Abdullah *et al.*, 1988), chickpeas (Legesse and Powell, 1992) and lima beans (Kannenberg and Allard, 1964). Adherence of the seed coat to the embryo has been reported as an important seed coat characteristic which results in low rates of water uptake in dwarf French beans (Powell *et al.*, 1986b).

In soybeans, there is no published information about the seed coat characteristics in relation to different levels of imbibition damage when the seed coat is intact. The objectives of this work were therefore (1) to assess imbibition damage in genotypes with a range of seed coat characteristics, and (2) to identify seed coat characteristics that correlated with minimum and maximum levels of imbibition damage.

3.2. Materials and methods

3.2.1. Seed characteristics of the twenty genotypes.

The twenty genotypes were selected to cover a wide range of seed size (Table 3.1) and different seed coat colour (Table 2.1). Seed characteristics of the twenty selected genotypes were determined, as described in section 2.2.

3.2.2. Imbibition behaviour of the twenty genotypes.

Soybean seed coat is often firmly attached to embryo thus making the removal of the seed coat difficult without damaging the embryo. In order to facilitate the removal of the seed coat, seeds imbibed first in 20% (w/v) polyethylene glycol (PEG) for 2 hours at room temperature. After the seed coat removal, embryos were left to dry for 2 days under room conditions and subsequently were dried with air-drier set at 30°C for 4 days. The final moisture content of these embryos was 8 % m.c. (±1.5% m.c.) on a fresh weight basis, determined as described in section 2.2. Subsequently, water uptake and leachate conductivity after 6h of imbibition of the intact seeds and embryos were measured, as described in sections 2.5.1 and 2.6 respectively. The time course of water uptake of intact seeds was based on weight changes of individual seeds as described in section 2.5.2. Assessment of the vital

staining in the surface layers of the cotyledons with tetrazolium chloride was made as described in section 2.7. Scarification of the seed coat was carried out according to Powell *et al.* (1986a). Precision of the scarification was achieved using a razor blade, by carefully scratching the dorsal region of the seed (approximately 2 mm long). Low rate of water uptake was achieved by soaking seeds in 30% (w/v) PEG for 24 hours before staining with tetrazolium chloride.

3.2.3. Seed coat characteristics in relation to water uptake.

The hilar region of seeds of 4 genotypes was sealed by applying a nail varnish and leaving the seeds to dry overnight. Four genotypes were selected, one genotype was slow imbiber (line VLS-1) and the other three genotypes were fast imbibers (line SS 87040-2-1, line KWS-E and cv. Toreador). Water uptake in varnished and unvarnished seeds was measured during 4 hours, as described in section 2.5.2. Water uptake was expressed as the weight of water absorbed per seed. Each mean was the average of ten values. The effect of proximity to water of the different region of seed on the water uptake during imbibition was assessed using seeds of two genotypes. The two genotypes were selected because in seeds of cv. Sapporo the hilum was closed whereas in seeds of line KWS-E, the hilum was wide open. Ten individually weighed seeds were oriented so that the ventral, dorsal or abaxial side of the seed was in direct contact with water through a set of identical round holes (about $20mm^2$) cut in plastic discs. The plastic discs with the holes were glued on a 90mm Petri dish. The Petri dish was filled with distilled water. At pre-determined intervals, each seed was removed from the hole, blotted dry and weighed. Seeds were replaced in the same position until the end of the experiment. The water uptake was expressed as weight of water imbibed per seed. Each mean was the average of ten values.

The effect of wetting and drying of seeds on water uptake was investigated. Four genotypes were selected to cover different rates of water uptake. In particular, two genotypes were slow imbibers (line VLS-1 and line G2120) and the other two genotypes were fast imbibers (line SS 87040-2-1 and cv. Toreador). Seeds were soaked for 1hour in water, blotted dry and left to dry in ambient conditions for 2 days, and then for another 2 days with air-drier set at 30°C before placing them for soaking in water, as described in section 2.5.2. Control (untreated) seeds were also used. Water uptake was expressed as the weight of water absorbed per seed. Each mean was the average of ten values. The effect of wetting and drying of seeds on the physical characteristics of the coat was observed. Control (untreated) seeds were also used. The adherence of the seed coat to cotyledons was visually examined. The

surface of the seed coat after the wetting and drying was observed by a low magnification stereoscope.

3.3. Results

3.3.1. Seed characteristics of the twenty genotypes

Imbibition studies were conducted on seeds of 20 genotypes, selected from a total of 50 genotypes that were obtained from different sources (section 2.1). The selected 20 genotypes represented a wide range of seed coat colour (Table 2.1) and seed size (Table 3.1). Additionally, seeds of line GC 84128-17-2-1 and AGS-292 had a high proportion of seeds with split coats. The seed coat colour ranged from yellow (9 genotypes), green (4 genotypes), black (2 genotypes) and 1 genotypes with brown seed coat colour. Additionally, there were 4 genotypes with the seed coat partially black or brown coloured (Table 2.1).

There were differences ($P<0.001$) in seed dry weight between the genotypes (Appendix 1, Table 1.1). Line G2120 had the smallest seed weight (60 mg seed^{-1}) whereas line GC84128-17-2-1 had the largest seed weight (331 mg seed^{-1}) (Table 3.1). There were differences ($P<0.001$) in seed surface area between the genotypes (Appendix 1, Table 1.2). Line G2120 had the smallest area (69 mm^2 seed^{-1}) and line GC84128-17-2-1 had the largest area (223 mm^2 seed^{-1}). There were differences ($P<0.001$) in seed coat dry weight between the genotypes (Appendix 1, Table 1.3). Line G2120 had the smallest seed coat dry weight (5.8 mg seed^{-1}) whereas cv. Suwan-155 had the largest seed coat dry weight (18.3 mg seed^{-1}) (Table 3.1). There were differences ($P<0.001$) in seed coat dry weight as a percentage of seed dry weight between the genotypes (Table 3.1). Line GC84128-17-2-1 had the smallest value (5.4%) whereas the cv. Pusa-16 had the highest value (13.8%) (Table 3.1). There were differences ($P<0.001$) in seed coat dry weight per unit area between genotypes (Table 3.1). Line VLS-1 had the smallest value (0.075 mg mm^{-2}) whereas the cv. Pusa-40 had the highest value (0.15 mg mm^{-2}) (Table 3.1).

There were strong positive correlations between seed dry weight and seed coat dry weight and between seed dry weight and seed surface area. There was a strong negative correlation between seed dry weight and seed coat dry weight as percentage to seed dry weight. However, there was a weak negative correlation between seed dry weight and seed coat dry weight per unit area (Table 3.1).

Table 3.1. Seed characteristics of 20 soybean accessions.

	Accessions	Dry wt seed^{-1}	Surface area seed^{-1}	Seed coat dry wt seed^{-1}	Seed coat dry wt as % of seed dry wt	Seed coat dry wt per unit area
		(mg)	(mm^2)	(mg)	(%)	(mg/mm^2)
Ac 12	G 2120	60	69	5.8	9.7	0.084
Ac 16	Pusa-16	71	66	9.8	13.8	0.150
Ac 17	Pusa-40	84	65	9.8	11.7	0.150
Ac 3	KWS-5	102	104	9.8	9.6	0.094
Ac 8	Picador	127	110	9.7	7.6	0.088
Ac 13	CC 84051-32-1	131	114	10.7	8.1	0.094
Ac 5	KWC-C	132	116	12.2	9.2	0.100
Ac 18	VLS-1	134	130	9.7	7.4	0.075
Ac 4	KWS-A	155	127	12.3	7.9	0.097
Ac 7	Toreador	157	139	11.8	7.5	0.085
Ac 2	KWS-3	158	127	12.3	7.8	0.097
Ac 19	Pioneer-9581	160	156	12.7	7.9	0.081
Ac 1	KWS-2	168	142	13.2	7.9	0.093
Ac 9	Essor	190	142	13.2	6.9	0.093
Ac 15	GC 88037-38-2-2	199	147	13.2	6.6	0.089
Ac 14	SS 87040-2-1	201	154	13.2	6.5	0.086
Ac 6	KWS-E	204	143	14.2	6.9	0.099
Ac 20	Suwan-155	257	192	18.3	7.1	0.095
Ac 11	AGS 292	283	183	15.8	5.6	0.086
Ac 10	GC 84128-17-2-1	331	223	18	5.4	0.081
s.e.m.		*7.8*	*1.8*	*1.4*	*0.7*	*0.001*
correlation with seed dry wt			0.96***	0.93***	-0.81***	-0.44*

***: P<0.001, *: P<0.05

3.2.2. Imbibition behaviour of the twenty genotypes

The percentage weight increase after 6h of imbibition was measured in seeds with the seed coat and seeds without the seed coat (i.e. embryos). In general, there were differences ($P<0.001$) between the genotypes in the water uptake after 6 h of imbibition (Appendix 1, Table 1.4). Considerable differences ($P<0.001$) between the genotypes in percentage weight increase were observed when seeds with seed coats imbibed (Table 3.2). However, the differences on water uptake between the genotypes were smaller in the absence of the seed coat than in the presence of the seed coat. Seeds of cv. Pioneer-9581 had the lowest percentage weight increase (35%) and seeds of cv. Suwan-155 had the highest percentage weight increase (138%) after 6h of imbibition.

The 20 genotypes could be divided into four groups in relation to the percentage weight increase after 6h of imbibition. In the first group, there were 4 genotypes where the percentage weight increase of seeds was considerable lower (between 2 to 3.5 times) than that of embryos. In the second group, there were 11 genotypes were seeds imbibed 15-30% less ($P<0.001$) water than embryos. In the third group, there were 4 genotypes in which the percentage weight increase was the same in the seed as in the embryo. The fourth group consisted of 1 genotype in which seeds had a higher ($P<0.001$) percentage weight increase than embryos.

Figure 3.2 shows the leachate conductivity after 6h of imbibition of seeds with seed coat and seeds without seed coat. In general, there were differences ($P<0.001$) between the genotypes in the water uptake after 6 h of imbibition (Appendix 1, Table 1.5). Considerable differences ($P<0.001$) between the genotypes in leachate conductivity were observed when seeds with the seed coat imbibed (Table 3.2). However, the differences on leachate conductivity between the genotypes were smaller in the absence of the seed coat than in the presence of the seed coat. Seeds of cv. Pioneer-9581 had the lowest leachate conductivity (4 mS/g/cm) whereas those of line GC 84128-17-2-1 had the greatest conductivity (30 mS/g/cm) reading.

The 20 genotypes could be divided into three groups in relation to leachate conductivity. In the first group, there were 5 genotypes where the conductivity of seeds was largely lower (between 2 to 5.5 times) than that of embryos. In the second group, there were 10 genotypes in which seeds had 30-45% lower ($P<0.001$) leachate conductivity than that of embryos. In the third group, there were 4 genotypes in which the leachate conductivity was the same in the seed as in the embryo.

Table 3.2. Percentage weight increase and electrical conductivity after 6h of imbibition of intact seeds and embryos of the twenty genotypes (n=4).

	Genotypes	Weight increase at 6h (%) ± s.e.m. (n=4)		Electrical conductivity at 6h (mS/g/cm) ± s.e.m. (n=4)	
		seed	embryo	seed	embryo
Ac 19	Pioneer-9581	35 ± 1.4	123 ± 2.5	4 ± 0.7	22 ± 1.4
Ac 9	Essor	41 ± 1.9	119 ± 3.2	5 ± 0.3	22 ± 0.9
Ac 1	KWS-2	45 ± 4.3	123 ± 3.9	7 ± 0.4	21 ± 0.8
Ac 18	VLS-1	61 ± 1.2	127 ± 3.4	9 ± 0.4	21 ± 0.8
Ac 16	Pusa-16	94 ± 3.5	116 ± 3.3	19 ± 0.7	25 ± 0.9
Ac 12	G 2120	98 ± 2.1	121 ± 3.8	19 ± 1.2	25 ± 0.7
Ac 17	Pusa-40	98 ± 2.6	119 ± 3.6	15 ± 0.6	22 ± 1.2
Ac 2	KWS-3	99 ± 2.4	125 ± 1.6	14 ± 1.1	22 ± 1.2
Ac 13	CC 84051-32-1	99 ± 3.1	125 ± 1.8	17 ± 0.4	24 ± 0.7
Ac 8	Picador	100 ± 2.9	128 ± 3.8	19 ± 0.8	26 ± 0.6
Ac 3	KWS-5	102 ± 3.4	135 ± 1.9	18 ± 0.4	25 ± 0.9
Ac 5	KWC-C	102 ± 3.8	123 ± 3.1	12 ± 1.3	21 ± 0.9
Ac 7	Toreador	104 ± 3.3	123 ± 2.7	14 ± 0.9	22 ± 1.2
Ac 6	KWS-E	105 ± 2.7	130 ± 2.4	16 ± 1.2	23 ± 0.6
Ac 4	KWS-A	107 ± 3.6	122 ± 3.2	18 ± 0.4	24 ± 0.9
Ac 15	GC 88037-38-2-2	121 ± 3.4	125 ± 3.9	25 ± 0.8	26 ± 1.1
Ac 11	AGS 292	121 ± 3.7	123 ± 3.5	29 ± 0.6	31 ± 0.8
Ac 14	SS 87040-2-1	122 ± 3.8	127 ± 3.4	26 ± 0.7	28 ± 1.4
Ac 10	GC 84128-17-2-1	122 ± 4.1	126 ± 2.9	30 ± 0.8	30 ± 0.9
Ac 20	Suwan-155	138 ± 3.8	123 ± 3.1	27 ± 0.9	26 ± 0.6

The variation in both percentage weight increase and leachate conductivity suggested that there could be a relationship between them. A scatter diagram of percentage weight increase plotted against leachate conductivity showed a strong positive correlation between the two variables (Fig. 3.1).

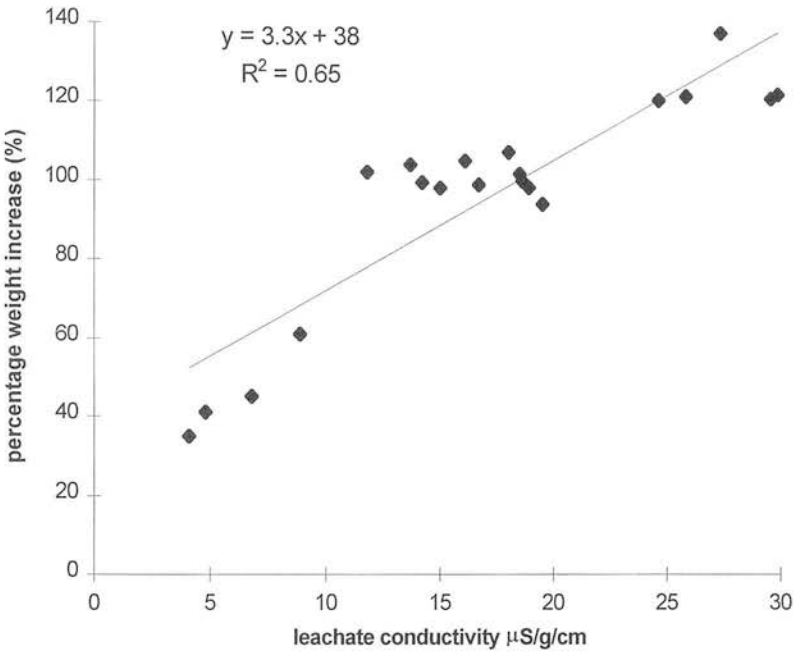


Figure 3.1. Relationship between mean leachate conductivity and mean percentage weight increase after 6h of imbibition of seeds with seed coat of the 20 genotypes.

The time course of water uptake during imbibition was measured. Results on the water uptake were presented for seeds of ten genotypes (Fig. 3.2). The selected genotypes were previously shown (Table 3.2) to have a low, medium and rapid water uptake after 6h of imbibition. In particular, seeds of cv. Pioneer-9581, Essor, line VLS-1 and KWS-2 had a low water uptake after 6h of imbibition. In seeds of line VLS-1, wrinkling of the seed coat was observed only on the dorsal region of the seed until after 2h of imbibition. Seeds of cv. Toreador and line KWS-E had a medium water uptake after 6h of imbibition. Seeds of line GC84128-17-2-1, AGS 292, GC 88037-38-2-2 and SS 87040-2-1 had a rapid water uptake after 6h of imbibition. In seeds of lines GC 88037-38-2-2 and SS 87040-2-1, wrinkling of the seed coat was observed in all regions of the seed within minutes of imbibition.

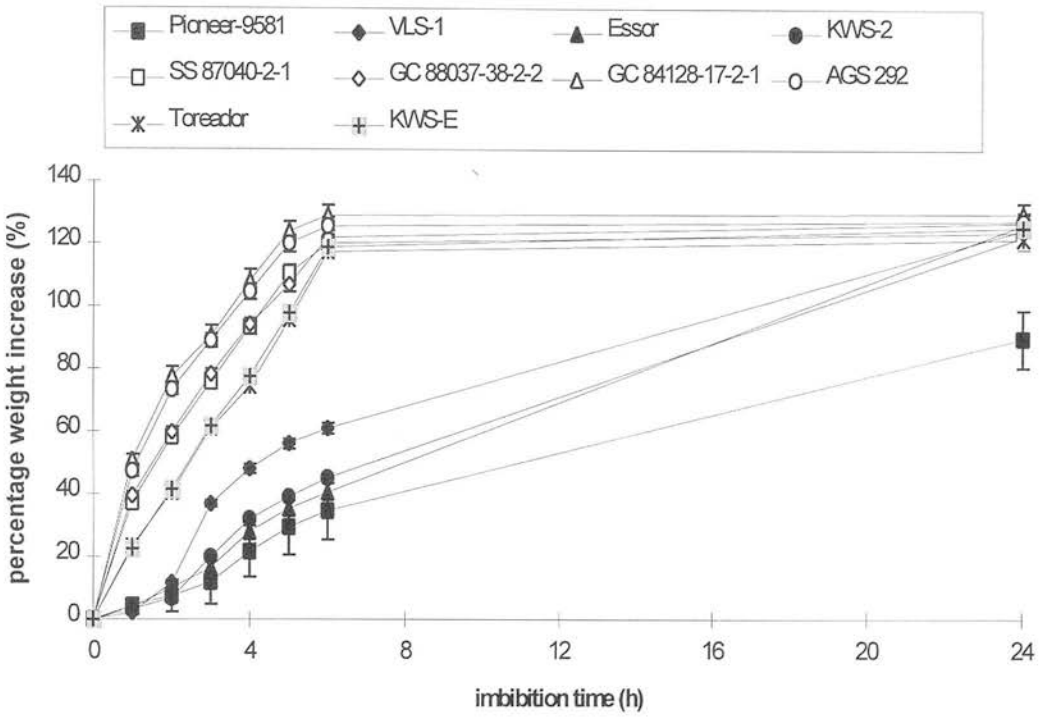


Figure 3.2. Percentage weight increase of ten individual seeds of the ten genotypes during imbibition; error bar, standard error of the mean, (n=10).

Figure 3.2 shows the percentage weight increase of individual seeds of the ten genotypes during imbibition. In general, there were differences ($P<0.001$) between the genotypes in the water uptake after 6 h of imbibition (Appendix 1, Table 1.6). The greatest rate of water uptake was observed in seeds of line GC84128-17-2-1 and AGS 292 which had a high proportion of seeds with splits in the seed coats. During the first hour they had a 50% weight increase and imbibition was completed at 6 hours. Seeds of line SS87040-2-1 and GC88037-38-2-2 had, also, a high rate of water uptake; during the first hour of imbibition they had a 40% weight increase. Seeds of cv. Toreador and line KWS-E had lower ($P<0.001$) water uptake than those of the previous four genotypes. However, after 6h of imbibition they had gained more than 90% of the weight increase at 24h imbibition. Seeds of line VLS-1, had a low water uptake during the first two hours of imbibition with a rapid increase (from 11-36% weight increase) between second and third hour of imbibition. Seeds of Essor and line KWS-2 had low water uptake during the first 6h of imbibition but the water uptake after 24h was similar to the previous genotypes. Seeds of cv. Pioneer-9581 had the lowest percentage weight increase during the whole imbibition period with, also, the lowest water uptake after 24h of imbibition.

Large variations in the water uptake were observed between individual seeds of cv. Essor, Pioneer-9581 and line KWS-2 as indicated by the large size of the standard error of the means (particularly evident in seeds of Pioneer-9581). The variation was due to the occurrence of hard seeds (Table 3.3). In cv. Pioneer-9581, there was a high percentage of hard seeds during the whole period of imbibition. In contrast, in cv. Essor and line KWS-2 a small proportion of those seeds was observed; all seeds of these two genotypes were became permeable to water after 24h of imbibition.

Table 3.3. The percentage of hard seeds remained during imbibition in seeds of the three genotypes.

Genotypes	percentage of hard seeds remained						
	1h	2h	3h	4h	5h	6h	24h
Essor	30	30	30	30	30	20	0
KWS-2	20	20	20	10	10	10	0
Pioneer-9581	70	70	70	70	70	60	50

The vital staining of the cotyledons with tetrazolium chloride was examined in seeds imbibed in either water or 30% (w/v) PEG with the seed coat intact or scarified (Table 3.4). In general, there were differences ($P<0.001$) between the genotypes in the water uptake after 6 h of imbibition (Appendix 1, Table 1.7). In most genotypes, scarified seeds soaked in water had a lower percentage of cotyledons fully stained in tetrazolium chloride that that of intact seeds soaked in water. When seeds soaked in 30% (w/v) PEG, the percentage of cotyledons fully stained was the same in the scarified seeds as in the intact seeds. A considerable variation ($P<0.001$) between the genotypes in the vital staining of the cotyledons was observed when intact seeds were soaked in water prior to staining. Seeds of line GC 84128-17-2-1 had 6% of cotyledons fully stained whereas seeds of line VLS-1 had 94% of the cotyledons fully stained. About half of the genotypes had more than 75% of the cotyledons fully stained, 6 genotypes between 20-60% of the cotyledons fully stained and 3 genotypes with less than 10% of the cotyledons fully stained (Table 3.4).

In most genotypes, seeds with intact coats imbibed in 30% (w/v) PEG , had a higher (P<0.001) percentage of their cotyledons that were fully stained than that of seeds imbibed in water; that was particularly evident in the genotypes with lower percentage of cotyledons stained. In contrast, the genotypes with higher percentage of cotyledons fully stained had little or no improvement when soaked in 30% PEG in comparison to water.

When seeds with scarified coats soaked in water, a lower (P<0.001) percentage of vital staining of the cotyledons than that of intact seeds was observed. The reduction in the percentage cotyledons stained was particularly evident in the genotypes which had a higher percentage of vital staining of the cotyledons rather than the genotypes with a lower percentage of vital staining of the cotyledons. In seeds of three genotypes (line G2120, KWS-5 and cv. Pusa-16), there was no difference in the vital staining of the cotyledons regardless of the treatment.

A scatter diagram of percentage weight increase and percentage of cotyledons fully stained with tetrazolium chlorite suggested that there could a relationship between them. Indeed, there was a negative correlation between the two variables (Fig. 3.3).

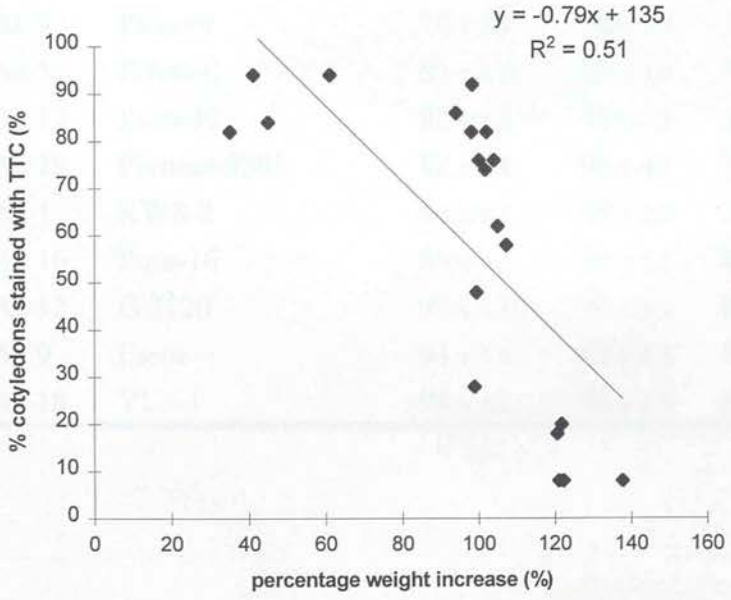


Figure 3.3. Relationship between mean percentage weight increase and mean percentage of cotyledons fully stained with tetrazolium chloride in seeds of the 20 genotypes.

Table 3.4. The percentage of cotyledons fully stained with tetrazolium chloride after 24h imbibition in water or 30% PEG with the seed coats intact or scarified, (n=4).

Accessions		Percentage of cotyledons fully stained with tetrazolium chloride (TTC) ± s.e.m. (n=4)			
		testa intact		testa scarified	
		water	30% PEG	water	30% PEG
Ac 10	GC 84128-17-2-1	6 ± 2.3	55 ± 3.3	6 ± 3.4	60 ± 4.6
Ac 11	AGS 292	8 ± 3.2	61 ± 4.3	6 ± 4.6	52 ± 5.8
Ac 20	Suwan-155	8 ± 2.8	51 ± 4.9	6 ± 3.5	59 ± 6.3
Ac 15	GC 88037-38-2-2	18 ± 5.9	47 ± 2.5	4 ± 3.4	39 ± 3.2
Ac 14	SS 87040-2-1	20 ± 6.1	66 ± 4.2	6 ± 2.7	72 ± 4.3
Ac 13	CC 84051-32-1	28 ± 5.8	54 ± 5.6	4 ± 3.8	59 ± 3.4
Ac 2	KWS-3	48 ± 4.6	60 ± 6.2	26 ± 3.1	54 ± 2.3
Ac 4	KWS-A	58 ± 5.4	82 ± 3.2	24 ± 4.3	76 ± 3.6
Ac 6	KWS-E	62 ± 6.3	88 ± 2.9	38 ± 4.9	88 ± 4.3
Ac 3	KWS-5	74 ± 3.9	76 ± 4.8	72 ± 3.7	80 ± 3.6
Ac 7	Toreador	76 ± 4.3	82 ± 5.3	56 ± 5.3	78 ± 4.6
Ac 8	Picador	76 ± 2.9	80 ± 5.7	54 ± 2.8	74 ± 5.3
Ac 5	KWC-C	82 ± 6.2	80 ± 3.6	58 ± 4.3	82 ± 6.2
Ac 17	Pusa-40	82 ± 4.6	78 ± 3.1	70 ± 3.6	85 ± 4.2
Ac 19	Pioneer-9581	82 ± 6.4	92 ± 4.5	72 ± 2.7	94 ± 2.8
Ac 1	KWS-2	84 ± 4.3	90 ± 3.9	36 ± 2.9	88 ± 3.7
Ac 16	Pusa-16	86 ± 3.5	88 ± 5.3	80 ± 4.3	84 ± 4.4
Ac 12	G 2120	92 ± 4.3	90 ± 3.1	86 ± 4.7	92 ± 5.1
Ac 9	Essor	94 ± 5.6	92 ± 4.2	54 ± 4.9	90 ± 3.6
Ac 18	VLS-1	94 ± 4.2	90 ± 3.3	42 ± 5.3	88 ± 3.9

3.2.3. Seed coat characteristics in relation to water uptake

The relationship between seed coat characteristics and water uptake during imbibition was investigated (Fig. 3.4). There was no correlation between seed coat dry weight and the percentage weight increase after 6h of imbibition. However, the scatter plot distribution indicated that three points were far removed from the rest. The three points represented the genotypes with a proportion of hard seeds (cv. Essor, Pioneer-9581 and line KWS-2). When these three genotypes were excluded from the calculation, a positive correlation was obtained ($y=3.8x + 59$, $R^2=0.53$).

There was no correlation between seed coat dry weight as a percentage to seed dry weight and the percentage weight increase after 6h of imbibition. Exclusion of the three genotypes with a proportion of hard seeds from the calculation, resulted in a weak negative correlation ($y=-3.2x + 132$, $R^2=0.17$).

There was no correlation between seed coat dry weight per unit area and percentage weight increase after 6h of imbibition. Exclusion of the three genotypes with a proportion of hard seeds had no affect in the correlation.

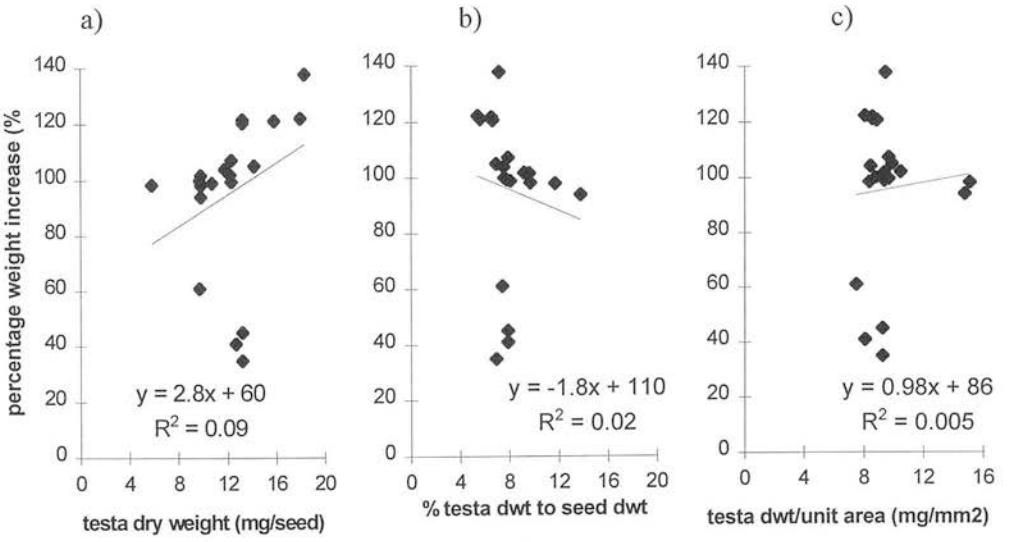


Figure 3.4. Relationship between mean percentage weight increase after 6h of imbibition and seed coat characteristics; a) seed coat dry weight, b) seed coat dry weight as a percentage to seed dry weight, and c) seed coat dry weight per unit area.

The water uptake through different regions of the seed coat was measured. In seeds of line KWS-E, the hilum was wide open whereas in seeds of cv. Sapporo it was closed.

Figure 3.5 shows the weight of water imbibed during the first 3h of imbibition when the dorsal, ventral or abaxial region of the seed was in contact with water. Little difference ($P>0.005$) between the three regions in the weight of water absorbed by seeds of cv. Sapporo was observed (Appendix 1, Table 1.8) whereas large differences ($P<0.001$) between the three regions in the weight of water absorbed by seeds of line KWS-E was observed (Appendix 1, Table 1.9). In both genotypes, the lowest water uptake was observed when the ventral region of the seed was exposed to water. Also, in both genotypes, there was no difference in the water uptake between the dorsal and abaxial region of the seed during the first 3h of imbibition. In seeds of line KWS-E, with the hilar fissure wide open, the water uptake from the ventral region was 17-33% lower ($P<0.001$) that from the dorsal region during the first 2h of imbibition. The differences in the water uptake between the dorsal and the ventral region were greater in seeds of cv. Sapporo (hilar fissure closed) than in seeds of line KWS-E (hilar fissure wide open). In seeds of cv. Sapporo the water uptake from the ventral region was from 2 to 4.5 times lower than that from the dorsal region during the first 2h of imbibition.

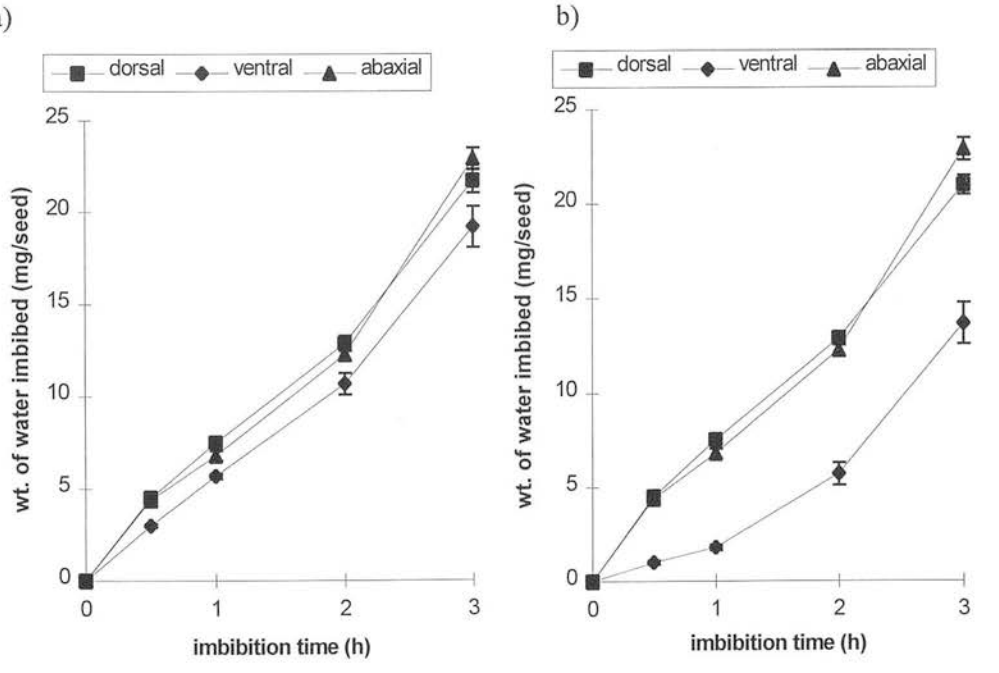


Figure 3.5. The weight of water imbibed during the first 3h of imbibition when the dorsal, ventral or abaxial region of the seed was exposed to water, a) seeds of line KWS-E, and b) seeds of cv. Sapporo; error bar, standard error of the mean, (n=10).

The hilar region of the seed coat in seeds of four genotypes was sealed with nail varnish, and then the amount of water imbibed during 4h of imbibition was measured. In seeds of line KWS-E, the hilum was wide open whereas in seeds of the other genotypes it was closed.

In seeds of genotypes with the hilum closed no difference between the control and the varnished seeds was observed (Appendix 1, Table 1.10). In contrast, in seeds of line KWS-E, varnished seeds had a 30% lower ($P<0.001$) and 12% lower ($P<0.001$) water uptake than that of control seeds, after 1h and 2h imbibition respectively (Fig. 3.6). However, no difference in the water uptake between varnished and control seeds was observed after the 3h of imbibition.

The effect of wetting and drying on the water uptake of seeds of four genotypes was examined (Fig. 3.7). In all four genotypes examined, wetting and drying resulted in an increased ($P<0.001$) water uptake in comparison to the untreated control seeds during the 4h period of imbibition (Appendix 1, Table 1.11).

Untreated control seeds of line VLS-1 had a low water uptake particularly during the first 1h of imbibition (5mg of water seed^{-1}). In contrast, treated seeds had 4 times and 2 times more water uptake than the untreated control seeds after 1h and 2h of imbibition respectively.

Untreated control seeds of line SS 87040-2-1 had a high water uptake; after 1h of imbibition seeds absorbed 77mg of water seed^{-1}. Treated seeds imbibed 43% more ($P<0.001$) water uptake than the untreated control seeds. In seeds of cv. Toreador, treated seeds had a higher ($P<0.001$) water uptake than untreated seeds, during the 4h imbibition period.

Untreated control seeds of cv. Toreador had a medium water uptake; after 1h of imbibition seeds absorbed 35mg of water seed^{-1}. Treated seeds imbibed 86% more ($P<0.001$) water uptake than the untreated control seeds.

Untreated control seeds of line G 2120 after 1h of imbibition seeds absorbed 13mg of water seed^{-1}. Treated seeds imbibed 2.5 times more water uptake than the untreated control seeds.

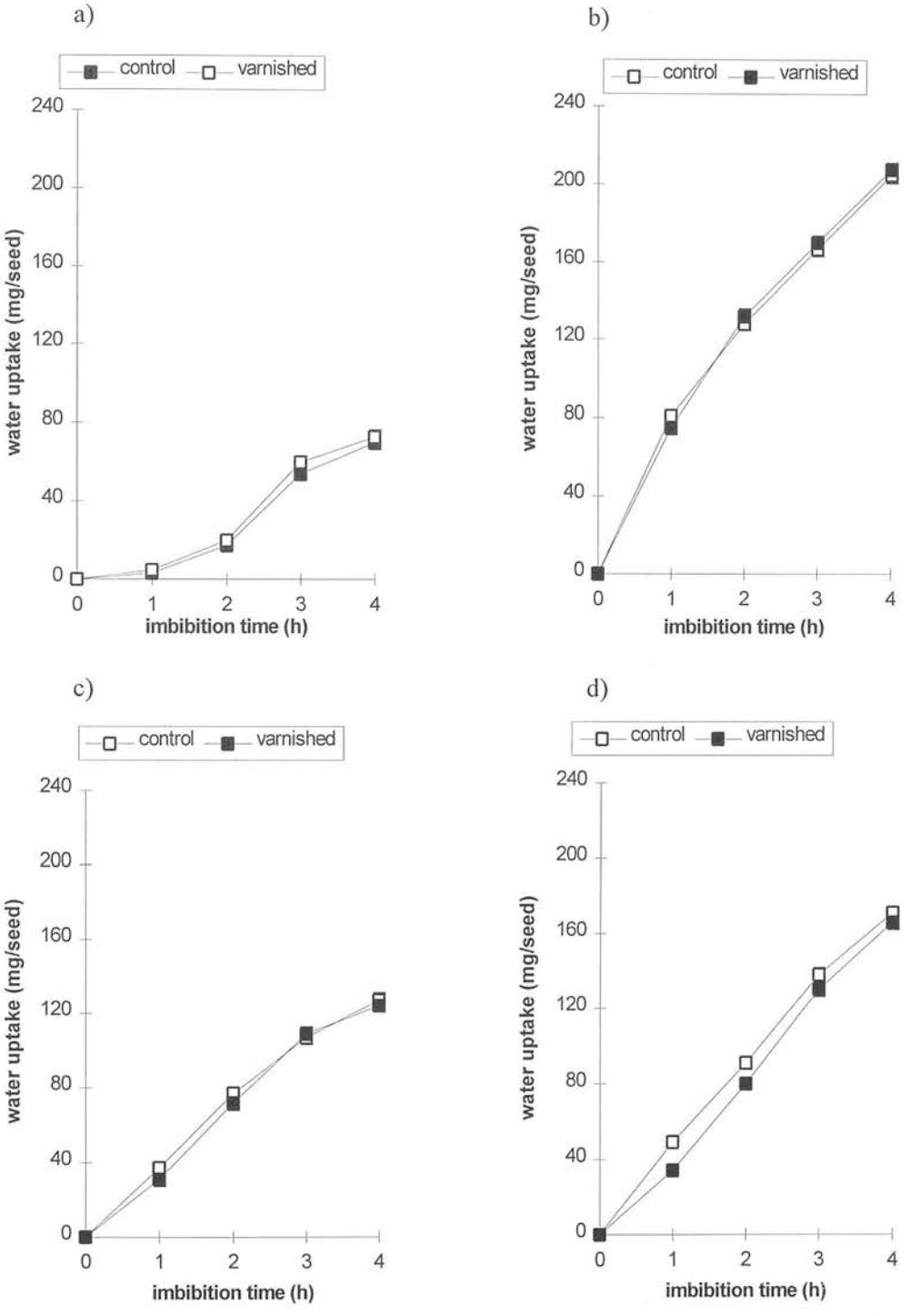


Figure 3.6. The weight of water imbibed during the first 4h of imbibition in control and varnished (hilar region) seeds, a) line VLS-1 b) line SS 87040-2-1 c) cv. Toreador d) seeds of line KWS-E; error bar (smaller than the symbols), standard error of the mean, (n=10).

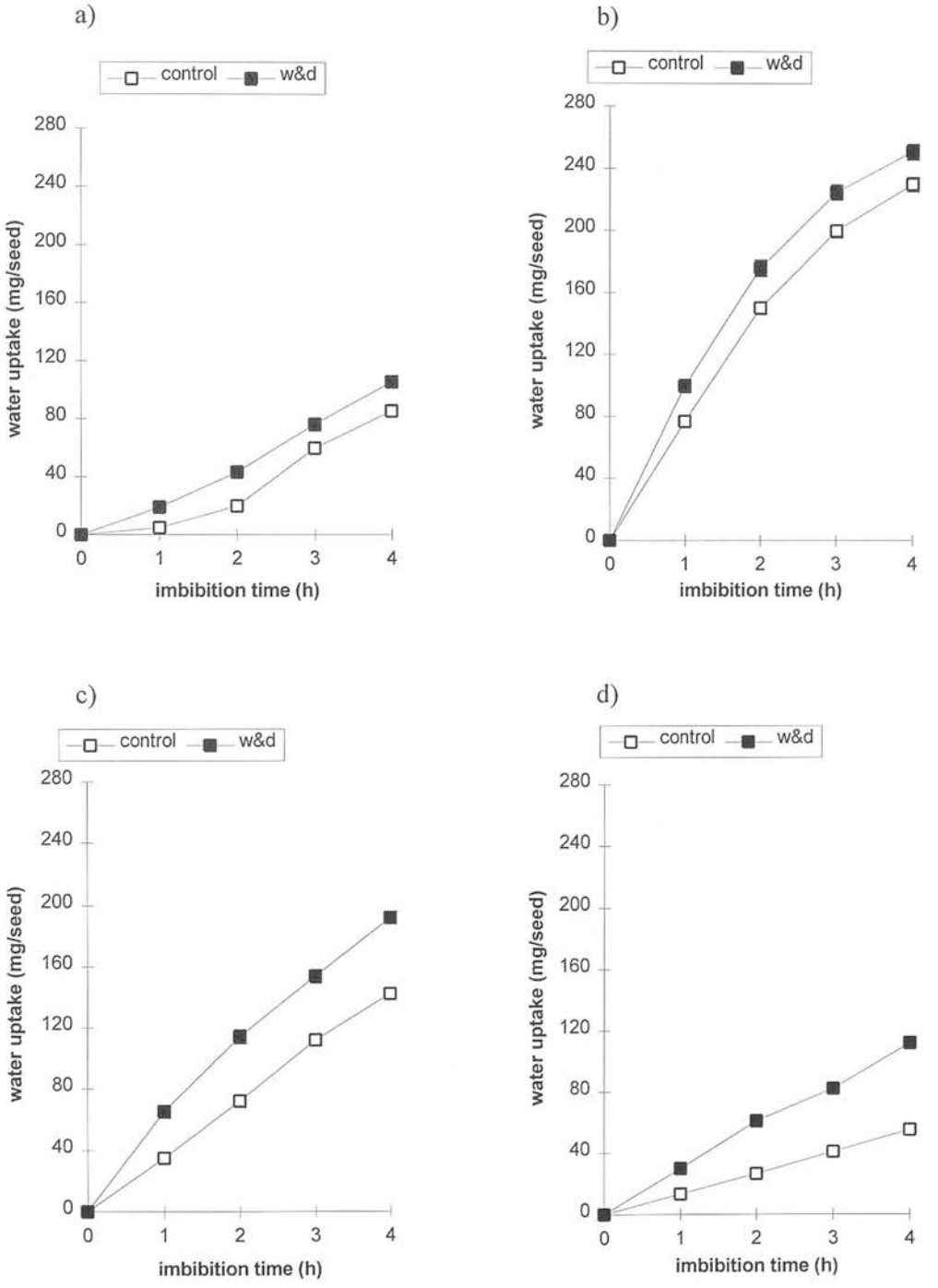


Figure 3.7. The weight of water imbibed during the first 4h of imbibition by control seeds and seeds which had undergone wetting and drying (w&d), a) line VLS-1 b) line SS 87040-2-1 c) cv. Toreador d) line G 2120; error bar (smaller than the symbols), standard error of the mean, (n=10).

The adherence of the seed coat to the embryo was examined visually. In seeds of cv. Toreador and line VLS-1, the seed coat adhered tightly to the embryo thus making difficult the separation of the two structures from each other (Fig. 3.8a). On the other hand, in seeds of cv. Suwan-155, the seed coat adhered loosely to the embryo, thus the embryo was completely separated from the seed coat (Fig. 3.8b). In addition, in most seeds of cv. Suwan-155, the seed coat in the dorsal region of the seed appeared to be highly wrinkled thus allowing additional extra free space between the seed coat and the embryo.

The effect of wetting and drying on the physical relationship between the seed coat and the cotyledons was examined visually. In seeds of cv. Toreador, after the wetting and drying treatment, the seed coat appeared to be detached from the embryo, particularly in the dorsal and abaxial region of the seed. In contrast, in seeds of line VLS-1, there was no clear difference between treated seeds and the untreated control seeds in the adherence of the seed coat to the embryo.

Surface view of the seed coat of untreated control seeds and seeds after wetting and drying under low magnification stereoscope was made. In seeds of cv. Toreador, soaking for 1h followed by drying resulted in the appearance of ellipsoidal-shaped ruptures in the surface of the seed coat (Fig. 3.9a). Some of the ruptures were of significant size but smaller ruptures appeared as well (Fig. 3.9a). In most cases, the large ruptures appeared in the abaxial and ventral region of the seed. In many seeds of line GC 88037-38-2-2, soaking for 1h followed by drying resulted in the appearance of long slit-shaped ruptures which were found predominantly in the abaxial region of the seed coat (Fig. 3.9b).

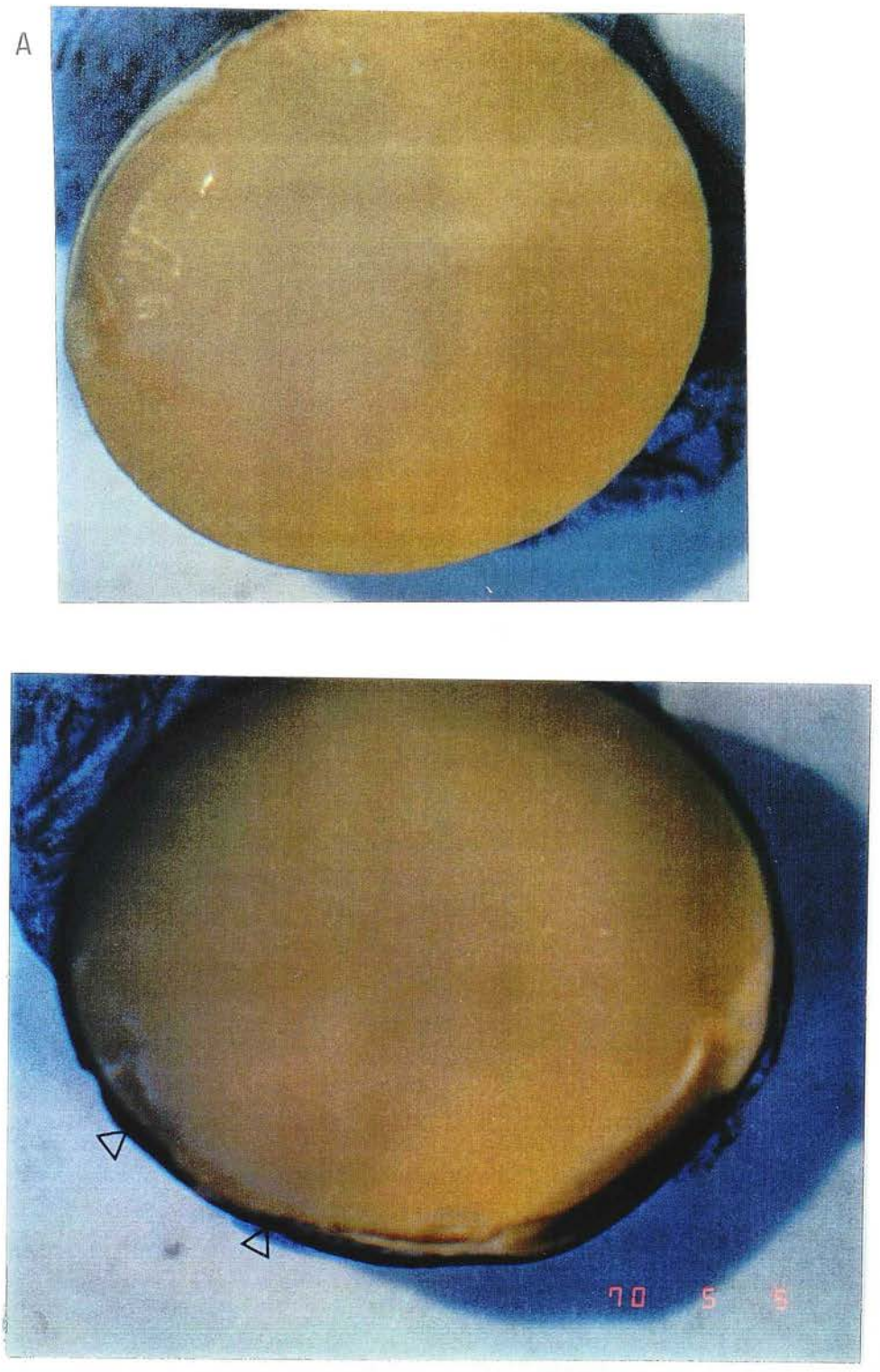
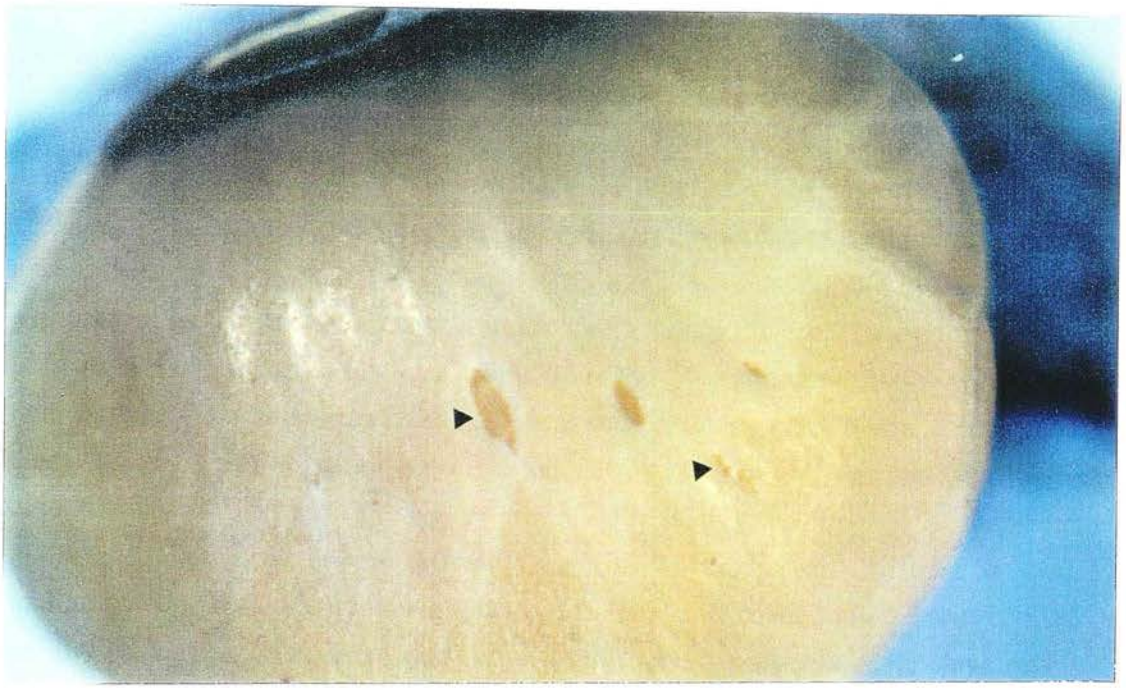


Figure 3.8. Micrographs showing the adherence of the seed coat to the embryo in a) seed of cv. Toreador and b) seed of cv. Suwan-155, 10x magnification; arrows indicate the gap between the seed coat and the embryo.

A



B

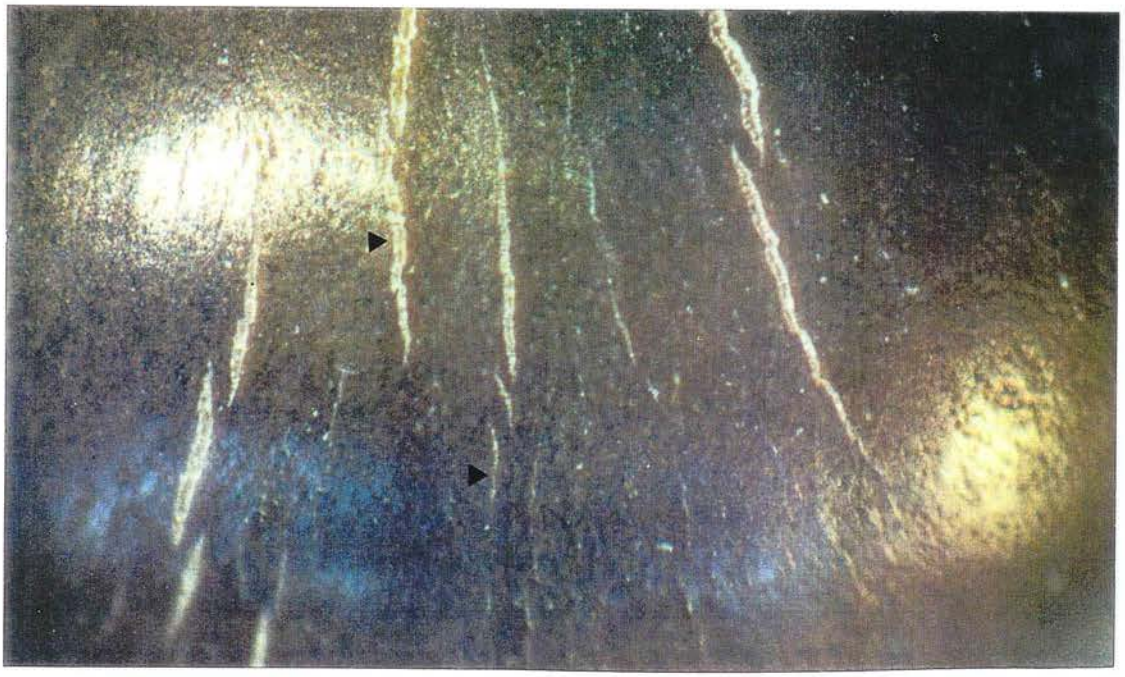


Figure 3.9. Micrographs showing the surface of the seed coat after wetting and drying in a) seed of cv. Toreador, 20x magnification, b) seed of line GC 88037-38-2-2, 30x magnification; arrows indicate the ruptures in the surface of the seed coat.

3.2. Discussion

In this study, the seed coat was clearly shown to be a regulator controlling the rate of water uptake in soybeans since seeds without a seed coat imbibed greater amounts of water than those with a seed coat. In addition to the role of the seed coat in controlling the rate of water uptake, the seed composition and seed microstructure have been proposed as other factors regulating the water diffusivity into seeds. Vertucci, (1989) reported that sweet maize had a higher diffusivity than dent maize. Phillips, (1968) reported that soybeans had about 10 times more water diffusivity than cotton (*Gossypium hirsutum* L.). However, Vertucci, (1989) in a comparative study of six grain legumes reported that seed composition was not the major determinant of diffusitivity since seeds with similar composition displayed remarkably different water diffusitivities. In a number of studies in both monocotyledons and dicotyledons species, the seed microstructure has been reported to be critical to the seed diffusitivity; *Phaseolus* embryos with low water diffusitivities had small, tightly packed starch granules whereas wheat grains with vitreous endosperms had a slower hydration than grains with mealy endosperms (Vertucci, 1989).

In this study, the rate of water uptake, the level of leachate conductivity and the extent of staining of the cotyledons with tetrazolium chloride were closely related characteristics. High rates of water uptake resulted in imbibition damage as evidenced by high leachate conductivity and low percentage of cotyledons staining with tetrazolium chloride. This observation is in agreement with two previous reports, in soybeans, that high rates of water uptake were associated with high solute leakage and low percentage of cotyledons fully stained with tetrazolium chloride (Semple, 1981; Oliveira *et al.*, 1984). Duke and Kakefuda (1981), reported additional evidence of the occurrence of imbibition damage due to rapid water uptake. They observed that when soybean embryos (seeds minus the testa) soaked in an aqueous solution of 1% Evans Blue, the outermost layers of cells absorbed the stain shortly after the soaking. They also reported that embryos had poor retention of solutes which was interpreted as being the result of cellular rupture during imbibition.

Further support for the incidence of imbibition damage in soybeans due to the rapid water uptake was the improved percentage of cotyledons fully stained with tetrazolium chloride when the genotypes were slowly imbibed in 30% PEG prior to

staining. The improvement in the percentage of cotyledons fully stained due to the low water uptake was particularly evident in the genotypes in which the imbibition damage was more pronounced. However, there were genotypes that even after the slow imbibition in 30% PEG a considerable number of the cotyledons remained unstained with the tetrazolium chloride. Also, there were genotypes where slow imbibition resulted in little or no improvement in comparison to the water soaking indicating that there were genotypes in which imbibition damage did not occur. Scarifiaction of the seed coat resulted in aggravation of imbibition damage in most cases, indicating that the seed coat offers a significant protection to the embryo in relation to imbibition damage.

Powell and Matthews (1979), in peas, reported that slow imbibition did not improve the vital staining of the cotyledons in all cases; slow imbibition in 30% Carbowax of seeds of cv. Sprite resulted in about 30% improvement in the vital staining of the cotyledons whereas the slow imbibition of seeds of cv. Kelvedon Wonder resulted in no improvement of the vital staining of the cotyledons. Powell *et al.*, (1986b) reported that, in dwarf French beans, slow imbibition in 40% PEG resulted in an improvement in the percentage of cotyledons fully stained with tetrazolium chloride only in the genotype with high imbibition damage. Legesse and Powell, (1992) reported in cowpeas improvement in the percentage of cotyledons fully stained with tetrazolium chloride when seeds were imbibed slowly from paper towel. They also reported that the improvement was particularly evident in genotypes with high rate of water uptake and low vital staining of the cotyledons. Abdullah *et al.*, (1992), in long beans, reported that in some genotypes even after slow imbibition in 30% PEG, a considerable proportion of cotyledons remained unstained with the tetrazolium chloride due to low vigour of seeds prior to testing. Similar results implicating the low vigour of seeds in cases where poor staining was observed after slow imbibition, were also reported in cowpeas (Legesse and Powell, 1992).

In this study, the condition of the seed coat appeared to be a very important factor in relation to the occurrence of imbibition damage. The two genotypes (line GC 84128-17-2-1 and AGS 292) with a high proportion of cracked seed coats had the highest rate of water uptake, the highest amount of leachate conductivity and the lowest vital staining of the cotyledons. Oliveira *et al.*, (1984) reported that cracks in the soybean seed coat resulted in a high rate of water uptake, leachate conductivity and low vital staining of the cotyledons.

Additionally, there were two genotypes (lines GC 88037-38-2-2 and SS 87040-2-1) in which although the testa was apparently intact (examined visually and under low magnification stereoscope), a high level of imbibition damage was observed. This observation indicated that there were situations where the presence of intact seed coat was not sufficient to reduce the rate of water uptake, and provide no protection against imbibition damage.

The slow mean rates of water uptake by three genotypes (cv. Essor, Pioneer-9581 and line KWS-2) was largely attributed to the presence of hard seeds. Thus, the mean rate of water uptake was related to the proportion of hard seeds present. Hard seeds became soft at different rates in the three genotypes. Similar effects of hard seeds on the water uptake have been reported in a number of grain legumes including chickpeas (Legesse and Powell, 1992), long beans (Abdullah *et al.*, 1992) and *Phaseolus* beans (Dickson and Boettger, 1982).

In this study, a number of genotypes with a wide range of seed sizes was examined. According to Nelson and Wang (1989), seed weight between 40 and 346mg per seed represents the 99% of the range of the seed weight of all accessions in the USDA Soybean Germplasm Collection. In the genotypes examined, the percentage seed coat dry weight to seed dry weight and the rate of water uptake were totally uncorrelated. This result is in agreement with several reports in the lack of correlation between the physical characteristics of the seed coat and the rate of water uptake (Calero *et al.*, 1981; Yaklich *et al.*, 1986).

In this study, water uptake did not occur uniformly through the soybean seed coat. Wrinkling of the seed coat started from the dorsal region, and then covered the abaxial and ventral region of the seed. This observation is in agreement with previous reports that wrinkling of the seed coat was the first visual sign of imbibition, and the dorsal region being the first one showing wrinkling of the seed coat as a sign of imbibition (Yaklich *et al.*, 1986; McDonald *et al.*, 1988). There was an association between the rate of water uptake and the time taken for wrinkles to appear after the start of imbibition.

From the results of the effect of seed proximity to the water uptake, it was shown that regardless of the openness of the hilar fissure, the ventral region facilitated the slowest rate of water uptake during the first 4h of imbibition. There

was no difference in the rate of water uptake between the dorsal and abaxial region of the seed coat. In contrast, McDonald *et al.*, (1988) in an experiment where soybean seeds orientated so that either the ventral or the dorsal region was exposed to water on paper towels, found that the ventral region facilitated a greater rate of water uptake than the dorsal region. Additionally, from the results in this study with nail varnish, it was shown that the hilar region played little or no role in water uptake in comparison to the permeability of the seed coat. Powell *et al.*, (1986a) reported that in dwarf French beans sealing of both hilum and micropyle resulted in a significant reduction in the rate of water uptake during the first 12h of imbibition. The hilum and micropyle has previously been shown to be a major route of the water entry into seeds of *Phaseolus* (Kyle and Randall, 1963; Schroth and Cook, 1964; Spurny, 1973; Korban *et al.*, 1981). However, in cowpeas, sealing of both hilum and micropyle in most genotypes resulted in no reduction in the rate of water uptake (Legesse and Powell, 1992).

The lower rate of water uptake in the hilar area in comparison to the other regions of the seed coat, may be explained on the basis that water could only penetrate through the tracheid bar but not through the palisade layers (outer and inner palisade). As a result, although the hilum fissure was wide open, the rate of water uptake was lower in comparison with the dorsal side. Ragus, (1987), on the basis of microscopical studies, suggested that testa and micropyle were better sites of entry of water than the hilum in soybeans. Hyde, (1954) proposed that the hilar fissure in Papilionoidae seeds acted as a hygroscopic valve, permitting water loss from, but preventing water entry into, the maturing, dehydrating seed. Lersten, (1982) in an anatomical survey of the tracheid bar of 232 species in Papilionoidae seeds supported the idea that the tracheids in the tracheid bar have lost their water conductive significance in favour of gas exchange capability.

In this study, there was no clear relationship between the colour of the seed coat and the rate of water uptake since it was observed that one genotypes with black coat imbibed water at high rate and one genotype with black coat imbibed water at a low rate of water uptake. The genotype with a brown seed coat imbibed water at a high rate. In the literature, there have been several reports showing a close association between dark coloured seed coat and low rate of water uptake. However, in this study, there were examined only 3 genotypes with black or brown seed coat. For a better assessment of the relationship between testa colour and water

permeability a larger number of genotypes with pigmented seed coat is required. Kuo, (1989) investigated the relationship between seed coat colour and water permeability in 20 soybean genotypes of which 15 had black or brown seed coats. A low rate of water uptake was clearly associated with the black seed coats although not all black genotypes had a low rate water uptake (Kuo, 1989). Powell *et al.*, (1986a), in dwarf French beans, reported a close association between the testa colour and a low rate of water uptake, in an investigation of 30 genotypes of which 20 had black or brown seed coats. Similar close associations between the dark coloured seed coat and a low rate of water uptake has been reported in several grain legumes including snap beans (Deakin, 1974; Wyatt, 1977), long beans (Abdullah, 1988), chickpeas (Legesse and Powell, 1992) and lima beans (Kannenberg and Allard, 1964).

In grain legumes, the close association between dark coloured seed coat and low water uptake has been explained on the basis of the quantity of the phenolic material, the adherence of the seed coat to the embryo and the high degree of seed coat cracking.

Firstly, low rate of water uptake by pigmented seeds due to the high quantity of phenolic material within the seed coat was proposed by Marbach and Mayer (1974, 1975). They reported that phenolic material, in pigmented *Pisum* seeds, could be oxidised in the presence of catechol oxidase during dehydration of the seeds following maturation. Other authors have been reported a similar indirect relationship between the development of the pigmentation (during seed development and maturation) and the rate of water uptake measured by those seeds. Legesse and Powell (1992) reported results in *Phaseolus* beans, chickpeas and cowpeas. They reported that in the absence of pigmentation at the early stages of maturation, seeds imbibed rapidly. However, as soon as pigmentation started to develop, the rate of the water uptake by the pigmented seeds was reduced. Additionally, the close association of pigmentation with reduced rates of water uptake has been previously reported in isogenic lines of peas (Powell, 1989). Seeds having the dominant A gene for seed coat colour were usually pigmented and imbibed slowly in comparison to the unpigmented seeds having the recessive gene (Powell, 1989).

Secondly, the rate of water uptake by pigmented seeds has been associated with the degree of the seed coat adherence to the embryo. In the present investigation, the existence of a narrow space between the seed coat and the embryo was observed which could provide an important pathway of water movement during imbibition.

This observation is in agreement with earlier studies which have shown that the seed coat of many genotypes detached from the embryo leaving a free narrow space in between (Moore, 1971). The importance of the close adherence of the seed coat in relation to the low water uptake has been reported in French beans (Powell *et al.*, 1986b), peas (Powell, 1989) and snap beans (Wyatt, 1977). Powell *et al.*, (1986a, 1986b) reported a close association between rapid water uptake, loose testa adherence and high imbibition damage in *Phaseolus* seeds. The water could move freely in the gap between the coats and the cotyledons (loose adherence of seed coat to the embryo) whereas prevention of water movement was observed in seeds in which there was a tight adherence of the seed coat to the embryo. Powell *et al.*, (1986a)suggested that changes in the rate of water uptake during imbibition after previous wetting and drying could indicate the contribution of the seed coat adherence to the water uptake in dwarf French beans. In this study, it was shown that the increased rate of water uptake of seeds subjected to one cycle of wetting and drying could not be attributed entirely to a loosening of the adherence of the seed coat to the embryo. Seeds subjected to wetting and drying appeared to have extensive ruptures in the surface of the seed coat. This observation is in agreement with previous results such as those of Wolf *et al.*, (1981) who soaked intact seeds of soybean cv. Beeson in water for 1h and then air-dried the seeds. They reported that this pre-treatment resulted in large splits in the seed coat and additional smaller cracks in the surface of the seed coat. They suggested that the seed coat could dry more rapidly than the cotyledons which resulted in fissures that closely resemble those found in field-harvested seeds as well. Also, an earlier report by Moore (1971) indicated that alternate cycles of wetting and drying progressively resulted in loosened, wrinkled seed coats and an increased number of fissures in soybeans. Adherence of the testa to the embryo could play an important role in the water uptake in soybeans but it appears to be difficult to measure or quantify it. As it was clearly shown, assessment of the role of testa adherence could not be made after one cycle of wetting and drying of the seeds.

Thirdly, the higher rate of water uptake in non-pigmented than pigmented seeds has been associated with the degree of cracking of the seed coat. Recently, Kantar *et al.*, (1996) reported that there was a greater number of cracks in the seed coat of zero-tannin faba beans than the high-tannins lines; the incidence of the cracks in the seed coats was clearly associated with low vigour in these lines.

In this study, one black seeded genotype (line VLS-1) had a low rate of water uptake during the first 2h of imbibition but a considerably greater rate of water uptake thereafter. This delayed-permeability character of the seed coat was associated with a low solute leakage and a high proportion of cotyledons fully stained with tetrazolium chloride. Kuo (1989) reported that 3 black seed coat genotypes had a similar delayed-permeability character. He suggested that this character could improve seed quality if successfully transferred to cultivated soybean genotypes.

In this study, one black seeded genotype (cv. Suwan-155) had a higher rate of the rate of water uptake when seeds soaked with the coat present than that of seeds without the coat present. This high rate of water uptake resulted in high solute leakage and very low percentage of cotyledons fully stained with tetrazolium chloride. A possible explanation for the previous anomaly may be the following: a loose adherence of the testa to the embryo was observed when seeds were visually examined. This loose adherence resulted in a wide gap between these two structures; the gap was greatly increased due to the wrinkling of the seed coat which was mainly concentrated in the dorsal region of the seed. As a result, water had penetrated the seed coat and trapped in the gap between the coat and the embryo. When seeds were blotted dry and then weighed, the weight increase referred not only to the weight of water absorbed but also to the amount of the water trapped in the gap. The presence of free water trapped in the gap between the seed coat and the embryo was visually confirmed during the imbibition experiments.

CHAPTER 4

Influence of the the condition of the seed coat on seed performance

4.1. Introduction

Seed longevity is markedly influenced by the ambient temperature, seed moisture content, pre-storage environment and genotype (Delouche, 1974; Ellis *et al.*, 1982).

There are several reports in the literature of differences amongst genotypes in seed deterioration characteristics in the field and during storage. Resistance to seed deterioration in the field has been associated with small seed size, short reproductive growth period and black seed coat colour (Dassou and Kueneman, 1984; Mugnisjah *et al.*, 1987). A large proportion, also, of hard seeds within a seed lot has been reported to offer resistance in the mother plant particularly when harvest was delayed and/or adverse environmental conditions occurred (Potts *et al.*, 1978; Minor and Pascal, 1982). Low infection by seed-borne fungi has been also associated with increased percentage germination and seedling emergence (Pascal and Ellis, 1978; TeKrony *et al.*, 1984). Deterioration of soybean seeds may start on the mother plant in the field prior to harvesting (Green *et al.*, 1965; Mondragon and Potts, 1974) and poor seed vigour, as revealed by low percentage of normal seedlings after 3 days of accelerated ageing, has been reported even at the physiological maturity (TeKrony *et al.*, 1980).

Seed moisture and temperature are the primary environmental determinants of seed longevity in storage (Roberts, 1960; Roberts, 1972). Ellis and Roberts (1980) proposed a quantitative model for the decline in the percentage viability during storage which could be applied in orthodox seeds under a wide range of seed moisture and temperature levels. Poor storability, in the tropics, has been a major biological constraint to expanding soybean production (Delouch et al., 1973; Singh and Rachie, 1987). According to a report by IITA (1979), hot temperatures (above 35 °C) and large relative humidity values (above 75% r.h.) accelerate seed deterioration and loss of viability; germination declined sharply after only 60 to 90 days in storage under ambient conditions. However, there have been several reports about consistent

differences in the storage potential among genotypes. The lines with high storage potential had seeds with smaller seed sizes than the lines with low storage potential but not all small seeded lines stored well (Wien and Kuenerman, 1981; Dassou and Kuenerman, 1984). In addition, Wien and Kuenerman (1981) reported that seed coat colour was not consistently correlated with storability, although, the best three lines in terms of longevity had black seed coats. Delayed permeability of the seed coat has been proposed to be a promising character for producing seeds of good quality (Kuo, 1989).

Seed coat splitting occurred in the outer layers of the seed coat and involved the separation of cuticle, palisade and hourglass layer of cells (Wolf *et al.*, 1981). Seed coat splitting could be a severe problem in soybeans, especially in genotypes with large seeds (Lassim and Delouche, 1981). This imperfection of the seed coat may affect seed quality either as an avenue for pathogens or increase deterioration particularly under adverse environmental conditions before or after harvest (Yaklich and Barla-Szabo, 1993). The occurrence of seeds with split coat within seed lots has occasionally been associated with low levels of seed germination and vigour, but not always (Burchett *et al.*, 1985).

There is still a lack of conclusive information about the influence of the condition of the seed coat in seed performance. Therefore, the objectives of this study were: (1) to measure the influence of the intact or split seed coat in seed, and (2) to present additional evidence of the effectiveness of the hard seed coat in maintaining high seed performance.

4.2. Materials and methods

One seed lot of each of the five cultivars that received from Agricultural Research Station, Mingora, Pakistan were provided by Dr. Ehsanullah Khan.

The genotypes were grown in the field under similar conditions, were of medium seed size and required similar time to flowering and maturity (Table 4.1). However, the condition of the seed coat was different. In particular, approximatelly 60% of the seeds of cv. Douglas were noticed to have seed coat splitting whereas in all other genotypes few seeds (discharged) with seed coat splitting were observed (Table 4.1). Separation of seeds with split or intact seed coat was visually made. Also, a high proportion of hard seeds (about 65%) were observed in cv. Pioneer-9581

whereas in all other genotypes no hard seeds were observed (Table 4.1). Separation of hard from soft seeds was made as described in section 2.2. Seeds of the five genotypes were placed for the accelerated ageing, as described in section 2.8. Cracked and intact seeds of cv. Douglas and hard and soft seeds of cv. Pioneer-9581 were placed separately for ageing. Seed moisture content during the ageing test was measured as described in section 2.2. Leachate conductivity from seeds was measured as described in section 2.6.

Table 4.1. Characteristics of the five soybean genotypes which were grown in the field, in Pakistan.

Genotypes	Dry weight seed^{-1} (mg)	Time to 50% flower (days)	Maturity time (days)
Forrest	182	42	134
Douglas	165	39	128
HSC-591	154	38	133
HSC-401	140	44	134
Pioneer-9581	153	41	137

The germination tests were performed as described in section 2.3. Seeds of cv. Pioneer-9581 that remained hard (about 10-15% of the initial amount) after the 8th day were carefully scarified and subjected to another germination test. The total percentage of normal seedlings was expressed as the sum of the two tests. Seedling evaluation at the end of the germination test was made as described in section 2.3. The fresh weight of all normal seedlings was measured before taking separate measurements for shoot and root fresh weight. Results from fresh weight were based in normal seedlings from the first germination test with unscratched seeds. The vigour index was calculated by multiplying the percentage of normal seedlings by seedling fresh weight, as proposed by Woodstock (1973)..

4.3. Results

Figure 4.1 shows the effect of accelerated ageing 1 to 4 days on the percentage of normal seedlings produced. Analysis of variance results were presented in

Appendix 2, Table 2.1. In the unaged control seeds, there was no significant difference between the genotypes in the percentage of normal seedlings. In general, as the ageing period increased, the percentage of normal seedlings declined. However, there were differences between genotypes in the rate of decline in the percentage of normal seedlings. After 1 day of ageing, there was no significant difference between the aged seeds and the unaged control seeds in the percentage of normal seedlings in most genotypes. However, in seeds of cv. Douglas an immediate and steady decline in the percentage of normal seedlings from the first day of ageing afterwards was measured. After 2 days of ageing, there was no difference between aged and unaged control seeds, in the percentage normal seedlings, only in seeds of cv. HSC-401. After 3 days of ageing, all genotypes had a decline ($P<0.001$) in the percentage normal seedlings in comparison to the unaged control seeds. The lowest decline in the percentage normal seedling was measured in seeds of cv. Pioneer-9581 and HSC-591; aged seeds had about 12% ($P<0.05$) lower percentage normal seedlings than that of the unaged control seeds. After 4 days of ageing, seeds of cv. Pioneer-9581 retained the highest percentage of normal seedlings; about 70% of aged seeds produced normal seedlings.

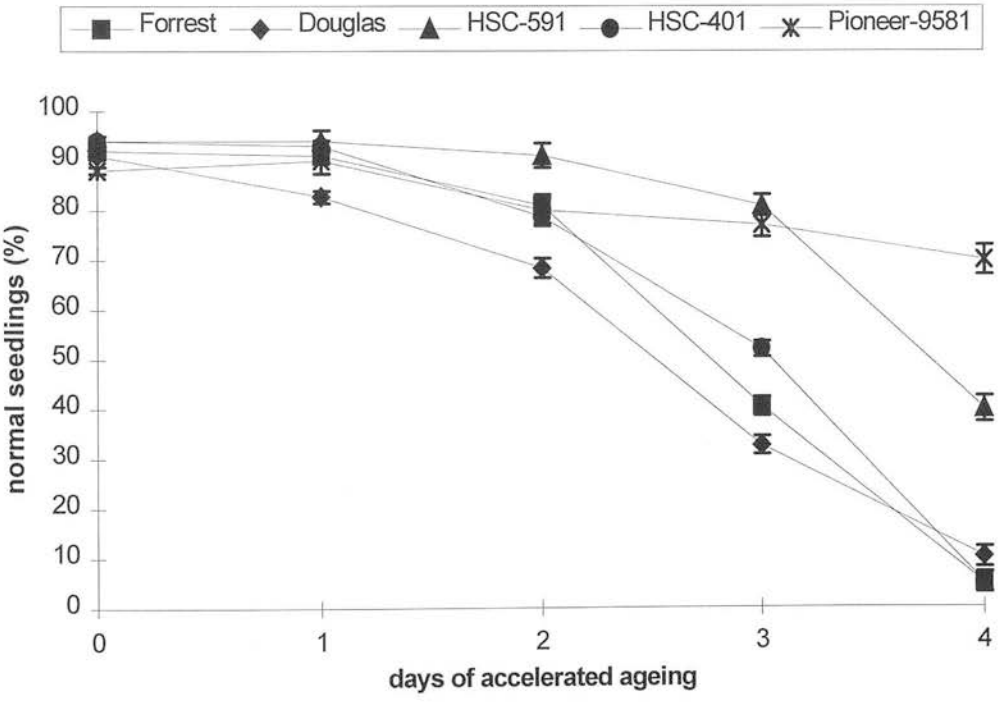


Figure 4.1. Effect of accelerated ageing on the percentage of normal seedlings produced from seeds of five genotypes; error bar, standard error of the mean, (n=4).

Figure 4.2a shows the effect of accelerated ageing on the percentage of normal seedlings produced by seeds with intact or split seed coats of cv. Douglas. In the unaged control seeds and seeds aged for 4days, there was no significant difference between seeds with intact or split coats in the percentage of normal seedlings. After 2 days of ageing, however, seeds with split coats had a 22% lower ($P<0.001$) percentage of normal seedlings than that of seeds with intact coats.

Figure 4.2b shows the effect of accelerated ageing on the percentage of normal seedlings produced by hard or soft seeds of cv. Pioneer-9581. In the unaged control seeds, there was no significant difference between hard or soft seeds in the percentage of normal seedlings. In hard seeds, ageing had no significant effect in the decline of percentage of normal seedlings. However, soft seeds produced 12% and 55% lower ($P<0.001$) percentage of normal seedlings than hard seeds at 2day and 4day of ageing respectively.

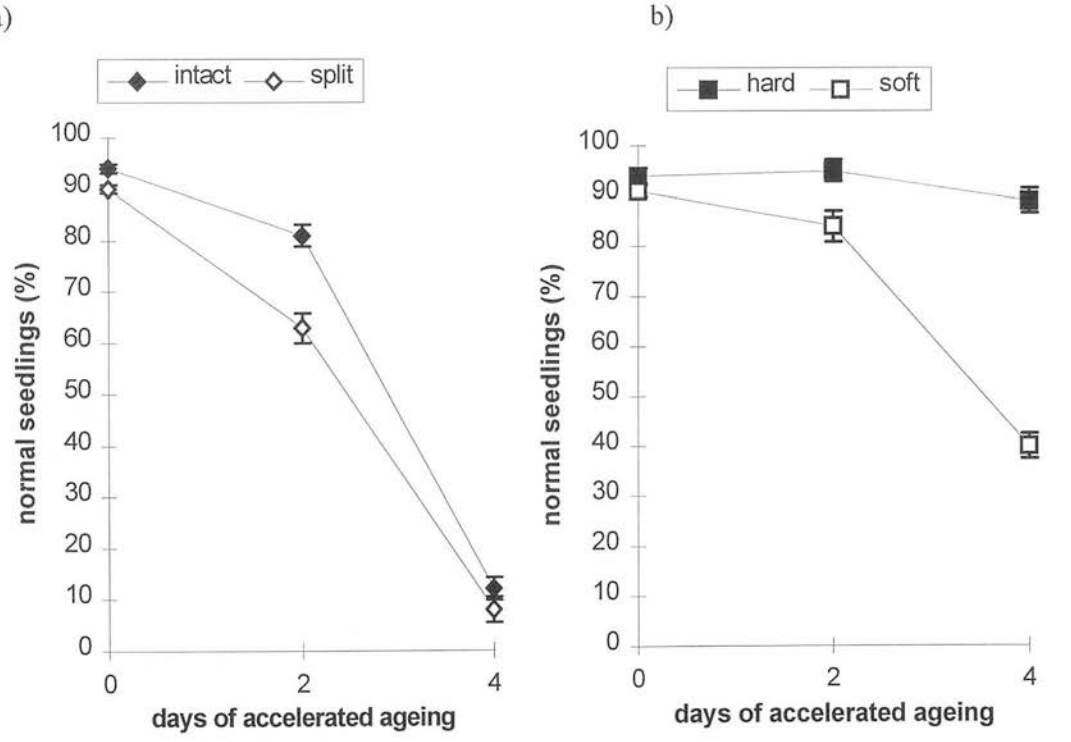


Figure 4.2. Effect of accelerated ageing after 2 and 4 days on the percentage of normal seedlings produced from seeds of a) cv. Douglas and b) cv. Pioneer-9581; error bar, standard error of the mean, (n=4).

Figure 4.3 shows the increase in the percentage moisture content during the ageing in seeds of the five genotypes. Analysis of variance results were presented in Appendix 2, Table 2.2. In unaged control seeds, difference in the percentage moisture content were only between seeds of cv. Pioneer-9581 and seeds of cv. Douglas. As the ageing period increased, the percentage moisture content also increased. However, there were differences between genotypes in the percentage moisture content. Seeds of cv. Pioneer-9581 had the lowest increase in the percentage moisture content, reaching 25% moisture content at the end of the ageing. Seeds of cv. Douglas had the highest increase in the percentage moisture content. At the 4 day of ageing seeds of all but cv. Pioneer-9581, reached about 32% moisture content.

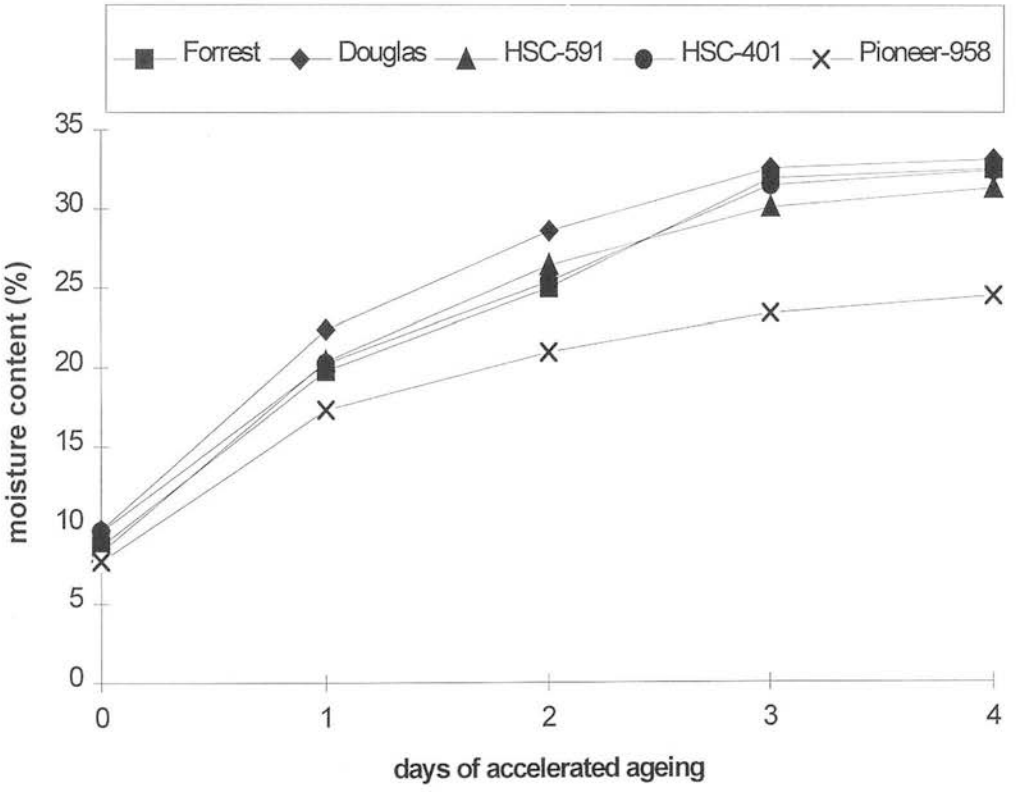


Figure 4.3. The percentage moisture content during the accelerated ageing of seeds of five genotypes; error bar (smaller than the symbols), standard error of the mean, (n=4).

Figure 4.4a shows the effect of accelerated ageing on the percentage moisture content in seeds with intact or split seed coats of cv. Douglas. In the unaged control seeds and seeds aged for 4days, there was no significant difference between seeds with intact or split coats in the percentage moisture content. After 2 days of ageing, however, seeds with split coats had a 3% higher ($P<0.001$) percentage of moisture content than that of seeds with intact coats.

Figure 4.4b shows the effect of accelerated ageing on the percentage moisture content in hard or soft seeds of cv. Pioneer-9581. In the unaged control seeds, soft seeds had a 14% higher ($P<0.001$) percentage moisture content than hard seeds. Soft seeds had also a 2.7 and 1.6 times higher percentage moisture content after the 2 day and 4 day of ageing respectively.

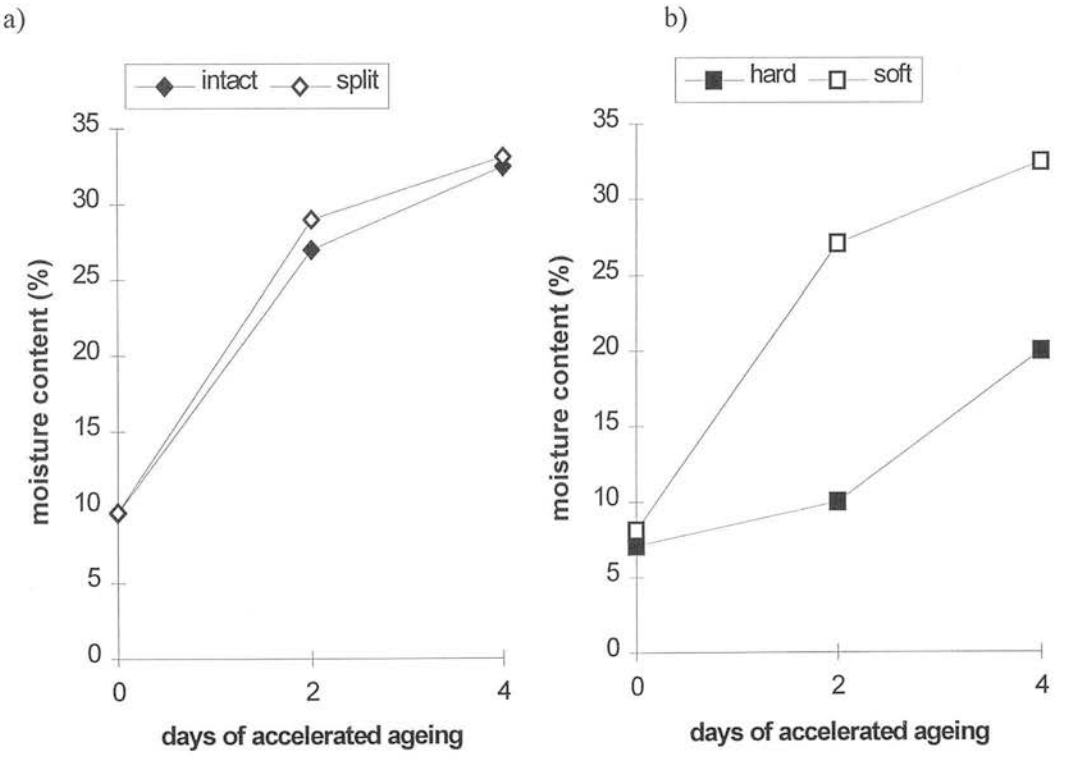


Figure 4.4. The percentage moisture content after 2 and 4 days of accelerated ageing of seeds of a) cv. Douglas and b) cv. Pioneer-9581; error bar (smaller than the symbol), standard error of the mean, (n=4).

Figure 4.5 shows the effect of accelerating ageing on the leakage from seeds of the five genotypes. Analysis of variance results were presented in Appendix 2, Table 2.3. Leakage from unaged seeds of cv. Douglas was 3 times more than that from seeds of cv. Pioneer-9481 was. There was no difference between the three other genotypes in the leakage conductivity. In general, as ageing increased the leachate conductivity increased. However, there were differences between genotypes in the conductivity values. Seeds of cv. Douglas had the highest leakage throughout the ageing period whereas seeds of cv. Pioneer-9581 had the lowest leakage throughout the ageing period. Aged seeds of the other three genotypes differed little in leakage when tested.

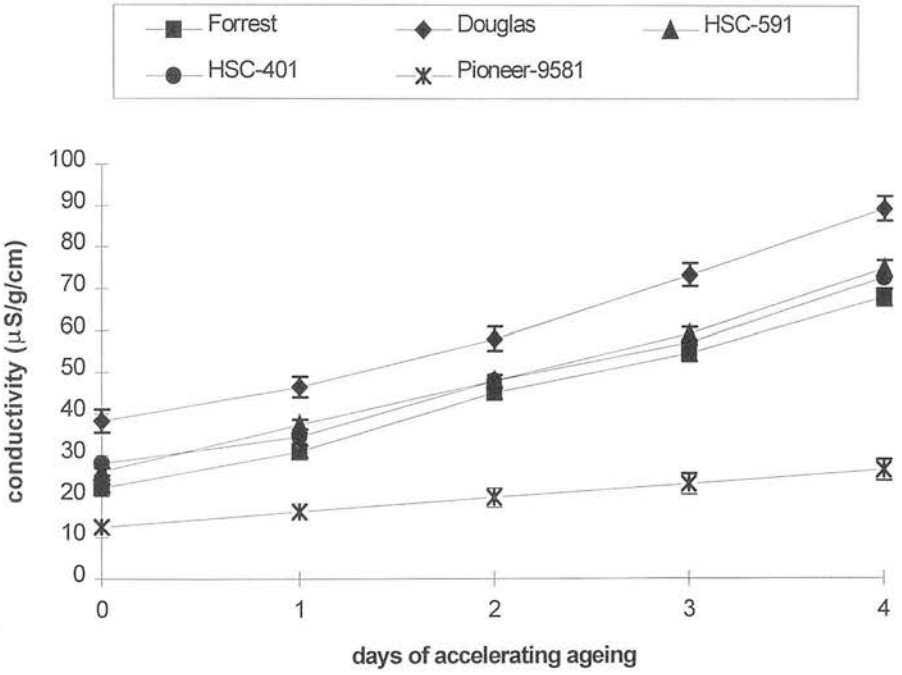


Figure 4.5. Effect of accelerated ageing 1 to 4 days on the conductivity of the leachate from seeds of five genotypes; error bar, standard error of the mean, (n=4).

Figure 4.6a shows the effect of accelerated ageing on the leakage from seeds with intact or split seed coats of cv. Douglas. Analysis of variance results were presented in Appendix 4, Table 4.4. In general, seeds with split coats had a higher leakage than intact seeds. In the unaged control seeds, seeds with split coat had an 84% higher ($P<0.001$) leakage than intact seeds. However, after 4days of ageing, seeds with split coats had a 15% higher ($P<0.001$) leakage than intact seeds.

Figure 4.6b shows the effect of accelerated ageing on the solute leakage from hard or soft seeds of cv. Pioneer-9581. In general, leakage from hard seeds was negligible whereas solute leakage from soft seeds was high.

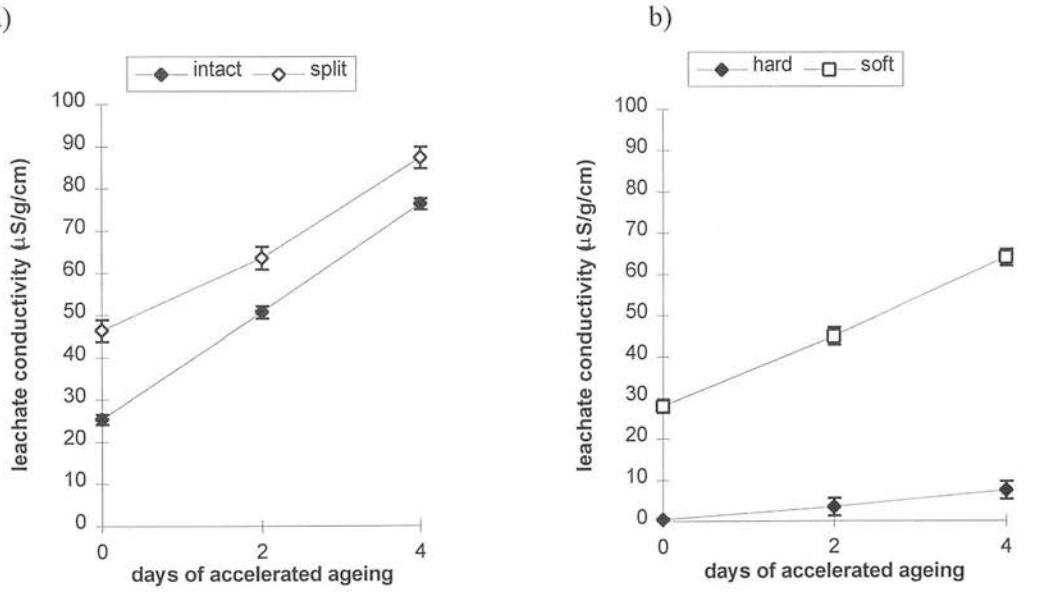


Figure 4.6. Effect of accelerated ageing at 2 and 4 days on the conductivity of the leachate from seeds of a) cv. Douglas and b) cv. Pioneer-9581; error bar, standard error of the mean, (n=4).

Figure 4.7 shows the effect of accelerating ageing 1 to 4 days on the fresh weight per normal seedling produced by seeds of the five genotypes. Analysis of variance results were presented in Appendix 2, Table 2.4. In general, ageing had a negative effect on the fresh weight per normal seedling. However, there were differences between the genotypes on the fresh weight per normal seedling produced by aged seeds. Unaged seeds of cv. Pioneer-9581 had the lowest seedling fresh weight (409 mg per normal seedling) whereas unaged seeds of cv. HSC-591 had the highest seedling fresh weight (706 mg per normal seedling) ($P<0.001$). Seeds of cv. Pioneer-9581 showed the lowest decline in the seedling fresh weight due to ageing whereas seeds of cv. Douglas showed the highest decline in the seedling fresh weight due to ageing.

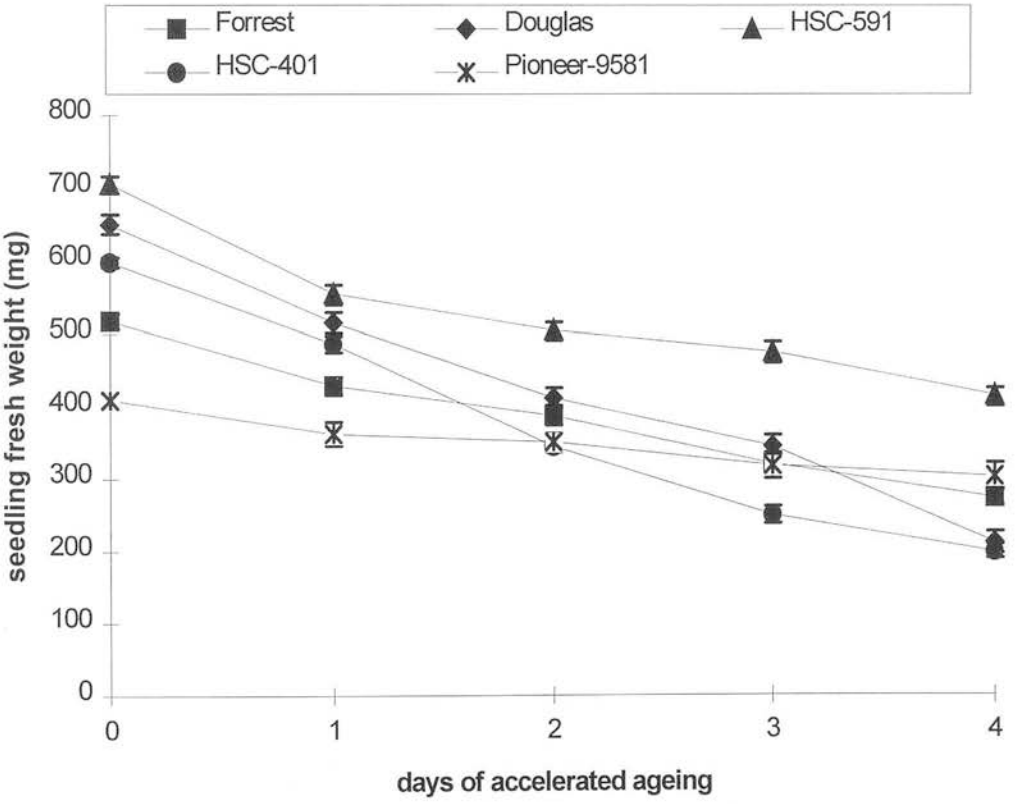


Figure 4.7. Effect of accelerated ageing 1 to 4 days on the fresh weight per normal seedling produced by seeds of five genotypes; error bar, standard error of the mean, (n=4).

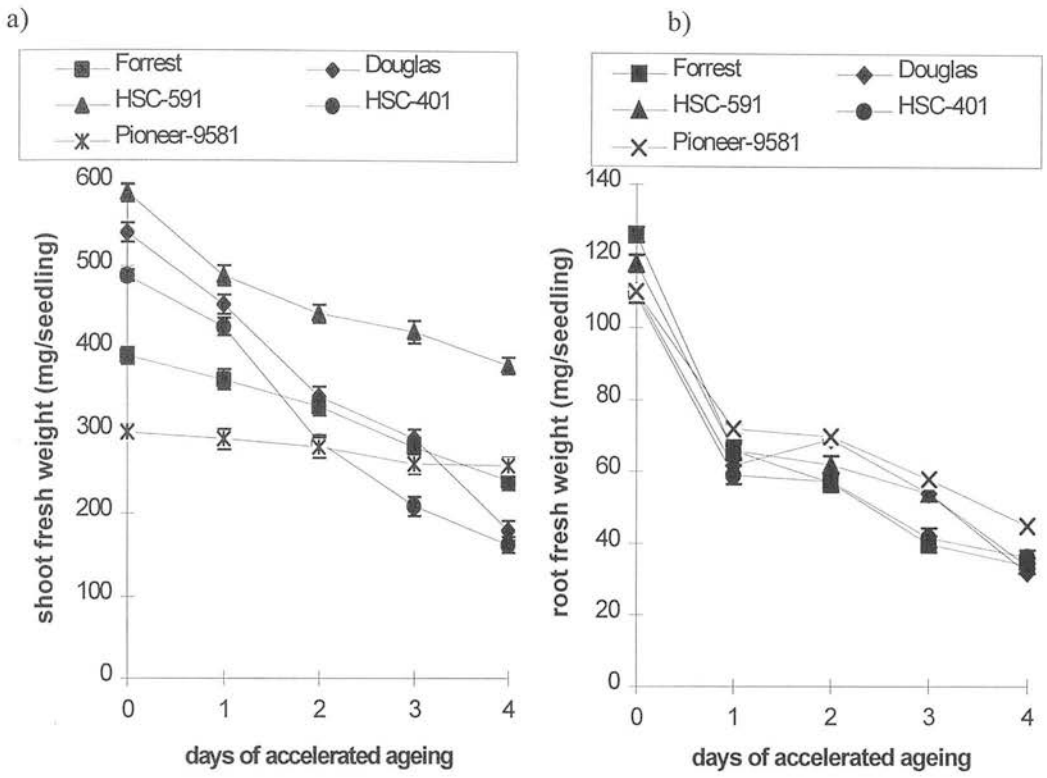


Figure 4.8. Effect of accelerated ageing 1 to 4 days on a) the shoot fresh weight per normal seedling, b) the root fresh weight per normal seedling, produced by seeds of five genotypes; error bar, standard error of the mean, (n=4).

Figure 4.8a shows the effect of the accelerating ageing 1 to 4 days on the shoot fresh weight per normal seedling produced by seeds of the five genotypes. Analysis of variance results were presented in Appendix 2, Table 2.5. In general, ageing had a negative effect in the shoot fresh weight and the decline in shoot fresh weight was similar to that in the seedling fresh weight. There were, however, differences between the genotypes on the shoot fresh weight produced by aged seeds. Unaged seeds of cv. Pioneer-9581 had the lowest shoot fresh weight (299 mg per normal seedling) whereas unaged seeds of cv. HSC-591 had the highest shoot fresh weight (588 mg per normal seedling) ($P<0.001$). Seeds of cv. Pioneer-9581 showed the lowest decline in the shoot fresh weight due to ageing whereas seeds of cv. Douglas showed the highest decline in the seedling fresh weight due to ageing.

Figure 4.8b shows the effect of the accelerating ageing 1 to 4 days on the root fresh weight per normal seedling produced by seeds of the five genotypes. Analysis

of variance results were presented in Appendix 2, Table 2.6. In general, root fresh weight was more affected than the shoot fresh weight by the ageing. Seeds after 1 day of ageing produced 35-50% lower ($P<0.001$) root fresh weight than the unaged seeds. After 4 days of ageing, seeds of all genotypes had about 3 times less root fresh weight than the unaged control seeds.

Ageing had a negative effect in the vigour index (normal seedlings x fresh weight per normal seedling) (Fig. 4.9). Analysis of variance results were presented in Appendix 2, Table 2.7. However, there were differences between the genotypes on the decline of the vigour index from aged seeds. Unaged seeds of cv. Pioneer-9581 had the lowest vigour index (9,000 mg) whereas unaged seeds of cv. HSC-591 had the highest vigour index (16,500 mg) ($P<0.001$). Seeds of cv. Pioneer-9581 showed the lowest decline (42% after 4 days of ageing) in the vigour index due to ageing whereas seeds of cv. Douglas and HSC-401 showed the highest decline (30 times after 4 days of ageing) in the vigour index due to ageing. Aged seeds of cv. HSC-591 for 4 days had about 3 times lower vigour index than that of the unaged seeds.

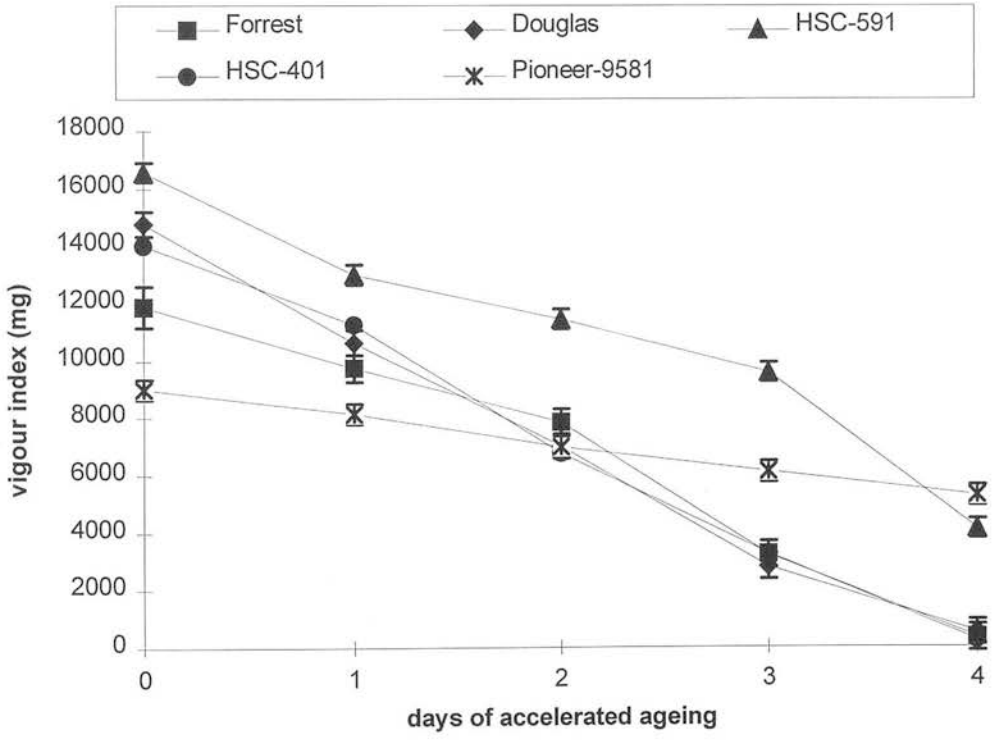


Figure 4.9. Effect of accelerated ageing 1 to 4 days on the vigour index (normal seedlings x fresh weight per normal seedling), from seeds of five genotypes; error bar, standard error of the mean, (n=4).

4.4. Discussion

The effect of accelerated ageing on decline of viability using seeds of five genotypes were assessed. The genotypes were of the same maturity group (group V), similar seed size, yellow seed coated and were grown under the same conditions. Genotypic differences in seed longevity during storage can be identified only when different genotypes are grown under the same conditions and treated identically before storage (Roberts, 1986). A large proportion of seeds with split coats were noticed in cv. Douglas whereas in all other genotypes few seeds with seed coat splitting observed. Also, a high proportion of hard seeds (about 65%) were noticed in cv. Pioneer-9581 whereas in all other genotypes no hard seeds were noticed. The cultivar Douglas has been reported as prone to seed coat splitting (Yaklich and Barla-Szabo, 1993).

In this study, although the initial germinability of the unaged seeds was similar across the genotypes, significant differences between the genotypes in the storage potential were revealed. During storage in hot and humid conditions, a high initial germinability in itself appeared to confer inadequate storage life. Seed coat could influence the storage life of seeds under unfavourable conditions. Differences in the storage potential could be associated with the influence of the seed coat to moisture absorption from the hot and humid atmosphere. A large proportion of hard seeds in cv. Pioneer-9581 resulted in a low moisture absorption during accelerated ageing and high viability in the subsequent germination test. On the other hand, a large proportion of seeds with split coats in cv. Douglas resulted in a high moisture absorption during accelerated ageing and low viability in the subsequent germination test. Additionally, the seed coat could vastly influence the leachate conductivity by regulating the water uptake; a high proportion of hard seeds resulted in very low leachate conductivity whereas a high proportion of seeds with splitted coat resulted in high leachate conductivity.

Hardseedness has been previously reported to maintain high storage potential in seeds. Prolonged storage potential (as revealed by accelerated ageing test) and resistance to field weathering (deterioration in the mother plant due to late harvesting) was found in a soybean genotype with a high percentage of hard seeds (Potts *et al.*, 1978). Minor and Paschal (1982) reported variation in storability of 253 soybean genotypes; seeds were produced in a tropical environment and stored in a simulated tropical environment (30°C and 80% r.h.). One genotype (cv. Barchet)

with 60% hard seeds had a storage half-life 16 weeks whereas all the other genotypes had a storage half-life shorter than 8 weeks. Prolonged storage potential due to a large percentage of hard seeds was also reported in long bean (*Vigna sesquipedalis* L.) (Abdullah *et al.*, 1992). In a comparative study of eleven genotypes, they found small decrease in percentage of normal seedlings after 4days of accelerated ageing in seed lots which had above 30% hard seeds whereas in seed lots with no hard seeds, the viability was completely lost after 4 days of ageing.

In the present work, large difference between seeds with split coats and seeds with intact coats in the percentage of normal seedlings produced by 2 days aged seeds were observed. In addition, accelerated ageing had a similar negative effect on leachate conductivity in seeds with split and intact coats. Burchett *et al.* (1985), in a study of the importance of the presence of seeds with split coats within soybean seed lots on germination and seed quality, reported that seeds with split coats were inferior to both germination and vigour (use of accelerated ageing).

In this study, the large difference between seeds with split coats and intact coats in the percentage of normal seedlings produced by 2 days aged seeds could be ascribed to either the effect of the seed coat in the moisture absorption during the accelerated ageing test or difference between splitted and intact seeds in the initial seed quality. There was about 3% difference between splitted and intact seeds in the moisture content at the end of the first two days of the accelerated ageing. In ageing tests where the seed moisture is kept constant during the whole period of the test, even small differences in the seed moisture could result in different rates of deterioration (Matthews, 1980). For the same reasons, Ellis and Roberts (1980) reported that differences of more than 2% in moisture content during the controlled deterioration test, had a considerable effect on seed longevity. However, Tomes *et al.* (1988) reported that a variation of up to 3% in soybean seed moisture at the end of the fourth day of accelerated ageing had no significant effect on the percentage of normal seedlings.

It is difficult, however, to ascribe all the difference between seeds with split coats and intact coats to the influence of the seed coat on moisture absorption during the accelerated ageing. Seeds with split coats might have a lower initial seed quality in comparison to the intact seeds for the following reasons:

a) It is in fact very difficult to achieve the same initial level of seed quality, although every care was made. The selected genotypes were of the same maturity

group, medium seed size, same seed coat colour, grown under similar field conditions, stored and treated in the same way before the experiments started. However, it is possible that seeds with split coats may have suffered more mechanical damage and pathogen infection than the seeds with intact coats. Mechanical damage during handling operations was found to be very common in soybean seeds (TeKrony *et al.*, 1987). Burris (1980) suggested that seeds with split coats may not offer optimum protection to the embryo particularly when increased stress forces were placed on seeds during handling.

b) The initial seed quality was found to be influenced by the position of the pod in the mother plant; seeds from upper pods possessed more initially quality than those from lower pods (Adam *et al.*, 1983). It may possible that seeds with split coats are produced exclusively or predominately in lower pods in the mother plant. Unfortunately, there has been no report about the occurrence of seeds with split coat in relation to the position of the pod in the mother plant.

c) It is possible that the seeds with split coats may have undergone more deterioration in comparison to the seeds with intact coats before harvesting in the mother plant. Seed coat splitting started just before physiological maturity when seeds attained their maximum weight (Yaklich and Barla-Szabo, 1993). Seed quality can deteriorate prior to harvest in the mother plant depending on the environmental conditions (TeKrony *et al.*, 1980; Horlings *et al.*, 1991).

One possible way to assess whether differences in the storage potential are entirely ascribed to the influence of the seed coat on moisture absorption during accelerated ageing and/or reflect differences in the initial seed quality, is to measure the initial seed quality, as Ellis and Roberts (1980) in their equation for the prediction of storage potential suggested. They reported that the construction of a survival curve from germination results (fitted by probit analysis) after controlled deterioration gave an accurate estimate of the initial seed quality. The construction of the survival curve requires a controlled deterioration of seeds of 16-10% moisture content for about 50-100 days. During this period, a considerable number of germination tests (from 7 to 10 germination tests) should be performed (Zanakis, 1993). In this experiment, however, this method was not performed because of the limited seed supply.

In the present work, the influence of the presence of seeds with split coats on seed quality was assessed in seeds of one seed lot of one genotype. However, for more conclusive results, seeds of more genotypes or different seed lots of the same genotype, grown under different locations and years should be assessed. It may be

also important to investigate under which environmental conditions seed coat splitting is pronounced. Burchett *et al.* (1985) reported that supplemental irrigation or delayed planting resulted in a three-fold increase in seed coat splitting.

In the present work, seedling growth characteristics were measured to provide further information about genotypic differences in vigour decline due to accelerated ageing. In seeds of cv. Pioneer-9581 and Douglas, a common assessment of the seedling growth after the ageing was made regardless of the type of the seed coat (hard or soft, intact or split). It was planned to answer questions about differences between genotypes rather than differences between hard and soft or seeds with intact and seeds with split coats. It is has been reported that in seed lots where hard seeds appeared, only a proportion of seeds appeared to be hard (Potts *et al.*, 1978; Hartwig and Potts, 1987). Additionally, in seed lots where seeds with split coats appeared, only a proportion of the seeds had split coats (Burchett *et al.*, 1985). Based on seedling growth after ageing seeds for different periods of time, an estimation of vigour could be made. Seeds of cv. HSC-591 showed their superiority in vigour in comparison to seeds of other genotypes, whereas seeds of cv. Douglas possessed a low vigour. Hardseedness greatly reduced the decline of seedling growth during the ageing but a significantly lower seedling growth and higher variation in comparison to the other genotypes was measured. Based on the vigour index, hardseedness greatly reduced the vigour decline. However, final statements about genotypic differences in the vigour level could be made only when more than one seed lot of a particular genotype is examined (Wien and Kueneman, 1981). In this study, root growth was generally more affected by ageing than shoot growth. Chauhan (1985) using tetrazolium staining reported that in soybeans the most sensitive tissues to ageing were radicle and plumule.

Hardseedness (in cv. Pioneer-9581) greatly reduced the decline of seedling growth during ageing but a lower seedling growth than that of the other genotypes was observed. Also, hardseedness greatly reduced the decline in vigour during ageing. Despite the clear potential of successful regulation of water uptake in soybeans by introducing the hardseeded characteristic to agronomicaly important genotypes, some problems may also emerge. One of the major disadvantages of introducing the hardseedness into soybean cultivars would be the non-uniform rate of imbibition and germination, indicating that scarification of the seed coat might be necessary before planting. Potts *et al.*, (1978) reported that a high percentage of the hard seeds were scarified in the normal commercial harvesting therefore seeds for

planting would not require added treatments in the normal seed conditioning process. They reported that the hardseeded line had a 87% emergence at the time the normal seeded genotype had a 100% emergence. However, additional studies concerning means and effects of scarifiacation during harvesting and/or processing should be made. Another disadvantage of introducing the hardseedness into soybean cultivars might be the volunteer plants in the year following the seed production year. Duangapatra (1978) reported that the hardseedness caused numerous volunteer plants in the year following the field experiments.

CHAPTER 5

Effect of polymer application on imbibition, germination and seedling growth in soybeans during soaking and two different soil water regimes

5.1. Introduction

In tropical and subtropical countries soybean seeds frequently experience waterlogged conditions due to rainfall immediately after planting which could result in severe imbibition damage and poor crop stands (Troedson *et al.*, 1983; Hwang and Sung, 1991). For similar reasons, Ragus (1987) suggested that selection of those soybean cultivars that are less affected by imbibition damage is the first step to successful germination under saturated soil moisture conditions.

It has been suggested that improved germination, seedling emergence and growth could be achieved when imbibition damage was largely prevented by slowing down the initial rate of water uptake during imbibition (West *et al.*, 1985; Priestley and Leopold, 1986; Hwang and Sung, 1991). The lower rate of water uptake during imbibition could be achieved by three ways. Firstly, by using a imbibition medium with a low osmotic potential (Hobbs and Obendorf, 1972; Woodstock and Tao, 1981). Secondly, by breeding a delayed-permeable seed coat that protects the embryo from imbibition damage during the first hours of imbibition (Kuo, 1989), or by coating the seeds with hydrophobic polymers (West *et al.*, 1985; Priestley and Leopold, 1986). There has been only one report that soybean seeds of cv. Leu-kuang coated with ethyl cellulose reduced imbibition damage and improved germination after soaking for 48h in distilled water (Hwang and Sung, 1991).

However, there is no published information about the effect of polymer application on seedling emergence and growth particularly under flooded conditions. In addition, there is no published information about the effectiveness of polymer application in genotypes prone to imbibition damage due to either seed coat splitting or low seed vigour. The objectives of this study were, therefore: (1) to investigate the effectiveness of polymer application in preventing imbibition damage in genotypes prone to imbibition damage (2) to present evidence that polymer application could prevent imbibition damage and improve germination and seedling growth during soaking in water, and (3) to investigate the effect of polymer application on seedling emergence and growth under normal or flooded soil conditions.

5.2. Materials and methods

5.2.1. Polymer application

Two polymers were obtained from Vinamul Ltd, Surrey, England. The basis of the selection was the hydrophobic nature of the polymers. From preliminary experiments, one polymer (VINAMUL 3650) containing vinyl acetate, vinyl chloride, ethylene and acrylate with cellulose ether as a stabilising system was further selected for application to seeds. Application suggestions from the company were that the polymer should be applied at about 20°C with the drying air temperature at least 10°C above the filming temperature for good film integration.

The polymer was diluted to 80% (w/w) in water and was added to seeds by sequential coats of application and drying. About 40g of seeds were briefly (about 15 seconds) soaked in 200ml of polymer solution in a 500ml conical flask. The polymer solution with the seeds were manually shacked to help the even distribution of the polymer on the seeds. Subsequently, seeds were removed from the solution and placed for drying on a single layer on a muslin cloth inside an air-dryer set at 35°C.

Seeds were manually separated to avoid agglomeration and left for drying at the air-dryer for 4hours before a second coat of polymer was applied. Every 3 coats of polymer, seeds were left overnight for drying in the air-dryer set at 35°C. In every coat of polymer, seeds were both visually or under low magnification microscope examined to check how good the application was.

5.2.2. Effect of polymer application on imbibition and germination

The amount of polymer added to seeds was measured by the weight of 10 individual seeds before and after the removal of the film coating. Three levels of polymer application in seeds of cv. Forrest and Douglas were produced. The time course of water uptake of the polymer-coated seeds at the three levels of polymer application was measured, as described in section 2.5.2. The percentages of normal seedlings produced from coated seeds of cv. Forrest and Douglas were measured, as described in section 2.3. Aged seeds were produced after 2 days of the accelerated ageing treatment, as described in section 2.8.

5.2.3. Effect of 24h soaking in water on germination and imbibition damage of unaged and aged polymer coated seeds

Aged seeds were produced after 2 days of the accelerated ageing treatment, as described in section 2.8. Polymer coating (24 mg of polymer per seed) was applied to seeds, as described in section 5.2.1. Unaged (uncoated and polymer coated) and aged (uncoated and polymer coated) seeds were placed into 350 ml beakers, and then 200 ml of water was added. The beakers were incubated at 25°C for 24 hours. Subsequently, seeds were placed for germination in paper towels, as described in section 2.3. Four replications of 25 weighed seeds were used. The leachate conductivity from unaged (uncoated and polymer coated) and aged (uncoated and polymer coated) was measured, as described in section 2.6. The percentage of cotyledons fully stained with tetrazolium chloride was also measured, as described in section 2.7. In addition, seeds soaked in 30% PEG for 24h before staining with tetrazolium chloride was made, as described in section 2.7.

5.2.4. Seedling emergence and growth of unaged and aged polymer coated seeds during two different soil water regimes

Aged seeds were produced after 2 days of the accelerated ageing treatment, as described in section 2.8. Polymer coating (24 mg of polymer per seed) was applied to seeds, as described in section 5.2.1. Unaged (control and coated) and aged (control and coated) seeds were sown in pots with compost, as described in section 2.4. For each treatment four replications of 25 seeds were sown in plastic pots, and placed at random inside a controlled environment cabinet at 25°C with 12h light and 12h dark. Irradiance was 100mE PAR $m^{-2}s^{-1}$. The pots were watered with tap water, and the compost held at moisture levels between 70-80% of the field capacity (control water regime). The flooding water regime was produced as follows: pots were watered at the beginning of the experiment at 100% of the field capacity without any watering for the next four days. At the end of the 4 day, the compost moisture level had dropped to about 70% of the field capacity. Subsequently, pots were treated as the pots in the control water regime. A second control water regime was also used; pots were kept at a flooding conditions (about 100% of the field capacity) for the whole period of the emergence test. Seedling emergence was counted daily up to day 16 when plants were cut at the base just above the soil surface. Seedling emergence from pots that were kept at 100% of the field capacity was lower than 10%, and

therefore results were not presented. The shoot length of all emerged seedlings was measured. The fresh weight of the plants was determined per pot, and average single plant values were calculated by dividing the fresh weight by the total number of plants harvested. After harvesting, pots were kept in the Fisons cabinets for another week (day 23). During that period, no other seedling emergence was observed.

5.3. Results

5.3.1. Effect of polymer application on imbibition and germination

From preliminary experiments, it was concluded that the best technique for the polymer application was a gradual increase in the amount of polymer applied to seeds, as described in section 5.2.1. There was a linear increase in the amount of polymer applied to seeds; every coat of application added about 5mg polymer per seed (Fig. 5.1). After 10 coats of polymer, the total amount of polymer applied was 52 mg per seed or about 32% of the initial seed weight (Fig. 5.1).

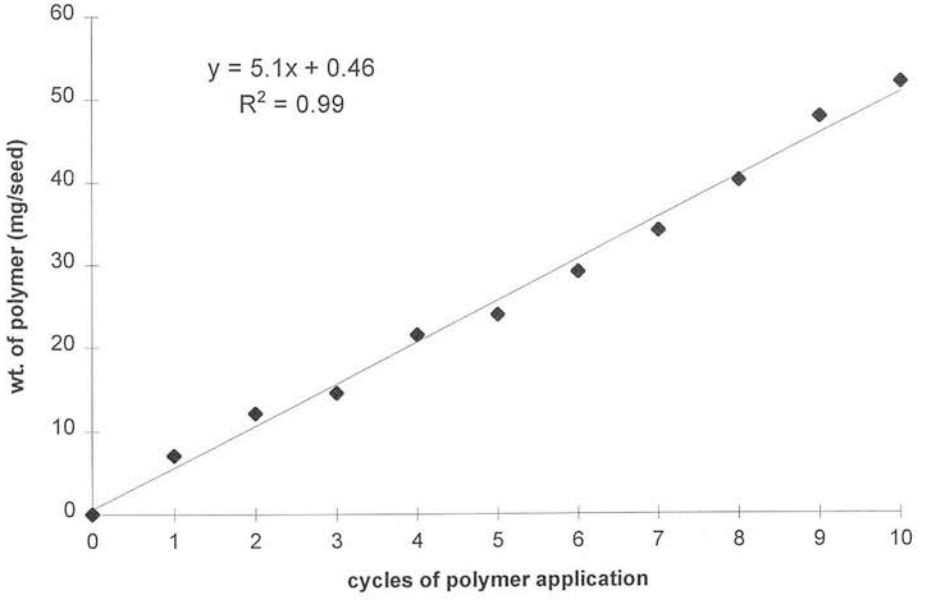


Figure 5.1. The weight of polymer applied to seeds of cv. Forrest in every coat of polymer application.

Figure 5.2 shows the time course of water uptake of seeds of cv. Forrest coated with 12, 24 and 48 mg of polymer per seed. Analysis of variance results were presented in Appendix 3, Table 3.1. In general, polymer coated seeds absorbed lower amount of water during imbibition than uncoated seeds. Water uptake of uncoated control seeds was initially high and eventually terminated around 24 hours after the start of imbibition. There was no difference between uncoated and coated with 12 mg of polymer per seed in the water uptake during the 72h imbibition period. Seeds coated with 24 and 48 mg of polymer per seed absorbed water more slowly than the uncoated control seeds. Coating seeds with 24 mg of polymer per seed resulted in a water uptake which was lower ($P<0.001$) than that of the uncoated seeds during the 48h of imbibition. However, after 60h of imbibition the amount of water which had been taken up by the coated seeds was the same as that which had been taken up by the uncoated seeds. The total water uptake of seeds coated with 48 mg of polymer per seed at 72h was lower ($P<0.001$) than that the uncoated control seeds.

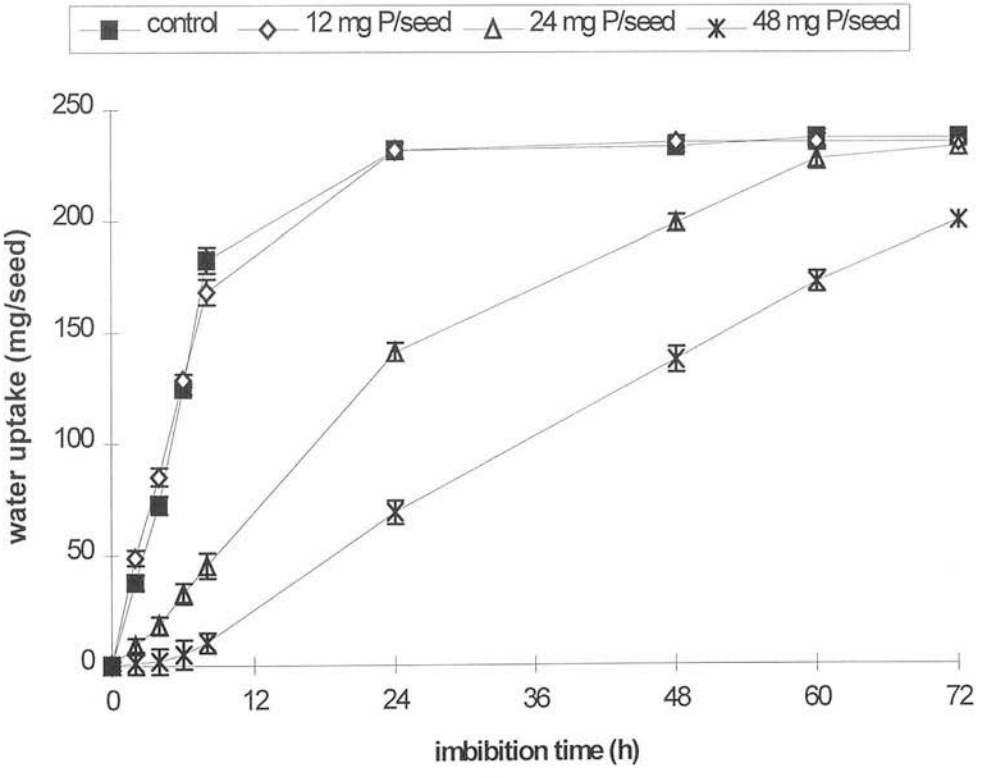


Figure 5.2. The time course of water uptake of seeds of cv. Forrest coated with three different levels of polymer (P) (12mg, 24mg and 48 mg per seed); error bar, standard error of the mean, (n=10).

Figure 5.3 shows the time course of water uptake of seeds of cv. Douglas coated with 12, 24 and 48 mg of polymer per seed. Analysis of variance results were presented in Appendix 3, Table 3.2. In general, polymer coated seeds absorbed lower amount of water during imbibition than uncoated seeds. The rate of water uptake of uncoated seeds and seeds coated with 12 mg of polymer per seed was initially very high; seeds absorbed 100 mg of water during the first two hours of imbibition. The very high initial water uptake was reflected in the short time taken to terminate imbibition; around 8 hours after the start of imbibition. Seeds coated with 24 and 48 mg of polymer per seed absorbed lower water than the uncoated control seeds. Coating seeds with 24 mg of polymer per seed resulted in a water uptake which was lower than that of the uncoated seeds during the 48h of imbibition but after 60h of imbibition the amount of water that had been absorbed by the coated seeds was the same as that which had been absorbed by the uncoated seeds. However, the total water uptake of seeds coated with 48 mg of polymer per seed at 72h was lower ($P<0.001$) than that of the uncoated control seeds.

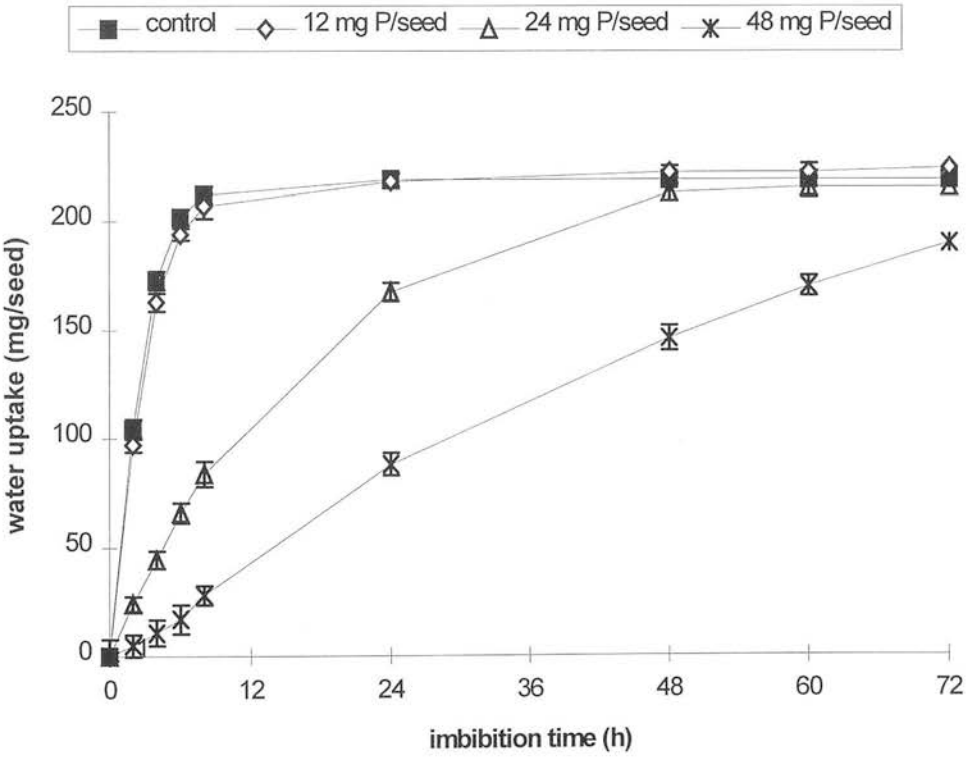


Figure 5.3. The time course of water uptake of seeds cv. Douglas coated with three different levels of polymer (P) (12 mg , 24 and 48 mg of polymer per seed); error bar, standard error of the mean, (n=10).

Figure 5.4 shows the percentage of normal seedlings produced from seeds of cvs Forrest and Douglas coated with the three different levels of polymer. Analysis of variance results were presented in Appendix 3, Table 3.3. There was no difference between the uncoated control seeds and those coated with 12 and 24 mg of polymer per seed in the percentage of normal seedlings produced. However, seeds of cv. Forrest and Douglas coated with 48 mg of polymer per seed produced 38% and 34% fewer ($P<0.001$) normal seedlings than the uncoated control seeds respectively.

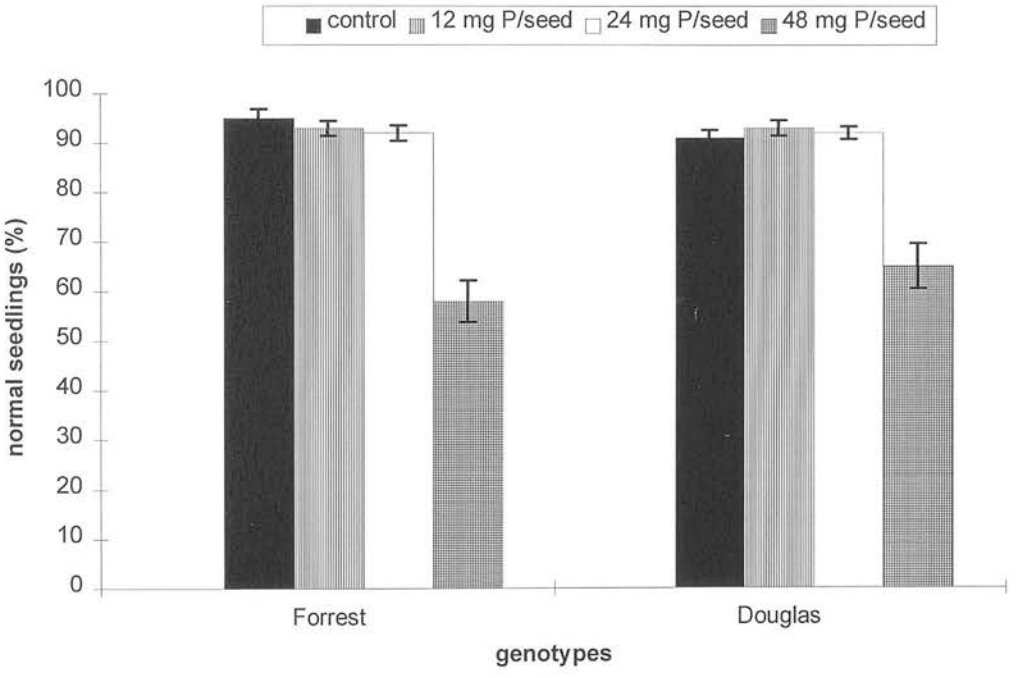


Figure 5.4. The effect of three different levels of polymer (12 mg, 24 and 48 mg of polymer per seed) on the percentage of normal seedlings produced from seeds of cv. Forrest and Douglas; error bar, standard error of the mean, (n=4).

In view of the results shown in Fig. 5.4, it was decided that 24 mg of polymer per seed was an appropriate level of polymer application.

Figure 5.5 shows the effect of the 24mg of polymer per seed on the percentage of normal seedlings, in unaged and aged seeds of both genotypes. Analysis of variance results were presented in Appendix 3, Table 3.4. Unaged seeds in both genotypes had the same high percentage of normal seedlings in both coated and uncoated seeds. Ageing reduced the percentage of normal seedlings by 12% ($P>0.05$) and 33% ($P<0.001$) in comparison to the unaged control in seeds of cvs Forrest and Douglas respectively. However, in neither genotypes, there was any difference between the aged control and the aged coated seeds in the percentage of normal seedlings produced.

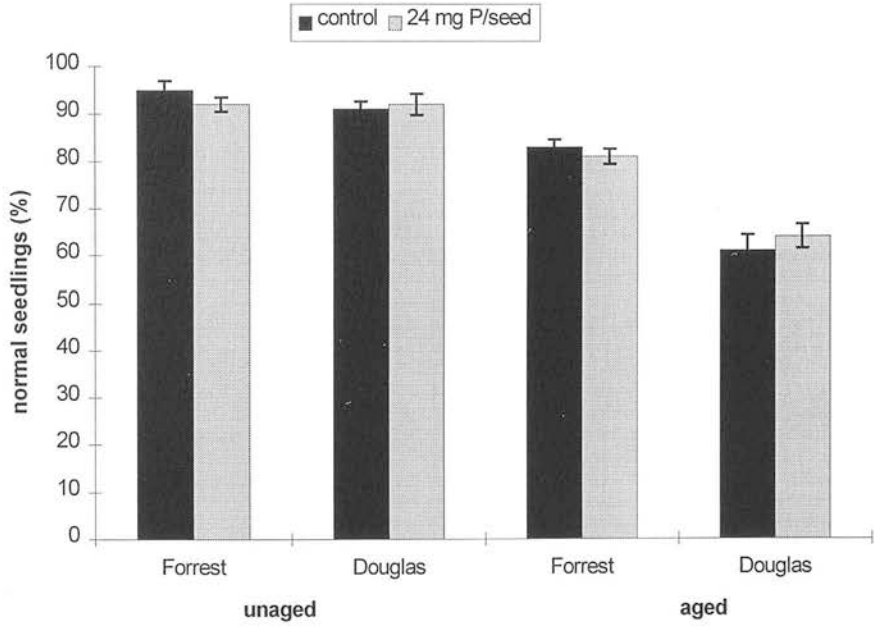


Figure 5.5. The effect of a 24 mg of polymer (P) per seed on the percentage of normal seedlings produced from unaged and aged seeds of cvs Forrest and Douglas; error bar, standard error of the mean, (n=4).

5.3.2. Effect of 24h soaking in water on germination and imbibition damage of unaged and aged polymer coated seeds

Figure 5.6 shows the effect of 24h soaking in water on percentage normal seedlings of unaged and aged polymer coated seeds with 24mg of polymer per seed. Analysis of variance results were presented in Appendix 3, Table 3.5. In general, 24h soaking in water resulted in a reduction of the percentage of normal seedlings. Coating although resulted in an improvement of the percentage of normal seedling, it was lower ($P<0.001$) than that from the paper towel germination tests.

Unaged coated seeds of cvs Forrest and Douglas had 56% and 115% higher ($P<0.001$) percentage of normal seedling that the unaged uncoated seeds. Aged coated seeds cvs Forrest and Douglas had 96% and 121% higher ($P<0.001$) percentage of normal seedling that the aged uncoated seeds.

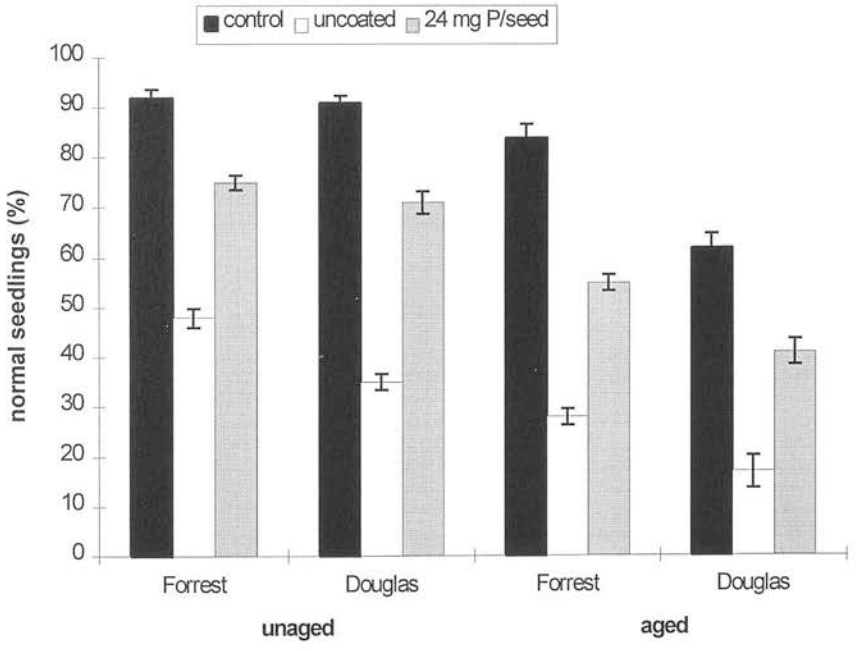


Figure 5.6. Effect of 24h soaking in water on percentage of normal seedlings of unaged and aged polymer coated seeds (24 mg of polymer per seed) of cvs Forrest and Douglas; error bar, standard error of the mean, (n=4).

Figure 5.7 shows the effect of the 24h soaking in water on leachate conductivity of unaged and aged polymer coated seeds with 24 mg of polymer per seed. Analysis of variance results were presented in Appendix 3, Table 3.6.

Unaged and aged coated seeds had a lower ($P<0.001$) conductivity than unaged and aged uncoated seeds. The leachate conductivity of unaged control seeds of cv. Douglas was 78% higher ($P<0.001$) than that of unaged control seeds of cv. Forrest. Coating of seeds reduced the conductivity in comparison to the uncoated seeds to a greater extent in seeds of cv. Douglas than in seeds of cv. Forrest. Aged seeds of cvs Forrest and Douglas had 52% and 17% higher ($P<0.001$) leachate conductivity than that of the unaged control seeds respectively. However, in either genotypes, aged coated seeds had a lower ($P<0.001$) leachate conductivity than that of the uncoated aged seeds.

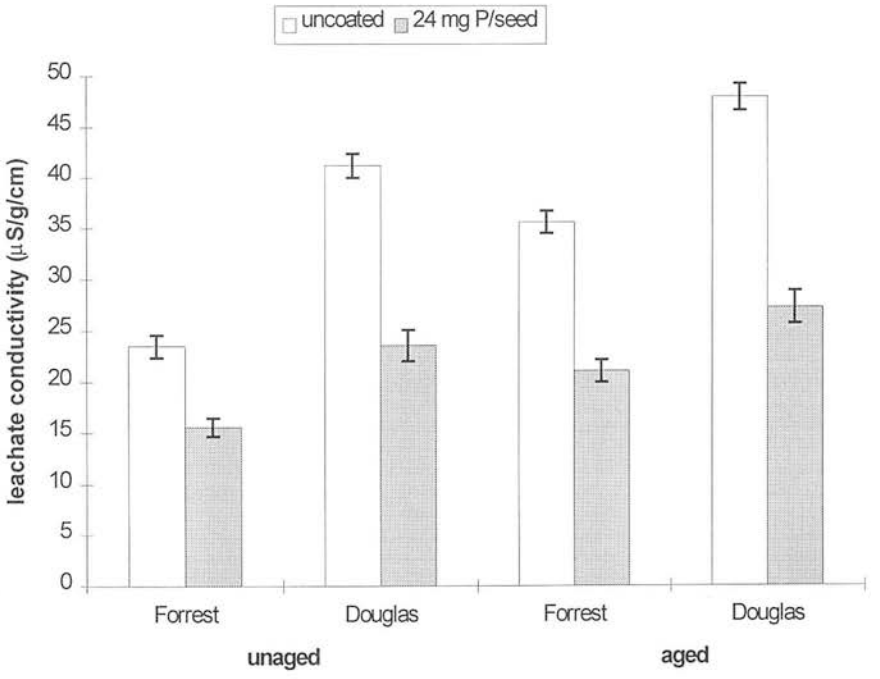


Figure 5.7. Effect of 24h soaking in water on leachate conductivity from unaged and aged polymer coated seeds (24 mg of polymer per seed) of cvs Forrest and Douglas; error bar, standard error of the mean, (n=4).

Figure 5.8 shows the effect of the 24h soaking in water on the percentage of cotyledons fully stained with tetrazolium chloride of unaged and aged polymer coated seeds with 24 mg of polymer per seed. Analysis of variance results were presented in Appendix 3, Table 3.7.

Unaged and aged coated seeds had a higher ($P<0.001$) percentage of cotyledons that were fully stained that the unaged and aged uncoated seeds. Unaged control seeds of cv. Douglas had a 2.6 times lower percentage of cotyledons that were fully stained than seeds of cv. Forrest. Coating of seeds increased the percentage of cotyledons stained to a greater extent in seeds of cv. Douglas than in seeds of cv. Forrest. Aged seeds of cvs Forrest and Douglas had 59% and 80% lower ($P<0.001$) percentage of cotyledons fully stained than the unaged control seeds respectively. However, in either genotypes, aged coated seeds had a higher ($P<0.001$) percentage of cotyledons fully stained than the uncoated aged seeds.

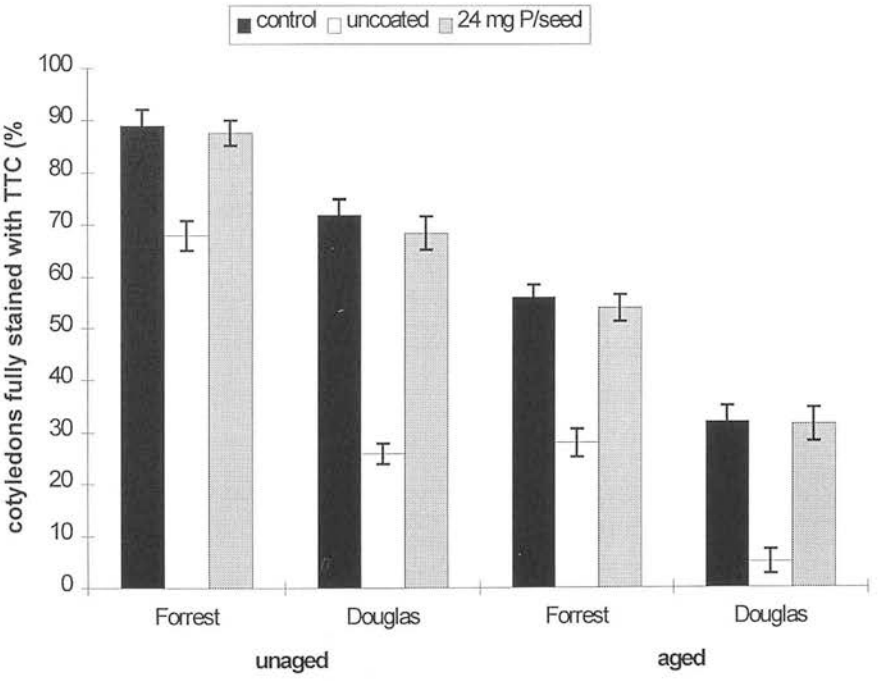


Figure 5.8. Effect of 24h soaking in water on percentage of cotyledons fully stained of unaged and aged polymer coated seeds (24 mg of polymer per seed) of cvs Forrest and Douglas; error bar, standard error of the mean, (n=4).

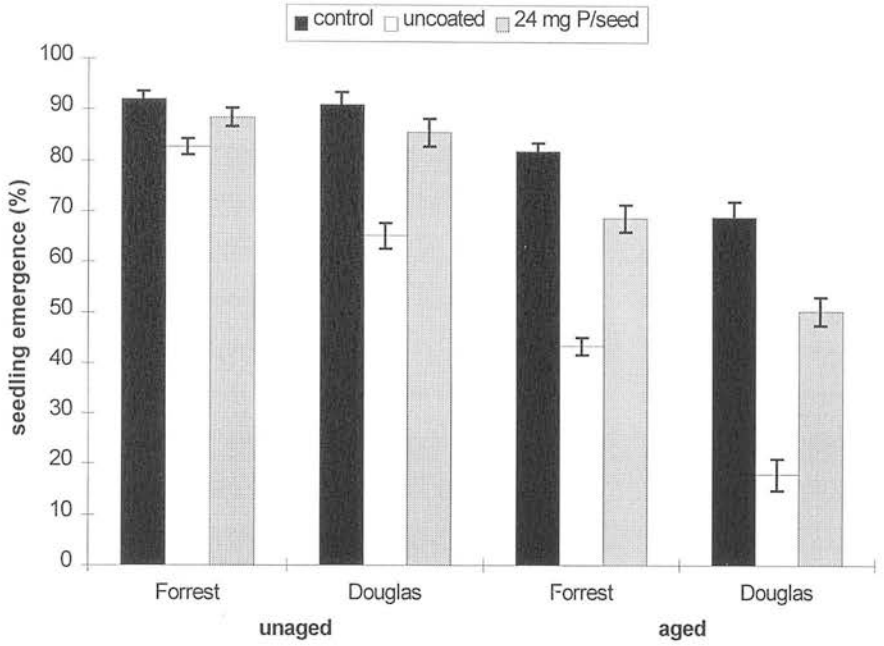
5.3.3. Seedling emergence and growth of unaged and aged polymer coated seeds during two different soil water regimes

5.3.3.1. Seedling emergence

The effect of 24 mg of polymer per seed on seedling emergence, establishment and growth of unaged and aged seeds of the two genotypes was studied. Control and flooding water regimes were applied to the pots as described in section 5.2. Figure 5.8 shows the effect of the 24 mg of polymer per seed on the percentage seedling emergence in the two water regimes of unaged and aged seeds of both genotypes. Analysis of variance results were presented in Appendix 3, Table 3.8. The percentage seedling emergence in compost was lower ($P<0.001$) than the percentage normal seedlings from the paper towel germination tests. Coated seeds had a higher ($P<0.001$) percentage seedling emergence than the uncoated seeds. Ageing reduced ($P<0.001$) the percentage seedling emergence. Flooding reduced ($P<0.001$) the percentage seedling emergence.

In the control water regime, unaged coated seeds and unaged control seeds of cv. Forrest had similar percentage seedling emergence. However, unaged coated seeds of cv. Douglas had higher ($P<0.001$) percentage seedling emergence than the uncoated seeds. This improvement resulted in a percentage seedling emergence which was at a similar level with the percentage normal seedlings from the paper towel germination test. Aged uncoated seeds of either genotypes produced lower percentage seedling emergence than the unaged control in seeds. Coating of aged seeds although resulted in a percentage seedling emergence which was higher than that of the uncoated aged seeds, it was lower than the percentage normal seedlings from the paper towel germination tests. In the flooding regime, unaged seeds of either genotypes had lower percentage seedling emergence than that of the seeds in the control water regime. Coating of unaged seeds although resulted in a percentage seedling emergence which was higher than the uncoated aged seeds, it was lower ($P<0.001$) than that from the paper towel germination test. In aged seeds, a further decline in the percentage seedling emergence was observed such that all seeds of cv. Douglas failed to emerge. Aged coated seeds of cvs Forrest and Douglas had a 1.6 and 30 times higher ($P<0.001$) percentage seedling emergence than that of the uncoated seeds. This improvement, however, resulted in a percentage seedling emergence which was at a lower level ($P<0.001$) with the percentage normal seedlings from the paper towel germination test.

a)



b)

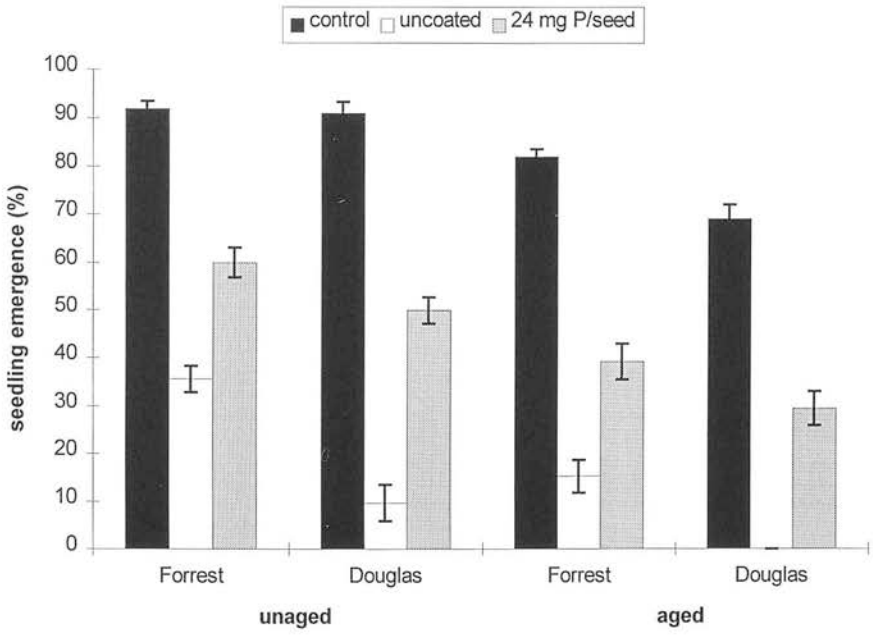


Figure 5.9. Percentage seedling emergence of unaged and aged polymer coated seeds (24 mg of polymer per seed) of cvs Forrest and Douglas; a) control water regime and b) flooding water regime; error bar, standard error of the mean, (n=4).

5.3.3.2. Time to 50% seedling emergence

The effect of 24 mg of polymer per seed on time of 50% seedling emergence in the two water regimes produced from unaged and aged seeds of the two genotypes was studied (Fig. 5.10). Analysis of variance results were presented in Appendix 3, Table 3.9. In the control water regime, unaged coated seeds of cv. Forrest had a 1.4 day delay ($P<0.05$) in the time of 50% seedling emergence in comparison to the unaged control seeds. However, no difference between unaged coated and control seeds in cv. Douglas in the time of 50% seedling emergence was observed. Little increase ($P>0.05$) due to ageing in the time of 50% seedling emergence was observed in seeds of either genotypes. Aged coated seeds of cv. Douglas had a 1.6 day delay ($P<0.05$) in the time of 50% seedling emergence in comparison to the aged uncoated seeds. In the flooding water regime, unaged seeds of cv. Forrest and Douglas had a 1.6 and 1.4 day delay ($P<0.05$) in the time to 50% seedling emergence in comparison to that of the seeds in the control water regime respectively. However, little increase ($P>0.05$) due to coating in the time to 50% seedling emergence was observed in both unaged and aged seeds of the two genotypes.

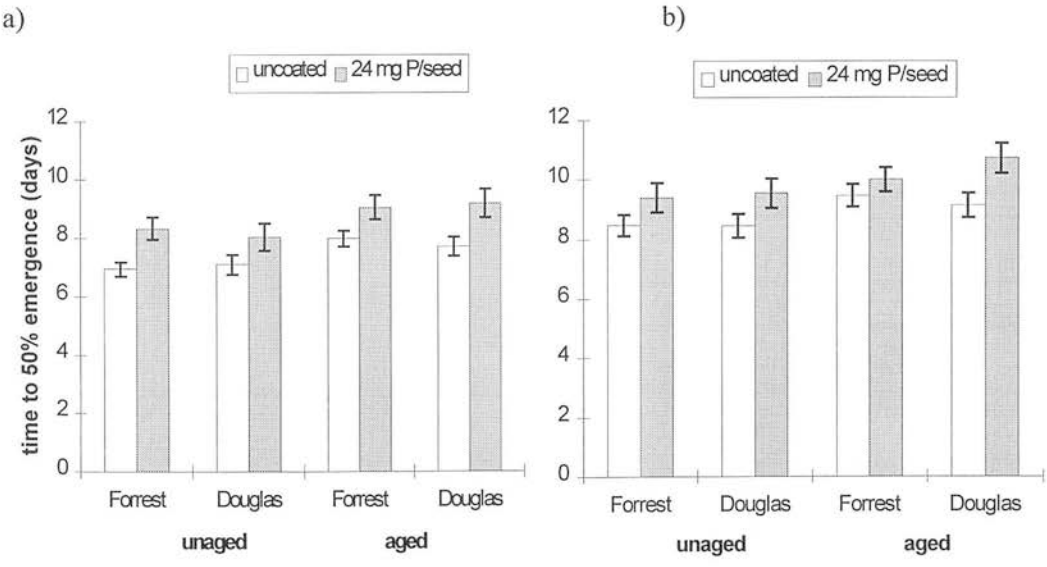


Figure 5.10. Time to 50% seedling emergence of unaged and aged polymer coated seeds (24 mg of polymer per seed) of cvs Forrest and Douglas; a) control water regime and b) flooding water regime; error bar, standard error of the mean, (n=4).

5.3.3.3. Seedling shoot length

The effect of 24 mg of polymer per seed on shoot length in the two water regimes produced from unaged and aged seeds of the two genotypes was studied (Fig. 5.11). Analysis of variance results were presented in Appendix 3, Table 3.10. In the control water regime, little difference ($P>0.05$) between unaged coated seeds and unaged uncoated seeds in the shoot length in seeds of either genotypes was observed. Ageing resulted in 16% and 18% lower ($P<0.05$) shoot length than that of unaged control seeds. Coating resulted in similar shoot length in seedlings produced from unaged or aged seeds in either genotypes.

In the flooding water regime, unaged seeds of cvs Forrest and Douglas produced seedlings which had 16% and 14% lower ($P<0.001$) shoot length respectively than that of the unaged seeds in the control water regime. Coating resulted in similar shoot length in seedlings produced from unaged or aged seeds in either genotypes.

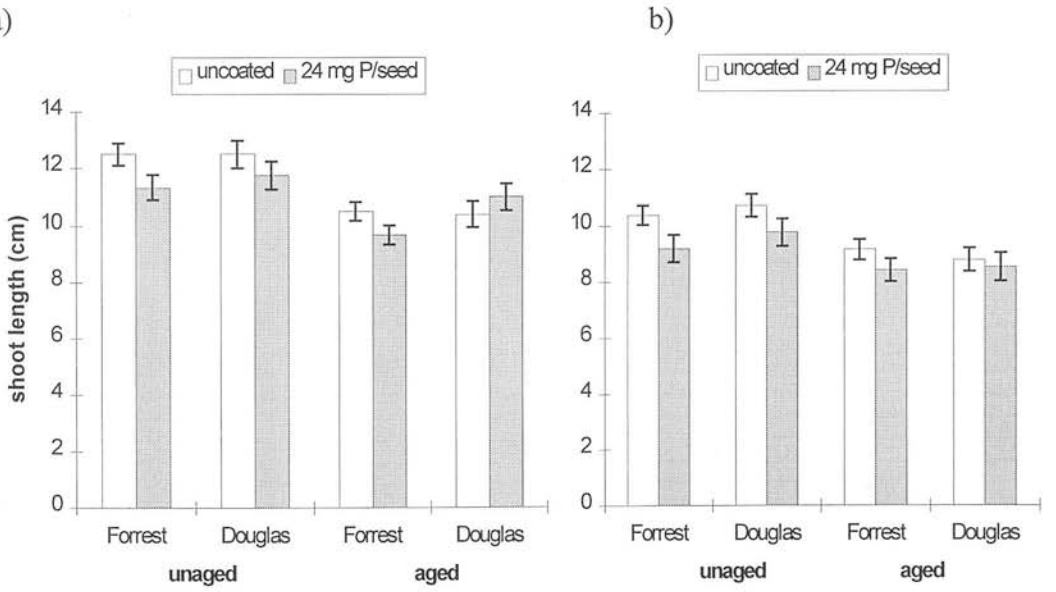


Figure 5.11. Shoot length of polymer coated (24 mg of polymer per seed) unaged and aged seeds of cvs Forrest and Douglas; a) control water regime and b) flooding water regime; error bar, standard error of the mean, (n=4).

5.3.3.4. Seedling shoot fresh weight

The effect of 24 mg polymer per seed on shoot fresh weight in the two water regimes produced from unaged and aged seeds of the two genotypes was studied (Fig. 5.12). Analysis of variance results were presented in Appendix 3, Table 3.11. In the control water regime, unaged coated seeds of cv. Forrest had 15% lower ($P<0.05$) shoot fresh weight than that of the control seeds. However, no difference between unaged coated and control seeds in cv. Douglas in the shoot fresh weight was observed. Ageing resulted in 19% and 24% lower ($P<0.001$) shoot length than that of unaged control seeds. Coated seeds of cv. Forrest had 17% lower ($P<0.05$) shoot fresh weight than that of the control seeds. However, no difference between unaged coated and control seeds in cv. Douglas in the shoot fresh weight was measured.

In the flooding water regime, unaged seeds of cv. Forrest and Douglas had 17% and 22% lower ($P<0.001$) shoot length respectively than that of the seeds in the control water regime. Ageing resulted in 20% and 18% lower ($P<0.001$) shoot length than that of unaged control seeds. Coating resulted in similar shoot length in seedlings produced from unaged or aged seeds in either genotypes.

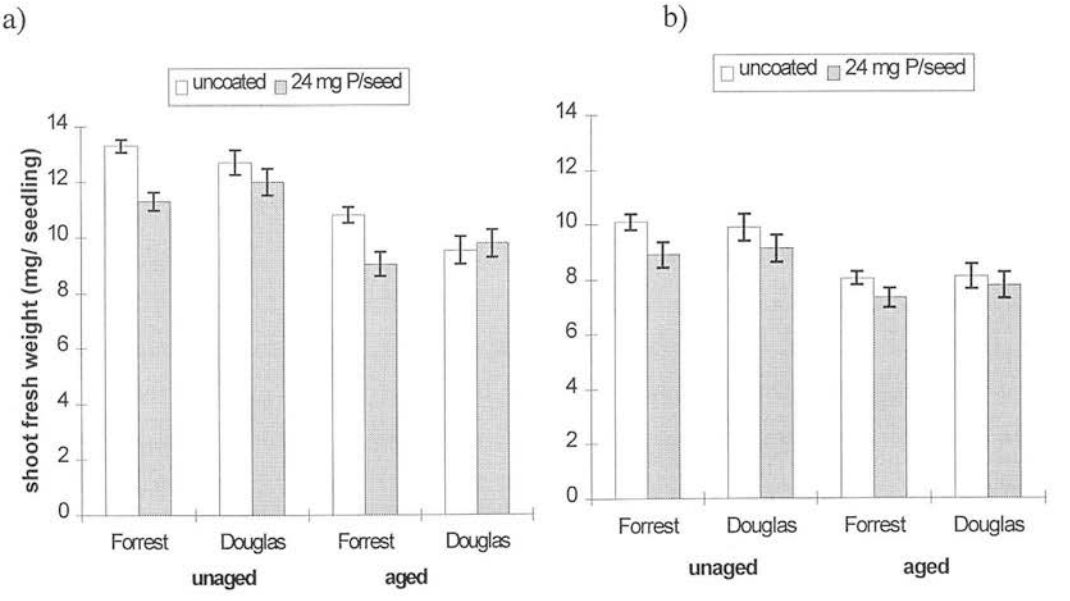


Figure 5.12. Shoot fresh weight of polymer coated (24 mg of polymer per seed) unaged and aged seeds of cvs Forrest and Douglas; a) control water regime and b) flooding water regime; error bar, standard error of the mean, (n=4).

5.4. Discussion

Polymer application to soybean seeds resulted in the formation of a film coating on the surface of seed. The amount of polymer was gradually added to seeds by sequential cycles of application and drying. Coated seeds were examined both visually and under low magnification stereoscope to ensure that optimum coating was achieved. It was observed that proper drying of the polymer-coated seeds was a very important factor in achieving the best coating. In each application cycle about 5mg of polymer was added to each seed.

The polymer used in the present study has a relatively hydrophobic nature and is different from those that are widely used in the film coating of seeds of several vegetables, and of peas and oilseed rape. These polymers are highly permeable to water and their main purpose is to offer fungicide and insecticide protection during the very early stage of seedling growth which is the most sensitive to pathogen attack (Powell and Matthews, 1988). West *et al.*, (1985) coated soybean seeds with two types of polymers (polyvinylalchohol and polyvinylidene chloride) in order to regulate moisture absorption and maintain seed quality during storage at high temperature and relative humidity. They applied an 80% aqueous solution of polymer to seeds at one step in a rotating chamber. Huang and Sung (1991) coated soybean in order to regulate water uptake and prevent soaking injury. They applied a solution of polymer diluted in ethanol together with an adhesive to seeds in a rotating inclined pan. Duan and Burris (1997) coated sugar beet (*Beta vulgaris* L.) seeds with a commercial polyvinyl polymer using a batch coater. In commercial scale, film coating is normally applied with the Wurster Air Suspension Technique in which seeds are fluidised in an upward moving column of air, continuously circulated in a fountain where seeds dry almost immediately (Powell and Matthews, 1988). Film coating materials are usually inert and not phytotoxic to seed (Robani, 1994). In this study, no attempt was made to identify any possible phytotoxic effects of the polymer to the seed.

In the present study, it was found that 24mg of polymer per seed was sufficient to reduce the rate of water uptake without affecting the final total water uptake. The highest amount of polymer tested (48 mg of polymer per seed) caused a significant decrease in the percentage of normal seedlings of seeds and this was considered undesirable. The reduction in the percentage of normal seedlings produced from polymer coated seeds with 48 mg of polymer per seed, was likely to be due to the

lower total water uptake. However, restricted oxygen supply might be another reason for the reduced germinability at high levels of polymer application.

Although, in most horticultural crop seeds the germination is not adversely inhibited by polymer film-coating (Robani, 1994), adverse effect of coating on germination of lettuce probably due to a temporary occurrence of thermodormancy (*Lactuca sativa* L.) seeds has been reported (Valdes and Bradford, 1987). Recently, Duan and Burris (1997) reported that polymer film coating (20 g Kg^{-1} of polymer) induced germination reductions in sensitive sugar beet cutlivars. They suggested that the reduction was likely to be due to restricted oxygen supply to the enclosed embryo and to the retention of water soluble germination inhibitors which would normally be leached into the germination medium.

In this study, coating with 24mg of polymer per seed, proved to be highly efficient in reducing the solute leakage and the percentage of cotyledons unstained with tetrazolium chloride particularly in the genotype (cv. Douglas) with a high proportion of seeds with split coats. Huang and Sung (1991) reported a reduction in leachate conductivity when 18 mg of ethyl cellulose per seed was applied to soybean seeds. In soybeans, some commercial seed lots appear to have a proportion of seeds with split coat. This has been reported as a significant factor contributing to low vigour as revealed by high leachate conductivity and the low percentages of cotyledons fully stained with tetrazolium chloride (Oliveira *et al.*, 1984). The beneficial effect of coating in relation to imbibition damage could be proposed for several grain legumes where splits in the seed coat have been reported as an important factor related to low vigour (Powell *et al.*, 1986a; Legesse and Powell, 1992; Kantar *et al.*, 1996).

In this study, the effects of both soaking in water and two different soil water regimes on germinability, seedling emergence and growth in seeds of high and low vigour were studied. Seeds of low vigour were produced after 2 days of accelerated ageing. This threshold was selected because it was found to be appropriate to produce seeds of low vigour without a high reduction in seed viability. Therefore, aged seeds of cvs Forrest and Douglas produced 84% and 69% normal seedlings respectively (section 4.3). In this study, ageing resulted in some well-known effects on seedling emergence and growth (Priestley, 1986); a lower percentage seedling emergence, a greater time to 50% emergence, a lower shoot length and shoot fresh weight than the unaged control seeds than the unaged seeds.

In this study, the seedling growth of polymer coated seeds in two different soil water regimes was studied. Coated seeds had a delay in the time to 50% emergence, a lower shoot length and fresh weight than the uncoated seeds regardless of the vigour level in either genotypes. The lower seedling growth of the polymer coated seeds was expected since imbibition proceeded at a slower rate in coated than in the uncoated seeds. Although coating resulted in significant lower rate of imbibition, there was little difference between uncoated and coated seeds in the seedling growth indicating that uncoated seeds were caught up.

Pre-emergence flooding was reported as a major problem causing severe reduction in seedling emergence in soybeans (Hwang and Sung, 1991). In this study, zero seedling emergence was observed from aged seeds of cv. Douglas in the flooding water regime. As a result, a combination of low vigour, high proportion of seeds with split coats and flooding soils could represent the worst scenario in relation to seedling emergence in soybeans. Under optimum conditions (high vigour seeds and control water regime), a low seedling emergence was only observed in seeds of cv. Douglas where a high proportion of seeds with split coats was observed. However, in the flooding water regime, a low seedling emergence was observed regardless of the seed vigour of the seeds. Ferriss and Baker (1990) studied the seedling emergence of 11 soybean seed lots under different soil conditions; they reported that the lowest percentage of seedling emergence (about 20%) was observed in the flooded soils.

In the present study, imbibition damage in low vigour seeds and seeds with split coats was inferred from both an increase in the percentage of cotyledons with a region of dead tissue and an increased leakage of solutes. One explanation for poor field emergence in seeds of grain legumes having low vigour and splitted seed coats is the incidence of infection by soil-borne fungi particularly of the *Pythium* species. As a result, higher levels of *Pythium* infection may explain the poor seedling emergence occurred particularly in the flooding soils in genotypes which were prone to imbibition damage. In this study no direct measurement of pre-disposition of seeds in infection of *Pythium* species was measured. Oliveira *et al.*, (1984) suggested a similar connection between imbibition damage, seed exudates and pathogen attack to explain the poor seedling emergence of several soybean seed lots. Dickson and Boettger (1982), in *Phaseolus vulgaris* seeds, reported that seeds with low water

uptake exhibited low imbibition damage and leachate conductivity. They suggested that low infection of *Pythium* was the reason for the observed vigorous seedling emergence and growth in the field. Similar results showing a relation between imbibition damage, seed exudates and pathogen infection has been also reported for peas (Matthews and Bradnock, 1968; Powell, 1985), dwarf French beans (Powell *et al.*, 1986a), field beans (Rowland, 1981), chickpeas (Legesse and Powell, 1992). Keeling (1974), in soybeans, reported a direct relationship between seed exudation and susceptibility to pathogen attack. Similarly, Kaiser and Hannan (1981, 1983) found in chickpeas that certain lines were highly susceptible to *Pythium* infection and pre-emergence damping-off. Additionally, Chen *et al.*, (1983) reported a significant reduction in chickpea seeds as a result of combination of rapid imbibition and low temperatures. Similar direct evidence between seed exudates and susceptibility to pathogen attack was reported in peas (Perry 1973).

Improvement in germinability and seedling emergence due to coating during soaking in water and in the flooding soil water regime was measured. However, the final percentage of both normal seedlings and seedling emergence was only part of the percentage germination from the paper towel germination tests. This result opens the possibility of additional or alternative causes of low seedling emergence in flooded soils. For similar reasons, Ferriss and Baker (1990) suggested additional causes for low seedling emergence in flooded soils.

CHAPTER 6

Relationship between seed coat features and water uptake during imbibition in soybeans (*Glycine max* L. Merril)

6.1. Introduction

Deposits are present on the surface of the seed coat of most soybean genotypes. Earlier reports have classified the seed coat appearance as shiny, dull or coated with bloom, depending on the amount of deposits present (Wiliams, 1950; Bernard and Weiss, 1973). Several reports have described deposits as being material adhered to the surface of the seed most likely to be residues of the pod endocarp (Newell and Hymowitz, 1978; Wolf *et al*, 1981; Yaklich *et al.*, 1986). In contrast, other reports suggested that deposits were of waxy origin (cutins) and therefore had originated from the epidermal layer of the seed coat (Calero *et al.*, 1981; Ragus, 1987).

The existence of pits in the surface of the soybean seed coat is also well documented (Wolf and Baker, 1972; Calero *et al.*, 1981; Wolf *et al.*, 1981). However, the role of deposits and pits in the water uptake has not yet been clearly identified. Calero *et al.*, (1981), based on the information from SEM studies, reported that when deposits existed in the absence of pits, the soybean seed was hard and when both pits and deposits were present, water uptake was slow. However, there is no published information reporting direct evidence (using water soluble stains) that pits or/and deposits play a role in water uptake.

Organic solvents can be used to associate changes in the seed coat with changes in permeability to water. Traditionally, organic solvents have been used to overcome hardseedness in many species; in most of the previous reports, it was assumed that changes in the seed coat properties were related to changes in the cuticle (McDonald and Coopeland, 1989). There is no published information relating changes in the seed coat brought about by organic solvents to changes in water uptake in normal (soft) soybean seeds.

In this study, six contrasting genotypes were selected to investigate the relationship between seed coat features and water uptake during imbibition. The results presented in Chapter 3 revealed that there were large differences between the six genotypes in the regulation of the water uptake by the seed coat. The seed coats of seeds of lines SS 87040-2-1 and GC 88037-38-2-2 were highly permeable to water exhibited a high level of imbibition damage. In contrast, seeds of line VLS-1

possessed a delayed-permeable seed coat and the seeds exhibited a low level of imbibition damage.

The objectives of this study were therefore: (1) to identify differences between six contrasting genotypes in the surface structure (deposits and pits), (2) to identify the role that deposits and pits could play in water uptake using a water-soluble fluorescent stain, and (3) to identify the mechanism of water uptake regulation by the seed coat using organic solvents.

6.2. Materials and methods

6.2.1. The surface of the seed coat

The surface of the seed coat of seeds the six genotypes was studied. Seeds were processed for SEM as described in Section 2.11. For the estimation of the presence of deposits, micrographs were taken at 300x magnification in the middle of the abaxial region of the seed. Prior to the estimation of the density of pitting, the surface deposits had to be removed; twenty seeds were soaked for 24h in 50ml of methanol and chloroform solvents with continuous shaking. Seeds were then processed for SEM as described in Section 2.11. Micrographs were taken at 300x magnification in the middle of the abaxial side of the seed. The density of pitting was then arbitrary classified into 3 categories namely, 0 (no pits), 1 (low density of pits) and 2 (high density of pits).

6.2.2. Use of calcofluor as a water-soluble fluorescent stain

To demonstrate water penetration, whole seeds of cv. Sapporo were soaked in 0.1% calcofluor for 5 sec, 15 seconds, 1 min, 15 min and 30 minutes as described in section 2.10.1. Comparisons were made between the fluorescence image of the calcofluor stained seed coat and the light microscope image of the same area. To determine the depth of penetration of calcofluor into the seed coat, whole seeds were soaked in calcofluor for 2 min or 15 minutes. The experimental procedure was the same as described in section 2.10.2.

Seeds of the other five genotypes were used to demonstrate staining of the surface deposits. Additionally, seeds after the 24h methanol pre-treatment (removal of the deposits) were also used. Whole seeds were soaked in calcofluor for 5 seconds, and the experimental procedure was the same as described in section 2.10.1.

Water penetration through the hilar region of the seed coat was studied. Whole seeds of cv. Sapporo and KWS-C were soaked in calcofluor for 15 min or 60

minutes, and then segments of the seed coat of the pre-treated seeds were prepared as described in section 2.9. Hand-cut sections were also prepared.

6.2.3. Effect of methanol and chloroform pre-treatments on water uptake

Seeds of six genotypes (Table 6.1) were used. Organic solvent pre-treatments were applied as described in section 2.12. Four pre-treatments were used: 1) 1min chloroform (TR1), 2) 2h periods successively in methanol, chloroform:methanol (1:1) and chloroform with continuous shaking throughout (TR2), 3) 2h methanol with continuous shaking (TR3) and 4) 2h chloroform with continuous shaking (TR4). After the pre-treatments, seeds were blotted dry. Subsequently, 10 seeds were placed for measurement of the water uptake after 30 min of imbibition as described in Section 2.5.2.

Seeds cv. Sapporo were used to measure the effect of the prolonged methanol or chloroform pre-treatments on the water uptake. Seeds were soaked in methanol or chloroform for 2h, 24h, 48h, 72h, 96h as described in Section 2.12. After the pre-treatments, seed surface solvent removed. Subsequently, 10 seeds were placed for measurement of the water uptake after 30 and 60 min of imbibition as described in Section 2.5.2. The amount of solvent absorbed by seeds after 72h pre-treatment was indirectly calculated as the seed weight increase. The amount of solvent absorbed was expressed as mg per seed, and each mean is the average of ten values.

Seeds of the five genotypes were used to measure the effect of the 72h methanol and chloroform pre-treatment on the water uptake. Seeds were soaked in methanol and chloroform for 72h as described in Section 2.12. After the pre-treatments, seeds were blotted dry. Subsequently, 10 seeds were placed for measurement of the water uptake after 30 minutes of imbibition as described in Section 2.5.2.

6.2.4. Absorbance spectrum of supernatant

Seeds of cv. Sapporo were used. The absorbance spectrum of the supernatant obtained from the 24h and 72h methanol and chloroform supernatant was measured as described in section 2.13.

6.2.5. Effect of seed drying after prolonged methanol and chloroform pre-treatments on water uptake

Seeds of the six genotypes were used. Seeds were soaked in methanol and chloroform for 72h as described in section 2.12. After the pre-treatments, seeds were blotted dry, left to dry for 4 days at room conditions. The final moisture of the seeds

was 7.5% (±1.5%) m.c. on fresh weight basis. Subsequently, 10 seeds were placed for imbibition, and the water uptake was measured after 30 minutes as described in section 2.5.2.

6.2. Results

6.2.1. The surface of the seed coat

Scanning electron microscopy (SEM) of the surface of the seed coat revealed the presence of deposits and pits in seeds of most of the genotypes studied.

Deposits were observed as material attached to the surface of the seed coat and were visible even at low magnification. Pits were observed as indentations of the surface of the seed coat and could also be seen at low magnification. However, in order to observe the structure of deposits and individual pits, higher magnifications were used. Pits and deposits were often occurred together in the surface of the seed coat Fig. 6.2. In cases like these, the presence of deposits makes the study of pits very difficult unless a method for effectively removing the deposits is developed first.

Soaking for 24h in methanol with continuous shaking caused the surface of the seed coat to change from dull (Fig. 6.1a) to shiny (Fig. 6.1b) whereas little difference was observed in the appearance of seed coats of seeds that had been pre-treated for 24h with chloroform (Fig. 6.1c). Images obtained from SEM, revealed that the methanol pre-treatment had removed the deposits (Fig. 6.2b) whereas the chloroform pre-treatment had little effect upon them (Fig. 6.2c). SEM images, from the abaxial region of the seed, revealed that the surface of the seed coat in seeds of cv. Sapporo was free from deposits whereas deposits were present in seeds of all other genotypes.

The 24h methanol pre-treatment was applied to seeds of cv. Sapporo to see whether any changes, other than the removal of the deposits, had occurred in the surface of the seed coat due to the pre-treatment. At both low and high magnification, no other changes in the surface of the seed coat were observed.

Deposits could be seen in the form of a thick granular-like material present on the surface of the seed coat (Fig. 6.3a). At a higher magnification, granules of a smaller size were also observed (Fig. 6.3b). In most cases, a great part of the dorsal region had fewer deposits than the abaxial region.

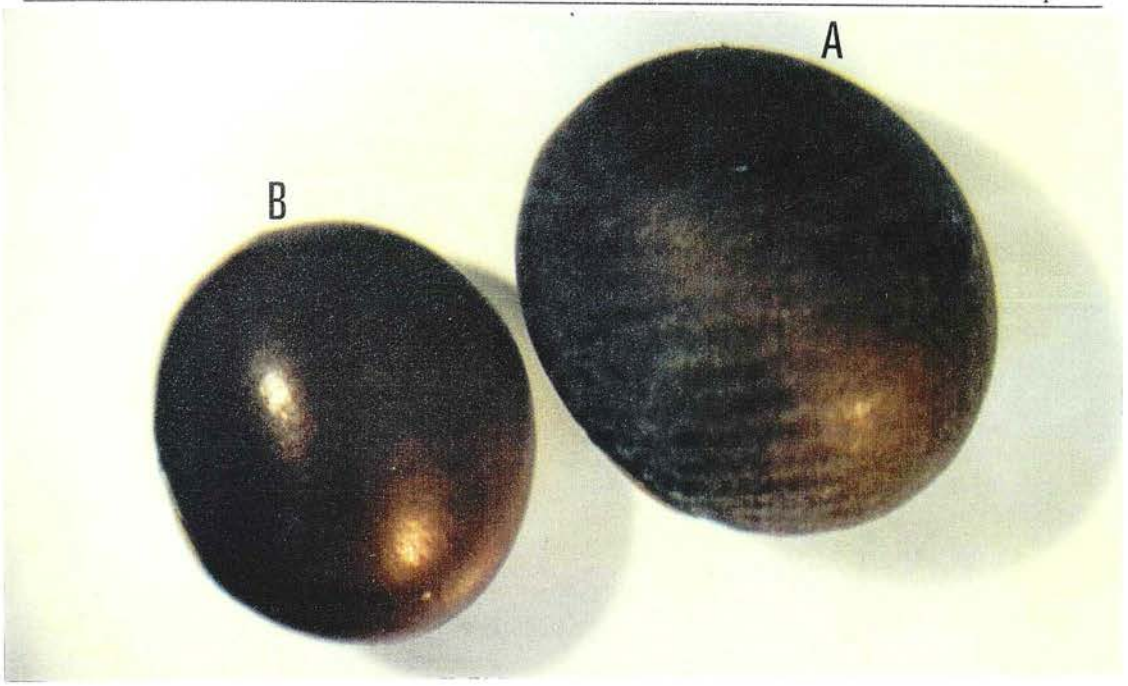


Figure 6.1. Light micrographs showing the effect of methanol pre-treatment on the appearance of the abaxial region of seeds of line GC 88037-38-2-2. A) control, no pre-treatment, B) after 24h methanol pre-treatment; 7x magnification.

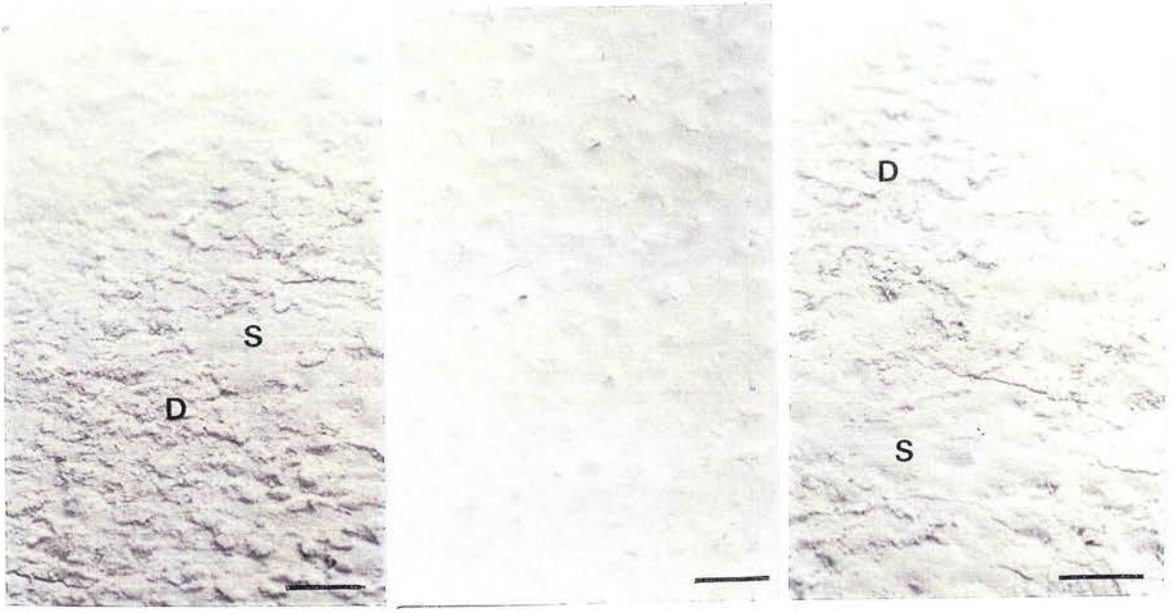


Figure 6.2. SEM micrographs showing the effect of methanol and chloroform pre-treatments on deposits in the abaxial region of seeds of GC 88037-38-2-2. A) control, no pre-treatment, B) after 24h methanol pre-treatment and C) after 24h chloroform pre-treatment. D: deposits, S: surface. Scale bar 100μm.

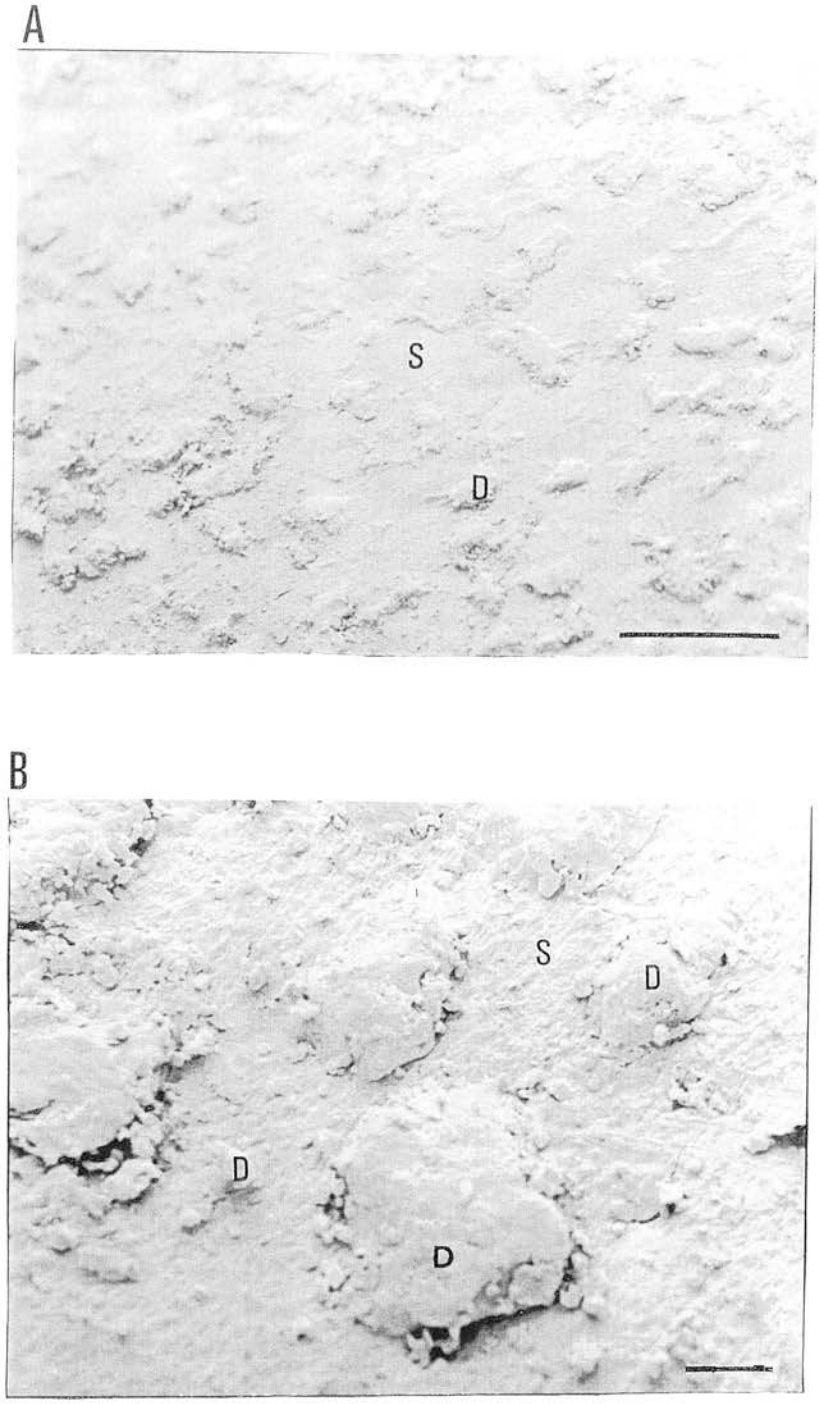


Figure 6.3. SEM micrographs of the deposits on the surface of seeds in the abaxial region of seeds of VLS-1. A) scale bar, 100μm; B) scale bar, 20μm D: deposits, S: surface.

Pitting of the surface of the seed coat was assessed after the removal of deposits by methanol. For the evaluation of the number of pits, SEM micrographs were taken from the abaxial region of the seed coat at 300x magnification for the arbitrary classification from zero to high density of pits (Table 6.1).

Table 6.1. The colour of the seed coat, presence of deposits and density of pits in the abaxial region in the six genotypes.

Genotypes	Testa colour	Presence of deposits	density of pits*
VLS-1	black	yes	0/+
KWS-C	yellow	yes	++
Sapporo	yellow	no	++
SS 87040-2-1	green	yes	++
GC 88037-38-2-2	brown	yes	++
Suwan-156	black	yes	+

*scale, 0: zero; +:medium; ++: high.

There was a variation among the genotypes in the density of pits observed . Figure 5.4 shows examples illustrating the range of pitting observed. Seeds of line VLS-1 had very few pits (Fig. 6.4a), seeds of cv. Suwan-156 medium density of pits (Fig. 6.4b) and seeds of cv. Sapporo (Fig. 6.4c) and line GC 88037-38-2-2 (Fig. 6.4d) high density of pits . Additionally, in seeds of line SS 87040-2-1 and GC 88037-38-2-2, there was a high proportion of pits which appeared to be very deep and highly open (Fig. 6.4d).

In seeds of line VLS-1, there was a variation in the number of pits observed within the different regions of the seed coat; very few pits in the abaxial region but significantly more in the dorsal region. In several seeds, in the abaxial region, small areas were observed that were extensively pitted surrounded by areas with no pitting.

Three types of pits were observed, namely; deep-elongated (Fig. 6.5a) or deep-circular (Fig. 6.5b) and shallow (Fig. 6.5c). In seeds of line VLS-1, most of the pits were shallow whereas in all other genotypes a combination of all types of pits occurred.

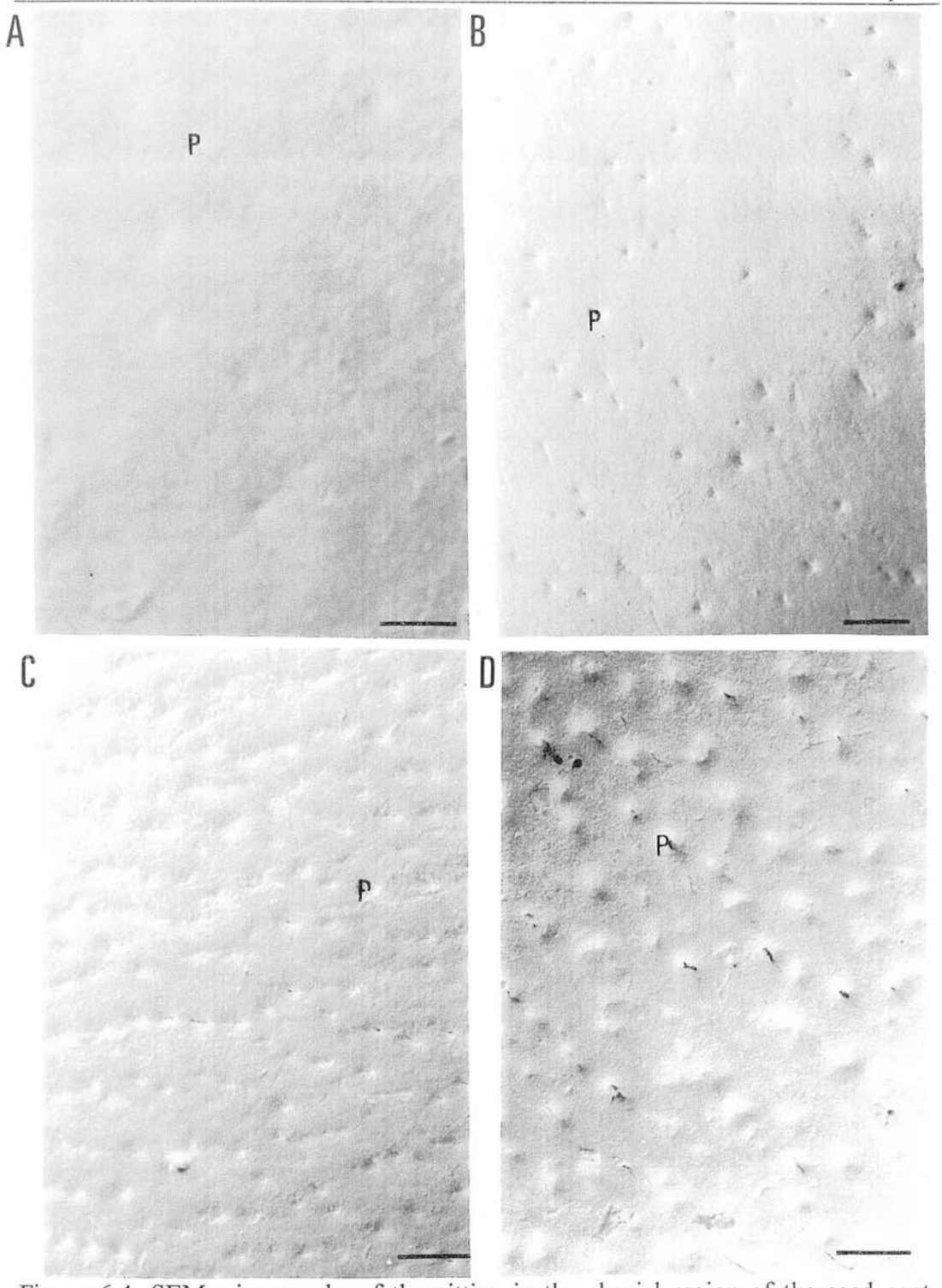


Figure 6.4. SEM micrographs of the pitting in the abaxial region of the seed coat within different genotypes. A) seeds from line VLS-1; B) seeds from cv. Suwan-156; C) seeds from cv. Sapporo and d) seeds of line SS 87040-2-1. P: pits. Scale bar, 100μm.

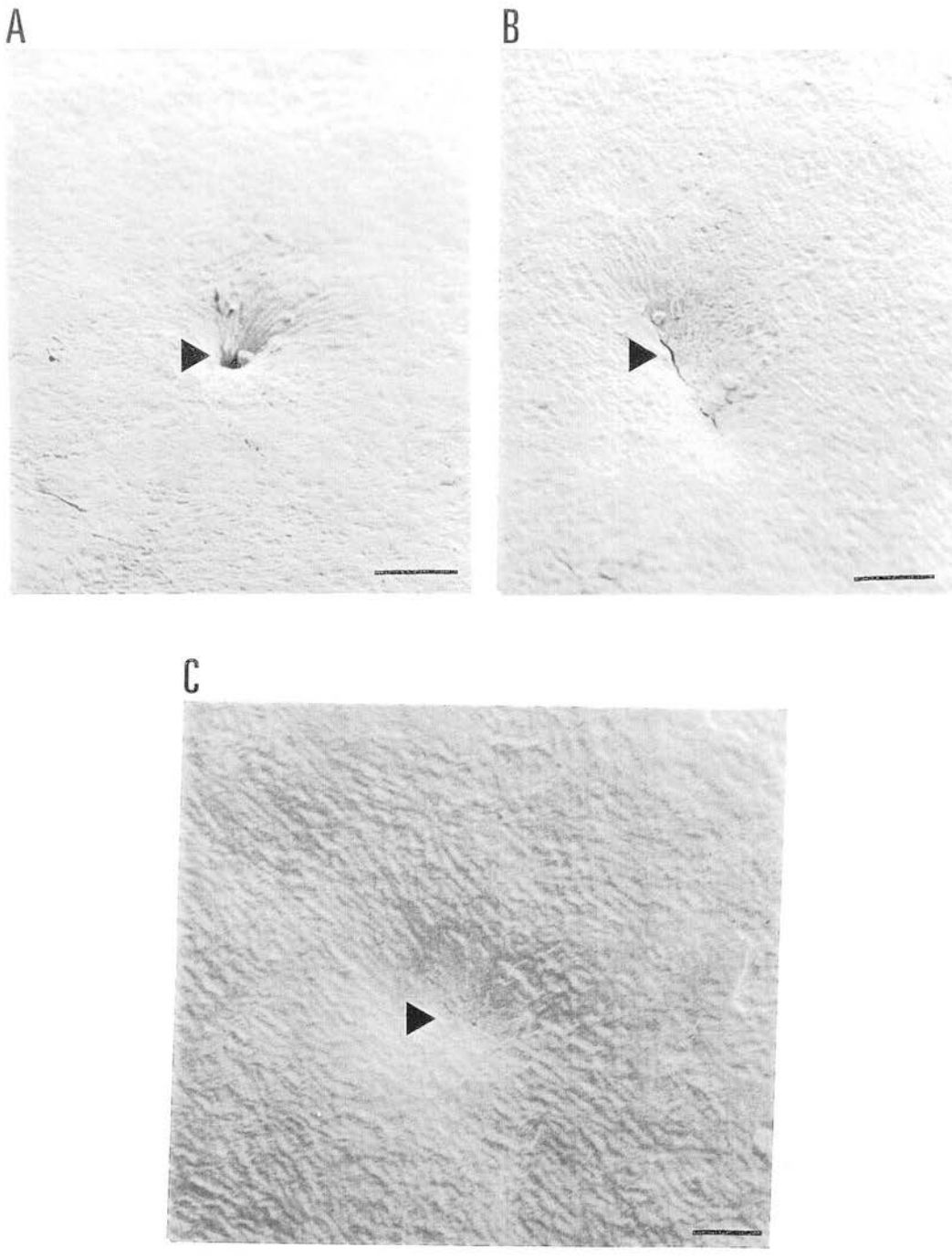


Figure 6.5. SEM micrographs of the structure of individual pits. A) deep and elongated-shaped pit; B) deep and circular-shaped pit and C) shallow pit; (arrow indicate pit), scale bar, 20μm.

6.2.2. Use of calcofluor as a water-soluble fluorescent stain

Soaking whole seeds in calcofluor was used to stain the surface deposits, and as a method of demonstrating water penetration mapping into the seed coat. The surface of the abaxial region of the seed coat was observed using fluorescence microscopy.

To observe water penetration mapping, whole seeds of cv. Sapporo were used because it was previously shown that their surface was free from deposits. After 5sec of soaking whole seeds in calcofluor no staining was observed in the abaxial region. In seeds soaked in calcofluor for 15 sec, a patchy staining pattern could be seen. In seeds soaked in calcofluor for 1 min, a similar patchy staining pattern could be seen (Fig. 6.6). In seeds soaked in calcofluor for 15 and 30 mins, the stain diffused throughout the layers of the seed coat, and the resolution was lost. The light microscope image of the same area was also observed in order to correlate the distribution of the patchy areas to the distribution of the pits. Comparison of the two images revealed the closed association of the distribution of the patchy areas to the distribution of the pits (Fig. 6.7).

To observe the staining of the surface deposits seeds of the line VLS-1 were used. In seeds of line VLS-1, deposits with few pits were present in the abaxial region of the seed coat. After soaking whole seeds of line VLS-1 for 5 sec in calcofluor, a strong staining was observed (Fig. 6.8a). When seeds of the line VLS-1 that had been pre-treated with 24h methanol (deposit removed) soaked in calcofluor for 5 seconds little staining was observed (Fig. 6.8b). The staining of the untreated control seeds and methanol treated seeds of the other genotypes was similar to that observed in seeds of line VLS-1.

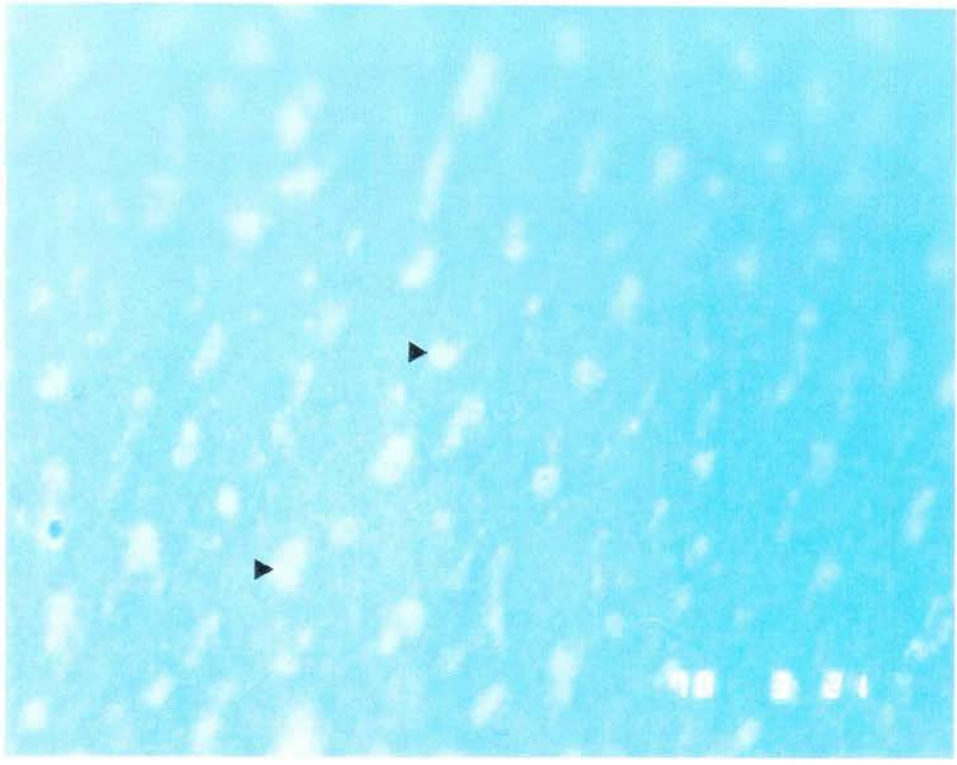


Figure 6.6. Fluorescence micrograph of the staining of the abaxial region of the seeds coat after soaking seeds of cv. Sapporo in 0.1% calcofluor for 1min; (arrows indicate sites of water penetration), 600x magnification, .

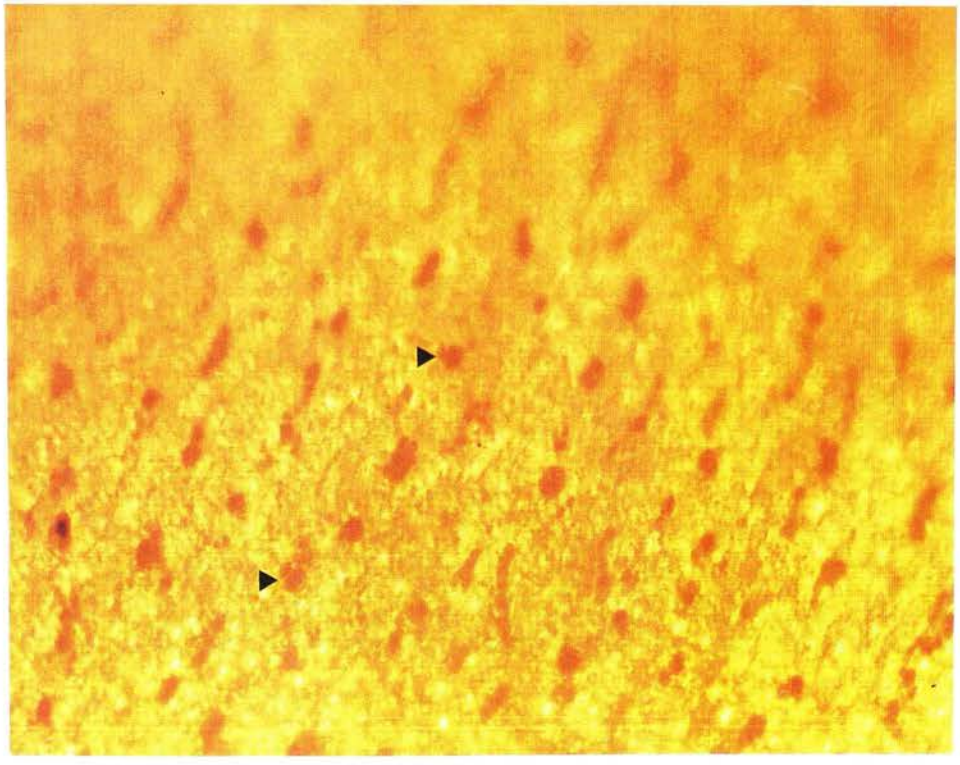


Figure 6.7. Light microscope micrograph of the same area as in the above microrgaph; (arrows indicate pits), 600x magnification.

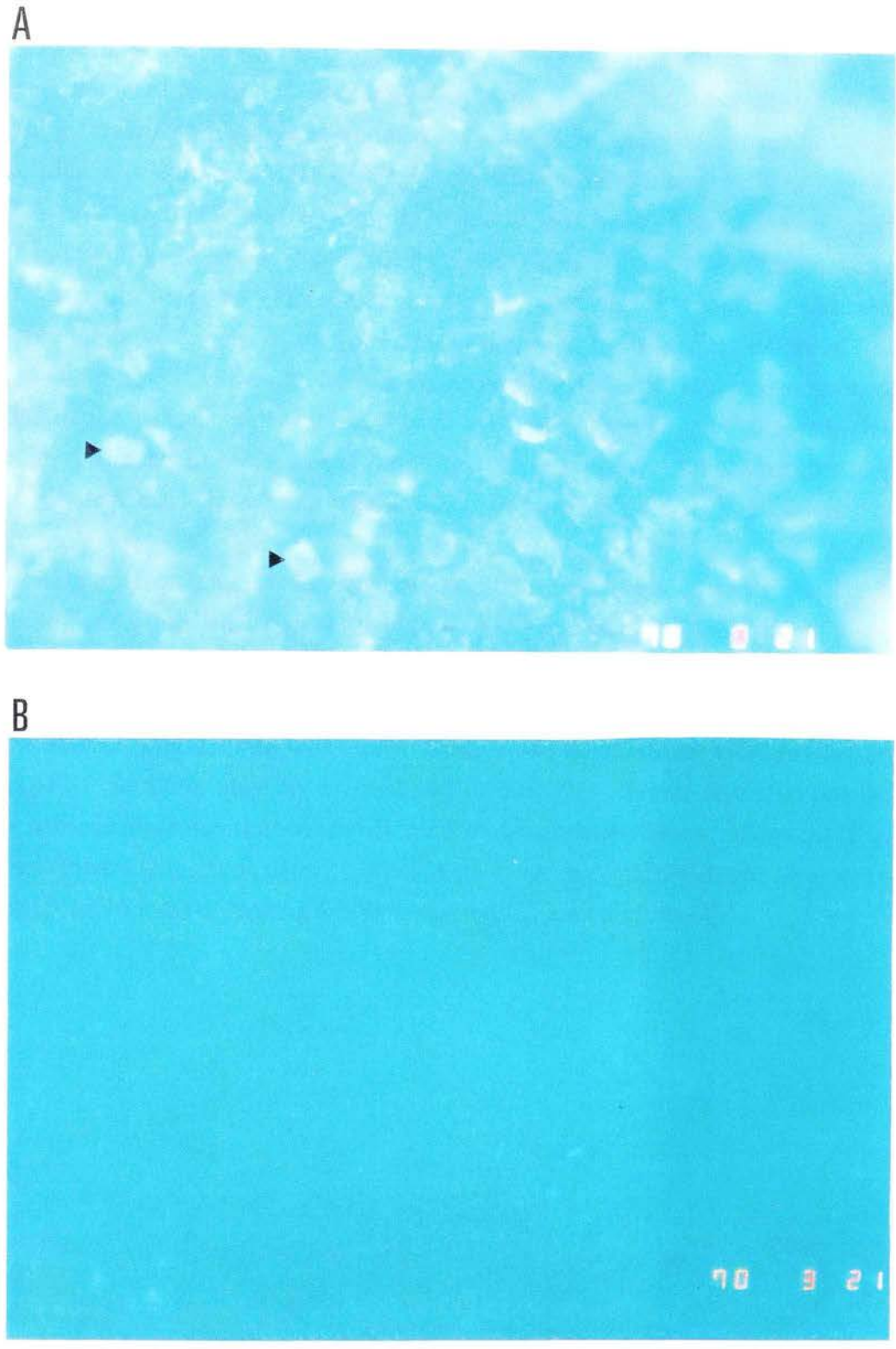


Figure 6.8. Fluorescence micrographs of the staining of the abaxial region of the seeds coat after soaking seeds of line VLS-1 in 0.1% calcofluor for 5sec. A) control, untreated seeds (arrows indicate staining), B) 24h methanol pre-treated seeds (no staining). 600x magnification.

Anticlinal sections of resin-embedded seed coats from dorsal, abaxial and ventral regions of the seed were examined in order to identify the penetration of the calcofluor staining within the layers of the seed coat. In seed coats of seeds soaked for 2 mins in calcofluor, a light staining of the outer parts of the palisade layer in all regions of the seed coat was evident (Fig. 6.9a). In seed coats of seeds soaked for 15 mins in calcofluor, a strong staining of the whole palisade layer in all regions of the seed coat was observed (Fig. 6.9b). It was not possible to identify differences between the three regions of the seed coat in the depth of the stain penetration into the palisade layer . However, frequently, in sections from all regions, there were groups of the palisade cells which showed a stronger stain reaction than adjacent cells. In seed coats of seeds soaked for 30 mins in calcofluor, a very strong staining of the whole palisade layer in all regions of the seed coat was observed (Fig. 6.9c).

Anticlinal sections of the hilar region of the seed coat of seeds soaked in calcofluor were studied in order to observe the penetration of the stain in these regions. In seeds of cv. Sapporo, the tracheid bar was only partially stained after 30 mins of soaking (Fig. 6.10a) whereas in seeds of line KWS-C the tracheid bar was totally stained after 15 min of soaking whole seeds in calcofluor (Fig. 6.10b). In seeds of cv. Sapporo, only the external part of the outer palisade layer but not the inner palisade layer was stained after 30 mins soaking whole seeds in calcofluor (Fig. 6.10a). However, in seeds of cv. KWS-C both outer and inner palisade layer were stained after 15 mins of soaking whole seeds in calcofluor (Fig. 6.10b).

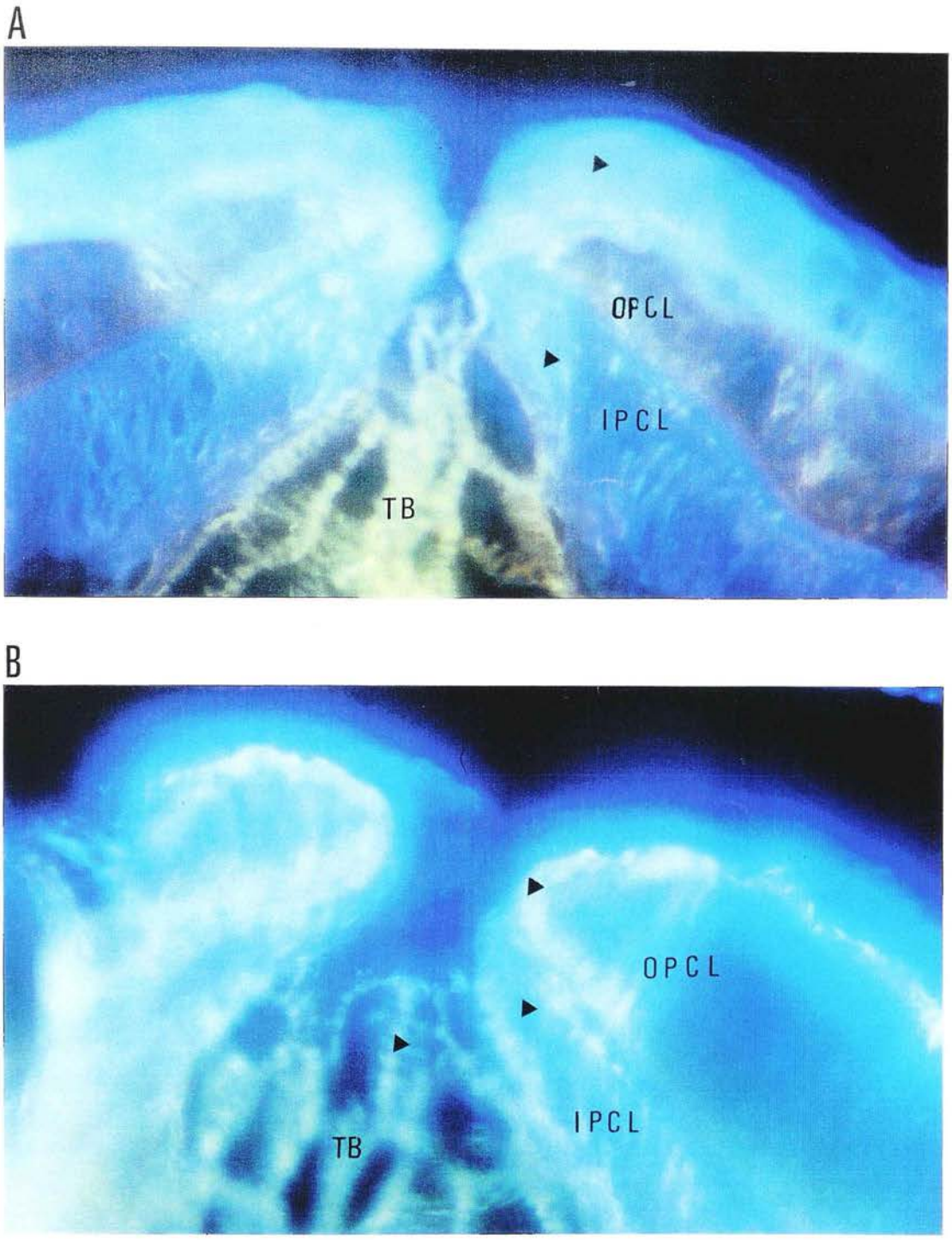


Figure 6.9. Fluorescence micrographs of anticlinal hand-cut sections of the hilar region of the seed coat after soaking seeds in 0.1% calcofluor for 15sec. A) seeds of cv. Sapporo; B) seeds of line KWS-C. TB: tracheid bar; OPCL: outer palisade cells layer; IPCL: inner palisade cells layer; (arrows indicate staining) 2,000 x magnification.

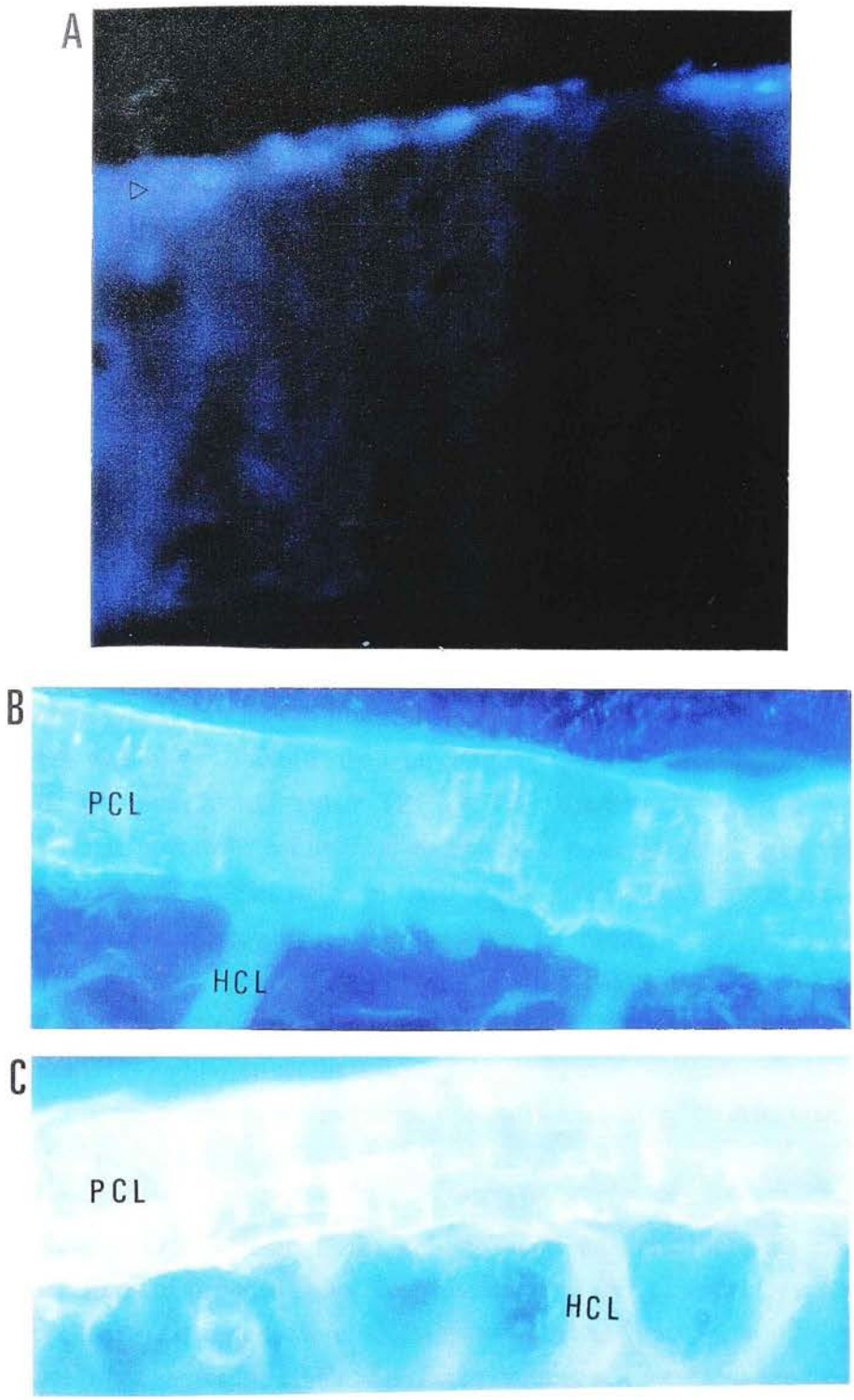


Figure 6.10. Fluorescence micrographs of anticlinal resin-embedded sections of the abaxial region of the seed coat after soaking seeds of cv. Sapporo in 0.1% calcofluor. A) seeds soaked for 2min, (arrows indicate staining) 4,000x magnification; B) seeds soaked for 15min, 1,500x magnification; C) seeds soaked for 30mins, 1,500x magnification. PCL: palisade cells later; HCL: Hourglass cells layer.

6.2.3. Effect of methanol and chloroform pre-treatments on the water uptake

The weight of water imbibed by seeds of the six genotypes that had been subjected to four different pre-treatments immediately prior to the start of imbibition was measured. The four pre-treatments were: 2min in chloroform (TR1), 2h periods successively in methanol, methanol:chloroform (1:1) and chloroform (TR2), 2h in methanol (TR3) and 2h in chloroform (TR4).

Figure 6.11 shows the water imbibed in 30 mins by untreated seeds and seeds subjected to pre-treatments TR1, TR2, TR3 and TR4. Analysis of variance results were presented in Appendix 4, Table 4.1. There was a variation ($P<0.001$) among the genotypes in the water imbibed in 30 mins by the untreated controls. Untreated seeds of line VLS-1 imbibed 2.2 mg of water per seed whereas those of line GC 88037-38-2-2 imbibed 51.1 mg of water per seed ($P<0.001$). Brief pre-treatment with chloroform (TR1) had no effect on the water uptake in comparison to the control in most genotypes; only in seeds of line GC 88037-38-2-2 the water uptake of the pre-treated seeds was lower ($P<0.001$) than the control.

Seeds pre-treated with either TR2 or TR3 consistently showed increased ($P<0.001$) water uptake in comparison to untreated controls in all genotypes; the increase varied from 10mg of water, in seeds of line KWS-C, to 27mg of water as in seeds of line VLS-1. In most genotypes, there was no difference between treatments TR2 and TR3. However, seeds of cv. Suwan-156 pre-treated with (TR3) imbibed less ($P<0.001$) water than those pre-treated with (TR2). Two hours pre-treatment with chloroform (TR4) had no effect on water uptake in most genotypes; only in Suwan-156 was the water uptake of the pre-treated seeds was higher ($P<0.001$) than that of the control seeds.

In view of the effect of the 2h pre-treatments on imbibition, the effects of prolonged periods of pre-treatments were investigating using seeds of cv. Sapporo. The five pre-treatment periods were: 2h, 24h, 48h, 72h and 96h.

Figure 6.12a shows the effect of different durations of methanol pre-treatments on water uptake in seeds of cv. Sapporo. Analysis of variance results were presented in Appendix 4, Table 4.2. In general, the larger the duration of the methanol pre-treatment the greater the water uptake ($P<0.001$). Seeds given a 2h methanol pre-treatment had a 60% higher ($P<0.001$) water uptake that the untreated control seeds. Seeds given a 96h methanol pre-treatment had a 4 times greater water uptake than the untreated control seeds.

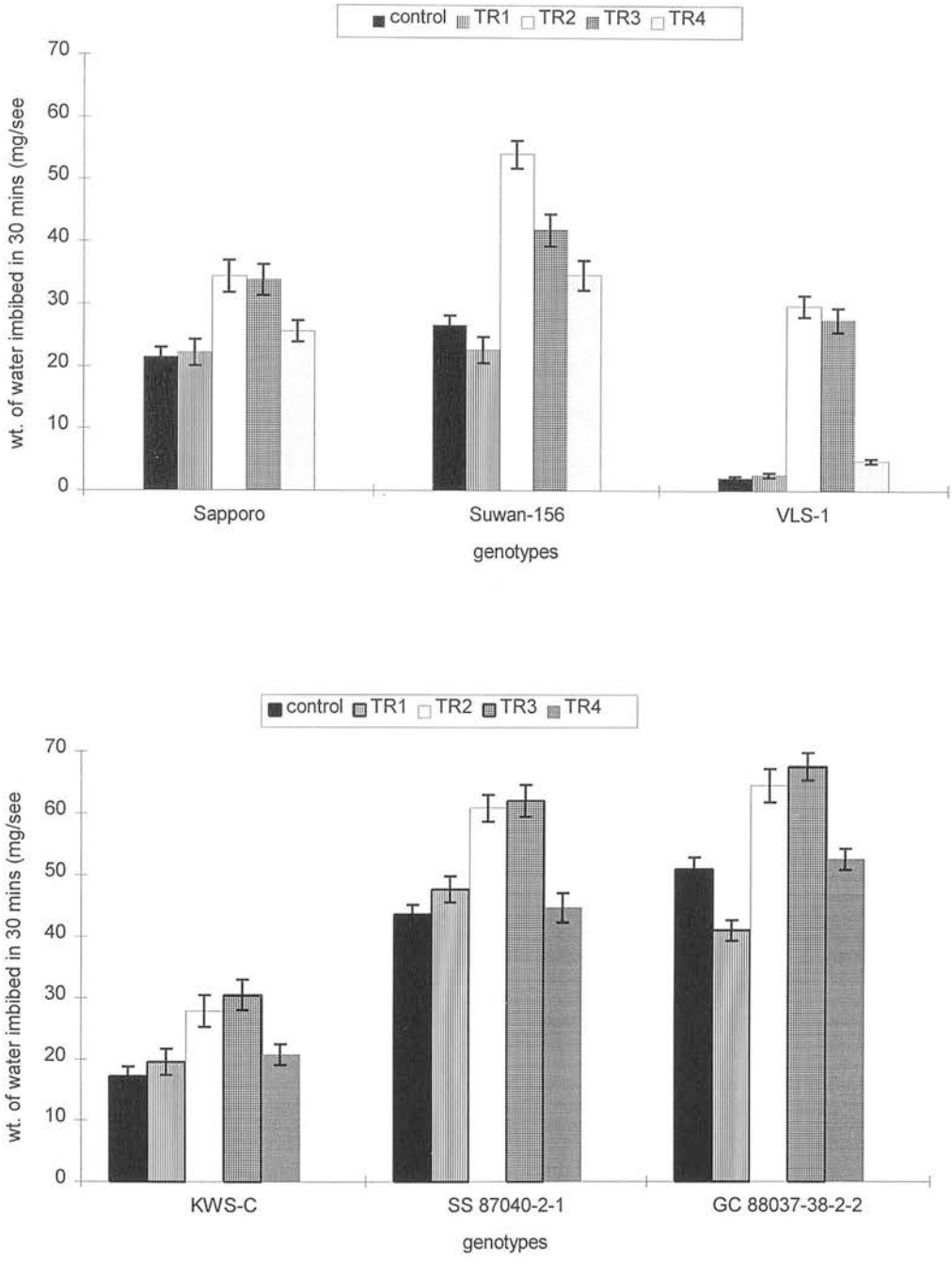


Figure 6.11. Weight of water imbibed in 30 min by seeds of six genotypes that had been pre-treated with different organic solvents. control (untreated), TR1 (2min chloroform), TR2 (2h methanol, 2h methanol:chloroform (1:1), 2h chloroform), TR3 (2h methanol) and TR4 (2h chloroform); error bars, standard error of means (n=10).

During imbibition, there was a linear increase in the water imbibed by the untreated control seeds. In contrast, seeds which had methanol pre-treatment imbibed more water in the first 30 mins than in the second 30 minutes. This effect was particularly evident in seeds given a 96h methanol pre-treatment.

Figure 6.12b shows the effect of different durations of chloroform pre-treatments on water uptake on seeds of cv. Sapporo. Analysis of variance results were presented in Appendix 4, Table 4.3. In general, seeds given chloroform pre-treatments imbibed more ($P<0.001$) water than the untreated control seeds. The water uptake of seeds given 2h chloroform pre-treatment was about 60% higher ($P<0.001$) than the untreated control seeds. Seeds given a 96h chloroform pre-treatment had a 2 times greater water uptake than the untreated control seeds.

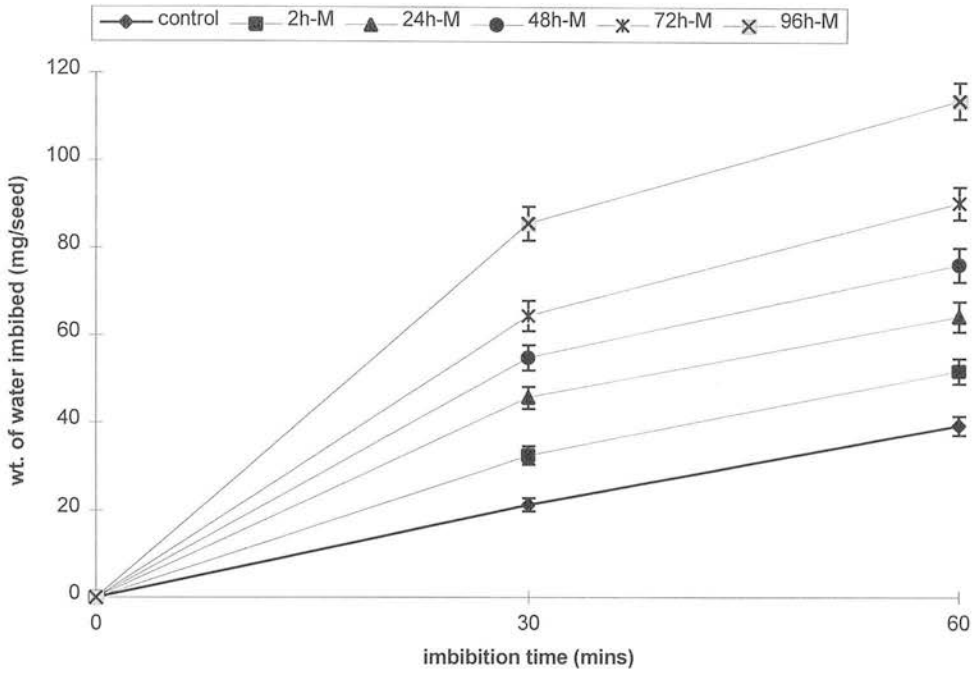
The amount of methanol and chloroform absorbed during the 72h pre-treatment was calculated from the seed weight increase. Table 6.2 shows that the amount of methanol absorbed was about 2 times greater ($P<0.001$) than the amount of chloroform absorbed.

Table 6.2. Weight of solvent absorbed by seeds of cv. Sapporo during the 72h pre-treatment (n=10).

Pre-treatments	weight of solvent absorbed mg per seed ± s.e.m. (n=10)
72h methanol	6.1 ± 0.9
72h chloroform	2.2 ± 0.5

Figure 6.13 shows the effect of 72h methanol and chloroform pre-treatments on the water uptake during 30 mins of imbibition, in seeds of all genotypes. Analysis of variance results were presented in Appendix 4, Table 4.4. There was a variation ($P<0.001$) among the genotypes in the water imbibed in 30 mins by the untreated controls.

a)



b)

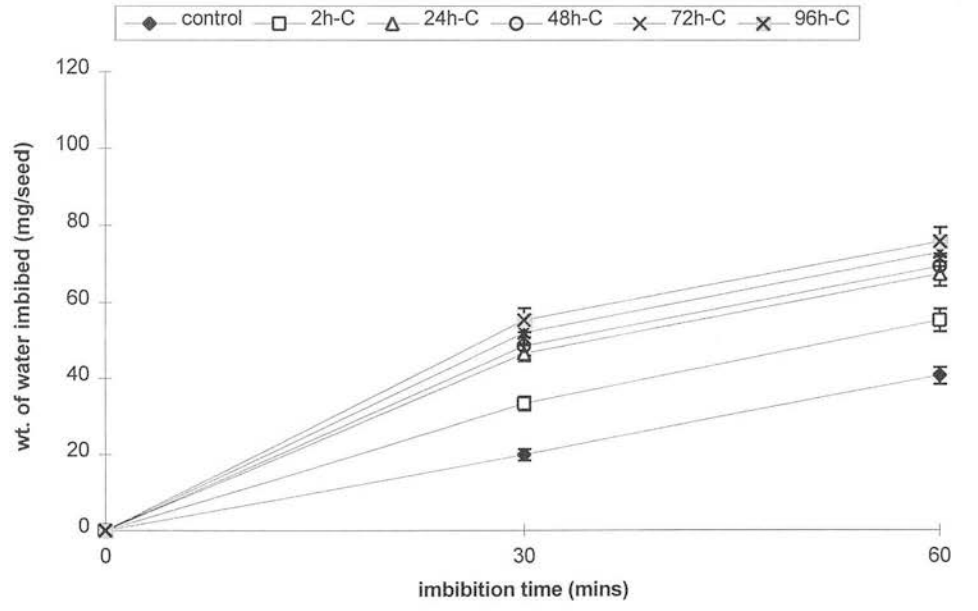


Figure 6.12. Weight of water imbibed in 30 and 60 mins by seeds of cv. Sapporo that had been pre-treated for control (untreated), 2h, 24h, 48h, 72h, 96h with a) methanol (M), b) chloroform (C); error bar, standard error of means (n=10).

In general, seeds with the methanol and chloroform pre-treatment had a greater ($P<0.001$) water uptake than the untreated control seeds. However, the methanol pre-treatment induced a greater ($P<0.001$) increase in the water uptake than the chloroform pre-treatment. In seeds of cv. Suwan-156, the water uptake of methanol and chloroform pre-treated seeds was 2.3 times and 53% ($P<0.001$) greater than that of the untreated control seeds respectively. In seeds of line VLS-1, the water uptake of methanol and chloroform pre-treated seeds was 21 and 5 times greater than that of the untreated control seeds respectively. In seeds of line KWS-C, the water uptake of methanol and chloroform pre-treated seeds was 3.5 and 2 times greater than that of the untreated control seeds respectively. In seeds of line SS 87040-2-1, the water uptake of methanol and chloroform pre-treated seeds was 2 times and 36% greater ($P<0.001$) than that of the untreated control seeds respectively. In seeds of line GC 88037-38-2-2 the water uptake of methanol and chloroform pre-treated seeds was 85% and 27% greater ($P<0.001$) than that of the untreated control seeds respectively.

6.2.5. Effect of seed drying after prolonged methanol and chloroform pre-treatments on the water uptake

After the 72h methanol and chloroform pre-treatment, seeds were allowed to dry for 48h at room temperature. Subsequently, the weight of water imbibed in 30 mins was measured.

Figure 6.14 shows the effect of different pre-treatments on water uptake. Analysis of variance results were presented in Appendix 6, Table 6.5. In general, seeds placed for imbibition directly after the 72h methanol and chloroform pre-treatments, showed an increase ($P<0.001$) in the water uptake in comparison to the untreated control seeds. Methanol pre-treatment had a higher increase ($P<0.001$) in the water uptake in comparison to the chloroform pre-treatments, in seeds of all genotypes. Seeds dried after the pre-treatments had a lower ($P<0.001$) water uptake in comparison to that of the undried seeds. In seeds of cv. Sapporo, the water uptake of methanol and chloroform pre-treated seeds which placed immediately for imbibition was 3 and 2 times greater than that of the untreated control seeds respectively. However, seeds dried after the pre-treatments had a similar water uptake with untreated control seeds.

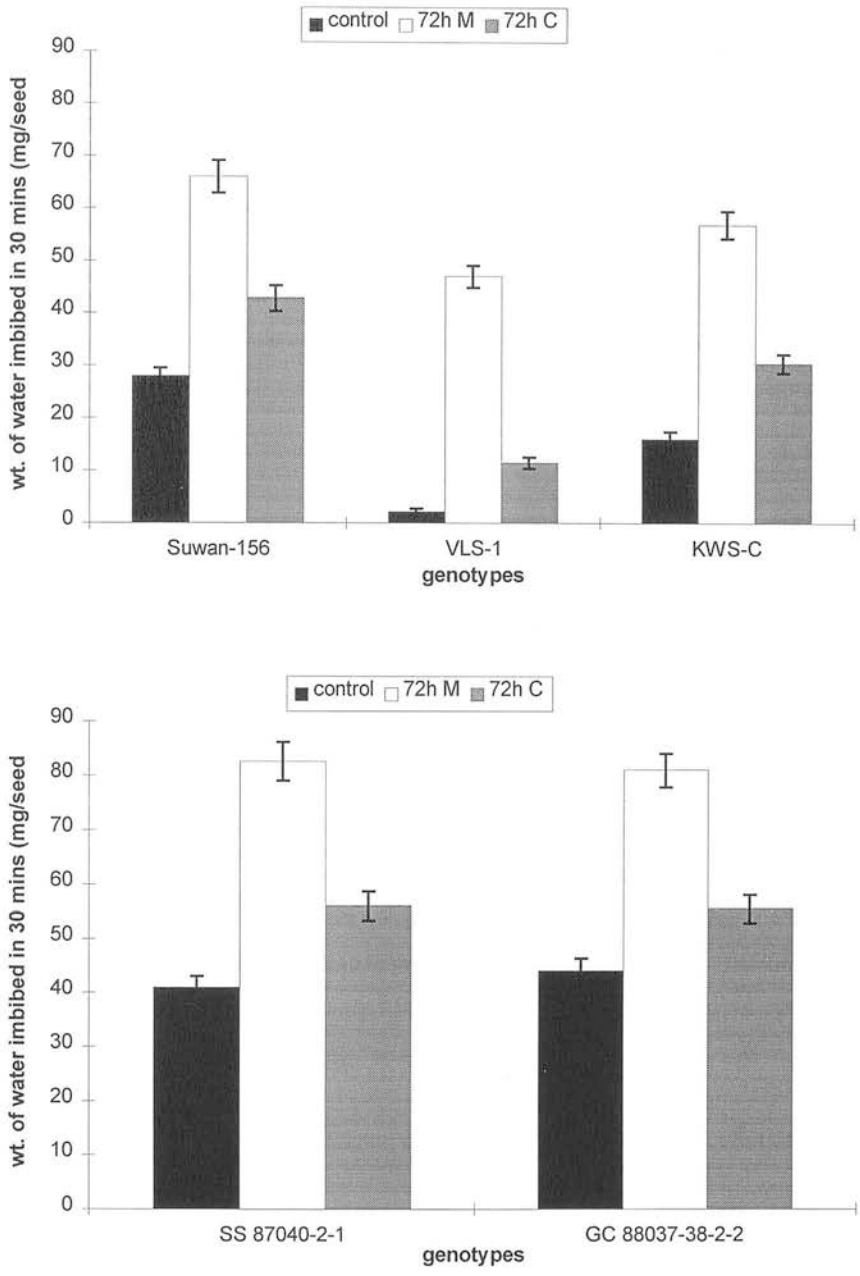


Figure 6.13. Weight of water imbibed in 30 min by seeds of six genotypes that had been pre-treated with different organic solvents, control (untreated), 72h M (72h in methanol) and 72C (72h in chloroform); error bars, standard error of means (n=10).

In seeds of cv. Suwan-156, the water uptake of methanol and chloroform pre-treated seeds which placed immediately for imbibition was 2.7 times and 65% ($P<0.001$) greater than that of the untreated control seeds respectively. Seeds dried after the methanol pre-treatments had a 44% greater ($P<0.001$) water uptake than the untreated control seeds whereas seeds dried after the chloroform pre-treatment had a similar water uptake with the untreated control seeds.

In seeds of line VLS-1, the water uptake of methanol and chloroform pre-treated seeds which placed immediately for imbibition was 16 and 7.6 times greater ($P<0.001$) than that of the untreated control seeds respectively. Seeds dried after the pre-treatments had a 2 times greater ($P<0.001$) water uptake than the untreated control seeds.

In seeds of line KWS-C, the water uptake of methanol and chloroform pre-treated seeds which placed immediately for imbibition was 3.2 and 2.2 times greater than that of the untreated control seeds respectively. However, seeds dried after the pre-treatments had a similar water uptake with untreated control seeds.

In seeds of line SS 78040-2-1, the water uptake of methanol and chloroform pre-treated seeds which placed immediately for imbibition was 90% and 39% greater ($P<0.001$) than that of the untreated control seeds respectively. However, seeds dried after the pre-treatments had a similar water uptake with untreated control seeds.

In seeds of line GC 88037-38-2-2, the water uptake of methanol and chloroform pre-treated seeds which placed immediately for imbibition was 80% and 35% greater ($P<0.001$) than that of the untreated control seeds respectively. Seeds dried after the chloroform pre-treatments had a 24% greater ($P<0.001$) water uptake than the untreated control seeds whereas seeds dried after the methanol pre-treatment had a similar water uptake with the untreated control seeds.

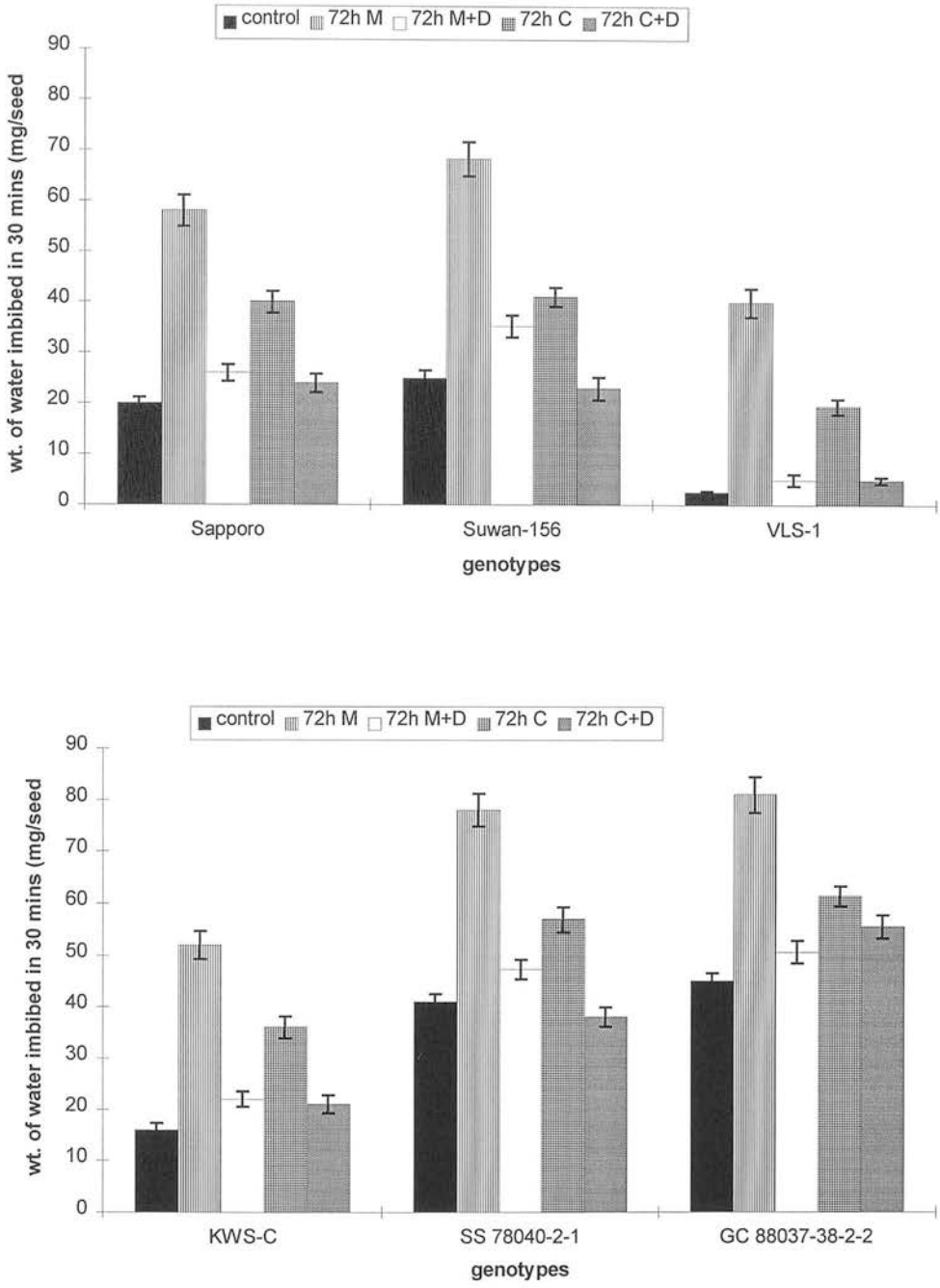


Figure 6.14. The effect of seed drying after the 72h methanol and chloroform pre-treatments on the water uptake during 30 min imbibition: control (untreated), 72h M (72h methanol), 72h M+D (72h methanol followed by 48h drying), 72h C (72h chloroform) and 72h C+D (72h chloroform followed by 48h drying); error bars, standard error of means (n=10).

6.2.4. Absorbance spectrum of supernatant

Figure 5.14 shows the absorbance spectrum in the range of 330-215nm of the supernatant obtained from the 24h and 72h methanol and chloroform extracts. In most of the range scanned, the absorbance of the methanol supernatant was lower than that of chloroform supernatant. In both extracts, the 72h supernatant had a higher values that the 24h supernatant in the whole range of spectrum. The absorbance of the methanol supernatant had constantly very low values up to 240nm with an increase in the absorbance thereafter. The chloroform mixture absorbance had several peaks between 220-275nm with no absorbance at values lower than 220nm.

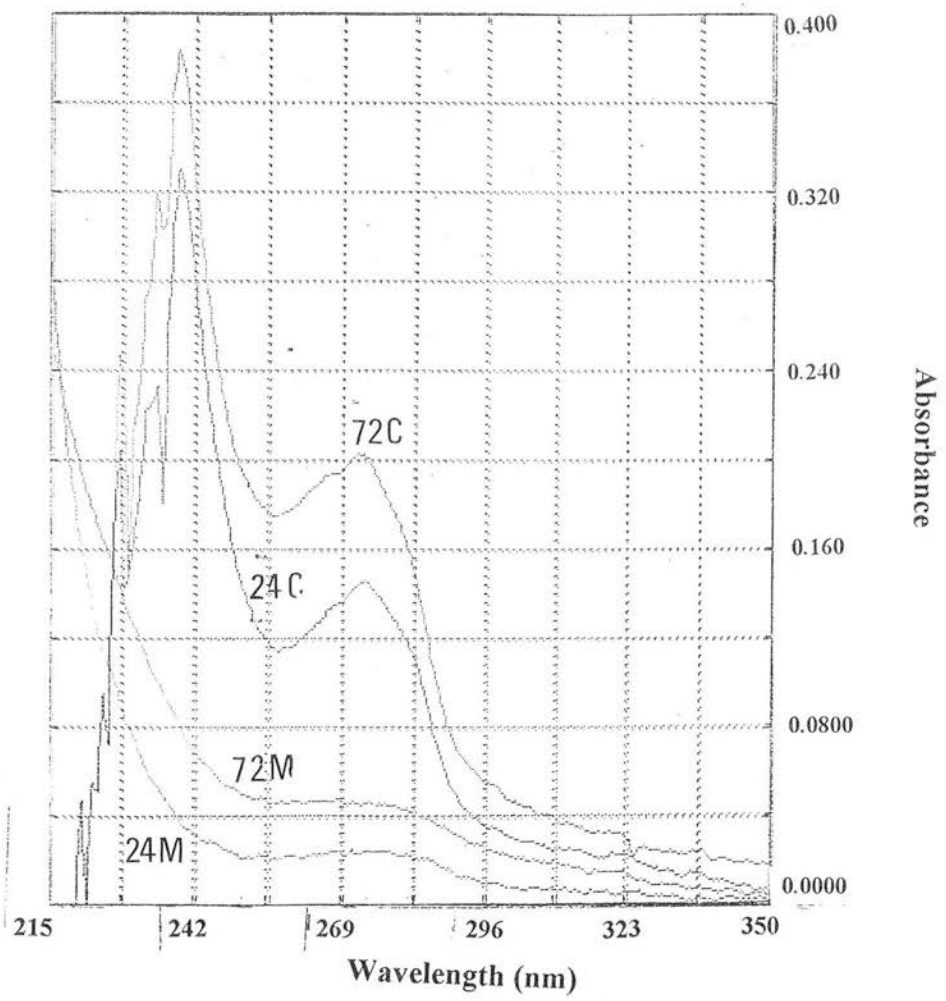


Figure 6.15. Absorbance spectrum of the methanol and chloroform supernatant from the 24h and 72h extracts; 24h M (24h methanol), 24h C (72h chloroform), 72h M (72h methanol), 72h C (72h chloroform).

6.4. Discussion

In the this study, deposits were observed on the surface of the seed coat in most genotypes examined. Similar observations were reported by Wolf *et al.*, (1981), in a survey of 33 soybean genotypes. In some genotypes, they observed that the surface of the seed coat was densely covered. In many cases, the deposits had a honeycomb-like appearance, visible to the naked eye.

As far as the nature of the deposit material is concerned, there is some contradiction in the literature. Newell and Hymowitz (1978), in a seed coat survey of *Glycine* Wild subgenus *Glycine* seeds (relatives of the cultivated soybean *Glycine max* (L.) Merr.) suggested that the deposits appeared to result from adherence of the endocarp to the seed coat. Also, Wolf *et al.*, (1981) having in several cases found similarities of size and shape between the seed coat deposits and the cell wall ridges of the endocarp, suggested a common origin for the two structures. Yaklich *et al.*, (1986), in a developmental investigation of the soybean seed coat reported that surface deposits were present in all four genotypes examined and they were derived from the endocarp of the pod wall. They, also, reported that deposits were present at the last stage of the seed development which described as the transversely broadly elliptic stage in seed development. Contrary to the previous reports, some authors have referred to deposits as being cutins (waxy material) which is a structural component of cuticle and therefore originates from the epidermal layer of the seed coat (Calero *et al.*, 1981; Ragus, 1987). In the previous reports, deposits were implicated in causing impermeability to water; however, the basis of these suggestions was the mere presence of deposits on the surface of the seed coat of hard seeds as revealed in SEM images.

In the present work, there were several pieces of evidence to include that the deposits were residues of the pod endocarp. Firstly, deposits were shown to be hydrophilic material with polysaccharide composition since calcofluor binds only with b-1,4- and mixed 1,3- and 1,4 glucans (Heslop-Harrison and Heslop-Harrison, 1982). When deposits were removed from the surface of the seed coat, the staining was significantly reduced. Secondly, there were a larger amounts of deposits in the abaxial than in dorsal region of the seed surface. Deposits were more likely to adhere to the abaxial region during seed development and maturation (Yaklich *et al.*, 1986). Thirdly, 24h soaking in methanol with continuous shaking was an effective method in removing the deposits whereas as the same pre-treatment with chloroform little

change induced in the deposits. If deposits were of wax nature, the chloroform should have been more effective in removing them than the methanol. However, Wolf *et al.*, (1981) reported that soaking soybean seeds in water, hexane or methanol:chloroform (2:1) for either 5 mins or 17h at room temperature had little effect on the deposit material present on the surface of the seed coat. Also, the methanol pre-treatment had a substantial change in the visual appearance of the seeds from dull to shiny. Reports have long indicated that soybean cultivars vary in seed appearance (classified as shiny, dull and bloomy) depending in the amount of material present in the surface of the seed coat (Williams, 1950; Bernard and Weiss, 1973).

In this study, both deposits and pits were present on the surface of the seed coat in most genotypes. There was no obvious relationship between the two features. For example, seeds of cv. Sapporo were highly pitted but had no surface deposits whereas seeds of line VLS-1 had plenty of deposits but few pits. A similar lack of relationship was observed in a survey of the surface structure of 33 soybean genotypes (Wolf *et al.*, 1981).

Assessment of pitting was done after the removal of deposits. The methanol pre-treatment for removal of deposits had no other obvious effect on the surface of the seed coat as could be seen in either low or high magnification. Individual pits appeared in three types, deep (round and elongated) and shallow. In most cases, pits were a combination of all types but it was not possible to identify a predominant type of structure of individual pits (round or elongated). There was a clear relationship between the water uptake and the pitting in two of the genotypes examined. As it was previously shown (Chapter 3, section 3.2.2), seeds of line VLS-1 had a low water uptake and wrinkling of the seed coat was observed in only the dorsal region of the seed until after 2h of imbibition. Wrinkling of the seed coat was the first visible sign of imbibition by seeds (Yaklich *et al.*, 1986). Seeds of line VLS-1, also, had few and shallow pits in the abaxial region. Seeds of line SS 87040-2-1 and GC 88037-38-2-2 had a high water uptake and seed coat wrinkling was observed in all regions of the seed within 30 mins of imbibition. Seeds of the of lines SS 87040-2-1 and GC 88037-38-2-2 had a high proportion of very deep and wide open pits.

Several reports have documented the existence of pits in the surface of the soybean seed coat (Wolf and Baker, 1972; Newell and Hymowitz, 1978; Calero *et al.*, 1981; Wolf *et al.*, 1981, Yaklich *et al.*, 1986). Although, pits definitely increase the seed coat surface area, there is no direct evidence so far, that they play a role in water imbibition.

In the present work, there was a clear correlation between the sites of initial water penetration to the distribution of pits in the surface of the seed coat. Comparisons between the fluorescence image of the calcofluor stained seed coat and the light microscope image of the same area, revealed that the distribution and size of the spots were closely corresponded to pits (compare Fig. 6.7 to Fig. 6.6). Heslop-Harrison and Heslop-Harrison, (1982) have successfully used the calcofluor staining for mapping the permeability of stigma surfaces. They suggested that 30-60 seconds was appropriate to provide an unambiguous map of the water penetration in stigmas.

In this study, in addition to seed surface calcofluor staining, anticlinal sections of the seed coat from seeds that had been soaked in calcofluor were studied. As imbibition progressed, the staining of the palisade layer was changed from light to intense. It was not possible to identify differences, in sections from all three regions, in the extent of water penetration through the palisade layer since the depth and density of calcofluor staining was similar. However, regardless of the region examined, it was clear that the water penetration did not occur uniformly through the palisade layer since groups of palisade cells showed a stronger staining than the adjacent cells. In the hilar region, water penetration was observed through the tracheid bar but not through the outer and inner palisade layer.

In the present work, different methanol and chloroform pre-treatments were applied to whole seeds and then the water uptake of the pre-treated seeds was measured.

Brief (2min) immersion of seeds in chloroform at room temperature did not affect the water uptake in comparison to the untreated controls. Brief chloroform pre-treatment was used as a method to remove epicuticular waxes. In studies of epicuticular waxes in leaves and stems, brief chloroform immersion of the tissue has been widely used as an effective method of removal these waxes (Silva Fernandes *et al.*, 1964; Baker *et al.*, 1982). In this study, the efficacy of the previous pre-treatment in removing epicuticular waxes was not established. However, epicuticular waxes on the surfaces of leaves, flowers and stems are thought to influence only the wetting of the surface (Holloway, 1970; 1984).

Two hours successive immersions of whole seeds, at room temperature, in methanol, methanol: chloroform (1:1) and chloroform was used to remove both epicuticular and intracuticular waxes. The same procedure was used in waxes extraction from separated tomato fruit cuticles (Baker, 1982). Seeds that had been pre-treated with the previous combination showed a significant increase in the water

uptake in comparison to the untreated control seeds. However, the increase in the water uptake could not be attributed to intracuticular waxes for several reasons.
Firstly, the efficacy of the pre-treatment in removing the intracuticular waxes was not established. In other plant cuticle studies, the experimental procedures used for the extraction of soluble cuticular waxes vary tremendously. In a comparative study of the different extraction methods, Riederer and Schneider (1989) reported that the solvents used for extraction cover a wide range of polarities from methanol to *n*-hexane, and they were applied at varying temperatures (ambient to boiling point) and for periods of time ranging from 2sec to 2hours. They also reported that different extraction methods resulted not only to different yields of waxes but also to different proportions of the various classes of waxes.
Secondly, it was not known if the pre-treatment affected only the cuticle or if it also affected the subcuticular or/and the palisade layer. It is, therefore, likely that changes in the subcuticular and palisade layer to be wrongly attributed to the extraction of the intracuticular waxes. Most of the studies in the relationship between the water permeability and intracuticular waxes in plant material have been performed on separated cuticular membranes rather whole tissues. In seeds, it is not known the relative effect of changes in cuticular water permeability in comparison to the changes in subcuticular and palisade layer. Treatment of separated leaf cuticular membranes with chloroform extracted intracuticular waxes and increased the water permeability by 2 to 3 orders of magnitude (Schönherr, 1976; Schönherr and Schmidt, 1979; Hass and Schönherr, 1979). However, no simple relation has been found between the amount and/or composition of soluble waxes and the permeability of cuticles (Schönherr and Schmidt, 1979).
Thirdly, it was the methanol and not the chloroform component of the previous pre-treatment that had the greatest impact in the increase of the water uptake. However, several reports have shown that chloroform was a better extraction medium of the intraciticuticular waxes than methanol (Tulloch, 1976; Baker, 1982; Holloway, 1984). Riederer and Schneider (1989) reported in isolated *Citrus* leaf cuticles that chloroform extraction yielded high amounts of intracuticuticular waxes and even the more polar intracuticuticular waxes were extracted.

The greatest increase, however, in water uptake was achieved when prolonged methanol and/or chloroform pre-treatments applied to seeds. Methanol pre-treatments, in particular, were highly effective in increasing the water uptake during imbibition. The seed coat of methanol pre-treated seeds observed to be highly permeable to water and within the first 30 min of imbibition seeds had already

absorbed a significant amount of water. There was no region of the seed that observed to be a point of rapid water penetration but the whole seed coat was highly permeable to water. The effectiveness of the methanol pre-treatment was particularly evident in seeds with low water uptake rather than that of seeds with high water uptake.

One possible explanation of these observations is that the organic solvent pre-treatments were extracting phenolic material from the subcuticular and palisade layer. Lower amount of phenolic material might give rise to higher water uptake. A close association of low rates of water uptake with seed coat pigmentation has been reported in isogenic lines of peas (Powell, 1989). In this case, seeds having the dominant A gene for seed coat colour were usually pigmented, and imbibed slowly in comparison with the unpigmented seeds having the recessive gene. Also, other reports have shown a close association between the development of pigmentation during seed development and low water uptake in chickpeas and cowpeas (Legesse and Powell, 1992). They reported that in the absence of pigmentation at early stages of maturation, all seeds imbibed rapidly whereas as pigments started to develop the rate of water uptake was significantly reduced. Although, the development of pigmentation may be associated with low rates of water uptake, no direct evidence exists that phenolics *per se* play an important role in reducing the water permeability of the seed coat. During seed development and maturation other changes in the seed coat may have taken place thus making the low water permeability an independent process. One possibility is that during the last stages of seed development, and due to a great loss of moisture, a marked shrinkage of the palisade cells give rise to a compaction of cells. Yaklich *et al.*, 1986 reported a compaction of the seed coat in the last stage of the soybean seed development. Similar observations have been reported for pigmented *Phaseolus vulgaris* seeds (Corner, 1951; Yeung and Cavey, 1990).

The fact that methanol was far more effective in increasing the water uptake than chloroform supported the possibility that extraction of phenolics may have resulted in high water uptake. Phenolics are well known to be highly soluble to polar solvents (Witham, 1983) and traditionally, methanol and ethanol are the most widely employed solvents in identification by spectrophotometric techniques (Waterman and Mole, 1994). The absorption spectrum of the supernatant, therefore, obtained from methanol and chloroform extracts was measured. From the absorption spectrum results, it was clear that prolonged methanol pre-treatments extracted little material

with UV absorbance in the range of 220-320nm. Phenolics are well documented to absorb in the previous range of with varied maxima depending on type of phenolic compounds (e.g. simple phenolics 265-280nm, simple aldehydes and acids 250-325nm) (Waterman and Mole, 1994). In contrast, the absorption spectrum of the supernatant from the prolonged chloroform pre-treatments extracted substances with UV absorbance in the range of 220-320nm. However, the nature of the exctracted substance(s) is not known and needs further investigation.

In the present work, drying of seeds after the application of the pre-treatments restored the water permeability to the levels observed in the untreated control seeds. Egley and Paul (1993), in a study of the effect of 28 different organic solvents in overcoming the water impermeability of prickly sida (*Sida spinosa*) seeds, reported that seed drying after the methanol pre-treatment restored the seed coat permeability to water in similar levels to the untreated control seeds. The following sequence of events could explain the action of particularly the methanol solvent pre-treatments: Organic solvents could penetrate the cuticle reaching the subcuticular and palisade layer. As the duration of the treatment increased, the presence of solvent has reached inner parts of the palisade layer. Cellulosic material in the subcuticular and palisade layer have hydrated in organic solvent, these carbohydrates swell and created forces among the palisade cells that caused breaking or/and separation of the cells. The breakage/ separation of the palisade cells opened wide avenues for the massive influx of water into seed that observed within minutes of imbibition. Drying of the seeds after the pre-treatment restored water permeability to the original levels because the disturbance was temporary induced. In the presence of methanol, water would reach all the seed coat cells invaded by methanol since water is soluble to methanol. When the methanol was dried off, there would be no open pathway for the water movement through the layers of the seed coat. The last possibility could explain the difference in the effectiveness between methanol and chloroform in changing the seed coat permeability to water since the seed coat was more permeable to methanol than to chloroform, and water is soluble in methanol but not in chloroform.

Egley and Paul (1993) reported results of the effects of 28 different organic solvents with a range of polarities, swelling factor and solubility to water in overcoming the water impermeability of prickly sida seeds. They reported that all the three previous physical properties of the solvents could interact each other, and finally change the seed coat permeability to water with the most active solvents those being non-polar, with high values of relative swelling factor and solubility to water.

They also reported that low values in one factor (e.g. polarity) could be compensated by high values in other factor (e.g. swelling factor) so that the final end result in the water permeability could be the same.

In the present work, it was not possible to identify the role of the physical properties of the solvents due to a small number of solvents used. However, the common imbibition behaviour of seeds of all genotypes tested indicated that the previous model for regulation of the water uptake is likely to be universal in soybeans.

CHAPTER 7

Comparative studies in the structure and histochemistry of the seed coat in hard and soft seeds in soybeans (*Glycine max* L. Merril)

7.1. Introduction

Deposits and pits occur in the surface of the hard seed coat in most soybean genotypes. However, the role of pits and deposits has been unclear since in some genotypes hard seeds were free of pits and deposits whereas other genotypes seeds were heavily pitted and/or had a lot of deposit material. The lack of clear description of the role of pits and deposits is mainly due to the fact that most of the published work about the hard soybean seed coat has been based in SEM studies of the surface or anticlinal sections of the coats (Calero *et al.*, 1981; Wolf *et al.*, 1981; Ragus, 1987; Yaklich *et al.*, 1987). There is no published information reporting a direct evidence (using water soluble stains) that pits or/and deposits could play a role in the water impermeability in soybeans. Additionally, there is no published information about the location of the water impermeability barrier in the hard soybean seed coat.

There is no published information about the nature of water impermeability barrier by comparative anatomical and histochemical studies between hard and soft seeds. Duanganparta (1977) reported no anatomical and histochemical difference between hard and soft soybean seed coats; hardseedness was attributed in the presence of a continuous layer of suberin in the inner palisade layer in the hilar region of the seed coat. Harris (1987) examined soybean seed coats of a hardseeded and softseeded genotype; no clear anatomical or histochemical difference between them was observed. However, a higher amount of phenolic material in seeds of the hardseeded genotype than in seeds of a softseeded genotype was observed.

In soybeans, the effectiveness of the organic solvents to overcome the hardseedness has been assumed to be related with changes in the cuticle due to removal of waxes (Ragus, 1987). Recently, Egley and Paul (1993), in hard seeds of *Sida spinosa*, reported that the effectiveness of the organic solvents to overcome the water impermeability barrier was related to changes in palisade layer of the seed coat as a combination of the hydration, swelling and polarity properties of the solvents. There is no published information that associates changes in the seed coat due to organic solvents with changes to water impermeability barrier.

The objectives of this study were, therefore: (1) to localise of the water impermeability barrier within the layers of the hard seed coat, (2) to identify of the nature of the barrier by comparative anatomical and histochemical studies between hard and soft seeds, and (3) to investigate the nature of the barrier when impermerability is overcome using organic solvents.

7.2. Materials and methods

7.2.1. Physical characteristics of hard and soft seeds

The percentage of hard seeds was calculated as described in section 2.2. After the separation, both hard and soft seeds were left to dry at ambient conditions for 4 days before placing them for air-drying set at 30°C for 2 days, and then the moisture content was measured (8% ±1.5% m.c.) as described in section 2.2. The seed characteristics of both hard and soft seeds were calculated as described in section 2.2.

7.2.2. Scanning electron microscopy of the seed coat of hard and soft seeds

The surface of hard seeds of the three genotypes was studied. Seeds were processed for the investigation of the surface of the seed coat by SEM as described in Section 2.11. For the assessment of the effects of wetting and drying on the surface structure of the seed coat, seeds of cv. Sapporo were soaked in water for 1hour. Subsequently, seeds blotted dry and left to dry at ambient conditions for 4 days. Sample preparation for the SEM studies was done as described in section 2.11. Unsoaked seeds were used as a control. For the estimation of the degree of pits, micrographs were taken at 300x magnification at the middle abaxial region of the seed coat. Anticlinal sections of hard and soft seeds of the three genotypes were made. Strips of the seed coat by a razor-blade were made. The experimental procedure was the same as described in section 2.11.

7.2.3. Use of calcofluor as a water-soluble fluorescence stain

Hard seeds of the three genotypes were used. For the calcofluor staining of the surface of the seed coat, hard seeds were soaked in calcofluor for 15 sec, 1 min, 15 min and 30 minutes. The experimental procedure was the same as described in section 2.10.1. For the identification of the calcofluor presence within the seed coat, hard seeds were soaked for 24h in stain solution. The experimental procedure was the same as described in section 2.10.2.

7.2.4. Light microscopy studies of the seed coat of hard and soft seeds

Hard and soft seeds of the three genotypes were used. Fixation, dehydration, vacuum infiltration, embedding and sectioning were done as described in section 2.9. The following histochemical reactions were carried out:

a) Toluidine Blue O (TBO): Sections were placed in 0.05% (w/v) aqueous Toluidine blue at pH 4.4 for 4 min as metachromatic stain, washed and mounted in distilled water (O'Brien and McCully, 1981).

b) Phloroglucinol: Sections were placed in a saturated solution of the stain in 20% (w/v) HCl. A cherry pink coloration taken to indicate the presence of lignin (O'Brien and McCully, 1981).

c) Vanillin test: Sections were placed in oversaturated solution of vanillin in HCl for staining of phenols (Ling-Lee *et al.*, 1977).

d) Ruthenium red: Sections were placed in an aqueous solution of 0.02% of the stain, washed and mounted in distilled water. Pink staining indicated pectins (O'Brien and McCully, 1981).

7.2.5. Fluorescence microscopy studies of the seed coat of hard and soft seeds

Hard and soft seeds of the three genotypes were used. Fixation, dehydration, vacuum infiltration, embedding and sectioning were done as described in section 2.9. The following histochemical reactions were carried out:

a) Autofluorescence: sections were mounted in glycerol and examined for autofluorescence by excitation with ultra-violet light (filter set I). A yellow colour indicated the presence of polyphenolic substances.

b) Calcofluor: Sections were stained in a 0.1% (w/v) aqueous solution of calcofluor white M2R for 30 sec., mounted in glycerol and examined with filter set II.

c) Fluorol Yellow: A 0.01%(w/v) solution of fluorol yellow 088 (Solvent Green 4) was prepared in polyethylene glycol by heating at 90 C for 1 hour. An equal volume of 90% (w/v) glycerol was added to the polyethylene glycol plus stain. Sections were stained in this mixture for 1h at room temperature, washed in water and mounted on slides in 75% (w/v) glycerol, and examined using filter set I.

d) Aniline blue: Sections were placed in a 0.005 (w/v) solution of the stain in 50% (v/v) alcohol for 24 hr. and mounted in 70% (w/v) glycerol. The presence of callose was examined (Jensen, 1962).

7.2.6. Effect of methanol and chloroform pre-treatments in the water uptake of hard seeds

Hard seeds of the three genotypes were used. Organic solvents pre-treatments were applied as described in Section 2.12. Four pre-treatments were used: 1) 2h methanol, 2) 24h methanol, 3) 2h chloroform and 4) 24h chloroform. After the pre-treatments, seeds were blotted dry. Subsequently, 10 seeds were placed for measurement of the water uptake after 30 min of imbibition as described in Section 2.5.2. Additionally, methanol and chloroform pre-treatments for 24h, 72h and 96h were used. After the pre-treatments, seeds were blotted dry. Subsequently, 10 seeds were placed for measurement of the water uptake after 30 min of imbibition as described in Section 2.5.2.

7.2.7. Absorbance spectrum of supernatant

Hard and soft seeds of cv. Pioneer-9581 were used. The absorbance spectrum of the supernatant obtained from the 24h and 72h methanol and chloroform supernatants was measured as described in section 2.13.

7.2.8. Effect of seed drying after prolonged methanol and chloroform pre-treatments on the water uptake

Hard seeds of the three genotypes were used. Seeds were soaked in methanol and chloroform for 72h as described in section 2.12. After the pre-treatments, seeds were blotted dry, left to dry for 4 days at room conditions. The final moisture of the seeds was 8% m.c. (± 1.5% m.c.) on a fresh weight basis. Subsequently, 10 seeds were placed for imbibition, and the water uptake was measured after 30 minutes as described in section 2.5.2.

7.3. Results

7.3.1. Physical characteristics of hard and soft seeds

The physical characteristics of both hard and soft seeds of the seeds of cv. Essor, Pioneer-9581 and line JS-7980 were measured.

In the seed lots examined, the percentage by weight of hard seeds varied ($P<0.001$) among genotypes; cv. Essor and line JS-7980 had a low percentage whereas cv. Pioneer-9581 had a high percentage of hard seeds (Table 7.1).

Seed dry weight was also variable ($P<0.001$) within genotypes. Seeds of line JS-7980 had the smallest size whereas seeds of cv. Essor had the largest size. In all cases, hard seeds were lighter than soft seeds but only in the seed lots of line JS-7980 and cv. Pioneer-9581 were the differences significant ($P<0.001$) (Table 7.1).

The testa dry weight was also variable ($P<0.001$) within genotypes. Seeds of line JS-7980 had the smallest testa dry weight whereas seeds of cv. Essor had the largest testa weight. Soft seeds had a higher testa dry weight than hard seeds in line JS-7980 and cv. Essor ($P<0.05$) whereas the difference was not statistically significant (Table 7.1). However, the testa dry weight was strongly positively correlated ($y=0.05x + 4$, $R^2=0.93$) to the initial seed weight therefore the ratio of testa to seed was calculated. In all cases, hard seeds had a higher ratio than soft seeds but the difference was significant ($P<0.05$) only in seeds of line JS-7980 (Table 7.1).

Table 7.1. Percentage hard seeds, seed and testa dry weight and ratio of seed coat dry weight to seed dry weight for the three genotypes.

Genotypes	Type	Percentage hard seeds (%)	Seed dry weight (mg)	Testa dry weight (mg)	Ratio testa to seed
Essor	hard	9	181	14.6	0.081
	soft		198	13.7	0.069
JS-7980	hard	17	77	7.9	0.102
	soft		109	9.5	0.087
Pioneer-9581	hard	67	150	11.1	0.074
	soft		179	12.8	0.071
s.e.m. (n=10)		*3.4*	*4.98*	*0.49*	*0.003*

7.3.2. Scanning electron microscopy of the seed coats

Scanning electron microscopy of the surface and of sections of the seed coat was carried out to find out whether hard and soft seeds in the three genotypes differed in morphology and chemical composition.

The surface of the seed coat was free of deposits, in both hard and soft seeds, in all three genotypes.

Before any attempt to investigate differences in the pitting between hard and soft seeds, evidence was needed that the process used to separate hard and soft seeds did not induce any changes in the surface of the seeds. Seeds of cv. Sapporo (100% soft) were used in this test. The seeds were covered in water for 30 mins at room temperature; wrinkling of the seed coat was observed particularly evident in the dorsal region of the seed. Seeds were dried and processed for the SEM studies as previously described. In many seeds, ruptures of the seed coat were observed which were similar to those reported in section 3.2.3. The surface of the seed coat of seeds that appeared to be intact was examined under SEM to investigate possible changes at high magnifications. SEM images revealed two differences in comparison to the untreated control seeds: Firstly, cracking of the seed coat was observed (Fig. 7.1b), and secondly the opening in the individual pits were about double the size of those in the pits of the untreated control seeds (Fig. 7.1d).

For this reason, valid comparisons between hard and soft seeds in the pitting were not valid, and therefore observations of hard seeds of the three genotypes were made. Observations of hard seeds, at the abaxial region, revealed that there was a clear difference between the genotypes in the number of pits present per unit area and structure of the individual pits. Hard seeds of line JS-7980 had few pits (Fig. 7.2a) whereas those of cv. Essor and Pioneer-9581 had numerous pits (Fig. 7.2b and 7.2c). The structure of the individual pits in hard seeds of line JS-7980 and cv. Essor was shallow (Fig. 7.2a) whereas pits in cv. Pioneer-9581 were deep (Fig. 7.2c). In many cases, at the dorsal region, hard seeds of cv. Essor appeared to have long and elongated (slit-like shaped) pits (Fig. 7.2b).

Anticlinal sections of the seed coat, in both hard and soft seeds in all genotypes, indicated the presence of passageways through the palisade layer. The passageways were wide or narrow, penetrating the whole or part of the palisade layer (Fig. 7.3).

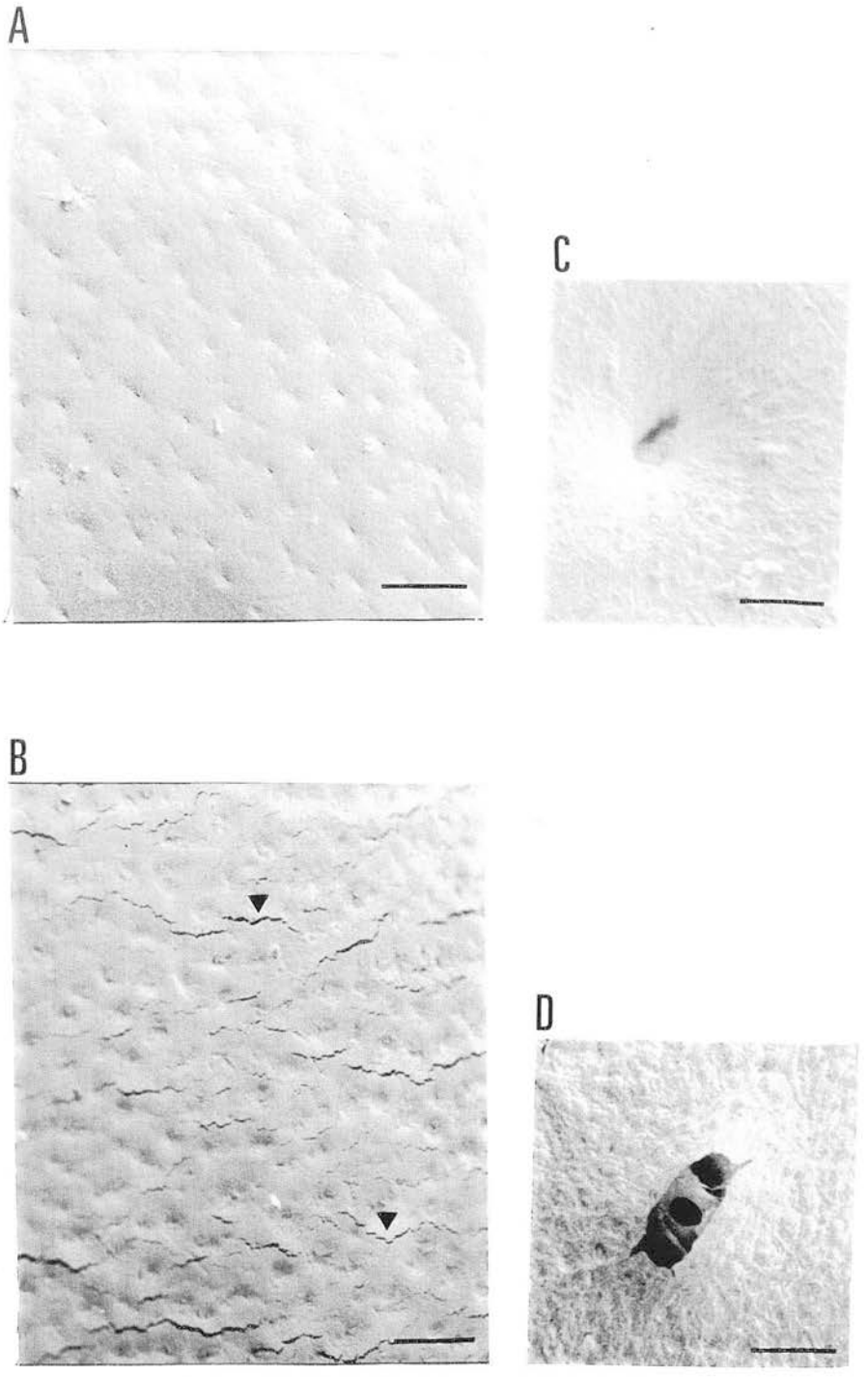


Figure 7.1. SEM micrographs of the dorsal region of the seed coat in cv. Sapporo. A) control, no pre-treatment, scale 100μm; B) after wetting and drying, scale 100μm; C) pit from control untreated seeds, scale 10μm and D) pit from seeds after wetting and drying; P: pits, (arrows indicate cracking), scale 10μm.

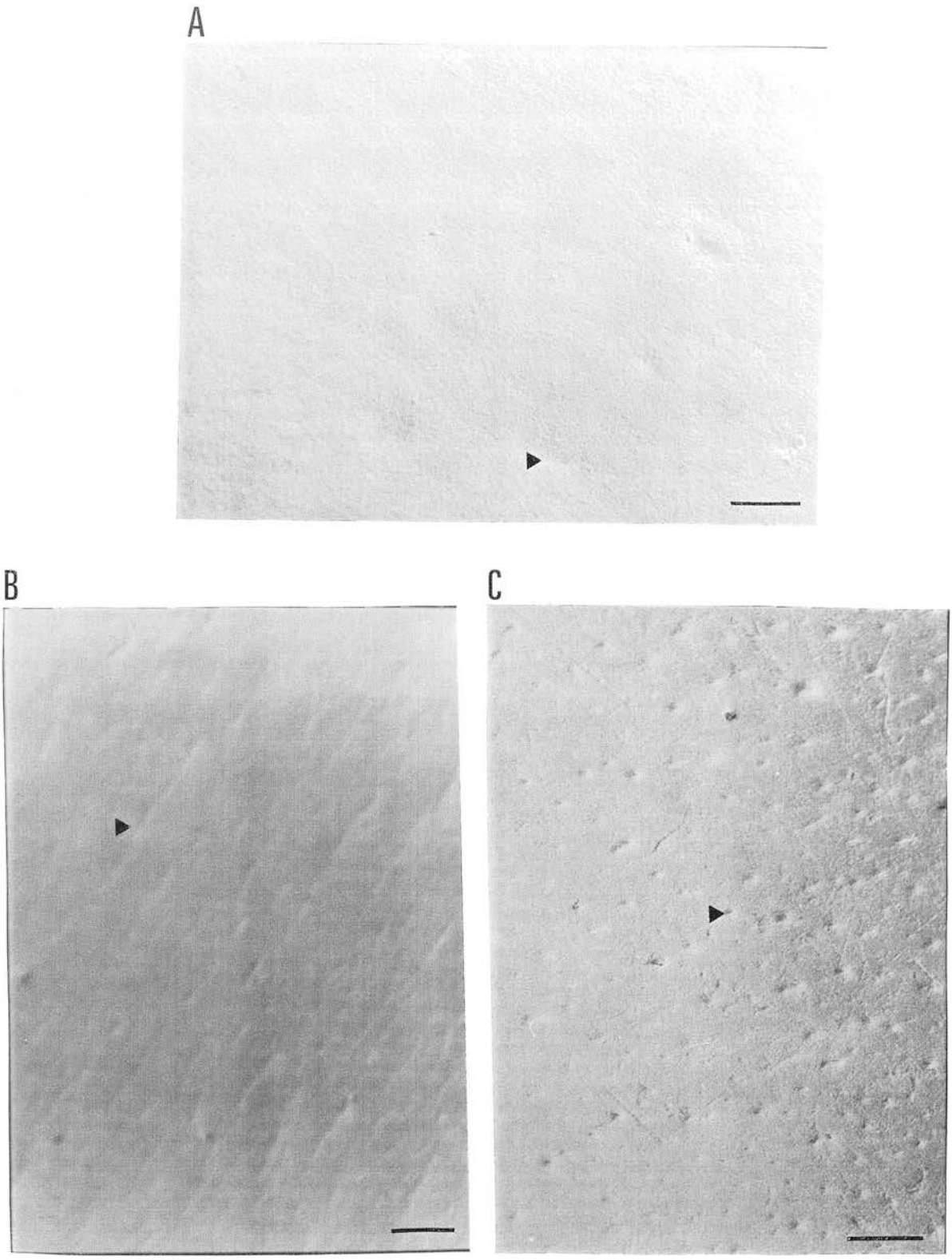


Figure 7.2. SEM micrographs of the surface of the seed coats, abaxial region, of different genotypes. A) seed of line JS-7980; B) seed of cv. Essor and C) seed of cv. Pioneer-9581, (arrows indicate pits) scale 100μm.

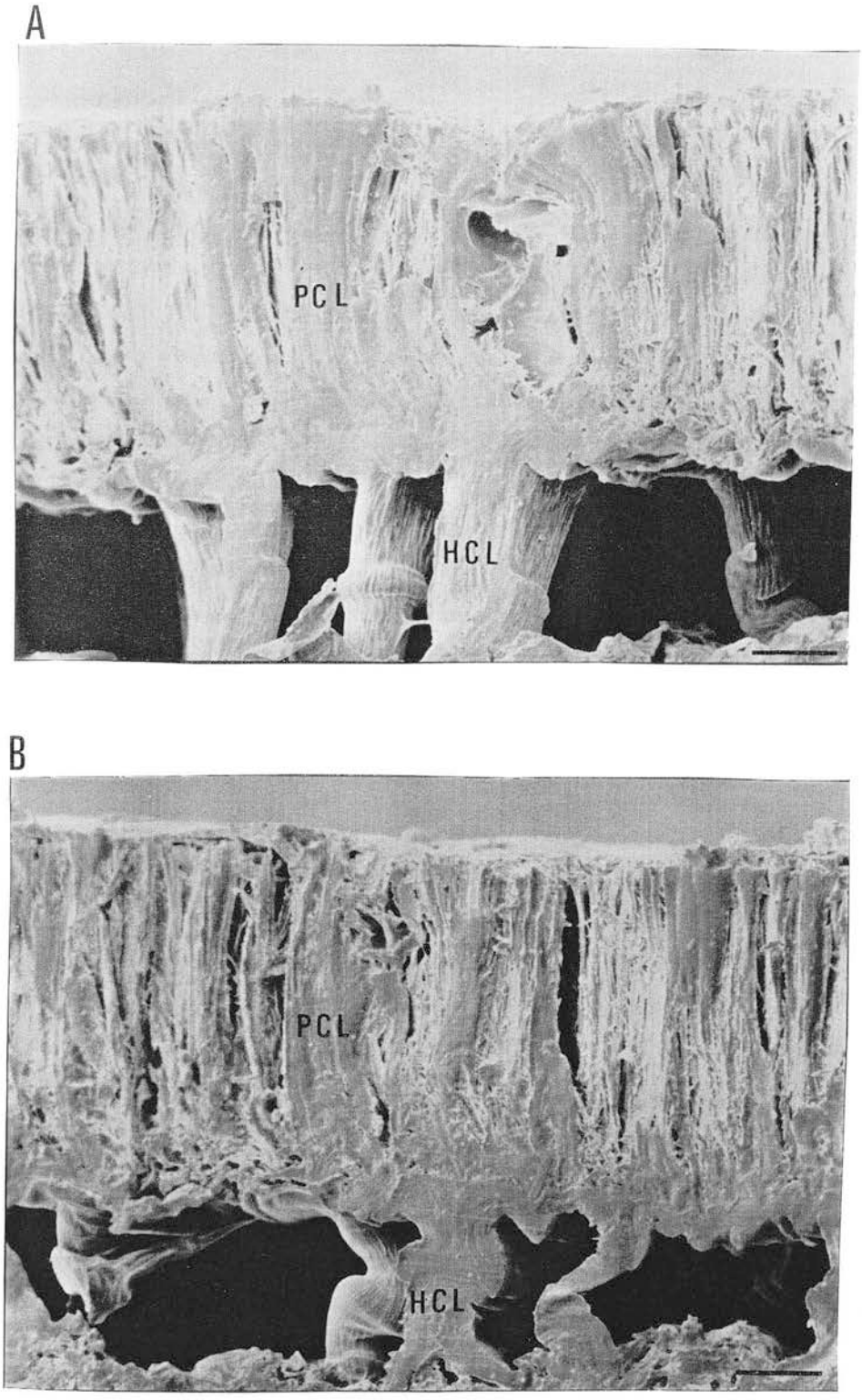


Figure 7.3. SEM micrographs of anticlinal sections of the seed coat A) hard and B) soft seeds of cv. Essor; PCL: palisade cell layer, HCL: hourglass cell layer, scale bar, 10μm.

7.3.3 Use of calcofluor as a water-soluble fluorescent stain

Soaking whole hard seeds in calcofluor was used as a method to demonstrate surface water penetration and localization of the calcofluor staining within the layers of the seed coat. In hard seeds of all genotypes soaked in calcofluor for 1min, there was a lack of calcofluor staining of the surface of the seed coat (Fig. 7.4). There was little difference on staining of the surface of the seed coat after soaking whole hard seeds for 15 and 30 min in calcofluor.

The presence of the calcofluor staining within of the seed coat was observed in anticlinal sections of the seed coat after soaking whole hard seeds in calcofluor for 24 hours. In the hilar region, after 24h soaking whole hard seeds in calcofluor, staining was evident only in the outer parts of the tracheid bar whereas the outer and inner palisade layer were totally unstained (Fig. 7.5). In hard seeds of all genotypes, the staining was clearly evident in the subcuticular layer of the seed coat (Fig. 7.6a). In higher magnifications, the staining was clearly confined in the subcuticular area bordering externally with the palisade cell layer whereas no staining was observed in the palisade layer itself (Fig. 7.6b). The same observation was made in all regions of the hard seed coats examined.

7.3.4. Light microscopy of the seed coats

Segments of the seed coat, from all regions of the seed, were fixed, dehydrated, infiltrated, embedded in resin and sectioned as previously described. Comparative anatomical and histochemical studies of anticlinal sections of the seed coat of hard and soft seeds of the three genotypes were made.

Figure 7.7a shows the general structure of the soybean seed coat. The cuticle was very thin and it was not possible to observe it properly with the light microscope. In most cases, it was difficult to identify it from the subcuticular layer. The subcuticular layer was also thin but clearly visible at higher magnifications of light microscope. The palisade layer was composed of elongated cells which had their long axes perpendicular to the surface. Each cell had thick cell walls and the lumen in the middle was comparatively small (Fig. 7.7a and Fig. 7.7b). The hourglass cells had a considerably thickened secondary cell wall with large spaces between the cells (Fig. 7.7a and Fig. 7.7c). The parenchyma layer consisted of six to eight layer of cells, lacking in cell content and flattened.

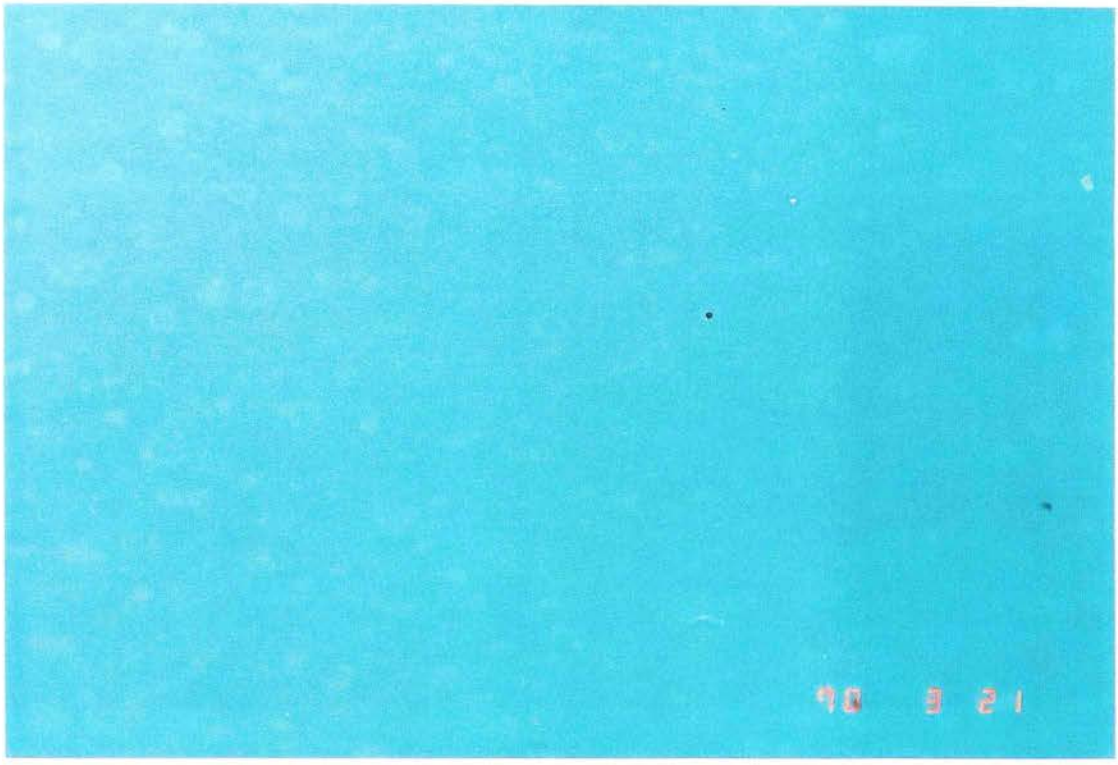


Figure 7.4. Fluorescence micrograph of the surface staining of the abaxial region of the seed coat after soaking whole seeds of cv. Pioneer-9581 in 0.1% calcofluor for 1min; (no staining), 600x magnification.

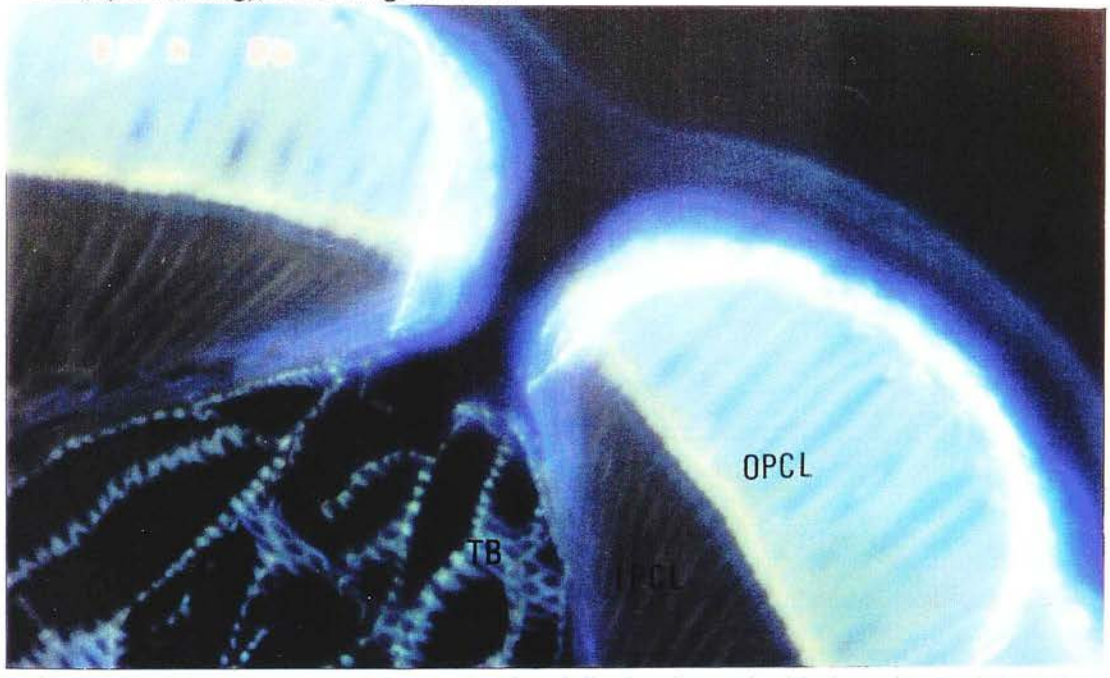


Figure 7.5. Fluorescence micrograph of anticlinal resin-embedded sections of the hilar region of the seed coat after soaking whole seeds of cv. Pioneer-9581 in 0.1% calcofluor for 24h, (arrows indicate staining); OPCL: outer palisade cell layer, IPCL: inner palisade cell layer, TB: tracheid bar, 2,000x magnification.

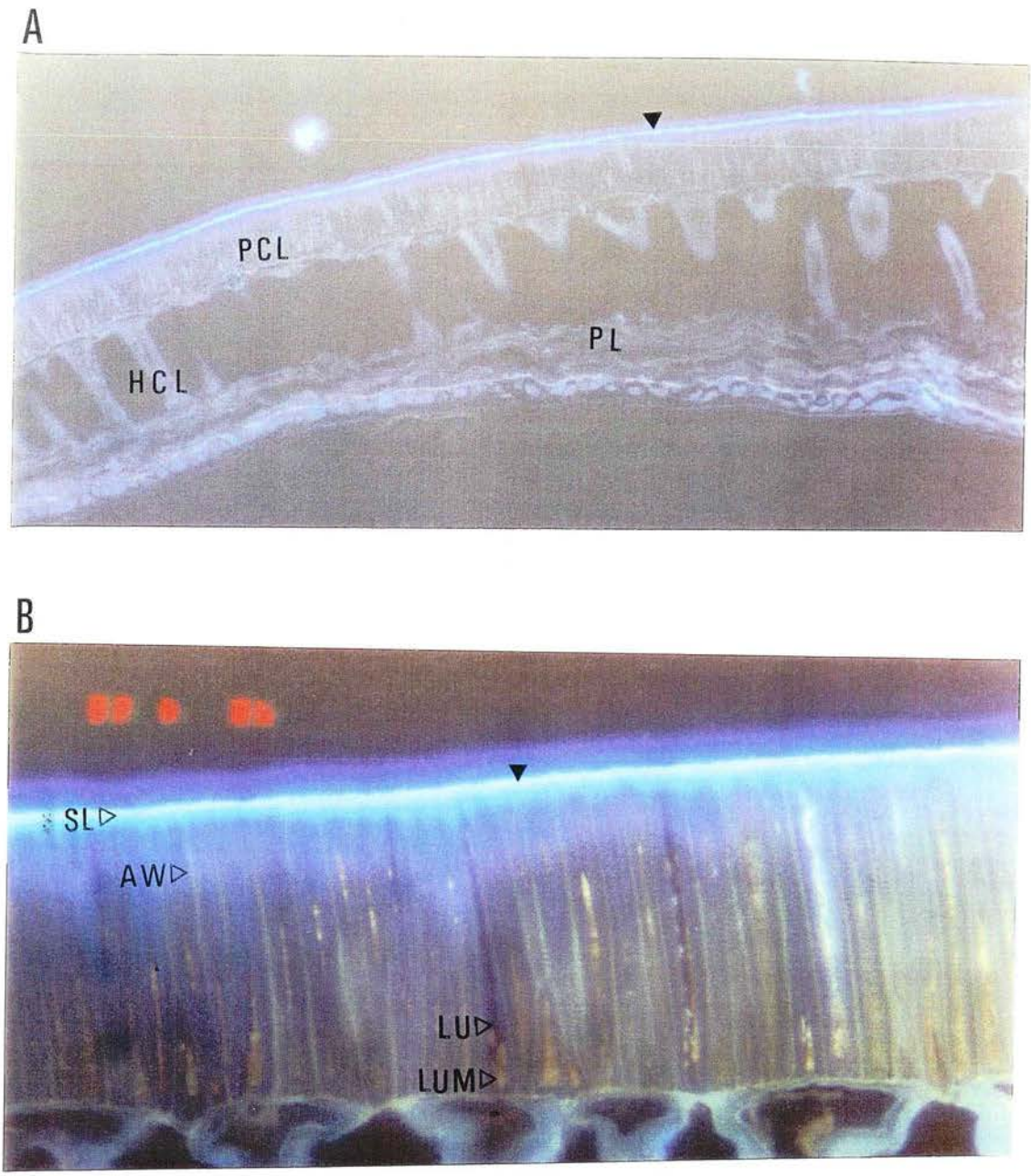


Figure 7.6. Fluorescence micrographs of anticlinal resin-embedded sections of the ventral region of the seed coat after soaking hard seeds of cv. Pioneer-9581 in 0.1% calcofluor for 24h, (arrows indicate staining). A) 500 x magnification, B) 2,000 x magnification. SL: subcuticular cell layer, PCL: palisade cell layer, HCL: hourglass cell layer, PL: parenchyma layer, AW: anticlinal walls, LU: lumen, LUM: lumina material.

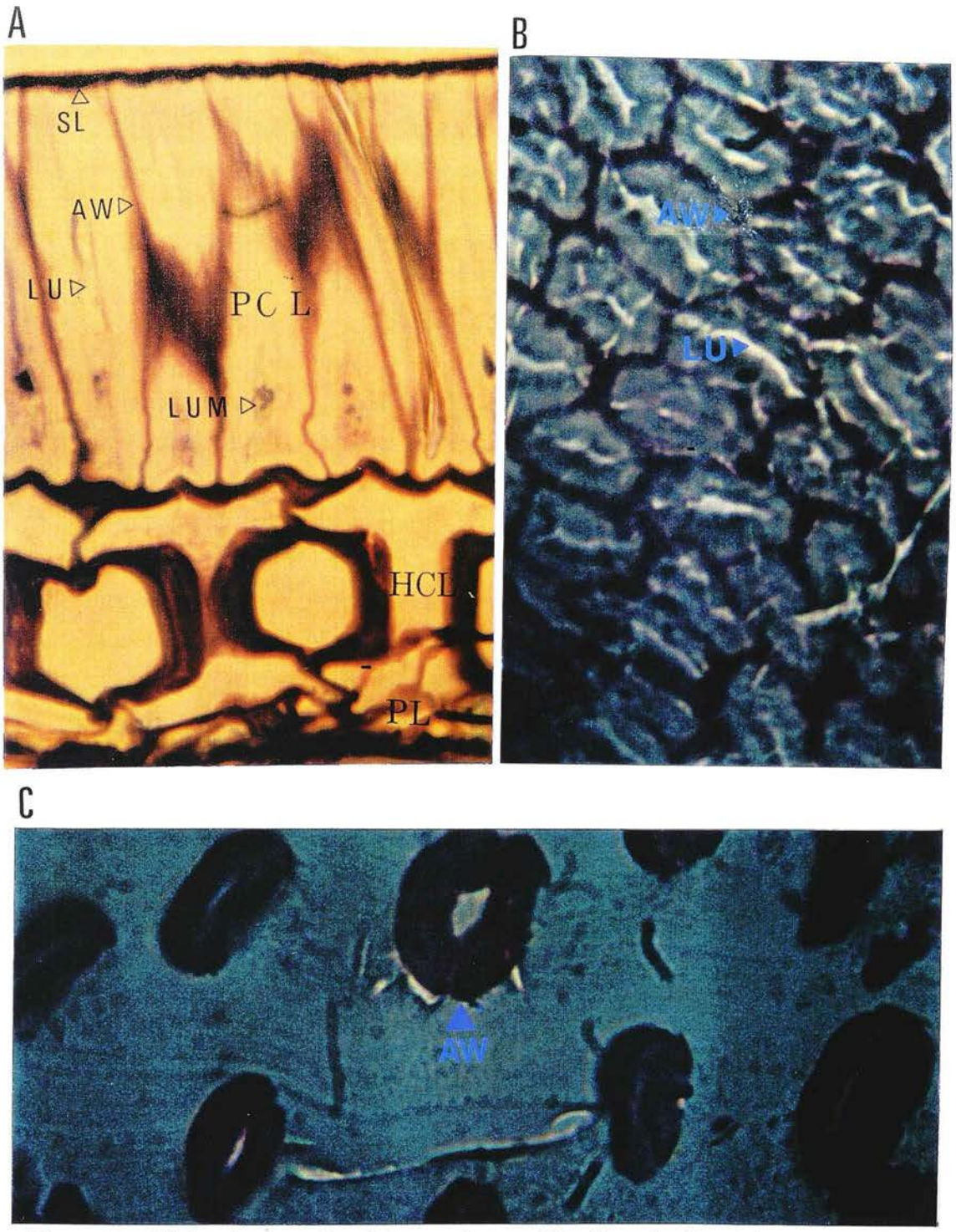


Figure 7.7. Light micrographs of the seed coat of hard seeds of cv. Pioneer-9581. A) anticlinal section of the whole seed coat; B) periclinal section of the palisade layer, and C) periclinal section of the hourglass cells; 5,000 x magnification. SL: subcuticular layer, PCL: palisade cell layer, HCL: hourglass cell layer, PL: parenchyma layer, AW: anticlinal walls, LU: lumen, LUM: lumina material.

The "light line" appeared as an indefinite transparent region in the outermost part of the palisade layer adjacent to the subcuticular layer (Fig. 7.8). The "light line" was identified in both hard and soft seeds in all of the genotypes (Fig. 7.8). There was no clear difference, between hard and soft seeds in any of the genotypes, in the position and the intensity of the "light line".

Frequently, fissures were observed in the palisade layer which were substantial covering the whole palisade layer (Fig. 7.9). The presence of fissures was identified in both hard and soft seeds in all genotypes (Fig. 7.9).

In anticlinal sections stained with Toluidine Blue O (TBO) at pH 4.4, cell walls of the subcuticular layer, anticlinal and inner tangential wall stained deep purple and the contents of the claviform lumina became dark. The secondary walls of the palisade cell layer stained light purple. No clear difference between hard and soft seeds, in the staining reaction of these cells was observed (Fig. 7.10). There was a difference between the genotypes in the amount of the material present in the lumina. Lumina material of seeds of cv. Essor showed a stronger stain reaction than that of seeds of line JS-7980 and cv. Pioneer-9581 (Fig. 7.10). However, no clear difference between hard and soft seeds, in the staining reaction of the lumina material was observed (Fig. 7.10).

The contents of the claviform lumina were stained red by vanillin/HCl. The lumina material stained strong red in seeds of cv. Essor but less red in seeds of line JS-7980 and cv. Pioneer-9581 (Fig. 7.11). There were no clear differences in the intensity of the red colour reaction between hard and soft seeds in any of the three genotypes (Fig. 7.11).

The cuticle failed to stain but the subcuticular layer was stained strongly pink-red by ruthenium red (Fig. 7.12). With ruthenium red, the anticlinal cell walls of the palisade layer had a weaker stain reaction than the subcuticular layer (Fig. 7.12). There were no clear differences either between the genotypes or between hard and soft seeds of any of the genotypes in the staining with ruthenium red.

With Phloroglucinol/HCl in both hard and soft seeds in all genotypes, no part of the seed coat showed any staining. However, in the hilar region, the tracheid bar showed a strong reaction to the stain and became red.

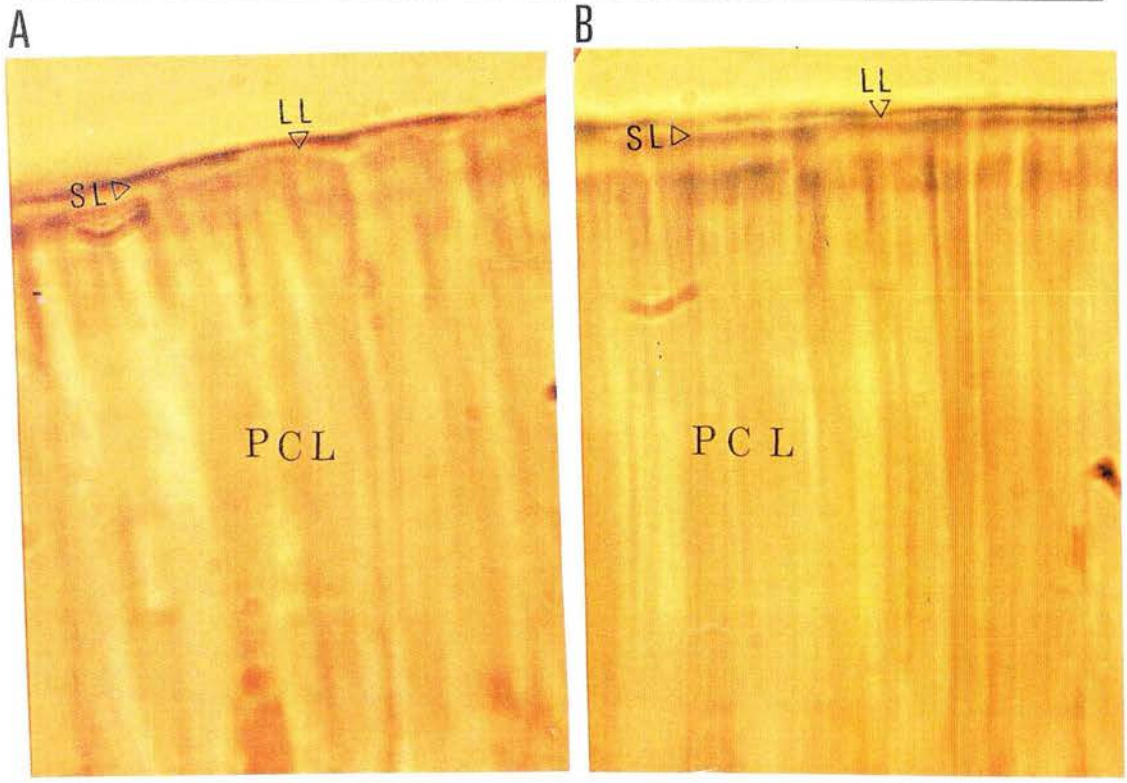


Figure 7.8. Light micrographs of anticlinal section the seed coat of cv. Pioneer-9581; 7,000 x magnification. A) hard seed, and B) soft seed. SL: subcuticular layer, PCL: palisade cell layer, LL: light line.

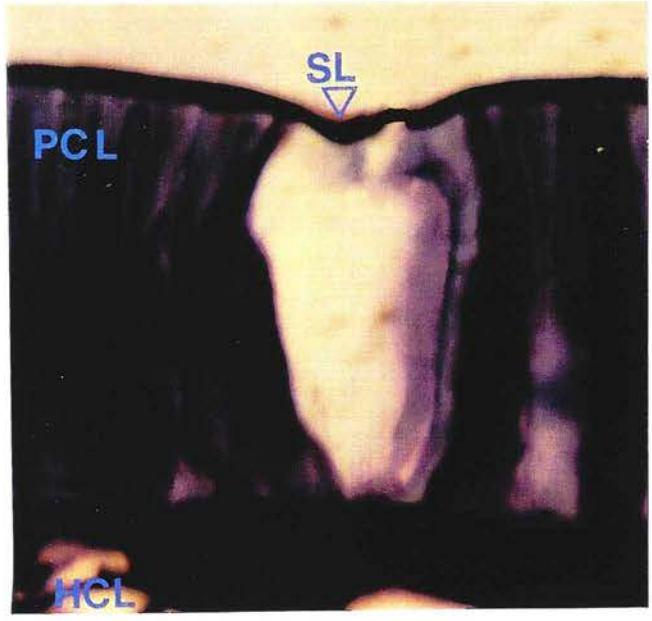


Figure 7.9. Light micrograph of anticlinal section of the seed coat stained with TBO of hard seed of cv. Pioneer-9581; 5,000 x magnification. SL: subcuticular cell layer, PCL: palisade cell layer, HCL: hourglass cell layer.

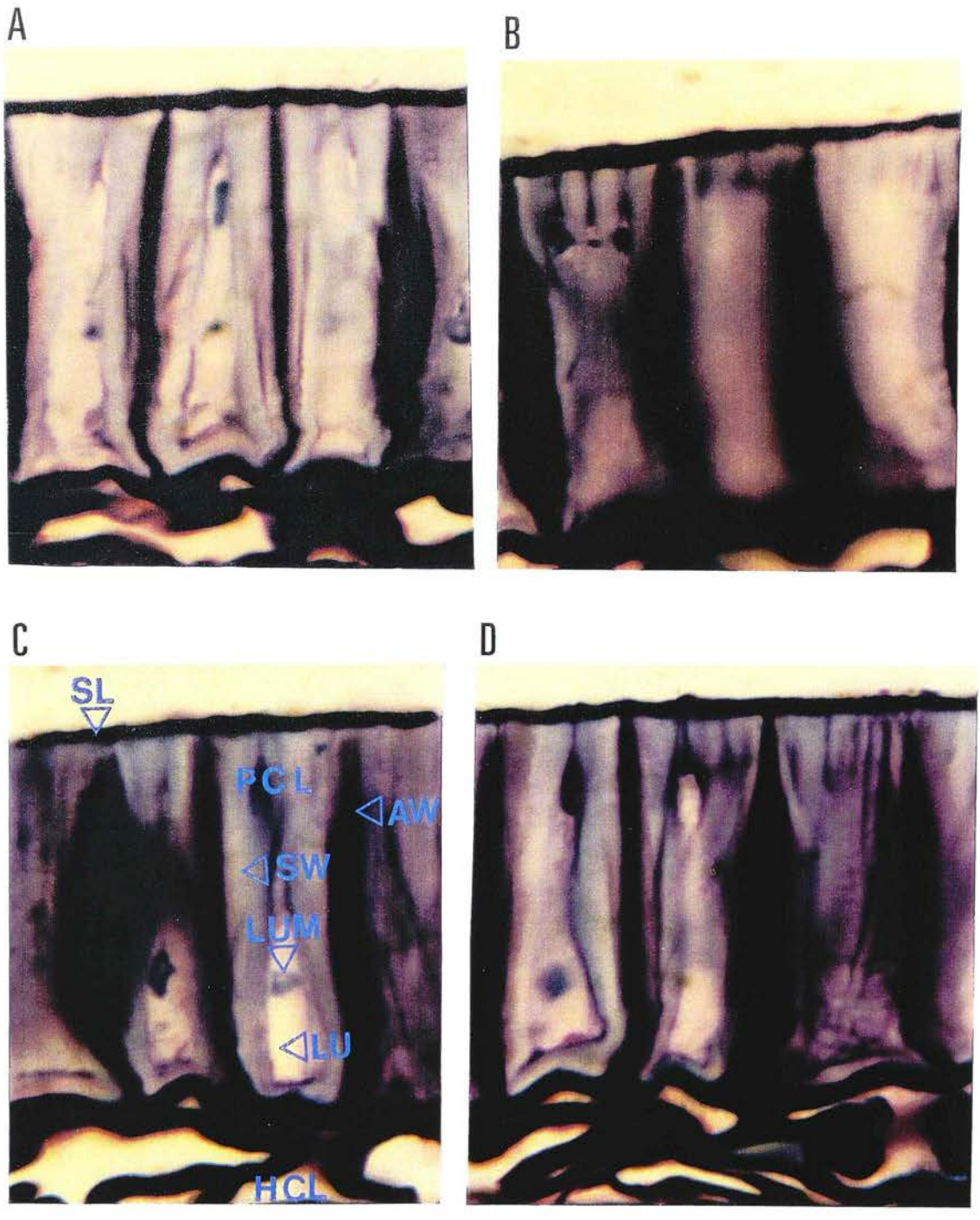


Figure 7.10. Light micrographs of anticlinal sections of the seed coat stained with TBO 5,000 x magnification. A) hard seed of cv. Pioneer-9581; B) soft seed of cv. Pioneer-9581; C) hard seed of cv. JS-7980 and D) soft seed of cv. JS-7980. SL: subcuticular cell layer, PCL: palisade cell layer, HCL: hourglass cell layer, AW: anticlinal walls, SW: secondary walls, LU: lumen, LUM: lumina material.

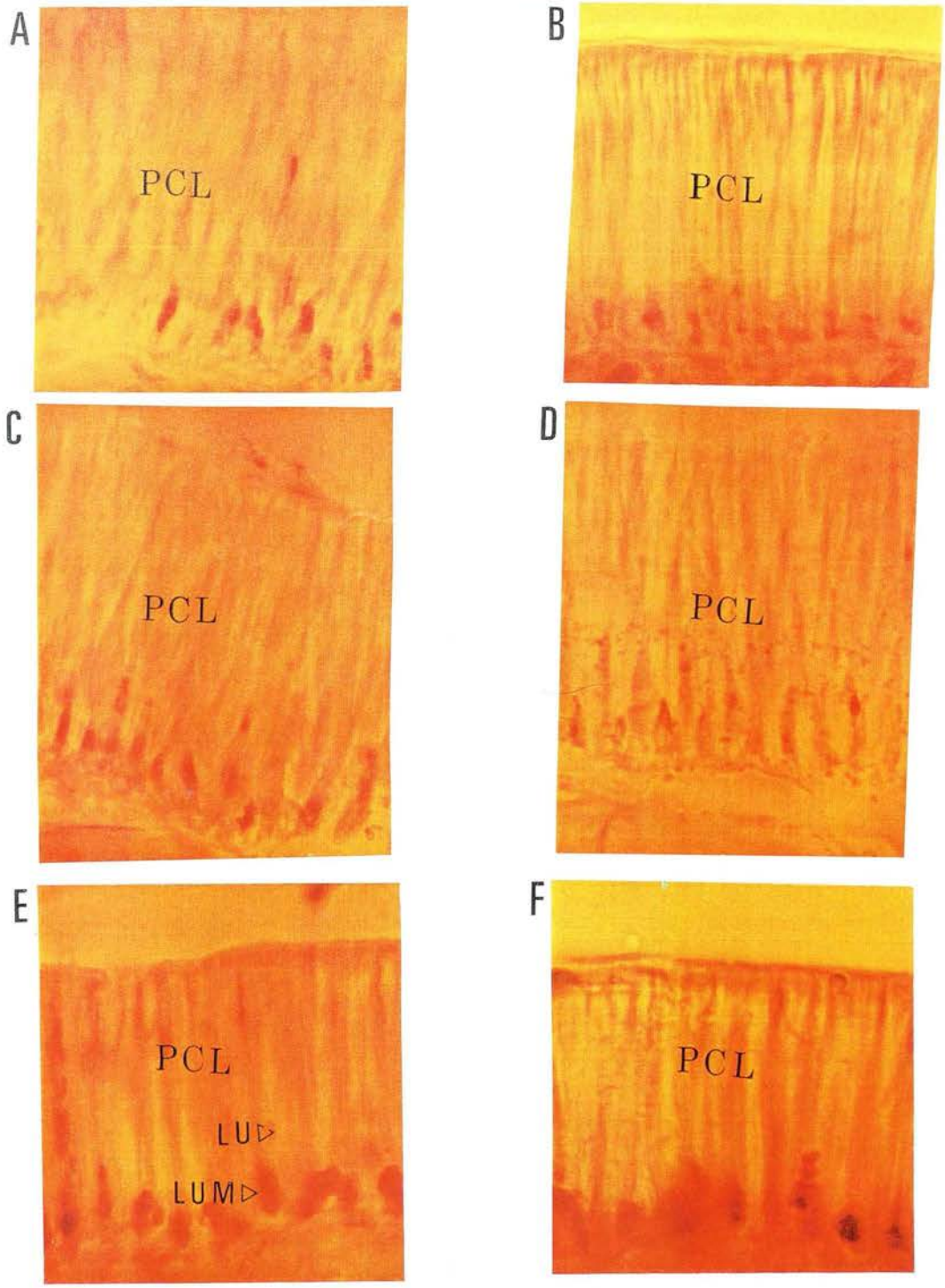


Figure 7.11. Light micrographs of anticlinal sections of the seed coat stained with vanillin/HCl, 2,000 x magnification. A) hard seed of cv. JS-7980; B) soft seed of cv. JS 7980; C) hard seed of cv. Pioneer-9581; D) soft seed of cv. Pioneer-9581; E) hard seed of cv. Essor and F) soft seed of cv. Essor. PCL: palisade cell layer, LU: lumen, LUM: lumina material.

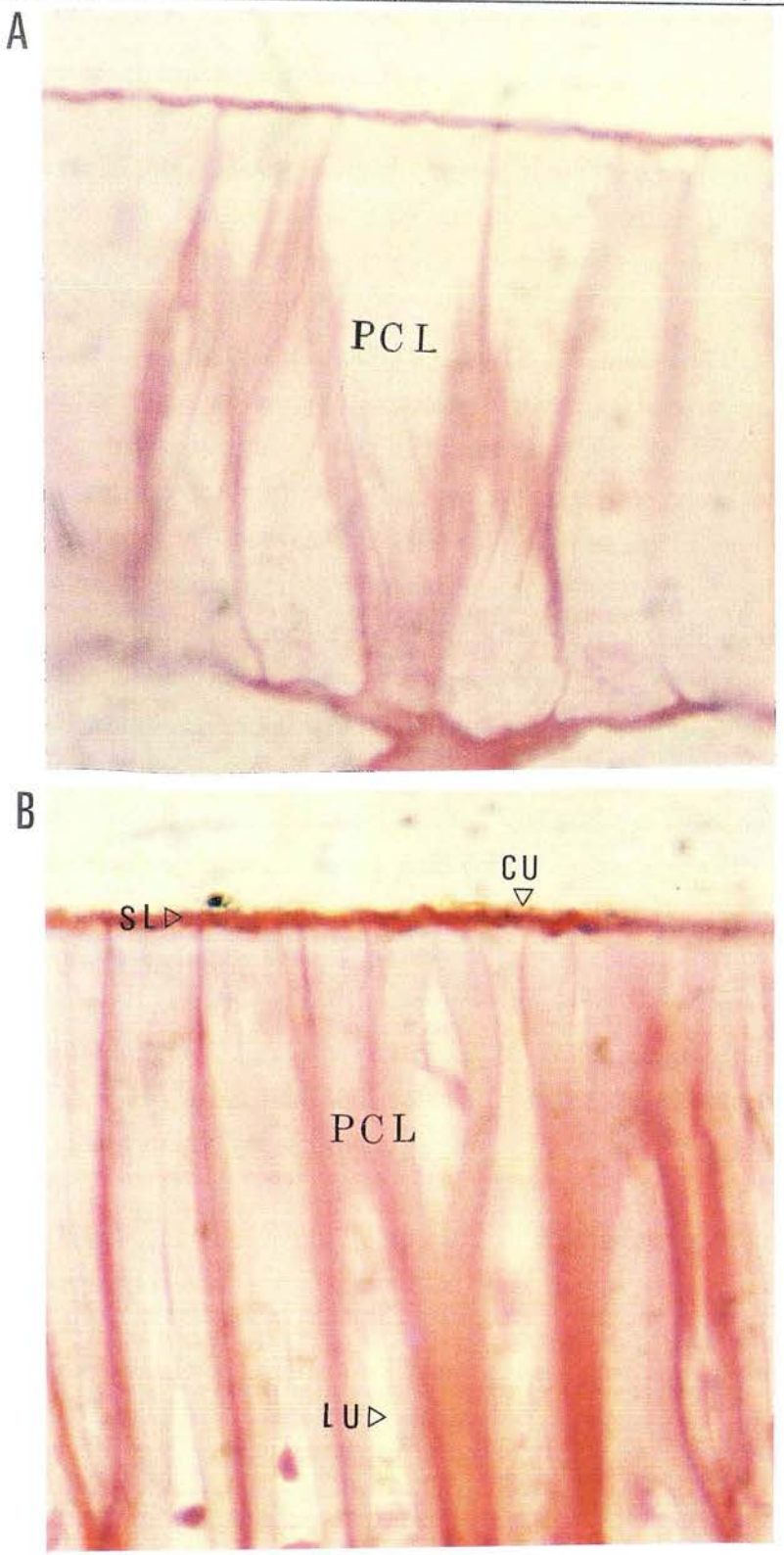


Figure 7.12. Light micrographs of anticlinal sections of the seed coat stained with ruthenium red of cv. Pioneer-9581, 7,000 x magnification. A) hard seed, and B) soft seed. CU: cuticle, SL: subcuticular layer, PCL: palisade cell layer, LU: lumen.

7.3.5. Fluorescence microscopy studies of the seed coats

Segments of the seed coat, from all regions of the seed, were fixed, dehydrated, infiltrated, embedded and sectioned as previously described. Hand-cut sections were also used. Comparative histochemical studies of the seed coat of hard and soft seeds of the three genotypes were made.

The subcuticular layer, the anticlinal palisade cell walls and the contents of the lumina showed a strong yellow autofluorence (Fig. 7.13). There appeared to be more autofluorescent material in the lumina of the palisade cells of cv. Essor than in the lumina of the palisade cells of the other two cultivars studied. However, no clear differences were observed between hard and soft seeds in any of the three genotypes (Fig. 7.13). Also, in many sections of seed coats of cv. Essor, material in the lumina showed an orange autofluorescence which formed a layer on the palisade cell layer (Fig. 7.14). There were no differences between hard and soft seeds in any of the genotypes in the presence and size of this orange autofluorescent layer.

The subcuticular layer, the anticlinal cell walls of the palisade layer and the contents of the lumina showed a strong gold-yellow fluorescence after staining with fluorol yellow (Fig. 7.15). Both hard and soft seeds in all the genotypes had similar fluorescence after staining by fluorol yellow.

The palisade cell layer was observed to fluorescence after staining by calcofluor (Fig. 7.16). Both hard and soft seeds in all the genotypes had similar fluorescence after staining by calcofluor.

No part of the seed coat observed to fluorescence after staining by aniline blue.

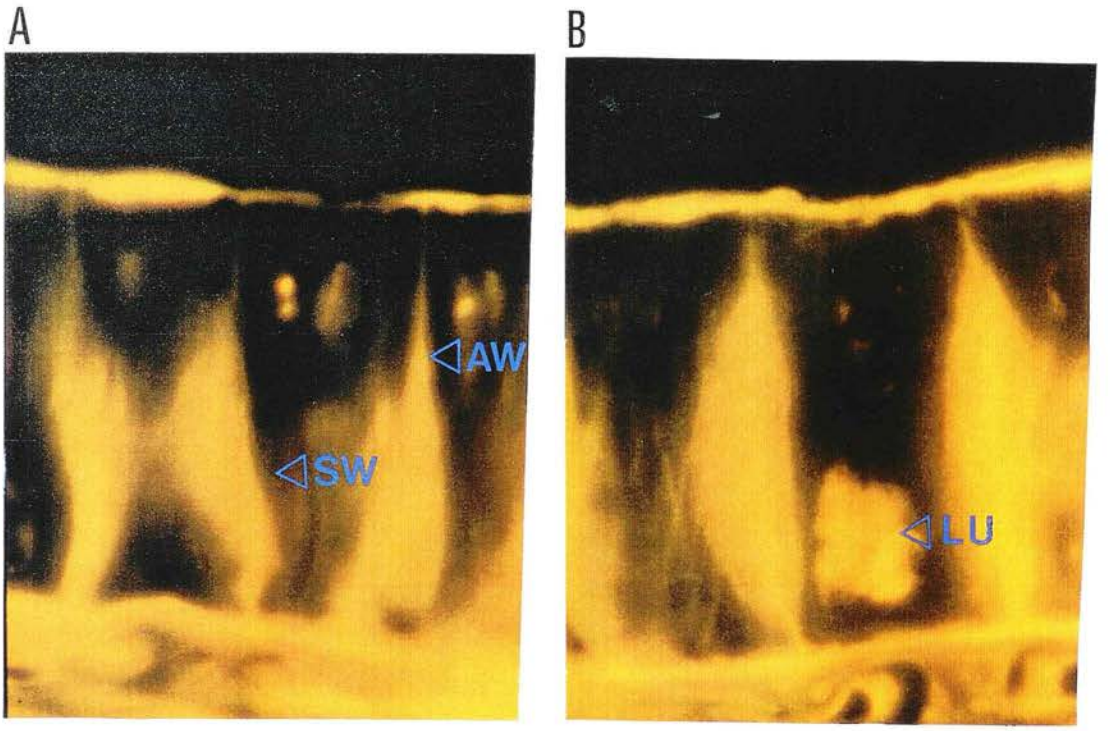


Figure 7.13. Micrographs showing autofluorescence in anticlinal sections the seed coat. A) hard and B) soft seed of cv. Pioneer-9581, 5,000 x magnification. SL: subcuticular cell layer, PCL: palisade cell layer, AW: anticlinal walls, SW: secondary walls, LU: lumen.

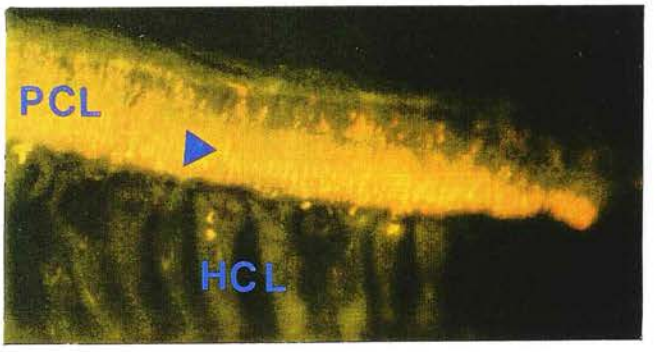


Figure 7.14. Micrograph showing autofluorescence of anticlinal section of hard seed of cv. Essor; 700 x magnification; (arrow indicates the "orange" autofluorenscence), PCL: palisade cell layer, HCL: hourglass cell layer.

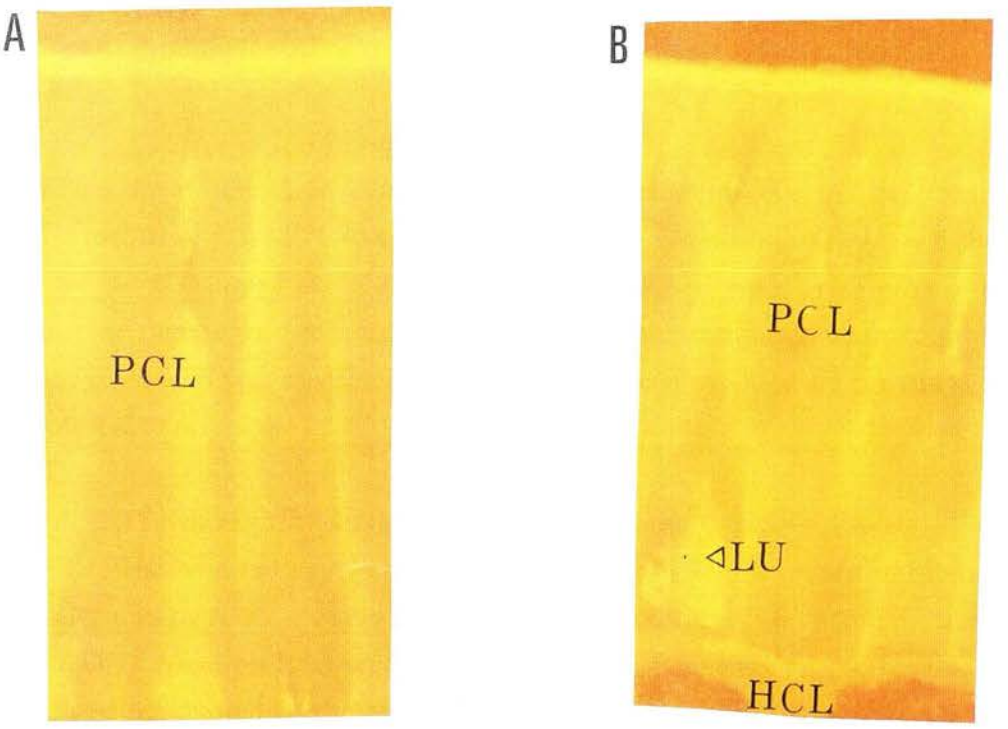


Figure 7.15. Fluorescence micrographs of the anticlinal sections of seed coat of cv. Essor stained with fluorol yellow. A) hard seed and B) soft seed; 7,000 x magnification. SL: subcuticular cell layer, PCL: palisade cell layer, AW: anticlinal walls, SW: secondary walls, LU: lumen, HGL: hourglass cell layer.

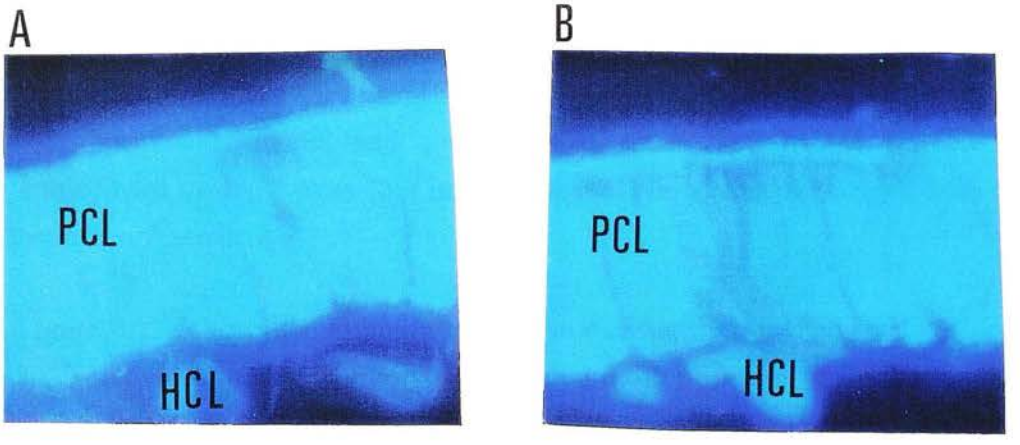


Figure 7.16. Fluorescence micrographs of the anticlinal sections of the seed coat of seeds of cv. Essor stained with calcofluor. A) hard seeds, and B) soft seeds; 1,500 x magnification. PCL: palisade cell layer, HCL: hourglass cell layer.

7.3.6. Effect of methanol and chloroform pre-treatments on water uptake of hard seeds

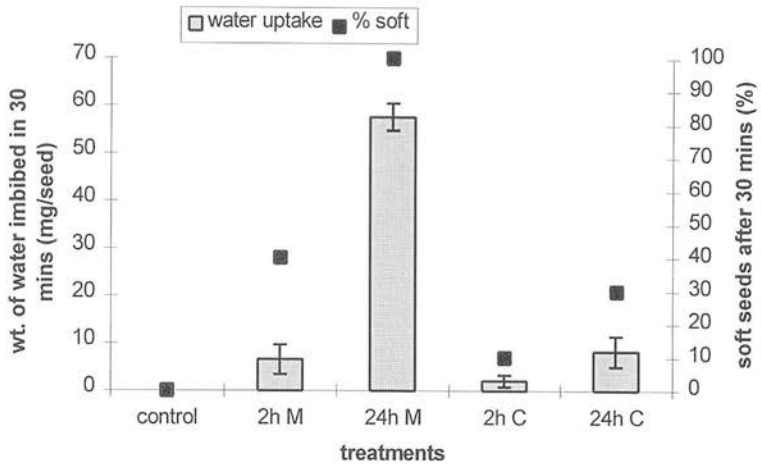
Hard seeds of the three genotypes were subjected to four pre-treatments, namely: 2h methanol (2h M), 24h methanol (24 M), 2h chloroform (2h C) and 24h chloroform (24h C). Immediately afterwards, the pre-treated seeds were placed for imbibition. The number of seeds that became soft and the weight of water imbibed by all seeds in 30 min was measured. Analysis of variance results were presented in Appendix 5, Table 5.1.

Figure 7.17a shows the imbibition behaviour of hard pre-treated seeds of cv. Pioneer-9581. The hard untreated controls remained hard after 30 min of imbibition thus no water uptake was measured. In general, the chloroform pre-treatments had a lesser ($P<0.001$) effect on hard seeds in comparison to the methanol pre-treatment. However, hard pre-treated seeds became soft in different numbers depending on the type of the pre-treatment. Forty percent of hard seeds with 2h methanol pre-treatment became soft. Twenty-four hours methanol pre-treatment had changed all hard seeds to soft seeds; a substantial amount of water was absorbed by these seeds. Chloroform pre-treatment for 24h changed 30% of the hard seeds to soft seeds.

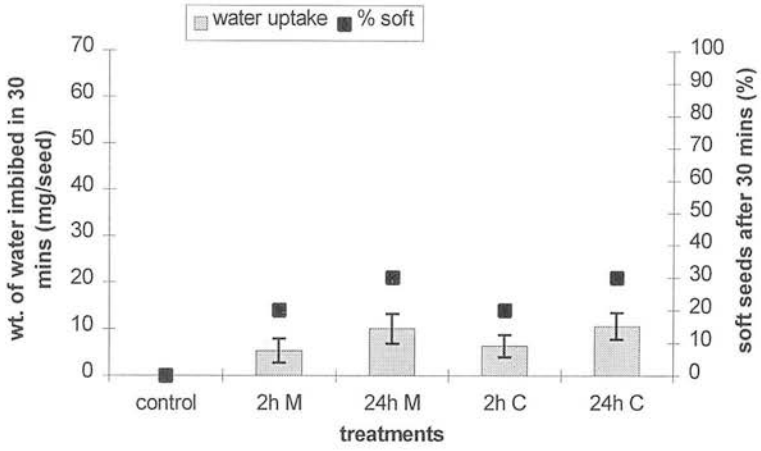
Figure 7.17b shows the imbibition behaviour of hard pre-treated seeds of cv. Essor. The hard untreated controls remained hard after 30 min of imbibition. In general, the different methanol pre-treatments had a smaller ($P<0.001$) effect on hard seeds than that observed in hard seeds of cv. Pioneer-9581. There was little ($P>0.05$) difference between methanol and chloroform pre-treatments in the imbibition behaviour of hard seeds. Twenty-four hours methanol pre-treatment changed 30% of hard seeds to soft seeds.

Figure 7.17c shows the imbibition behaviour of hard pre-treated seeds of line JS-7980. The hard untreated controls remained hard after 30 min of imbibition. In general, the imbibition behaviour of hard pre-treated seeds was similar to that of hard seeds of cv. Essor. There was little ($P>0.05$) difference between methanol and chloroform pre-treatments in the imbibition behaviour of hard seeds. Twenty-four hours methanol pre-treatment changed 30% of hard seeds to soft.

a)



b)



c)

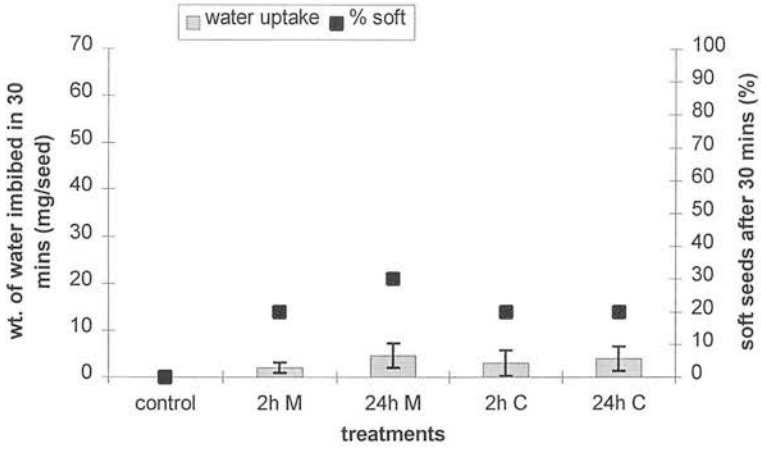


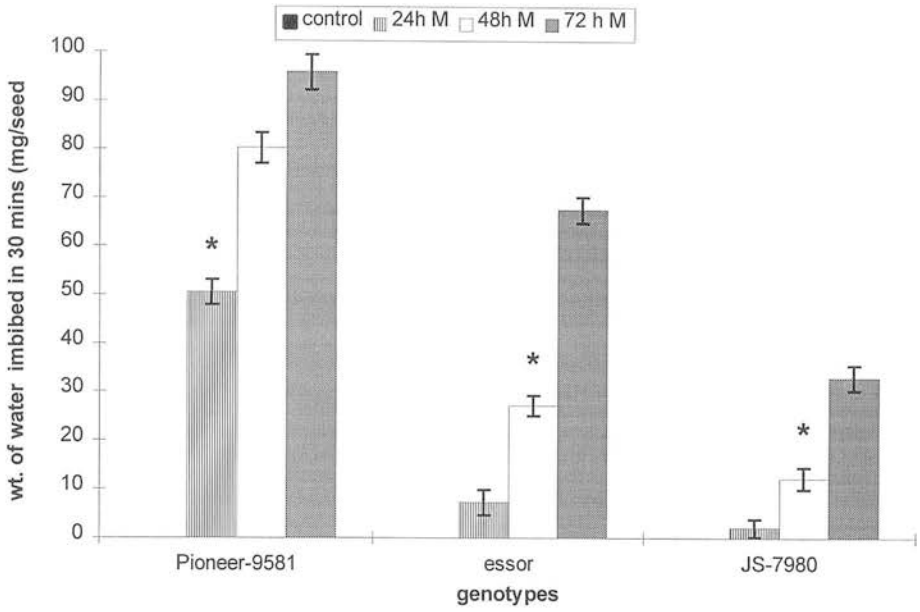
Figure 7.17. The effect of methanol and chloroform pre-treatments on water uptake and percentage of seeds became soft after 30 min of imbibition in water. a) hard seeds of cv. Pioneer-9581, b) hard seeds of line JS-7980 and c) hard seeds of cv. Essor; error bar, standard error of the mean, (n=10).

In view of the pre-treatment effects on the imbibition behaviour of the hard seeds, prolonged periods of methanol and chloroform pre-treatments were applied. In particular, three methanol and chloroform pre-treatments were used, namely: 24h, 48h and 72h. Immediately after the pre-treatments the seeds were placed for imbibition. The number of seeds that became soft and the weight of water imbibed by all seeds in 30 min was measured. Analysis of variance results were presented in Appendix 5, Table 5.2.

Figure 7.18a shows the imbibition behaviour of hard methanol pre-treated seeds of the three genotypes. In general, pre-treated seeds imbibed more ($P<0.001$) water than the untreated control seeds. All the hard, untreated control seeds remained hard after 30 min of imbibition, thus no water uptake was measured. All the hard seeds of cv. Pioneer-9481 had become soft after 24h methanol pre-treatment whereas complete conversion from hard to soft seeds of cv. Essor and line JS-7980 required 48h methanol pre-treatment. Hard pre-treated seeds of cv. Pioneer-9581 for 24h with methanol, imbibed about 50mg of water per seed during the first 30 mins of imbibition whereas 72h methanol pre-treated seeds imbibed about 100mg of water per seed ($P<0.001$). In hard seeds of cv. Essor, 24h pre-treatment with methanol resulted in 7mg of water per seed whereas 72h pre-treatment with methanol resulted in 68mg of water per seed ($P<0.001$). Hard pre-treated seeds of line JS-7980 for 24h with methanol, imbibed 2mg of water per seed whereas 72h methanol pre-treated seeds imbibed about 33mg of water per seed ($P<0.001$).

Chloroform pre-treatment had a smaller ($P<0.001$) effect than methanol pre-treatment on the imbibition behaviour of seeds of all the three genotypes (Fig. 7.18b). After 72h chloroform pre-treatment, most of the hard seeds remained hard during the first 30 mins of imbibition; 50% in line JS-7980 and cv. Essor and 40% in cv. Pioneer-9581. Hard pre-treated seeds of cv. Pioneer-9581 for 24h with chloroform, imbibed 8mg of water per seed during the first 30 mins of imbibition whereas 72h chloroform pre-treated seeds imbibed 28mg of water per seed ($P<0.001$). In hard seeds of cv. Essor, 24h pre-treatment with chloroform resulted in 12mg of water per seed whereas 72h pre-treatment with chloroform resulted in 30mg of water per seed ($P<0.001$). Hard pre-treated seeds of line JS-7980 for 24h with chloroform, imbibed 3mg of water per seed whereas 72h methanol pre-treated seeds imbibed about 12mg of water per seed ($P<0.05$).

a)



b)

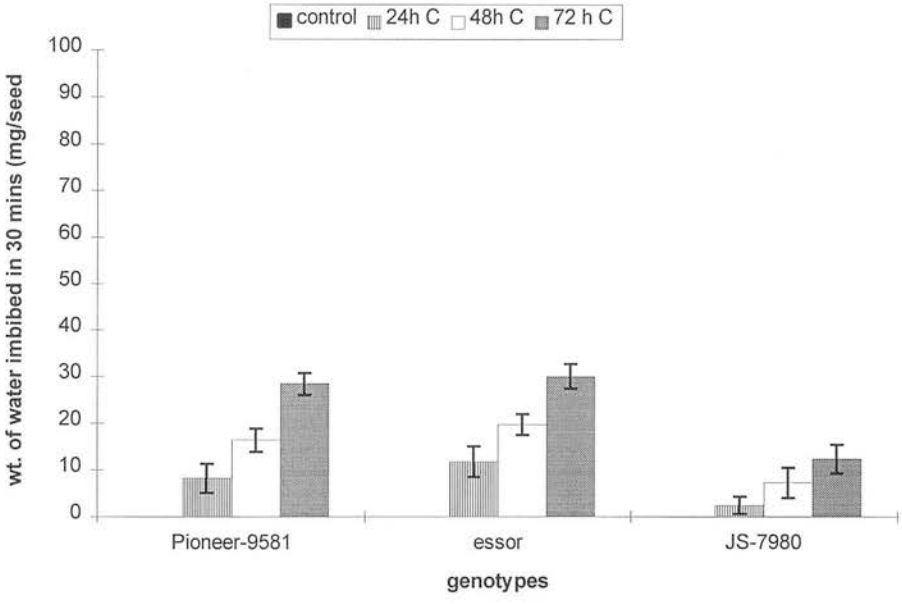


Figure 7.18. The effect of different periods of a) methanol and b) chloroform pre-treatments on the water uptake after 30 mins of imbibition; (*) indicates 100% soft seeds, error bars, standard error of the means (n=10).

7.3.8. Absorbance spectrum of supernatant

Figure 7.19 shows the absorbance spectrum of the supernatant obtained from the 24h and 72h methanol and chloroform extracts from hard and soft seeds of cv. Pioneer-9581. In most of the range scanned, the absorbance of the methanol supernatants was lower than that of chloroform supernatants in extracts from both hard and soft seeds. In all cases, the 72h supernatants had a higher values that the 24h supernatants. In general, the absorbance of chloroform supernatant from soft seeds was higher than that from hard seeds.

In all cases, the absorbance of the methanol supernatant had constantly very low values up to about 240nm with an increase in the absorbance thereafter. In all cases, in the methanol supernatants, there were no peaks in the absorbance in the whole range scanned. However, in the chloroform supernatants, there were several peaks in the absorbance between 220-275nm.

7.3.10. Effect of seed drying after the 72h methanol and chloroform pre-treatments

After the 72h methanol and chloroform pre-treatment, seeds were allowed to dry for 48h at room temperature. Subsequently, the imbibition behaviour of pre-treated seeds was monitored by measuring the amount of water imbibed and the number of seeds that became soft after 30 mins of imbibition. Analysis of variance results were presented in Appendix 5, Table 5.3.

Figure 7.20 shows the effect of the different pre-treatments on the water uptake. Hard untreated control seeds showed no imbibition after 30 min. In general, seeds placed for imbibition immediately after the pre-treatments had a higher ($P<0.001$) water uptake that those dried after the pre-treatments. However, genotypes responded differently ($P<0.001$) in relation to the water uptake due to the pre-treatments.

All hard seeds that had been pre-treated with 72h methanol, and placed immediately afterwards for 30 mins imbibition became soft. When seeds dried after the 72h methanol pre-treatment, and then placed for imbibition became again hard. During the 30 mins of imbibition, the hard pre-treated seeds with 72h methanol of cv. Pioneer-9581, Essor and line JS-7980 imbibed about 100, 70 and 40 mg of water per seed respectively. When seeds dried after the 72h methanol pre-treatment, and then placed for imbibition imbibed about 5, 4 and 1mg of water per seed respectively.

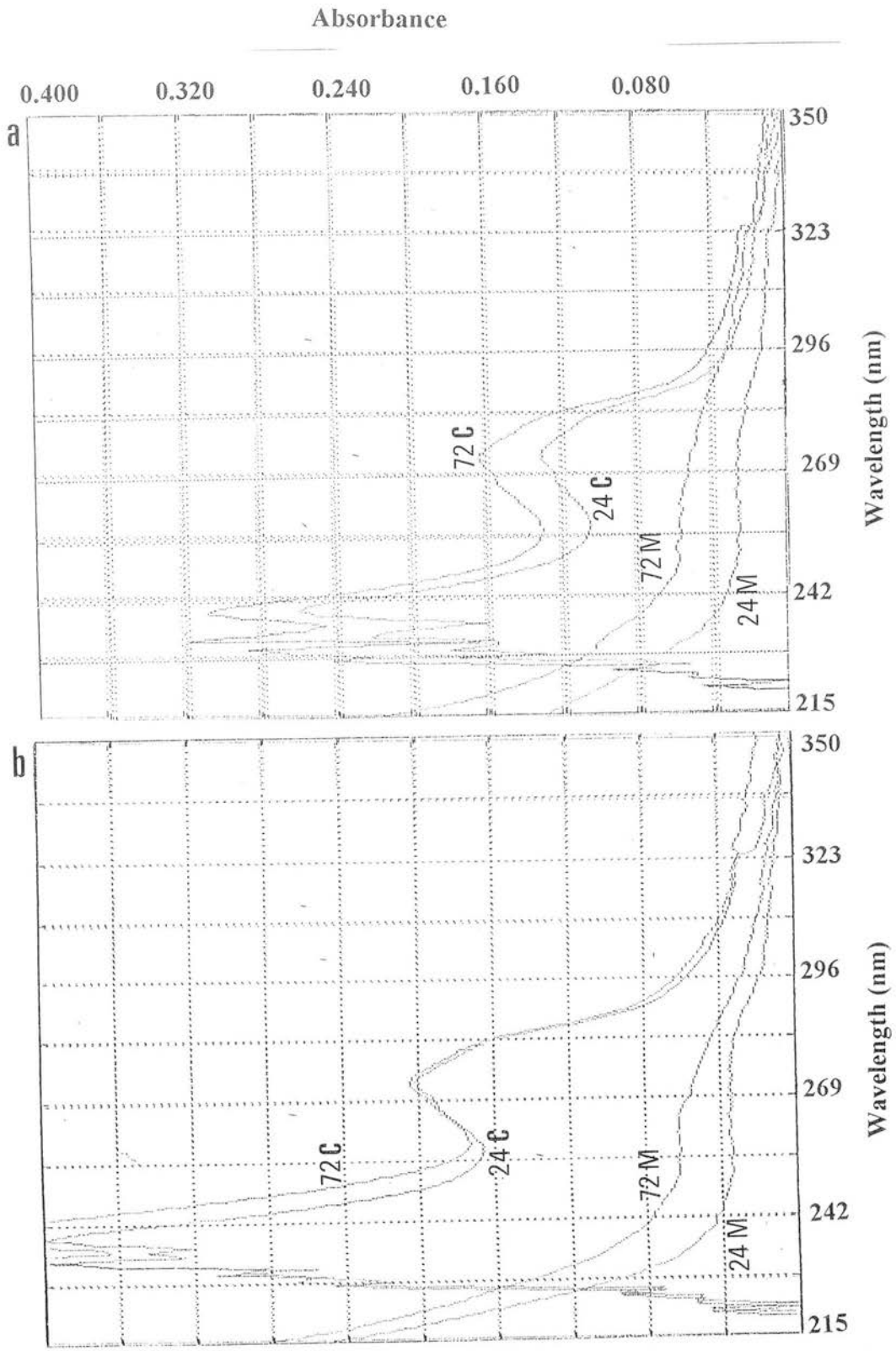


Figure 7.19. Absorbance spectrum from the 24h and 72h extracts methanol and chloroform supernatants obtained from a) hard seeds and b) soft seeds; 24 M (24h methanol), 24 C (72h chloroform), 72 M (72h methanol), 72 C (72h chloroform).

Few of the hard seeds that had been pre-treated with 72h chloroform, and placed immediately afterwards for 30 mins imbibition became soft. When seeds dried after the 72h chloroform pre-treatment, and then placed for imbibition became again hard. During the 30 mins of imbibition, the hard pre-treated seeds with 72h chloroform of cv. Pioneer-9581, Essor and line JS-7980 imbibed about 40, 24 and 15 mg of water per seed respectively. When seeds dried after the 72h methanol pre-treatment, and then placed for imbibition imbibed about 3, 2 and 1mg of water per seed respectively.

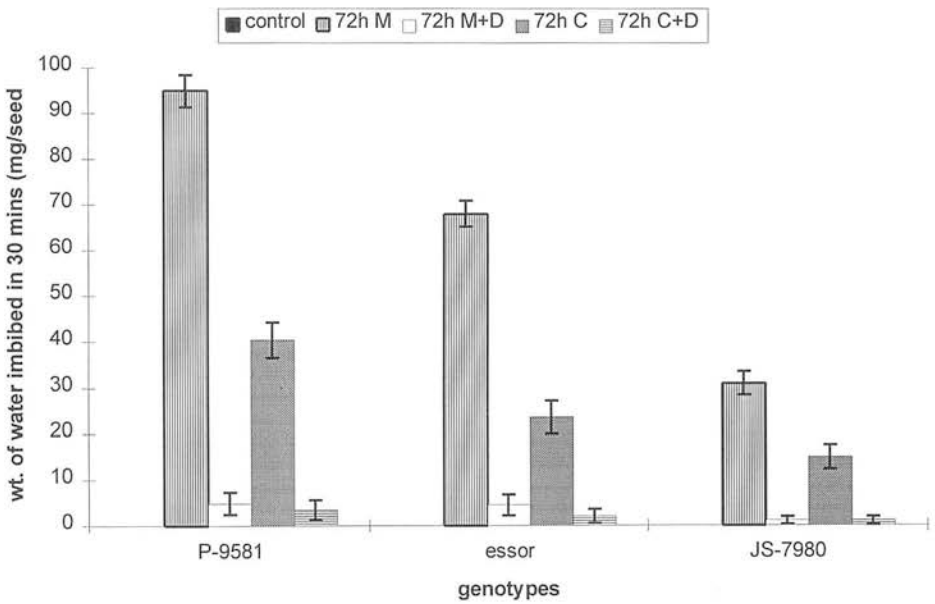


Figure 7.20. The effect of seed drying after the pre-treatments on water uptake during 30 mins of imbibition: control (untreated), 72h M (72h methanol), 72h M+ D (72h methanol followed by drying), 72h C (72h chloroform), 72h C + D (72h chloroform followed by drying); error bars, standard error of the means (n=10).

7.4. Discussion

The seed lots of three genotypes on which the comparative studies between hard and soft seeds were made, had a significant variation in the percentage hard seeds they contained. It was assumed that genotypic differences could be identified particularly in situations where a range of hardseedness occurred.

The incidence of hard seeds may be an inherited characteristic. In soybeans, Verma and Ram (1987) supported the view that impermeability was an inherited characteristic that was dominant over permeability, a theory proposed followed by earlier studies on soybeans (Ting, 1946; Shahi and Pandey, 1982). In contrast, Srinives and Hadley (1980), believed that permeability was a dominant seed coat characteristic. Other studies have related the induction of hardseedness to environmental factors during seed maturation and subsequent drying. In soybeans, Duangapatra (1977), reported a negative association between seed moisture and expression of hardseedness; the presence of hard seeds was first detectable only after the seed moisture content dropped to or below 14% and an increased proportion of seeds were hard when seeds were dried to 7.5% moisture content. Baciu-Miclaus (1970) considered that variation in hard seed percentages between seed lots was dependent on climatic conditions or the length of growing seasons.

In the present work, the comparison of the physical characteristics of hard and soft seeds revealed two features. First, hard seeds were shown to have a lower seed dry weight in comparison to the soft seeds and second, the ratios of seed coat dry weight to seed dry weight was higher in hard seeds than in soft seeds.

Some reports have shown that soybean lines with an impermeable seed coat generally have smaller seeds (Saio, 1976; Arechavaleta-Medina and Snyder, 1981) and seed lots with permeable seed coats occasionally produced impermeable seeds that were among the smallest in the seed lot. In contrast, Aitken (1939), reported that soft seeds of *Trifolium subterraneum* were about 20% lighter than hard. The higher dry matter content of the hard seed was attributed to a possible longer period of seed development on the plant, involving a higher nutrient concentration and consequently a high dry matter content (Aitken, 1939). Yaklich *et al.*, (1986) suggested that the ratio of the soybean seed coat to embryo was an important index related to hardseedness; the two genotypes with impermeable seed coat (cv. Sooty and line D67-5677) had ratios above 0.1 whereas the two genotypes with permeable seed coat had ratios below 0.1. However, early attempts in *Trifolium* and *Lotus* species to relate

the thickness of the testa to hardseedness were unsuccessful (Aitken, 1939; Watson, 1948).

In this study, the suggestion that in hard seeds the ratio of seed coat to seed was below 0.1 observed only for the hard seeds of line JS-7980 although in all genotypes hard seeds had a higher ratio of testa to seed in comparison to soft seeds.

Additionally, the ratio of seed coat to seed may be more important in breaking the hardseedness rather than inducing it. Russi *et al.*, (1992) in hard seeds of *Trifolium* and *Medico* species reported that the ratio of seed coat thickness to seed radius was important in breaking the hardseedness under natural soil conditions. They observed that cases where most of the hard seeds became soft after 3 months in soil conditions the ratio of testa thickness to seed radius was 0.06. In cases where all of the hard seeds remained hard after the 3 months period, the ratio of testa thickness to seed was 0.13. They suggested that the thinner the testa, the more likely it would be that the intense stresses under diurnal temperature fluctuations would lead to rupture at weak sites. Tran (1979) in a study of the effects of microwave energy on *Acacia* seeds reported a negative relationship between seed coat thickness and susceptibility to softening.

In this study, there was no relationship between the presence of deposits and hardseedness since all the genotypes were free of deposits. Although the presence of deposits on the surface of both hard and soft soybean seed coats has been well documented in a number of studies (Calero *et al.*, 1981; Harris, 1987; Ragus, 1987), there has been no real evidence of their involvement in hardseedness. If deposits are to play a role in hardseedness in soybeans, they must be of waxy origin (cutins) and also the cuticle to be the primary location for the impermeability barrier to water. As shown in Chapter 6, section 6.2.2., in all five genotypes examined, the deposits were hydrophilic material, probably remains of the pod endocarp.

It is not known whether the deposits that were observed in the surface of soft seeds are of the same origin as the deposits in hard seeds observed by other researchers. In images published by Calero *et al.*, (1981), however, the deposits on the surface of hard seeds appear to be very similar to the deposits reported in Chapter 6, section 6.2.1.

In the present work, the presence and distribution of pits on the surface of the seed coat of the hard seeds was documented. Unfortunately, valid comparisons between hard and soft seeds within the same genotype in relation to the surface

structure could not be made because the separation procedure (the cycle of wetting and drying) significantly affected the surface structure of the seeds. One way to eliminate the problem may be soaking only half of the seed (to test whether it is hard or soft) and observe the other half (to identify possible differences between them). However, observation of the surface structure of hard seeds of the three genotypes could indicate variation in the pitting between the genotypes.

In the genotypes studied in this investigation, pitting in hard seeds was varied. In hard seeds of two of the genotypes, pits appeared to be shallow but in the third genotype the pits appeared to be deep. Although, it is logical to assume that shallow pits are less suited to allowing water penetration, explanations are needed for the failure of deep pits to facilitate water uptake. Calero *et al.*, (1981) in a comparison of the surface structure and water uptake suggested that the size and shape of individual pits could play a role and that the elongated/shallow pits were those with minimum function in relation to water permeability. Few or total lack of pits was reported for hard soybean seeds of cv. Sooty and cv. Brachett (Yaklich *et al.*, 1986; Harris, 1987). Additionally, in other studies on the surface structure of soybean hard seeds (cv. Altona and line D67-5677), a significant degree of pitting was reported (Wolf *et al.*, 1981; Yaklich *et al.*, 1986). Apparently, the mere presence of numerous deep pits does not make the seed coat of a hard seed permeable to water.

A surface view of the hard seed coats after soaking whole hard seeds in calcofluor indicated that no staining of the surface of the seed coat had taken place. Unfortunately, valid comparisons between hard and soft seeds of the same genotype in relation to surface calcofluor staining could not be made. The separation procedure, between hard and soft seeds, created cuticular cracks in the surface of the soft seed that were entry points for calcofluor staining. However, comparisons could be made with soft untreated seeds of other genotypes.

As previously presented (Chapter 6, section 6.2.2.), in such soft seeds there was a clear correlation between the distribution and size of pits and the distribution, size of the patchy areas. In the hard seeds of genotypes studied in this investigation, this staining pattern was absent indicating that the pits did not function in the same way in relation to the water permeability. One possible explanation for the previous anomaly may be that the shallow-shaped pits which appeared in hard seeds of two genotypes, were not suited for water penetration. However, the reason that pits were not function in hard seeds of cv. Pioneer-9581 (pits were deep) is not known.

Localization of the presence of the barrier to water uptake within the seed coat of hard seeds was successfully achieved using calcofluor as a water-soluble fluorescent stain. In other studies, localization of the impermeability barrier to water had been also achieved by other water-soluble stains such as aqueous solution of ruthenium red, in *Leucaena leucocephala* seeds (Serrato-Valenti *et al.*, 1995), aqueous solution of crystal violet, in sugar maple seeds (Jannerette, 1979), aqueous solution of fast green FCF, in *Aspalathus linearis* seeds (Kelly and Van Staden, 1986).

The location of the barrier was very superficial near the surface of the seed coat in hard seeds. There was no difference between the different regions of the seed in the distribution of staining within the seed coat indicating the uniformity of the presence of the water barrier. In the hilar region, staining could be localized only at the outer parts of the tracheid bar but not at the outer and inner palisade layers indicating that only the tracheid bar could play a role in the imbibition of a hard seed. Anticlinal sections of the seed coat of hard seeds soaked for 24h in calcofluor, showed that the subcuticular layer was clearly stained. In addition, the uppermost part of the palisade cell layer, the region bordered by the "light line", appeared to be stained as well.

From the observations with the use of calcofluor as a water-soluble fluorescent stain, it was apparent that the cuticle was not the primary location of the water permeability barrier in soybeans. This observation is in agreement with other reports that in most species cuticle is not totally responsible for water impermeability. However, there have been some cases reported of cuticles exhibiting water impermeability. Seeds of *Ononis sicula*, a desert annual legume, has a well developed and thick cuticle that was shown to be the location of water impermeability (Gutterman and Heydecker, 1973). Similar cuticle characteristics have been described in *Trigonella arabica* (Gutterman, 1979). Although, the cuticle alone may not explain the different degrees of water impermeability for most legume species (Russi *et al.*, 1992), if it is well developed and thickened, it might delay imbibition by seeds. Russi *et al.*, (1992) in *Medicago orbicularis* observed that cuticle was well developed and 3.0-6.6mm thick.

In the present work, no clear anatomical differences between hard and soft seeds in any of the three genotypes were observed. SEM images from anticlinal sections revealed that both hard and soft seeds had abundant passageways penetrating most of the part of the palisade layer. These passageways were more likely to be the

lumen of the palisade cells as revealed by light microscope images as well. In 3 dimensions, the size of the passageways (e.g. wide or narrow, high or short) is more likely to be an effect of the cutting edge through the palisade layer in the seed coat. It is logical to assume that in hard seeds if water can penetrate the outer parts of the palisade layer, then the presence of passageways would provide an avenue for easy penetration in the base of the palisade cell layer. In this case, the water permeability barrier should be located at the base of the palisade layer; however, previous observations with the use of calcofluor as a water-soluble stain indicated that the later view was not true. Additionally, the "light line" was present at the same position in both hard and soft in all three genotypes examined. It was not possible to identify differences between hard and soft seeds in the intensity of the "light line".

Calero *et al.*, (1981) observed the presence of passageways only in soft-seeded soybean genotypes but not in hardseeded genotypes. In contrast, Wolf *et al.*, (1981) in a survey of 33 soybean genotypes observed passageways in both hard and soft soybean seeds. Duanagapatra (1978) in soybeans, observed the presence of the "light line" in both hard and soft seeds. She also reported that no clear difference between hard and soft seeds in the intensity of this region could be observed. In contrast, Harris (1987), in an anatomical comparison of a hardseeded to a softseeded genotype, using interference microscope reported a sharp difference in refractive index at the light line zone. He also reported that the "light line" was stronger in the hardseeded than in softseeded genotype.

Observations from the light and fluorescence microscopy studies showed that although there were some differences between genotypes in the histochemistry of the seed coat, there was no clear difference between hard and soft seeds within the same genotype.

In this study, sections stained with ruthenium red revealed the presence of pectin material in parts of all cell walls throughout the seed coat of both hard and soft seeds. Callose was not detected in sections of the seed coat stained with aniline blue of either hard or soft seeds in any of the genotypes. Lignin was not detected in sections of the seed coat stained with Phloroglucinol/HCl of either hard or soft seeds in any of the genotypes. However, lignin was detected in the tracheid bar from both hard and soft seeds. Suberin was not detected in sections of the seed coat stained with aniline blue of either hard or soft seeds in any of the genotypes. Phenolic material was stained with fluorol yellow but no difference between hard and soft seeds was observed.

Duanagapatra (1978) in soybeans observed pectins in the cells walls of the palisade layer in both hard and soft seeds. The presence of callose is well-known as a sealing or plugging compound (Currier and Strugger, 1956), and has been reported as an important permeability control factor in a number of studies; Bevilacqua *et al.*, (1989) in *Melilotus alba,* Bhalla and Slattery (1984) and Bevilacqua *et al.*, (1989) in *Trifolium subterraneum* seeds. Duanagapatra (1978), also, reported absence of lignin of both hard and soft seeds in soybeans. Contrary, Baciu-Miclaus (1973) high lignification level of the palisade cells in hard seeds. Duanagapatra (1978) reported the presence of suberin in the seed coat in the hilar region using safranin-o-fast green, zinc-chloro-iodide and IKI-H_2SO_4 stains. Suberin was detectable only in the inner palisade layer in the hilar region in both hard and soft seeds but in different forms; in soft seeds the substance appeared in the form of droplets scattered throughout the length of the palisade cells whereas in hard seeds the substance was compressed into an inverted cone shape, completely filling the basal portion of the cells. Based on the previous observation, she attributed the soybean hardseedness to this morphological difference in the suberin deposition in the inner palisade layer.

In this study, however, the inner palisade cell layer was partial stained with calcofluor. The water had penetrated through the tracheid bar since first, the outer palisade cell layer was preventing the water penetration and second, the areas of the inner palisade cell layer stained with calcofluor were those closed to the tracheid bar. Additionally, for the same reasons, even in soft seeds (Chapter 6, section 6.2.2.) the partial staining of the inner palisade cell layer with calcofluor was more likely to be due to water penetration through the tracheid bar rather than the outer palisade cell layer. It is more likely, therefore, that although that morphological difference in the deposition of suberin does exist, it has got no important connection to the water impermeability in soybeans.

In this study, no anatomical or histochemical differences between the seed coats of hard and soft seeds in any of the three genotypes examined were observed. It was not possible to detect the nature of the water impermeability barrier by the procedures used. However, the location of the barrier suggested its close proximity to the uppermost part of the palisade cell layer closed to the "light line".

Harris (1983) stated that the "light line" has been a subject of controversy since investigators have been studying seed coats microscopically. However, in soybeans, several reports have confirmed the existence of "light line" (Duangaptra, 1978; Harris, 1987). Harris (1987), in TEM studies of the soybean seed coat, reported that

the light line zone appeared as an osmiophilic layer more likely to be suberin or related substance(s) which was responsible for the optical phenomenon known as light line. However, in the same studies, the previous author did not report any difference between hard and soft seeds in the structure or density of the osmiophilic layer. Similar osmiophilic layer closely related to the occurrence of the light line was reported in normal seeds of *Pisum sativum* by the same author (Harris, 1983).

In the other species, there have been suggested several reasons of causing of the "light line" phenomenon. Miller (1967) in anatomical studies in *Crotalaria* seeds, suggested that it was an artifact of light resulting from differences of refractive indices in the cell walls. Hamly (1932, 1935), in *Melilotus* seeds, suggested that the juxtaposition of suberin and cellulose, which differ in refractive index, produced this optical phenomenon. Cavazza (1950), in *Gleditshia* seeds, stated that the occurrence of the light line was due to a compact layer of cellulose. Spurny (1964) reported that suberin or related compound(s) existed in the outer parts of the cell walls.

In other species, researchers have observed the presence of caps on top of the palisade layer, and frequently the structure and histochemistry of the palisade caps have been implicated with the cause of impermeability to water. However, the structure and histochemistry of the palisade caps have been highly disputed. Spurny (1964) suggested that the full development of suberin star-shaped structures on the apical part of the palisade layer was related to the water impermeability in *Pisum sativum* seed coat. Werker *et al.* (1979) reported in *Pisum* species reported that caps on the top of the palisade layer formed a continuous layer of pectins. He reported that pectic substances were forming a "hard" pectinaus layer which was suggested to be the cause of water impermeability. Cochrane (1985) reported that pectic substances could play an important role as substances hindering water penetration. She reported that pectic substances were filling the xylem elements in some barley ears; when the pectic substances were at high concentrations could completely stop the flow of water whereas removal of the pectic substances by enzymes allowed normal water penetration. Serrato-Valenti *et al.* (1994) in studies of testa structure and histochemistry related to water uptake in *Leucaena leucocephala*, observed a continuous of the thin palisade cap layer composed of polysaccharide hydrophilic material. Rangaswamy and Nandakumar (1985) in correlative studies on seed coat structure, chemical composition and impermeability in *Rhynchosia minima* observed that the palisade cap layer was a thin sheet covering the entire outer surface of the palisade area, and it was suggested that the cap layer prevented water uptake. Raleigh (1930) in anatomical studies of *Gymnocladus dioica,* Werker *et al.*, (1973) in

Prosopis farcata and Bhalla and Slattery (1984) in *Trifolium subterraneum* reported the occurrence of single suberised caps over each palisade cell. Bevilacqua *et al.*, (1989) observed in *Melilotus alba* separate auramine-fluorescent caps indicating heavily cutinized caps which could prevent water penetration.

In this study, there was a clear association between the amount of phenolic material indicated by staining with vanill/HCl in present in the lumina of the palisade cells and the colour of the seed coat.

Yellow-coloured seed coats of seeds of cv. Pioneer-9581 and line JS-7980 appeared to have less phenolic material than the partially-brown seed coats of seeds of the cv. Essor. Also, the observations from the Toluidine Blue O, vanillin test and the autofluorescence consistently stressed the previous relationship between seed coat colour and phenolics present in the lumens. Several other studies have shown a similar relation between the amount of phenolic material and the pigmentation of the seed coat in other species. Marbach and Mayer (1974,1975) in *Pisum* seeds reported that seeds with coloured seed coat had a higher amount of total phenolic material. Werker *et al.* (1979) in anatomical studies in *Pisum* seeds, reported that there was a clear association between the phenolic material detected by $FeCl_3$ in ethanol or $MaNO_2$ and the seed coat colour; coloured seed coats had a higher amount of phenolic material in comparison to the lighter colour seed coats. Slattery *et al.* (1982) in *Trifolium subterraneum* seeds, reported that dark coloured seed coats had a higher amount of total phenolic material that light coloured seeds.

In the present work, no clear difference between hard and soft seeds in any of the 3 genotypes studied in the amount of phenolic material, indicated by staining with vanill/HCl, was observed . Water impermeability has previously been associated with the quantity of phenolic material present within the seed coat. Harris (1987) studied the seed coat structure of a hard-seeded (cv. Brachett) and soft-seeded (cv. Hardee) in soybeans. He reported that the hard-seeded cultivar had more phenolic material (indicated by $FeSO_4$) in the palisade layer than the soft-seeded cultivar. Phenolic material developed during early maturation in cv. Brachett but not in cv. Hardee. Marbach and Mayer (1974, 1975) reported a high content of phenolic material and catechol oxidase in pigmented *Pisum* species. They suggested that during dehydration of the seeds following maturation, phenolic compounds in seed coats were oxidised in the presence of the catechol oxidase and this might render the seed coats impermeable to water. Werker *et al.*, (1979) also associated the presence of phenolic material, in *Trifolium subterraneum,* with seed coat impermeability to

water. However, Slattery *et al.*, (1982) in *Trifolium subterraneum* reported that although phenolic material played an important role in the pigmentation of the seed coat, no convincing evidence existed about the relation of the phenolic material to impermeability to water.

In the present study, there was a clear difference between the effect of methanol pre-treatment and the effect of chloroform pre-treatment when hard seeds were placed for imbibition immediately after the pre-treatments. Methanol effectively changed all hard seeds to soft and the duration of the pre-treatment that required for this effect was genotype-dependent. Once all hard seeds became soft, further methanol pre-treatment had a substantial effect in the amount of water absorbed during imbibition. On the other hand, a small proportion of hard seeds became soft following pre-treatment with chloroform regardless of the duration of the pre-treatment. Arechavaleta-Medina and Snyder (1981) in a study of water imbibition of soft and hard soybean seeds (cv. Amsoy 71) reported that 24h methanol or ethanol pre-treated seeds converted hard seeds to soft seeds. In contrast, 24h pre-treatment with chloroform, hexane or acetone had little effect on hard seeds. They suggested that cuticle was the site of water impermeability although no difference in the cuticle was observed between hard and soft seeds.

In view of the effect of organic solvents, particularly the methanol, in the imbibition behaviour of hard seeds explanations were sought. One possibility was that the organic solvent pre-treatments were extracting phenolics from the subcuticular or/and palisade layer. At the first step, the extraction of phenolics might have caused a disruption in the water impermeability barrier thus changing the seeds from hard to soft. At a second step, as prolonged pre-treatments were applied, the seeds showed a high amounts of water absorbed during imbibition. However, the suggestion that the extraction of phenolics was a cause for the loss of water impermeability was not substantiated. From the absorption spectrum results, it was clear that prolonged methanol pre-treatments extracted little material with UV absorbance in the range of 220-320mm which is the region in which phenolic material absorbs (Waterman and Mole, 1994). Additionally, it was observed that prolonged chloroform pre-treatments extracted a lot of material with UV absorbance in the range of 220-320mm.

Drying of seeds after the pre-treatments restored water impermeability to about 90% of seeds. If something which caused impermeability was extracted from the seed coat, it would not be restored on drying of the seeds after the pre-treatments.

Egley and Paul (1993) examined the effects of 28 different organic solvents with a range of polarities, swelling factor and solubility to water in relation to the nature of the water-impermeable barrier(s) in *Sida spinosa*. They observed that the most active solvents in overcoming the barrier were those with non-polar, high swelling factor and high solubility to water organic solvents (e.g. pyridine, diethylamine). Seed drying after the application of the pre-treatments, however, did not restore water impermeability due possibly to a permanent disturbance of the barrier. Some other polar (ethanol) and non-polar (chloroform) solvents were of medium activity in overcoming the barrier; seed drying restored to a great extent water impermeability probably due to a temporary disturbance of the barrier. They, also, suggested two points as a key factors in relation to the previous results: a) water impermeability was lost at few specific sites in the chalaza in Malvaceae seeds and a cascade of events resulted in a massive disruption of cell layers as cells hydrated and expanded, and b) a slight amount of moisture (3% increase in moisture content) in the seed coat helped to maintain the barrier and block further penetration of liquid water.

The following model could provide explanations in relation to the effects of organic solvents, particularly the methanol, on the water impermeability barrier in hard soybean seeds. Organic solvents could penetrate the cuticle reaching the subcuticular layer and the external part of the palisade layer where apparently was the location of the barrier. Cellulosic and/or pectic material in the subcuticular layer and very upper parts of the palisade layer hydrated, swelled and created forces that ruptured the barrier. Prolonged soaking in organic solvents created further breakages or/and separations in the whole palisade cell layer thus creating wide avenues for the massive influx of water into seed that was observed within minutes of imbibition. Drying of the seeds after the organic solvent pre-treatments restored water impermeability to the original levels because the disturbance was temporary induced. In the presence of methanol, water would reach more easily the location of the water impermeability barrier since water is soluble to methanol. When the methanol was dried off, there would be no open pathway for the water movement through the barrier in the uppermost part of the seed coat. The last possibility could explain the difference in the effectiveness between methanol and chloroform in changing the seed coat permeability to water since the seed coat was more permeable to methanol

than to chloroform, and water is soluble in methanol but not in chloroform. In this study, no weak sites were observed (as observed in prickly sida seeds) where water penetration started. The whole seed coat appeared highly permeable within minutes of imbibition.

Egley and Paul (1993) suggested that not one but all three properties (polarity, swelling factor and solubility to water) of the organic solvents could play a role in overcoming the impermeability to water barrier. In this study, it was not possible to identify the role of the physical properties of the solvents due to a small number of solvents used. However, the common imbibition behaviour of seeds of all genotypes tested indicated that the model proposed above for regulation of the water uptake is likely to be universal in soybeans.

CHAPTER 8

General Discussion

8.1. Introduction

In many countries, soybean planting is frequently followed by heavy rains that result in flooded soil conditions. In such conditions, seedling emergence, establishment and growth is poor. It is therefore important to investigate the response of different soybean genotypes to imbibition and to understand the mechanism of regulation of the water uptake during imbibition.

The aims of this study were to measure some physiological effects of the water uptake, and to investigate how the soybean seed coat regulates the water uptake. Special reference was given to: i) the imbibition behaviour of a wide range of genotypes with different seed coat characteristics, ii) the use of a polymer to regulate the rate of water uptake and iii) the mechanism of regulation of water uptake by the soybean seed coat. These aims were achieved by a combination of physiological and anatomical and histochemical studies using a range of soybean genotypes.

The experimental approaches have elucidated many physiological and anatomical points which were properly discussed at the end of each experimental chapter. The purpose of this General Discussion is, therefore, to provide a synthesis of the experimental chapters by summarising and discussing information under four themes; namely, imbibition damage, the use of a polymer to regulate the water uptake, seed coat characteristics related to different imbibition behaviour, and the mechanism of water uptake regulation by the seed coat. These themes were chosen because they pervade the aims and the objectives of this study.

8.2. Imbibition damage

In the present work, a close relationship was observed between the rate of water uptake, the level of leachate conductivity and the percentage of cotyledons fully stained with tetrazolium chloride (section 3.2.2). This situation has been

previously called imbibition damage (Powell and Matthews, 1978). Further support for the cause of imbibition damage in soybeans due to the rapid water uptake was gained either from the improved percentage of cotyledons fully stained with tetrazolium chloride when seeds were slowly imbibed in 30% PEG prior to staining or the aggravation of the percentage of cotyledons fully stained when the seed coat was scarified (Table 3.4). Imbibition damage, as previously described, was a common situation within a range of soybean genotypes. However, there were genotypes that even after the slow imbibition in 30% PEG, exhibited a considerable percentage of cotyledons that were unstained with tetrazolium chloride (Table 3.4). It was suggested, therefore, that seeds had already suffered a significant deterioration which could not be completely overcome by slow imbibition. In contrast, there were genotypes in which slow imbibition resulted in little or no improvement (Table 3.4) indicating that there were genotypes in which imbibition damage did not occur.

Powell *et al.* (1984) proposed that imbibition damage due to rapid water uptake during imbibition was a major physiological factor that caused decline in seed vigour, in many grain legumes. According to this theory, imbibition damage occurred as a combination of physiological damage of the surface of embryos and increased predisposition of seeds to infection by soil-borne fungi. They proposed that under field conditions, reduced seedling emergence and growth observed in flooded soil conditions could be explained on the basis of the imbibition damage that had occurred. However, it could be argued that imbibition damage was a decline in vigour (as measured by high solute conductivity and low staining of the cotyledons with tetrazolium chloride) that may not be entirely correlated with the lost final ability of seeds to germinate. In the literature, the low germination of seeds due to soaking has been frequently called soaking injury or damage. Although, in this study, it was not aimed to establish a relationship between these two situations, results indicated the possibility of alternative or additional theories. When polymer coated seeds were soaked in water, a lower rate of water uptake and lower levels of imbibition damage than corresponding uncoated seeds were observed (section 5.3.2). However, the germinability of the coated seeds was lower than that of the untreated control seeds (Fig. 5.6). This result suggested that although imbibition damage was prevented, the germinability was not restored to the untreated control seeds. In addition, total seedling emergence from coated seeds was lower than the percentage of normal seedlings from the paper towel germination tests (Fig. 5.9). Ferriss and

Baker (1990) also attributed the low soybean seedling emergence from flooded soils in reasons other than the imbibition damage.

In the literature, several other theories have been proposed as the basis of cause of germination failure during soaking of seeds. Some earlier reports have suggested that the fundamental cause of damage was the loss of essential cell constituents during soaking (Eyster, 1940; Barton, 1950; Barton and McNab, 1956). However, when lost constituents were added to water, prevention of imbibition damage did not occur. Another theory ascribed low germination of *Phaseolus vulgaris* seeds to a deficient oxygen supply at a critical early stage of germination. During soaking the cavity between the cotyledons was flooded with an excess of water that remained trapped unless forcibly removed (Orphanos and Heydecker, 1968). They reported that any treatment that increased oxygen supply had a beneficial effect in germination of water soaked seeds. Another theory ascribed the reduction in seedling emergence under flooding to damage by ethanol self-poisoning of seeds (McManmon and Crawford, 1971). They suggested that lactic and malic acids are nontoxic whereas ethanol was a toxic end product. In flooding-resistant genotypes, a malic enzyme activity was detected but not in flooding-susceptible genotypes. However, substances other than ethanol have been suggested as a cause of germination failure during soaking. Harman *et al.*, (1981) reported a close association between poor field seedling emergence and production of a volatile aldehyde during germination particularly in low vigour soybean and pea seeds. Small *et al.*, (1991) concluded that ethylene was not the cause of failure of germination during soaking since it was found to be partially required for the prevention of damage, in *Phaseolus vulgaris* seeds. Another recent theory proposed that failure of *Phaseolus* seeds to germinate during soaking was due to a lack of oxidative pentose phosphate pathway activity which resulted in a depressed protein synthesis (Pretorious and Small, 1992).

8.3. Use of a polymer to regulate water uptake

Polymer application resulted in formation of a film coating on the surface of seeds which was sufficient to reduce the rate of the water uptake without affecting the total water uptake (section 5.3.1). The suitability of the polymer for water uptake regulation is therefore indicated. The applicability, however, of polymer coating in

soybean seeds (or in other grain legumes which are prone to imbibition damage), is challenged by the following concerns:

Firstly, the reduction in water uptake due to polymer coating must be minimum and balanced in order to allow a rapid and uniform seedling emergence and establishment under commercial growing conditions. Enhancing the rate of germination to get the seedlings out of the soil as early as possible or before a soil crust has formed, was a priority in soybean agronomic practices (Hinson and Hartwig, 1982). This was particularly true in many tropical and sub-tropical soybean cultivation regions where although most of the seeds germinate, the seedlings were trapped within the soil structure (Dadson, 1982; Emerson, 1982). Coating seeds (24 mg per seed) with the polymer resulted in no significant difference between coated and uncoated seeds in the seedling growth in controlled conditions (section 5.3.3). It is, however, doubtful if this would be true under normal growing conditions. This requires further investigation by sowing coated seeds under range of growing conditions.

Secondly, polymer coating represents a seed treatment with an extra cost to seeds. In most tropical and sub-tropical soybean cultivation regions, this extra cost could not be afforded by farmers. From the economic point of view, only vegetable seeds could be so coated due to the greater profit margin in comparison to grain legumes.

8.4. Seed coat characteristics in relation to different imbibition behaviour

Water uptake did not occur uniformly throughout the soybean seed coat. Wrinkling of the seed coat started from the dorsal region, and then covered the abaxial and ventral region of the seed (section 3.2.2). Measurements of rate of water uptake through different regions of the seed coat indicated that regardless how open the hilar fissure was, the ventral region facilitated the lowest rate of water uptake whereas there was no observed difference between the dorsal and abaxial region of the seed coat in the rate of water uptake (Fig. 3.5). Additionally, from the results with nail varnish, it was shown that the hilar region played little or no role in water uptake in comparison to the permeability of the testa (Fig. 3.6). Calcofluor was used as a water-soluble fluorescent stain to directly follow the water penetration through the seed coat. From observations using calcofluor, it was concluded that water could only penetrate through the tracheid bar but not through the outer and inner palisade

layers (Fig. 6.9). As a result, although the hilar fissure was open, the rate of water uptake in the hilar side was lower than that of the dorsal side.

The condition of the seed coat appeared to be an important factor in relation to the occurrence of imbibition damage. Lines GC 84128-17-2-1 and AGS 292 that had a high proportion of seeds with split coats, had the highest rate of water uptake, the highest amount of leachate conductivity and the lowest percentage of cotyledons fully stained with tetrazolium chloride (section 3.2.1). However, the presence of intact seed coat was not always sufficient to reduce the rate of water uptake, thus offering no protection against imbibition damage. In seeds of lines GC 88037-38-2-2 and SS 87040-2-1, although the seed coat was apparently intact (examined visually and under low magnification stereoscope), a high level of imbibition damage occurred (section 3.2.1). A surface view of the seed with SEM, revealed the presence of a high proportion of very deep and wide open pits (Fig. 6.4). Wrinkling of the seed coat was observed in all regions of the seed within minutes of imbibition (section 3.2.2).

Kuo (1989) identified some soybean genotypes with black seed coats that possessed a delayed-water permeable seed coat during imbibition. In this study, a similar seed coat characteristic was identified in seeds of line VLS-1 that had possessed black testas. In addition, this delayed-permeability character was associated with low levels of imbibition damage (section 3.2.1). A surface view of such seeds with SEM, revealed that seeds of line VLS-1 had few and shallow pits in the abaxial region (Fig. 6.4). Also, wrinkling of the seed coat of such seeds was observed in only the dorsal region of the seed until after 2h of imbibition (section 3.2.2). It was, therefore, suggested that the lack of pits in the abaxial region of the seed coat could explain the delayed-permeability character in soybeans. This observation could open up the possibility of breeding soybean cultivars with seeds that possess the delayed-permeability character. Since seeds with black testas are not preferred by consumers (Wolf *et al.*, 1981), the delayed-permeable character (lack of pits in the abaxial region of the seed coat) would need to be transferred to agronomically important cultivars with yellow seed coats.

There was no clear relationship between the dark colour of the seed coat and the rate of water uptake since it was observed that genotypes with dark coloured coats imbibed water at a high and low rate (section 3.2.1). However, definite

conclusions about the association of the dark coat colour and the rate of the water uptake could not be made due to the small number of genotypes examined that had pigmented coats. Starzinger and West (1982) reported that pigmented seeds had a greater percentage germination than otherwise genetically similar yellow seeds due to high levels of tannins and phenolic material present in the seed coat. The black seed coat colour has been proposed as a characteristic to be closely associated with superior seed quality in comparison to genotypes with yellow seed coats (Dassou and Kueneman, 1984). However, Wien and Kueneman (1981) reported that some genotypes with black seed coats were found to possess poor longevity during storage.

In this study, seeds of cv. Suwan-155 (black testa) had a high rate of water uptake that resulted in high levels of imbibition damage (section 3.2.1). A loose adherence of the seed coat to the embryo was observed when seeds were visually examined (Fig. 3.8b). This loose adherence resulted in a wide gap between these two structures and water had trapped in this gap during imbibition. In this study, it was shown that the increased rate of water uptake of seeds subjected to one cycle of wetting and drying could not be attributed to a loosening of the adherence of the seed coat to the embryo. Seeds subjected to wetting and drying appeared to have coats with extensive ruptures (Fig. 3.9). In addition, a SEM surface view of seeds subjected to wetting and drying revealed seed coat cracking and an increased size of the individual pits due to the treatment (Fig. 7.1). Although, the adherence of the seed coat to the embryo could play an important role in the water uptake in soybeans, it was difficult to measure or quantify it.

It appeared that no single individual seed coat characteristic could provide a sound basis for selection of soybean genotypes for superior seed quality. This was probably because a clear separation amongst genotypes in relation to seed quality may not be based on a single individual seed characteristic but a combination of them.

8.5. Mechanism of regulation of water uptake by the seed coat

In this study, in order to elucidate the mechanism of the water regulation by the seed coat a number of different approaches were used. Water penetration was successfully followed by the use of calcofluor as a water-soluble fluorescent stain.

The seed coat structure (surface or anticlinal) was observed by SEM or light microscope techniques. The seed coat histochemistry was studied by using appropriate light and fluorescent staining techniques. Finally, changes in the seed coat due to the treatment with organic solvents were associated with changes in the water permeability of the seed coat. Studies were made using a wide range of genotypes with different imbibition behaviour. On one hand, studies were made on genotypes with normal (soft) seeds that had a low, medium or high rate of water uptake. In contrast, studies were also made on genotypes with different proportions of hard seeds. As a result, a whole range of imbibition behaviour was studied using a range of genotypes, therefore the results obtained are more likely to be of universal significance in soybeans.

Two aspects of water uptake by seeds are important in considering the mechanism of water regulation by the seed coat: the initial sites of water penetration and the subsequent water penetration through the seed coat.

8.5.1. Initial sites of water penetration

Deposits and pits were observed by SEM on the surface of the seed coat in most genotypes examined. In the literature, there is some contradiction in relation to the nature and role of deposits during imbibition. Deposits have been either described as a waxy material (cutins) which hinders the water uptake or as a residues of the pod endocarp which may not play any role in the water uptake. Results from this study, provide strong evidence that deposits were cellulosic material most likely to be residues of the pod endocarp which could not play any role in the water uptake during imbibition (section 6.3.1 and Fig. 6.8). The fibrous nature of deposits which contain calcium or silicate could provide a basis to understand why soybean-miso manufacturers prefer soybean seeds with glossy coats (Saio, 1976).

Assessment of pitting was performed after the removal of deposits by methanol and pits were present on the surface of the seed coat in most genotypes (section 6.3.1). Individual pits appeared in three types, namely deep (round and elongated) and shallow (Fig. 6.5). In most cases, there was a combination of all types without being possible to identify a predominant type of structure of individual pits. Using calcofluor as a water-soluble fluorescent stain, there was a clear correlation between the sites of initial water penetration to the distribution of pits in the surface of the

seed coat (Fig. 6.6 and Fig. 6.7). In hard seeds pitting was varied. In hard seeds of two of the genotypes, pits appeared to be shallow but in the third genotype the pits appeared to be deep (Fig. 7.2). Apparently, the mere presence of numerous deep pits does not make the seed coat of a hard seed permeable to water. Observations from the use of calcofluor as a water-soluble stain showed that the staining was absent indicating that the pits did not function in the same way as in soft seeds (Fig. 7.4). Although, it is logical to assume that shallow pits are less suited to allowing water penetration, explanations are needed for the failure of deep pits to facilitate water uptake.

8.5.2. Subsequent water penetration through the seed coat

Water uptake progresses in the seed with the water penetration through the seed coat itself. Using calcofluor as a water-soluble fluorescent stain, water penetration in the hilar, abaxial and ventral region of the seed coat was examined. In soft seeds, even after minutes of imbibition, water was present in the palisade cell layer (Fig. 6.10a). As imbibition was progressed, more water was present in the palisade layer as revealed by the strong calcofluor staining (Fig. 6.10b and Fig. 6.10c). It was not possible to identify clear differences in the water presence in the palisade layer within regions of the seed coat. However, regardless of the region examined, it was clear that water penetration did not occur uniformly through the palisade layer since groups of palisade cells showed a stronger staining than the adjacent cells (Fig. 6.10c). In the hilar region, water penetration was observed through the tracheid bar but not through the outer and inner palisade cell layers (Fig. 6.9).

In hard seeds, localisation of the presence of the barrier to water uptake within the seed coat was successfully achieved using calcofluor as a water-soluble fluorescent stain (section 7.3.3). Anticlinal sections of the seed coat of hard seeds soaked for 24h in calcofluor, showed that the subcuticular layer was clearly stained (Fig. 7.6). As a result, it was apparent that the cuticle was not the primary location of the water permeability barrier in soybeans. There was no difference between the different regions of the seed in the distribution of staining within the seed coat indicating the uniformity of the presence of the water barrier.

Changes in the seed coat due to the treatment with organic solvents were associated with changes in the water permeability of the seed coat.

In soft seeds, two hours of successive immersion of whole seeds, at room temperature, in methanol, methanol: chloroform (1:1) and chloroform was utilised to remove both epicuticular and intracuticular waxes (Fig. 6.11). Seeds that had been pre-treated with the previous combination of solvents showed a significant increase in the water uptake in comparison to the untreated control seeds (Fig. 6.11). However, the increase in the water uptake could not be attributed to intracuticular waxes. Methanol pre-treatments, in particular, were highly effective in increasing the water uptake during imbibition (section 6.3.3). There was no region of the seed that was observed to be a point of rapid water penetration but the whole seed coat was highly permeable to water. The effectiveness of the methanol pre-treatment was particularly evident in seeds with a low water uptake (section 6.3.3).

In hard seeds, there was a clear difference between the effect of methanol pre-treatment and the effect of chloroform pre-treatment when hard seeds were placed for imbibition immediately after the pre-treatments (Fig. 7.17). Methanol effectively changed all hard seeds to soft and the duration of the pre-treatment that was required for this effect was genotype-dependent (Fig. 7.18a). Once all hard seeds became soft, further methanol pre-treatment had a substantial effect in the amount of water absorbed during imbibition (Fig. 7.18a). In contrast, a small proportion of hard seeds became soft following pre-treatment with chloroform regardless of the duration of the pre-treatment (Fig. 7.18b).

Results from the absorption spectrum of the different methanol and chloroform supernatants indicated that the effect of the organic solvent pre-treatments on the water uptake were not due to the extraction of phenolic material from the seed coat (Fig. 6.15 and Fig. 7.19). Drying of seeds after the organic solvent pre-treatments restored water permeability to the original untreated control levels. In soft seeds, drying reduced the rate of water uptake to similar level of the untreated control seeds (Fig. 6.13). In hard seeds, drying restored impermeability to about 90% of seeds (Fig. 7.20). If something which caused either delayed water permeability or impermeability was extracted from the seed coat, it would not be restored on drying of the seeds after the pre-treatments.

The following model was suggested to provide an explanation in relation to the effects of organic solvents, particularly methanol, on the water permeability in

soybean seeds. Organic solvents could penetrate the cuticle reaching the subcuticular and palisade layer. In soft seeds, as the duration of the treatment increased, the presence of solvent reached the inner parts of the palisade layer. Cellulosic and/or pectic material in the subcuticular and palisade layer would have hydrated in the presence of organic solvent, these carbohydrates swell and created forces in the palisade cells that caused breaking and/or separation of the cells. In hard seeds, this region (outermost part of the palisade cell layer) was the location of the water impermeability barrier that has to be ruptured first. The breakage/separation of the palisade cells opened avenues for the influx of water into seed that was observed within minutes of imbibition. Drying of the seeds after the pre-treatment restored water permeability to the original levels because the disturbance was temporarily induced. In addition, in the presence of methanol, water would reach all the seed coat cells invaded by methanol since methanol is soluble to water. When the methanol was dried off, there would be no open pathway for the water movement through the layers of the seed coat. The last possibility could explain the difference in the effectiveness between methanol and chloroform pre-treatments. The common imbibition behaviour of seeds of all genotypes tested indicated that the model proposed above for regulation of the water uptake was likely to be universal in soybeans.

The nature of the impermeability barrier to water by comparative anatomical and histochemical methods was not detected. However, it is logical to assume that the cell walls in the uppermost region of the seed coat of soft seeds would be more brittle, less resilient than those in hard seeds thus offering less resistance to water penetration. What causes this situation is not known but several possibilities exist.

Firstly, it may be possible that phenolic and/or pectic material differ in the way they cross-link with the cell wall components thus creating differing degrees of resistance to water penetration. Briggs and Fry (1987) suggested that phenolics commonly occur as components of cell walls and the possible function of phenolic material may be to cross-link cell wall components, thus strengthening the wall. A possible difference between hard and soft seeds in the cross-linking of the above material with the cell walls may be due to reasons that are not detectable with microscopy techniques (e.g. methylation, ionic binding or degree of polymerization of the phenolic and/or pectic material).

Secondly, it may be possible that the soybean seed coat impermeability to water may be a sum of several factors related to changes in the physical

characteristics of the seed coat during the later stages of seed development. These factors may include a tightly packed palisade cells and tightly bonded cell layers. Several authors have reported the soybean seed coat became impermeable to water during the final stages of seed development when seed moisture levels fell (Duangapatra, 1978; Yaklich *et al.*, 1986). During this time, cells in the seed coat collapsed, the seed coat shrank and tightly bonded cell layers resulted (Yaklich *et al.*, 1986).

Thirdly, it may possible that drying of pectic material and other gel-like cellular material, during the later stages of seed development, to contribute in the development of the impermeability barrier to water (Egley, personal communication). This might be comparable to the drying of mucilage to form a "glue".

8.6. Conclusions

In this study, imbibition damage (inferred by high leakage conductivity and low percentage of cotyledons fully stained with Tetrazolium chloride) due to rapid water uptake was documented in a wide range of soybean genotypes. However, some genotypes were identified to be resistant (line VLS-1) or susceptible (lines GC 88037-38-2-2 and SS 87040-2-1) to imbibition damage due to low or rapid water uptake respectively. Therefore, the original aim whereby genotypes with different imbibition behaviour and hence different levels of imbibition damage were to be identified was accomplished. Seed size or seed coat colour were uncorrelated with the low or high levels of imbibition damage observed in these genotypes. The hilum region of the seed coat facilitated the slowest rate of the water uptake during the first 4h of imbibition whereas no difference between the dorsal and the abaxial region was observed. In seeds of cv. Suwan-156, that was shown to have a rapid-permeability seed coat characteristic, the seed coat was adhered only loosely to the embryo. However, the role of seed coat adherence *per se* in relation to rate of water uptake appeared to be difficult to measure or quantify. As a result, more work on this aspect needs to be done and appropriate methods developed to quantify the seed coat adherence to the embryo.

From structural studies performed in this thesis, the density and structure of pits appeared to be an important seed coat characteristic in relation to levels of imbibition damage. In seeds of line VLS-1, that possessed the delayed-permeability seed coat characteristic, there was a lack of pits in the abaxial region of the seed coat. In seeds of lines GC 88037-38-2-2 and SS 87040-2-1, both of which possessed a rapid-permeability seed coat characteristic, a high density of deep and wide open pits were observed. Therefore, the original objective set to identify seed coat characteristics that were correlated with different levels of imbibition damage was accomplished. The implication of the identification of a delayed-permeable seed coat characteristic (lack of pits in the abaxial region) is important to soybean breeding. As a result, genotypes resistant to imbibition damage due to the possession of a delayed-permeable seed coat characteristic could be selected. Careful screening for agronomically-important, yellow seed coated genotypes that are resistant to imbibition damage might be one option. Another option might be the transfer of the trait (if inheritable) from the black seeded genotype (line VLS-1) to other yellow seeded agronomically-important genotypes.

Coating seeds (24 mg per seed) with a polymer regulated the rate of water uptake and offered protection against imbibition damage during soaking in water. However, the germinability of the coated seeds was lower than that of the untreated control seeds. Percentage seedling emergence from polymer coated seeds was also lower than the uncoated seeds during flooding soil conditions. Although imbibition damage was prevented by application of a polymer, the germinability was not restored to the untreated control seeds. This result suggested that regulation of the rate of water uptake by using a polymer could only partially avoid the diminished seedling emergence in flooded soil conditions. Therefore, the original objective of investigating the effect of polymer coating on regulation of water uptake, as a mechanism to prevent imbibition damage and improve germination and seedling emergence and growth was shown to be problematical in practice. As a result, more work should be done to identify the causes of low germination in cases where imbibition damage was completely overcome.

From the structural studies, deposits and pits occurred in the surface of the seed coat in most genotypes. Deposits were shown to be hydrophilic material consisting of polysaccharide since staining with calcofluor was observed. Pits were shown to be the sites of the initial water penetration through the seed coat as indicated by calcofluor staining. In hard seeds, two genotypes had seeds with few and shallow pits whereas the third genotype had many and deed pits. Although, it is likely that shallow pits are less suited to allow water penetration, explanations are needed for the failure of deep pits to facilitate water uptake. Therefore, the original objective of identifying the role of deposits and pits on the regulation of water uptake was mostly achieved.

In hard seeds, the location of water impermeability barrier was near the outermost part of the palisade cell layer. The nature of the barrier was not identified by comparative anatomical and histochemical studies between hard and soft seeds. Therefore, the original objective of defining the locality and identification of the nature of the water impermeability barrier using histochemical methods were only partially accomplished. Future work, on the nature of the barrier, should be performed employing the facility of a confocal microscope.

In both hard and soft seeds, prolonged methanol pre-treatments were highly effective in increasing the water uptake when seeds placed for imbibition

immediately after the pre-treatments. It was concluded that the cuticle and its components (epicuticular and intracuticular waxes) played little role in regulating water uptake. Drying of seeds after the organic solvent pre-treatments restored permeability to water to the original untreated control levels. Results from the absorption spectrum of the methanol and chloroform supernatants, indicated that the effect of the pre-treatments were not due to the extraction of UV-absorption substances from the seed coat. It was suggested that organic solvent pre-treatments would cause hydration and swelling of cellulosic and/or pectic material in the subcuticular and palisade layer. The created forces would cause temporary disturbances in the cells to facilitate the rapid influx of water into seed. When the solvent was dried off, there would be no open pathway for the water movement through the layers of the seed coat.

A mechanism for the water uptake regulation by the soybean seed coat was proposed. The proposed mechanism involved: i) a diminished role of deposits, the cuticle and its components (epicuticular and intracuticular waxes), ii) a key role for pits as initial sites of water penetration, and iii) swelling or collapse of the cellulosic and/or pectic material in the subcuticular and palisade cell layer that could regulate water penetration through the seed coat. The common behaviour of a wide range of genotypes tested indicated that the above mechanism of regulation of water uptake by the seed coat is likely to be universal in soybeans. Therefore, the original aim of investigating the mechanism of regulation of water uptake by the soybean seed coat, using light, fluorescence and scanning electron microscopy techniques, was mostly accomplished. However, more work in this area is needed to elucidate other elements of the mechanism of water regulation by the soybean seed coat. Future work should concentrate on how cellulosic and/or pectic material could regulate the water penetration using either confocal microscope or non-microscopical techniques.

8.7. Suggestions for future research

8.7.1. Investigation of regulation of water uptake by the seed coat using Confocal Microscope

In soybeans, the imbibition process is greatly regulated by the seed coat. How the seed coat structure achieves this regulation is largely unknown although associations of seed coat characteristics with rate of water uptake have been reported. The confocal microscope is ideally suited to study imbibition process, and calcofluor would be an appropriate water-soluble fluorescent stain for this type of investigation. There has been no published information about the control of imbibition by seed coat using confocal microscope. It would also be interesting to combine changes due to the organic solvent pre-treatments with confocal microscopy studies. Finally, this type of study is ideally suited for the investigation of the breaking process of hardseedness in seeds.

8.7.2. Changes in the seed coat structure during seed development and maturation

In other grain legumes, there have been several reports on changes in water uptake during seed coat development which are correlated with changes in the pigmentation of the seed coat. It would be important to investigate whether such changes also exist in the soybeans. Additionally, changes in the structure and histochemistry that are related to the water uptake may provide a broad spectrum of unaddressed research questions. This type of study would require growing and harvesting soybeans at different growing developmental stages during seed maturation.

8.7.3. Investigation of the cause of soaking damage

Several reports have shown that soybean and other legume seeds would suffer diminished germination in flooded soil conditions. In most grain legumes, the relationship between imbibition damage and soaking damage is far from clear or straightforward. It is, therefore, important to separate the different elements during the imbibition process (rate of water uptake, oxygen availability, direct or indirect carbon dioxide effect). Additionally, it is important to separate the effects of imbibition and soaking damage on cotyledons and embryonic axis.

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APPENDICES

APPENDIX 1

1. Section 3.3.1.

Table 1.1. Analysis of variance in seed dry weight (mg per seed) in all genotypes (Table 3.1).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	269560	19	14187	77.6	S***
residual	7306	40	182		
total	276866	59			

Table 1.2. Analysis of variance in seed surface area (mm^2 per seed) in all genotypes (Table 3.1).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	494	19	26.1	29.1	S***
residual	36	40	0.9		
total	530	59			

Table 1.3. Analysis of variance in seed coat dry weight (mg per seed) in all genotypes (Table 3.1).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	93158	19	4903	478	S***
residual	410	40	10.4		
total	93568	59			

2. Section 3.3.2.

Table 1.4. Analysis of variance between seed weight increase (%) at 6h of imbibition, produced by intact seeds and embryos (seed coat) for all genotypes (Table 3.2).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	12735	19	670	39.2	S***
seed coat	20742	1	20742	1216.4	S***
Interaction	13243	19	697	40.8	S***
residual	2046	120	17		
total	48767	159			

Table 1.5. Analysis of variance in electrical conductivity (mS/cm/g) at 6h of imbibition, produced by intact seeds and embryos (seed coat) for all genotypes (Table 3.2).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	943	19	49	14.4	S***
seed coat	5906	1	5906	1723.4	S***
Interaction	633	19	33	9.7	S***
residual	411	120	3.2		
total	7894	159			

Table 1.6. Analysis of variance in seed weight increase (%) during imbibition, for all genotypes (Fig. 3.2).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	10756	19	566	37.9	S***
time	24865	1	24856	1668.2	S***
Interaction	14238	19	749	50.3	S***
residual	1795	120	15		
total	51645	159			

Table 1.7. Analysis of variance in percentage of cotyledons fully stained with Tetrazolium Chloride, produced after imbibition in water or 30% PEG with the seed coat intact or scarified (treatment) for all genotypes (Table 3.4).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	50584	19	2662	74.1	S***
treatment	75871	3	25290	704.1	S***
Interaction	24296	57	426	11.8	S***
residual	2871	80	36		
total	153624	159			

3. Section 3.2.3.

Table 1.8. Analysis of variance in the weight of water imbibed (mg/seed) during the first 3h of imbibition (time) when the dorsal, ventral or abaxial region of the seed was exposed to water (region) for seeds of cv. Sapporo (Fig. 3.5a).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
region	816	2	408	36.9	S***
time	4241	3	1413	128	S***
Interaction	111	6	18.6	1.6	NS
residual	1191	108	11.2		
total	6360	119			

Table 1.9. Analysis of variance in the weight of water imbibed (mg/seed) during the first 3h of imbibition (time) when the dorsal, ventral or abaxial region of the seed was exposed to water (region) for seeds of cv. KWS-E (Fig. 3.5b).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
region	147	2	73	3.9	S***
time	5067	3	1689	91.2	S***
Interaction	56	6	9.4	0.5	S***
residual	1999	108	18.5		
total	7270	119			

Table 1.10. Analysis of variance in the weight of water imbibed (mg/seed) during the first 4h of imbibition (time) when the hilum was warnished for seeds of all genotypes (Fig. 3.6).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotypes	16987	3	5662	77.6	S***
varnish	116	1	116	1.6	NS
time	55984	3	18661	255.6	S***
interaction gen. x varn.	7549	3	2516	34.5	S***
residual	1613	22	73		
total	82249	32			

Table 1.11. Analysis of variance in the weight of water imbibed (mg/seed) during the first 4h of imbibition (time) after wetting and drying (treatment) for seeds of all genotypes (Fig. 3.7).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotypes	19967	3	6655	80.2	S***
treatment	1345	1	1345	16.2	S***
time	61719	3	20573	247.8	S***
interaction gen. x treat.	9948	3	3316	39.9	S**
residual	1829	22	83		
total	94808	32			

APPENDIX 2

1. Section 4.3

Table 2.1. Analysis of variance between percentage of normal seedlings (%), produced by seeds after aging for all genotypes (Fig. 4.1).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	8870	4	2217	83.3	S***
aging	55984	4	13996	525.8	S***
Interaction	11635	16	727	27.3	S***
residual	2032	75	27.1		
total	78523	99			

Table 2.2. Analysis of variance between seed moisture content (%), produced by seeds during aging for all genotypes (Fig. 4.3).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	243	4	60	717.3	S***
aging	3241	4	810	9546.1	S***
Interaction	65	16	4	48.4	S***
residual	2	25	0.08		
total	3552	49			

Table 2.3. Analysis of variance between electrical conductivity (mS/g/cm), produced by seeds after aging for all genotypes (Fig. 4.5).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	6850	4	1712	74.4	S***
aging	35781	4	8945	388.9	S***
Interaction	10216	16	638		S***
residual	1729	75	23		
total	54576	99			

Table 2.4. Analysis of variance between fresh weight per normal seedling (mg), produced by seeds after aging for all genotypes (Fig. 4.7).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	1102485	4	275621	331.2	S***
aging	812459	4	203114	244.1	S***
Interaction	207095	16	12943	15.5	S***
residual	62439	75	832		
total	2184478	99			

Table 2.5. Analysis of variance between shoot fresh weight per normal seedling (mg), produced by seeds after aging for all genotypes (Fig. 4.8a).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	1100087	4	275021	341.1	S***
aging	750725	4	187681	232.7	S***
Interaction	202693	16	12668	15.7	S***
residual	58032	75	806		
total	2111537	99			

Table 2.6. Analysis of variance between fresh weight per normal seedling (mg), produced by seeds after aging for all genotypes (Fig. 4.8b).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	2386	4	596	9.8	S***
aging	61719	4	15429	254.9	S***
Interaction	4391	16	274	4.5	S***
residual	4500	75	60		
total	72996	99			

Table 2.7. Analysis of variance between vigour index (mg), produced by seeds after aging for all genotypes (Fig. 4.9).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	1383880	4	345970	49.2	S***
aging	35797020	4	8949255	1273.5	S***
Interaction	2546780	16	159173	22.6	S***
residual	527060	75	7027		
total	7299600	99			

APPENDIX 3

1. Section 5.3.1

Table 3.1. Analysis of variance between weight of water (mg/seed), absorbed by seeds of cv. Forrest coated with polymer during imbibition (Fig. 5.2).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
polymer	26246	3	8748	56.8	S***
time	678909	8	84863	551.1	S***
pol. x tim.	48301	24	2021	13.1	S***
residual	47927	311	154		
total	801383	359			

Table 3.2. Analysis of variance between weight of water (mg/seed), absorbed by seeds of cv. Douglas coated with polymer during imbibition (Fig. 5.3).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
polymer	22253	3	7417	39.2	S***
time	573529	8	71691	379.3	S***
pol. x tim.	38989	24	1624	8.59	S***
residual	59029	311	189		
total	693800	359			

Table 3.3. Analysis of variance between percentage of normal seedlings, produced by seeds of the two genotypes coated with polymer (Fig. 5.4).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	1.5	1	1.5	0.1	NS
polymer	5815	3	1938	27.6	S***
gen. x pol.	116	3	38	0.5	NS
residual	1683	24	70		
total	7617	31			

Table 3.4. Analysis of variance between percentage of normal seedlings, produced by seeds of the two genotypes, with different ageing regimes, coated with polymer (Fig. 5.5).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	957	1	957	22.2	S***
polymer	2.5	1	2.5	0.1	NS
ageing	3180	1	3180	73.9	S**
Interactions					
gen. x pol.	26	1	26	0.6	NS
gen. x ag.	693	1	693	16.1	S***
pol. x ag.	5.2	1	5.2	0.1	NS
gen. x pol. x ag.	3.7	1	3.7	0.1	NS
residual	1032	24			
total	5901	31			

2. Section 5.3.2

Table 3.5. Analysis of variance between percentage of normal seedlings, produced by seeds of the two genotypes, with different ageing regimes, coated with polymer after soaking in water for 24h (Fig. 5.6).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	520	1	520	9.2	S***
ageing	5002	1	5002	89.3	S***
polymer	18275	2	9137	163.2	S**
Interactions					
gen. x ag.	33	1	33	0.5	NS
gen. x pol.	205	2	102	1.8	NS
ag. x pol.	69	2	35	0.6	NS
gen. x ag. x pol.	263	2	131	2.3	NS
residual	2015	36	56		
total	26384	47			

Table 3.6. Analysis of variance between electrical conductivity (mS/g/cm), produced by seeds of the two genotypes, with different ageing regimes, coated with polymer after soaking in water for 24h (Fig. 5.7).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	621	1	621	24.4	S***
polymer	488	1	488	19.2	S***
ageing	1875	1	1875	73.8	S**
Interactions					
gen. x pol.	81	1	81	3.1	NS
gen. x ag.	116	1	116	4.5	S*
pol. x ag.	34	1	34	1.3	NS
gen. x pol. x ag.	9.1	1	9.1	0.3	NS
residual	609	24	25.4		
total	3835	31			

Table 3.7. Analysis of variance between percentage of cotyledons fully stained with TTC, produced by seeds of the two genotypes, with different ageing regimes, coated with polymer after soaking in water for 24h (Fig. 5.8).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	6745	1	6745	95.3	S***
polymer	11029	2	5514	77.9	S***
ageing	11501	1	11501	162.5	S**
Interactions					
gen. x pol.	299	2	149	2.1	NS
gen. x ag.	130	1	130	1.8	NS
pol. x ag.	23	2	12	0.1	NS
gen. x pol. x ag.	59	2	29	0.4	NS
residual					
total					

5.3. Section 5.3.3

Table 3.8. Analysis of variance between percentage of seedling emergence, produced by seeds of the two genotypes, with different ageing regimes, coated with polymer subjected to two soil water regimes (Fig. 5.9).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	3690	1	3690	62.8	S***
soil	16900	1	16900	288.1	S***
polymer	10100	1	10100	172.1	S***
ageing	11610	1	11610	197.8	S**
Interactions					
gen. x soil.	3	1	3	0.5	NS
gen. x pol.	495	1	495	8.4	S**
gen. x ag.	16	1	16	0.2	NS
soil. x pol.	272	1	272	4.6	S*
soil. x ag.	1242	1	1242	21.1	S***
pol. x ag.	115	1	115	1.9	NS
gen. x soil. x pol.	0.5	1	0.5	0.1	NS
gen. x soil. x ag.	272	1	272	4.6	S*
gen. x pol. x ag.	100	1	100	1.7	NS
soil. x pol. x ag.	495	1	495	8.4	S**
gen. x soil. x pol. x ag.	2	1	2	0.1	NS
residual	2816	48	58.6		
total	48132	63			

Table 3.9. Analysis of variance between time to 50% seedling emergence (days), produced by seeds of the two genotypes, with different ageing regimes, coated with polymer subjected to two soil water regimes (Fig. 5.10).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	0.1	1	0.1	0.1	NS
soil	36	1	36	24.6	S***
polymer	20	1	20	13.7	S***
ageing	11	1	11	7.8	S**
Interactions					
gen. x soil.	0.5	1	0.5	0.3	NS
gen. x pol.	1.1	1	1.1	0.7	NS
gen. x ag.	0.1	1	0.1	0.1	NS
soil. x pol.	0.2	1	0.2	0.1	NS
soil. x ag.	0.7	1	0.7	0.5	NS
pol. x ag.	0.1	1	0.1	0.1	NS
gen. x soil. x pol.	0.1	1	0.1	0.1	NS
gen. x soil. x ag.	0.1	1	0.1	0.3	NS
gen. x pol. x ag.	0.5	1	0.5	0.1	NS
soil. x pol. x ag.	0.2	1	0.2	0.1	NS
gen. x soil. x pol. x ag.	0.6	1	0.6	0.4	NS
residual		48			
total		63			

Table 3.10. Analysis of variance between shoot length (cm), produced by seeds of the two genotypes, with different ageing regimes, coated with polymer subjected to two soil water regimes (Fig. 5.11).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	1.2	1	1.2	0.7	NS
soil	49	1	49	31.9	S***
polymer	7.6	1	7.6	4.9	S*
ageing	31	1	31	20.3	S***
Interactions					
gen. x soil.	0.1	1	0.1	0.1	NS
gen. x pol.	1.1	1	1.1	0.7	NS
gen. x ag.	0.1	1	0.1	0.1	NS
soil. x pol.	0.2	1	0.2	0.1	NS
soil. x ag.	0.5	1	0.5	0.3	NS
pol. x ag.	3.2	1	3.2	2.1	NS
gen. x soil. x pol.	0.2	1	0.2	0.1	NS
gen. x soil. x ag.	1.1	1	1.1	0.6	NS
gen. x pol. x ag.	0.8	1	0.8	0.5	NS
soil. x pol. x ag.	0.1	1	0.1	0.1	NS
gen. x soil. x pol. x ag.	0.4	1	0.4	0.3	NS
residual	74	48	1.5		
total	172	63			

Table 3.11. Analysis of variance between shoot fresh weight per normal seedling (mg), produced by seeds of the two genotypes, with different ageing regimes, coated with polymer subjected to two soil water regimes (Fig. 5.12).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	1.9	1	1.2	0.7	NS
soil	53	1	49	28.8	S***
polymer	9.6	1	7.6	4.4	S*
ageing	43	1	31	18.2	S***
Interactions					
gen. x soil.	0.2	1	0.1	0.1	NS
gen. x pol.	1.6	1	1.1	0.6	NS
gen. x ag.	0.2	1	0.1	0.1	NS
soil. x pol.	0.1	1	0.2	0.1	NS
soil. x ag.	0.9	1	0.5	0.3	NS
pol. x ag.	4.2	1	3.2	1.8	NS
gen. x soil. x pol.	0.2	1	0.2	0.1	NS
gen. x soil. x ag.	0.9	1	1.1	0.6	NS
gen. x pol. x ag.	0.6	1	0.8	0.5	NS
soil. x pol. x ag.	0.2	1	0.1	0.1	NS
gen. x soil. x pol. x ag.	0.3	1	0.4	0.2	NS
residual	81	48	1.7		
total	168	63			

APPENDIX 4

1. Section 6.2.3

Table 4.1. Analysis of variance between weight of water (mg/seed) after 30 mins, absorbed by seeds subjected to different organic solvent pre-treatments, for all genotypes (Fig. 6.11).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	47173	5	9434	138.8	S***
pre-treatment	28086	4	7021	103.3	S***
gen. x pre-tr.	6208	20	310	4.5	S***
residual	18339	270	67.9		
total	99808	299			

Table 4.2. Analysis of variance between weight of water (mg/seed), after 30 and 60 mins of imbibition, absorbed by seeds of cv. Sapporo subjected to different methanol pre-treatments (Fig. 6.12a).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
pre-treatment	66744	5	13348	131.5	S***
time	12979	1	12979	127.9	S***
pre-tr. x tim.	894	5	178	1.7	NS
residual	10959	108	101		
total	91577	119			

Table 4.3. Analysis of variance between weight of water (mg/seed), after 30 and 60 mins of imbibition, absorbed by seeds of cv. Sapporo subjected to different chloroform pre-treatments (Fig. 6.12b).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
pre-treatment	21237	5	4247	110.5	S***
time	6351	1	6351	165.2	S***
pre-tr. x tim.	109	5	22	0.5	NS
residual	4151	108	38		
total	31848	119			

Table 4.4. Analysis of variance between weight of water (mg/seed) after 30 mins, absorbed by seeds subjected to different organic solvent pre-treatments, for all genotypes (Fig. 6.11).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	35247	4	8811	94.7	S***
pre-treatment	19257	2	9628	103.5	S***
gen. x pre-tr.	7528	8	941	10.1	S***
residual	12412	133	93		
total	74444	149			

Table 4.5. Analysis of variance between weight of water (mg/seed) after 30 mins, absorbed by seeds subjected to different organic solvent pre-treatments, for all genotypes (Fig. 6.14).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	42138	5	8427	120.3	S***
pre-treatment	29846	4	7461	106.6	S***
gen. x pre-tr.	8258	20	412	5.8	S***
residual	18968	270	70		
total	99210	299			

APPENDIX 5

1. Section 7.3.6.

Table 5.1. Analysis of variance between weight of water (mg/seed) after 30 mins, absorbed by hard seeds subjected to different organic solvent pre-treatments, for all genotypes (Fig. 7.17).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	4261	2	2130	13.5	S***
pre-treatment	8447	3	2815	17.9	S***
gen. x pre-tr.	12560	6	2093	13.3	S***
residual	16985	108	157		
total	42255	119			

Table 5.2. Analysis of variance between weight of water (mg/seed) after 30 mins, absorbed by hard seeds subjected to different organic solvent pre-treatments, for all genotypes (Fig. 7.18).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	62318	5	12463	51.1	S***
pre-treatment	25327	2	12663	51.9	S***
gen. x pre-tr.	12367	10	1236	5.1	S**
residual	39678	162	244		
total	139690	179			

Table 5.3. Analysis of variance between weight of water (mg/seed) after 30 mins, absorbed by hard seeds subjected to different organic solvent pre-treatments, for all genotypes (Fig. 7.20).

Sourse of variation	sum of squares	d.f.	mean square	F-ratio	
genotype	50938	2	25469	100.5	S***
pre-treatment	29250	2	14625	57.7	S***
gen. x pre-tr.	3840	4	960	3.7	S**
residual	20512	81	253		
total	104542	89			