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Physiology of Chatham Island Forget-me-not (*Myosotidium hortensia*) Seed

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Craig Robert McGill
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ABSTRACT

Chatham Island forget-me-not (*Myosotidium hortensia* (Decne) Baillon) is endemic to the Chatham Islands where it is mainly confined to the outer islands. There is speculation that seed of *M. hortensia* is recalcitrant and reports that germination can be slow and erratic. Moreover there is little information on the seed biology of *M. hortensia* available.

In this study the seed structure and composition of the seed storage reserves of *M. hortensia* were determined. The seed is a dicotyledon. The embryo is predominantly cotyledonary tissue with a only small embryo axis present. There appears to be a single cell thick layer of endosperm tissue between the embryo and seed coat. Food reserves are stored as both protein and oil with no starch reserves apparent. The seed contains 24% oil and therefore can be considered an oilseed. These oil reserves include the commercially important γ-linolenic (*cis*, *cis*, *cis*-6, 9, 12-octadecatrienoic) acid (9% of the fatty acid content).

Seed of *M. hortensia* was evaluated for recalcitrant behaviour by determining if desiccation to low seed moisture content caused a loss of viability. Seed was harvested at two moisture contents, 47.4% (green seed) and 35.5% (black seed), and air dried to a final moisture content of 7.5%. Seed viability and germination performance were monitored at harvest and as moisture content declined. At 7.5% seed moisture content viability was 89% and germination 92% for seed harvested at 47% seed moisture content, and 82% and 78%, respectively, for seed harvested at 36% seed moisture content. Within each colour classification, after desiccation there was no significant difference in germination compared to that at harvest, indicating that *M. hortensia* seed can be desiccated to a low seed moisture content without loss of germination and is therefore not recalcitrant.

Seed stored at 5°C and 7.5% seed moisture content showed no decline in viability after 21 months, but, seed stored at the same temperature and 9.5% seed moisture content showed a significant loss of viability after 9 months storage. The loss of viability at this higher (9.5%) seed moisture content is characteristic of oilseeds, but it is not clear whether the high oil content of the seed alone can account for the loss of viability after nine months storage at a temperature of 5°C.

This study confirmed earlier reports that germination of *M. hortensia* seed is slow and erratic. At maturity seed of *M. hortensia* is dormant. Seed dormancy is a function of the seed coat rather than the embryo. The dormancy is likely to be a result of either physical constraint of embryo growth or restriction of gas exchange by the seed coat, or a combination of both. Removal or weakening of the seed coat allowed germination to proceed. However, some of the treatments used to weaken the seed coat resulted in an increase in abnormal seedling development. An effective and non-damaging technique for alleviating dormancy was to prick the seed coat with a 0.6-0.8mm diameter dissecting needle in the middle of the cotyledons.

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CHAPTER 1 INTRODUCTION

1.1 Introduction

We are the custodians of a New Zealand heritage of world interest

Sir Charles Fleming

New Zealand's long isolation from other land masses has led to the development of a distinct flora (Martin, 1961). Approximately 75 percent ((Laing and Blackwell, 1949) to around 80 percent (Mark and Adams, 1979) of the indigenous flowering plants in New Zealand are not encountered elsewhere. The flora of New Zealand represents a unique genetic resource (Fountain and Outred, 1991). At the Tauranga Native Plants Symposium, held in 1990, Given reported that between 10% and 15% of New Zealand flora is either threatened or restricted to localised areas. He comments "conservation of these plants will require an integrated approach involving on-site and off-site techniques as well as vegetation restoration." (Given, 1990). The success of attempts to conserve and regenerate flora in the natural environment will in part be dependent on our ability to store, germinate and propagate seeds of native species. Yet our knowledge of the germination characteristics and storage requirements of many of these species is at best limited and often nonexistent (Fountain and Outred, 1991; Bannister and Jameson, 1991). Information that is available is frequently anecdotal and found in nonscientific literature such as home gardening guides (Bannister and Jameson, 1991). Moreover, this information is often conflicting or inconclusive.

Chatham Island forget-me-not (*Myosotidium hortensia* (Decne) Baillon) is endemic to the Chatham Islands of New Zealand (Crisp *et al.*, 2000). Maloy (1992) reports the seed will germinate readily if fresh, but Metcalf (1995) comments that the germination is erratic and seedlings may not emerge from seed shed in December-January until the following spring. He also recommends the seed be germinated in cool conditions (10°C to 15°C). Wilson and Given (1989) confirm Metcalf's observations reporting that the seed can take up to twelve months to germinate. Metcalf (1995) also suggests the seed will store for twelve

months or more. Fountain and Outred (1991) comment that *M. hortensia* produces seedlings shortly after shedding and speculate that this may indicate recalcitrant behaviour but that the moisture status of seed would need to be determined to confirm this.

1.2 Aims of the Study

The aims of this study were therefore to:

1. determine if seed of *Myosotidium hortensia* is recalcitrant or orthodox

2. determine the longevity of seed of *Myosotidium hortensia* under storage conditions of low temperature and low seed moisture

3. determine the nature of the seed storage reserves in seed of *Myosotidium hortensia*

4. determine if the germination behaviour of seed of *Myosotidium hortensia* is erratic as reported by Metcalf (1995) and, if so, the reasons for this erratic behaviour.

CHAPTER 2 LITERATURE REVIEW

2.1 Chatham Island forget-me-not (*Myosotidium hortensia*)

Chatham Island forget-me-not (*Myosotidium hortensia* (Decne) Baillon) is a member of the Boraginaceae family (Wilson and Given, 1989). It is the only species in the genus and is endemic to the Chatham Islands of New Zealand (Crisp *et al.*, 2000). At one time the plant reportedly formed an unbroken ring around the shore line of the islands. It is now largely confined to the outer islands of Pitt, South East, Mangere and Little Mangere and a few sites on Chatham Island where it is out of reach of cattle, sheep and pigs which trample the plant. The plant is also palatable to sheep and pigs; the latter root out and eat the rhizomes (Wilson and Given, 1989). *M. hortensia* is also threatened by introduced weeds, in particular marram grass (*Ammophila arenaria*), (Crisp *et al.*, 2000). Crisp *et al.* (2000) also report that the only known wild population of the white-flowered *M. hortensia* was "recently" destroyed by "coastal development".

M. hortensia is a perennial herbaceous plant that can grow up to 1 metre across. It is characterised by large (up to 30 cm across) thick, fleshy, green leaves that are ovate-cordate to reniform in shape (Allan, 1982) with long thick petioles that develop from stout rhizomes. The upper surfaces of the leaves are deeply grooved and shiny. The under surfaces are evenly scattered with minute hairs. In late spring dense pyramidal flower clusters, surrounded by smaller leaves, appear. The flowers can range in colour from pale to dark blue. There is also a white form in cultivation which is sometimes referred to as "Alba" (Metcalf, 1993). Each flower contains a four-lobed ovary. This develops into four large nutlets 12-15 mm in diameter (Allan, 1982). The nutlets are flattened with an unevenly serrated wing all the way around. Each nutlet contains a single seed (Moore and Irwin, 1978). *M. hortensia* is found in coastal habitats. It prefers the inland edge of boulder beaches where it grows in peaty pockets amongst the kelp drift and a mulch of paua shells (*Haliotis iris*). It is also found, associated with peaty soil, on sand dunes, sandy beaches and rock outcrops (Wilson and Given, 1989; Metcalf, 1993).

2.2 The Ecology of the Chatham Islands

The Chatham Islands are located approximately 860 km east of Christchurch between latitudes 43°S and 45°S. The group consists of some forty islands covering a total area of around 97,000 hectares. Only the main island, Chatham Island (90,000 hectares) and, the second largest island in the group, Pitt Island (6,190 hectares), are permanently inhabited (Anonymous, 1996). The islands are the only exposed part of the Chatham Rise, a submarine extension of the New Zealand subcontinent. The group formed as a result of uplifting along several fault lines, beginning some 100 million years ago, and subsequent volcanic activity. The topography of the Chatham Islands has been shaped by continual emergence and submergence above and below sea level over the last 3.6 million years. The resulting topography is low-lying and gentle. Much of it is covered with peat that began accumulating 30,000 - 40,000 years ago and continues to accumulate. The peat can be up to 10 metres thick in places and has volcanic ash from the Taupo eruptions mixed in with it, as well as a widespread covering of sand (Campbell, 1996).

The climate of the islands is heavily influenced by their location in the "roaring forties". The prevailing wind direction is westerly. Strong winds (above 21 knots) are experienced 16% of the time; the average wind speed is around 14 knots. Calm days are rare occurring only 2-3% of the time. Rainfall is frequent but of short duration; most of the rain falls as light showers or drizzle. Rainfall of at least 0.1 mm occurs on 225 days of the year, compared to 162 days in Wellington, however there are only 134 wet days (1.0 mm or more of rain). Summer temperatures average 13.5°C to 15°C reaching a maximum of 17°C to 18°C. Minimum temperatures during winter are 5°C to 6°C. The mean temperature range is narrow, averaging 5°C to 6°C over the year. Cloud cover is high, the average amount of daylight cloud cover is 5.9 oktas (where an okta is defined as one-eighth of the visible sky), with little variation throughout the year. The high cloud cover means that sunshine hours are only 30% to 40% of the potential, averaging around 1350-1550 hours of bright sunshine annually (Thompson, 1983). The mean hourly relative humidity, at Waitangi, ranges from 81% (January) to 86% (July to September) over the year (Anonymous, 1983). The preceding climate data was recorded at Waitangi, the largest settlement, but is typical for the island group.

The flora and fauna of the Chatham Islands reflects the repeated emergence and

submergence of the islands. Many species found on the mainland of New Zealand are missing. It is likely that many of these mainland species, if ever present, could have disappeared during a period of submergence. The absence of mainland predators and the topography and climate on the islands has lead to the development of a distinct flora and fauna (Atkinson, 1996).

The islands have a greater proportion of shrubs, herbs (in particular large-leaved herbs) and ferns than mainland New Zealand, but comparatively few trees. However, most of the tree species are endemic (Given, 1996). There are 325 flowering plant species indigenous to the Chatham Islands with another 215 exotic species growing wild (Given, 1996). Given (1996) lists thirty-nine species, subspecies or varieties of plants, as endemic to the Chatham Islands although he notes that for some plants "critical comparisons still need to be made and . . . taxonomic opinion may differ on whether some plants are different from those found elsewhere". Crisp *et al.* (2000) identify 42 species and subspecies of plants considered endemic to the Chatham Islands and comment that these endemic species dominate the Chatham Island vegetation.

2.3 Boraginaceae

2.3.1 Taxonomy

The Boraginaceae family comprises some 2,400 species in 117 genera (Zomlefer, 1994) and includes herbaceous plants (commonly with fleshy roots or rhizomes), trees and sometimes shrubs or climbers (Hickey and King, 1988). The family is widely distributed (Lawrence, 1971). Zomlefer (1994) comments that is it well represented in temperate and subtropical regions with the Mediterranean region and western North America being centres of diversity.

The general characteristics of the family are often scabrous (rough) or hispid (with hairs) and sometimes glabrous (as in *M. hortensia*) herbs, sometimes shrubs or trees, (Lawrence, 1971; Zomlefer, 1994) usually with simple, alternate leaves (Hickey and King, 1988). The inflorescence is usually a helicoid or scorpoid cyme and the flowers usually perfect (Zomlefer, 1994) and regular (Hickey and King, 1988).The calyx comprises 5-free or basally connate sepals (Hickey and King, 1988), usually imbricate (Zomlefer, 1994) and

a corolla tube often with projecting scales in the throat (Zomlefer, 1994; Hickey and King, 1988). The ovary is superior and 2-locular (2 ovules in each loculus) often becoming 4-locular (1 ovule in each loculus) at maturity (Lawrence, 1971; Hickey and King, 1988). The fruit is a schizocarp that splits into four nutlets (Zomlefer, 1994), as in *M. hortensia*, or a 1- to 4-seeded nut or drupe (Lawrence, 1971). The style is usually gynobasic or terminal (Zomlefer, 1994). The seed is usually without endosperm but, if present, it is usually scanty and fleshy (Zomlefer, 1994). Lawrence (1971) comments that most 'taxonomic treatments' are based on fruit or nutlet characters.

Zomlefer (1994) lists the major genera in the Boraginaceae as *Cordia* (250 spp.), *Heliotropium* (250 spp.), *Tournefortia* (150 spp.), *Onosma* (150 spp.) and *Cryptantha* (100 spp.); Miller and Nowicke (1989) comment that "with about 300 species" *Cordia* is the largest genus in the Boraginaceae.

The classification of the Boraginaceae at the sub-family level is controversial. Zomlefer (1994) comments that the Boraginaceae are usually divided into two to five subfamilies. Hickey and King (1988) note that some taxonomists divide the family into five subfamilies, but others, using a narrower concept of the family, classify only two subfamilies, the Heliotropioideae and the Boraginoideae. However, the second family is further divided into five tribes (Cynoglosseae, Eriticheae, Borageineae, Lithospermeae and Echieae). These divisions are largely based on the characteristics of the style and fruit. However, some taxonomists (Diane *et al.*, 2002) accept the Heliotropioideae as a separate family (Heliotropiaceae).

Various taxonomic markers have been used to clarify the classification of and relationships between and within genera and species. Bigazzi and Selvi (1998) used pollen morphology in the *Borageineae* and compared this to the "taxonomic delimitations" of the tribe. They found that pollen types only matched currently accepted delimitations in seven out of 12 genera examined. Miller and Nowicke (1989) used pollen and floral/fruit morphology to clarify the sectional placement of some *Cordia* species. Molecular approaches have also been taken. For example, Diane *et al.* (2002) used sequence analysis of the nuclear ribosomal internal transcribed spacer region (ITS1) to evaluate the infrafamilial relationships of Heliotropiaceae.

2.3.2 Pollinators

Flowers of some Boraginaceae, for example, *Onosma gigantea*, *Trichodesma africana* and *T. boissieri* (Dukas and Dafni, 1990), *Echium plantagineum* (Corbet and Delfosse, 1984), *Borago officinalis* (Corbet *et al.*, 1988), contain nectar. Zomlefer (1994) lists the primary pollinators as bees, Lepidoptera (butterflies and moths) and flies, but notes that birds and bats have also been reported as pollinators for some species, for example *Cordia*, and that for species with inconspicuous flowers self-pollination has been reported. There are numerous reports in the literature of insect visitors to members of the Boraginaceae. Oberrath and Bohning-Gaese (1999) found that in lungwort (*Pulmonaria collina*) the main flower visitors were bees (*Anthophora acervorum*), bumblebees (*Bombus pascuorum*) and beeflies (*Bombylius major* and *B. discolor*), but visits were observed from 21 insect species including species from three Lepidopteran families. In contrast, Corbet and Delfosse (1994) found worker honey bees (*Apis mellifera*) were the only large insects to "abundantly visit" flowers of *Echium plantagineum*. Corbet *et al.* (1988) report that *Borago officinalis* is "buzz-pollinated" by bumblebees including *Bombus terrestris* and *B. pratorum* and that this "buzz-pollination" may be related to flower form.

2.3.3 Economically Important Boraginaceae

Some plants in the Boraginaceae are of economic importance, in particular, for use as ornamentals. These include *Echium* spp. for example *Echium pininana* (Robinson, 1992) and *Heliotropium* spp., like *Heliotropium arborescens* which is grown as a summer bedding plant (Park and Pearson, 2000). *Symphytum* spp. (Harris *et al.*, 1989), *Myosotis* spp. and *Mertensia pulmonarioides* (Virginia bluebells), and *Borago* spp. (Lawrence, 1971; Hickey and King, 1988; Zomlefer, 1994) are also grown as ornamentals. *Borago officinalis* is also commercially important as a source of γ-linolenic acid (Ruiz del Castillo *et al.*, 2002). *Borago* spp. (Hickey and King, 1988; Zomlefer, 1994) and *Lithospermum* spp. (Zomlefer, 1994) are grown as medicinal herbs. Comfrey (*Symphytum* spp.) is also well known as a medicinal herb and has been used as a poultice for sprains, bruises and abrasions and is also used as a green manure (Mabey, 1996).

2.4 Seed Storage Reserves

Seeds store their major food reserves either in the embryo or in tissues outside the embryo. Most reserves found in the embryo are stored in the cotyledons, but occasionally reserves are found in the hypocotyl/radicle. Non-embryonic storage reserve tissue includes the endosperm, megagametophyte (in gymnosperms) or the perisperm (Bewley and Black, 1994). The major storage reserves in seeds are fats, oils, proteins and carbohydrates (Bewley and Black, 1994). Fats and oils are collectively referred to as lipids (Copeland and McDonald, 1995).

2.4.1 Oils and Fats

In seeds, oil and fat reserves are mostly as oils, i.e. the reserves are liquid above 20°C (Bewley and Black, 1994). These reserves are usually in the form of triacylglycerols (TAG's) (Wallis *et al.*, 2000), but, appreciable quantities of phospholipids, glycolipids and sterols can be present in some seeds (Bewley and Black, 1994). In most oil storing seeds the oil reserves are stored as droplets in structures called lipid bodies. These lipid bodies are usually 1-2 μm in diameter and comprise a TAG core surrounded by a half unit membrane of phospholipid. The lipid bodies are usually surrounded by oleosin proteins which are believed to prevent the bodies from coalescing into larger droplets (Wallis *et al.*, 2000).

The triacyglycerol molecule is a polyester of glycerol; it contains a glycerol with a fatty acid esterified to each of the three glycerol groups. The fatty acids esterified to three positions on the glycerol can vary, however, the common fatty acids found in plants include palmitic, stearic, oleic, linoleic and linolenic acids (Wallis *et al.*, 2000).

2.4.1.1 Oil storage reserves in Boraginaceae

There are numerous reports of seed of members of the Boraginaceae family containing oil storage reserves. The most prominent being borage (*Borago officinalis*), with an oil content of 23-38%, (Quinn *et al.*, 1989). Others include *Lappula myosotis*, 18%, *Myosotis sylvatica*, 28.3% and *Myosotis caespitosa*, 27.4% (Tsevegsüren and Aitzetmüller, 1996), *Cynoglossum zeylanicum* 28%, (Nasirullah *et al.*, 1980), *Lappula redowskii*, 19% and

Paracaryum augustifolium, 31% (Kleiman *at al.*, 1964). However, not all members of the Boraginaceae store a high percentage of their food reserves as oil. Some members of the family contain 5% or less oil reserves, for example, *Lappula intermedia*, 4.6% (Tsevegsüren and Aitzetmüller, 1996), *Cordia obliqua*, 4% and *Ehretia aspera*, 5% (Kleiman *at al.*, 1964).

The oil storage reserves of seed are of interest because many contain commercially important fatty acids. One such acid is γ-linolenic acid ($C_{18:3}$, $\Delta^{6, 9, 12}$). γ-Linolenic acid is a metabolite of linoleic acid and is important in the biomedical, cosmetic and dietary industries. *Borago officinalis,* is one of only three commercially available plant sources of γ-linolenic acid, the other two are *Oenothera biennis* (evening primrose) and *Ribes nigrum* (black currant), (Ruiz del Castillo *et al.*, 2002). Neither are Boraginaceae. *Borago officinalis* contains 17-25% γ-linolenic acid (Quinn *et al.*, 1989). Several other Boraginaceae contain similar percentages of γ-linolenic acid, for example *Echium asperrimum*, 21.1%, (Guil-Guerrero *et al.*, 2001a), *Echium bonnetii* var. bonnetii, 21.4%, *E. gentianoides*, 27.4%, (Guil-Guerrero *et al.*, 2001b) and *Lappula myosotis*, 17.2%, (Tsevegsüren and Aitzetmüller, 1996).

Another fatty acid important in the dietary, biomedical and pharmaceutical industries is stearidonic acid ($C_{18:4}$, $\Delta^{6, 9, 12, 15}$), (Tsevegsüren and Aitzetmüller, 1996). γ-Linolenic acid has been reported in the seed oil of families other than the Boraginaceae, for example, Onagraceae and Scrophulariaceae (Guil-Guerrero *et al.*, 2001a), and Ranunculaceae (Aitzetmüller and Tsevegsüren, 1994; Ucciani *et al.*, 1996). However, both γ-linolenic and stearidonic acid have only been reported to date in the seed oils of Boraginaceae (Guil-Guerrero *et al.*, 2001a), Primulaceae (Aitzetmüller and Werner, 1991) and *Ribes* spp. (Wolf *et al.*, 1983; Johansson *et al.*, 1997; Ruiz del Castillo *et al.*, 2002).

Boraginaceae contain significant amounts of other unsaturated C_{18} fatty acids, oleic, linoleic and α-linolenic acid. For example, Guil-Guerrero *et al.* (2001b), found that *Echium bonnetii* var. bonnetii contained 14.5% oleic acid, 30.2% linoleic acid and 16.5% α-linolenic acid and *E. gentianoides*, 10.5% oleic acid, 20.1% linoleic acid and 21.8% α-linolenic acid. There is, however, variation between species. In contrast to the *Echium* spp., for example, Tsevegsüren and Aitzetmüller (1996) found that *Cynoglossum divaricatum* contained only 5.4% α-linolenic acid. The oleic and linoleic acid content of *C. divaricatum* was 24.7 and 30.4% respectively.

2.4.1.2 Engineering seed oils for commercial use

Many oil seeds are important sources of industrial oils, for example, surfactants, obtained from medium (8-14 carbon atoms) chain fatty acids, are used in soaps, shampoos and detergents. Most of these oils are obtained from tropical coconut or palm kernel oil (Wallis *et al.*, 2000). Many industrially important oils are derived from species that will not grow in temperate climates. Those that will grow in temperate climates are not suited to harvesting and processing procedures being used in temperate areas. There is, therefore, interest in modifying the fatty acid profile of oil seeds. To do this the genes that encode the enzymes of the TAG biosynthetic pathways in plant species that produce industrially important oils need to be identified and introduced into major temperate oil crop species such as *Brassica napus* (canola (oilseed rape)) and *Glycine max* (soybean), (Wallis *et al.*, 2000). This has already been done successfully, for example, *B. napus* was modified to include lauric acid ($C_{12:0}$) using *Umbellularia californica* (California bay laurel) as the gene donor. The lauric acid content of "laurate" *B. napus* was 38.8%, no lauric acid was detected in the unmodified *B. napus* (Del Vecchio,1996).

The commercial importance of γ-linolenic acid has already been indicated. Genetic engineering offers the possibility of modifying the fatty acid profile of oil seed crops to increase the γ-linolenic acid percentage. Biosynthesis of γ-linolenic acid occurs via Δ^6-desaturase which will introduce an additional double bond at the C-6 position of linoleic acid ($C_{18:2}$, $\Delta^{9,12}$) (Gunstone, 1996; Galle *et al.*, 1997). The inclusion of the Δ^6-desaturase gene in an oil crop high in linoleic acid may provide an economically competitive source of γ-linolenic acid (Stymme and Stobart, 1993). Sayanova *et al.* (1997) suggest that *Helianthus annuus* (sunflower) would be a suitable crop as the presence of 50-70% linoleic acid with little or no α-linolenic acid should allow synthesis of high levels of γ-linolenic acid. A cDNA clone encoding the Δ^6-fatty acid destaruase has been isolated from developing seeds of *Borago officinalis* and transferred into tobacco plants where it resulted in the accumulation of γ-linolenic acid in the leaves of transgenic plants (Sayanova *et al.*,1997). In a subsequent study (Sayanova *et al.*, 1999) this group of workers found that expression of a *B. officinalis* Δ^6-fatty acid desaturase in transgenic tobacco plants resulted in accumulation of γ-linolenic acid in both the leaf and seed tissue of the transgenic plants.

2.4.2 Proteins

Seeds will also store food reserves as proteins where they act as sources of carbon, nitrogen and sulphur to be utilised by the growing seedling (Shewry, 2000). Storage proteins in seed are the main source of protein for human nutrition, stock feed and as an industrial raw material (Shewry, 1993).

Seed storage proteins are classified into groups based on their solubility and extraction in a sequence of solvents (the four "Osborne fractions": water (albumins), dilute salt solutions (globulins), aqueous alcohols (prolamins) and dilute acid or alkali (glutelins)), and their sedimentation coefficient. These groups are the 2S albumins, 7S and 11S globulins and the prolamins (Shewry, 2000).

The 11S globulins are the most widely distributed type of seed storage protein. The 7S globulins are characteristic of legumes where with the 11S globulins they form the majority of the storage proteins. Both the 7S and 11S globulins are found in both dicotyledons (for example *Glycine max* and *Vicia faba* (broad bean)) and monocotyledons (for example, *Avena sativa* (oat)). In contrast the 2S albumins have only been confirmed in dicotyledons (for example, *Brassica napus*). Prolamins are restricted to cereals (for example, *Triticum aestivum* (wheat) and *Oryza sativa* (rice)) and other grasses (Shewry, 2000).

Seed storage proteins are usually deposited in special storage organelles called protein bodies which range in diameter from 0.1-25μm (Bewley and Black, 1994). Some protein bodies are surrounded by a membrane but it can be incomplete or absent from some bodies. Protein bodies often contain inclusions. These include crystalloids (insoluble proteinaceous inclusions embedded in the soluble protein matrix) or, more commonly, globoids (noncrystalline, globular structures) (Bewley and Black, 1994). Globoids are the sites of deposition of phytin. Phytin or phytate is the insoluble mixed potassium, magnesium and calcium salt of *myo*-inositol hexaphosphoric acid (phytic acid) (Bewley and Black, 1994; Cochrane, 2000). Other minerals found in phytin include iron, manganese, and zinc (Chen and Lott, 1992) and copper and sodium (Bewley and Black, 1994). In seeds 50-80% of the phosphate present is found in the phytin (Cochrane, 2000).

Druse crystals of calcium oxalate are another inclusion found, more rarely (Bewley and Black, 1994), in the protein bodies of some seeds, for example in *Eucalyptus* (Buttrose

and Lott, 1977).

Shewry (2000) lists the storage proteins present in seeds of important crops. For the Dicotyledonae these are *Helianthus annuus*, *Glycine max*, *Vicia faba*, *Pisum sativum* (pea), *Phaseolus vulgaris* (French bean), *Brassica napus* and *Gossypium hirsutum* (cotton). None listed are members of the Boraginaceae. This may be one reason why the seed protein content of members of the Boraginaceae appears to have been poorly studied. The only report on the protein content of seed of Boraginaceae found in the literature is that of the seed of an edible fruit from Mexico, *Cordia boissieri* (anacahuita) (Alanis-Guzman *et al.*, 1998). The crude protein content found in seed of this species was 20% but no details on specific proteins present are given.

2.4.3 Carbohydrates

The third type of storage reserves found in seeds are carbohydrates. Starch is the most common carbohydrate found in seed storage reserves (Bewley and Back, 1994) and is synthesised in the plastids (Cochrane, 2000). In some seeds the major carbohydrate reserves may be hemicelluloses, and the raffinose series of oligosaccharides (Bewley and Black, 1994). The raffinose series contributes 2-6% of the dry weight in seeds of most Leguminosae and are present in significant quantities in some Gramineae (Cochrane, 2000). Hemicelluloses form the major carbohydrate reserve in the seeds of some Leguminosae (Bewley and Black, 1994; Cochrane, 2000). Hemicelluloses include mannans (such as galactomannans and glucomannans) and xyloglucans (Bewley and Black, 1994). Xyloglucans are also called "amyloids" because they stain blue with iodine/potassium iodide. Amyloids have been identified in the seed 230 species from a large range of families but it is not certain if all the amyloids identified are xyloglucans (Cochrane, 2000).

In cereals (*Hordeum vulgare* (barley), *T. aestivum*, *Secale cereale* (rye) and *Zea mays* (maize)) over 75-80% of the food reserve is stored as carbohydrates (Bewley and Black, 1994), much of which is in the form of starch (Cochrane, 2000). For example, Abdel-Aal *et al.* (1997) found that in hard red spring common *Triticum aestivum* cv. Katepwa, starch averaged 62.0% of dry matter and was similar to that of *Phalaris canariensis* (canarygrass) seed, 61%, grown in adjacent plots. A similar percentage of starch was found by Hoover *et al.* (1996) in grains of long grain brown rice (*Oryza sativa indica* var.

IR64) which yielded 64.4% starch on a dry weight basis.

Legumes, though primarily consumed as a source of protein, are also important sources of starch and other carbohydrates (Cochrane, 2000). Legume seeds typically yield 18-45% starch (Hoover and Sosulski, 1991). However, the percentage of starch reserves can vary between species, for example, *Glycine max*, has only 1-3% starch at maturity (Chen *et al.*, 1998). In contrast, Ratnayake *et al.* (2001) found that in four cultivars (Carneval, Carrera, Grande and Keoma) of field pea the yield of starch was 33.7%, 33.2%,32.7 and 33.5% respectively on a whole seed dry weight basis.

Starch is usually found in seeds in two forms, amylose and amylopectin. Both these are polymers of glucose. In the seed, starch is found in sub-cellular structures called starch granules (Cochrane, 2000). These starch granules can range is diameter from 2 to 100 µm even in the same seed (Bewley and Black, 1994). Hoover *et al.* (1996) reported that starch granules in wild rice (*Zizania palustris*) were round, angular and polygonal in shape and were typically 2-8 µm in diameter. Ratnayake *et al.* (2001) found that starch granules in four cultivars (Carneval, Carrera, Grande and Keoma) of field pea varied in shape from round (5-7 µm in diameter) to elliptical (10 µm on the shorter diameter and 25 µm on the longer).

Although carbohydrate reserves are well documented in some cereals and legumes (Bewley and Black, 1994; Cochrane, 2000), there are no reports in the literature identifying carbohydrate reserves in Boraginaceae.

2.5 Seed Storage

Many seeds have the ability to retain viability at least until the next growing season and often longer but, as with any organism, this viability cannot be retained indefinitely and all seed will eventually deteriorate and die (Copeland and McDonald, 1995). The longevity of seeds in storage is determined by genetic factors, pre-storage stresses experienced by the seed during development, harvesting and processing, and the storage environment into which the seed is placed (Thomson, 1979).

Roberts (1973) used the terms *orthodox* and *recalcitrant* when describing the storage

behaviour of seeds. These were based on seed survival during storage in response to seed moisture content and temperature. In orthodox seed the moisture content can be reduced to 2-5% without loss of viability, and over a wide range of storage environments longevity in storage increases with a decrease in seed moisture content and temperature (Roberts, 1973). In contrast, in recalcitrant seed a reduction in seed moisture content below a relatively high value, depending on species, decreases longevity (Roberts, 1973). Recalcitrant seed may also be susceptible to chilling injury (King and Roberts, 1980a). Since Roberts' (1973) original classifications of seed storage behaviour Ellis *et al.* (1990) have identified a third category of storage behaviour, intermediate between the orthodox and recalcitrant categories. The term intermediate has been used for this category of storage behaviour. The "essential feature of intermediate seed storage behaviour is that the negative relation between seed longevity in air-dry storage and moisture content is reversed at seed moisture content values below those in equilibrium (at 20°C) with about 40-50% relative humidity" (about 10% seed moisture content for *Coffea arabica* (arabica coffee) and 7% for *Citrus* spp.) (Hong *et al.*, 1998). Ellis *et al.* (1991) found that the desiccation-tolerant seeds of *Carica papaya* (papaya) could be stored for 12 months at 15°C and 7.9-9.4% seed moisture content without loss of viability but with storage at lower temperatures or lower seed moisture contents viability declined. They comment that another feature of seed showing intermediate storage behaviour is that longevity of dry seeds (around 10% seed moisture content) is reduced at temperatures below around 15°C.

There are some suggestions in the literature (Finch-Savage, 1992; Pammenter and Berjak, 1999) that rather than the division of storage behaviour into discrete categories the concept of a continuum of behaviour across species may be more appropriate. This later suggestion and the additional, intermediate, storage category recognise, that not all seed of all species can be classified as simply recalcitrant or orthodox. Nonetheless these terms remain a useful means of describing the storage behaviour of seed of most species and will be used here.

Hong *et al.* (1998) suggest that seed storage behaviour in the Boraginaceae appears to be orthodox, but note that for some species (for example *Myosotis cespiosa*, *Ehretia acuminata*, and *Cordia goeldiana*), further investigation is required before final confirmation of storage behaviour can be made.

2.5.1 Orthodox Seed

Even within seed considered orthodox there is considerable variation in storage behaviour. Several species have been authenticated as long-lived, for example, 147 years for *Albizia* (Bewley and Black, 1994). Many of the seeds that have been authenticated as long-lived (greater than 100 years) such as *Albizia*, *Trifolium* and *Canna* have hard impermeable seed coats. Species specific properties of the seed coat, perhaps resistance to water, gases or microorganisms may contribute to the seeds' longevity (Smith and Berjak, 1995). There is evidence in the literature to support this idea, for example, Flood (1978) found that in *Trifolium subterraneum* (subterranean clover) stored in a filing cabinet hard ("water impermeable") seed retained more than 95% viability after 20 years compared to 58-63% in seed permeable to water. There is also evidence for seed of some cereals being long-lived in storage. Steiner and Ruckenbauer (1995) found that *Avena sativa* seed and *Hordeum vulgare* seed that had been stored sealed (by melting the glass) in a glass cylindrical vial for 110 years showed 81% and 90% germination respectively.

Seed composition can affect the storage life of seed with seed species containing a high oil content generally storing more poorly than those with a lower oil content (Copeland and McDonald, 1995). However, both Copeland and McDonald (1995) and Priestley (1986) comment that for some species the analysis of total oil content may be misleading and that the oil content of the tissue "responsible for germination" may be more important.

Vertucci and Roos (1990) suggest that seeds with higher lipid content have lower moisture contents for optimum storage. Sacandé *et al.* (2000b) offer an explanation for this from their work on *Azadirachta indica* (neem) seed. They suggest the much more rapid decline in germination of *A. indica* seed stored at 20°C and 11.7% (75% relative humidity) seed moisture content after 26 weeks storage compared to seed stored at 5.5% (32% relative humidity) seed moisture is because the water content of the cytoplasm is much higher than the 11.7% overall seed moisture. They suggest that because 50% of the dry weight is in the form of oil the water content of the cytoplasm is 20.9% on a fresh weight basis. Citing previous workers (Sacandé *et al.*, 2000a; Sun, 1997), these authors suggest that at 20.9% moisture content the cytoplasm is non-glassy (liquid) state but at 32% relative humidity the cytoplasm is in the glassy state (Sacandé *et al.*, 2000a). In the glassy state the rate of deterioration in the cytoplasm is considerably slower than in the liquid state (Sun, 1997) hence the much slower decline in germination at 5.5% seed moisture.

Justice and Bass (1978) developed a "Relative Storability Index" which divided orthodox species into three categories based on their expected storage life. Species in category 1 could be expected to have 50% or more germination after 1-2 years storage at favourable ambient conditions (between latitudes of 35° and 45°N), those in category 2, 50% or more germination after 3-5 years and category 3 greater than 5 years. Amongst the Boraginaceae listed, *Borago officinalis*, heliotrope and myosotis, have a relative storability index of 1 and *Anchusa officinalis* (bugloss) a relative storability index of 2. This suggests that seed of Boraginaceae are relatively short-lived in storage under ambient conditions. This is supported by Priestley (1986) who also lists the mean number of years that horticultural and agronomic species can be expected to maintain high germination under favourable commercial storage conditions in Germany. Boraginaceae listed include *Anchusa capensis* (3 years), *Borago officinalis* (2-3 years), *Heliotropium arborescens* (1-3 years), *Myosotis alpestris* (2-3 years) and *M. scorpoides* (2-3 years).

2.5.1.1 Seed moisture content and temperature

A limitation with looking at longevity in storage under ambient conditions is that storage temperature and relative humidity (or seed moisture content) are not constant. Over the period of the storage it is likely that ambient conditions will vary considerably. Relative humidity (seed moisture content) and temperature are the two most critical factors in determining the longevity of orthodox seed in store (Priestley, 1986). In open storage the ambient relative humidity will determine the seed moisture content, but in air-tight (sealed storage) the ambient relative humidity will not affect the seed moisture content and the moisture content the seed is placed in store at is critical for longevity. The lower the relative humidity and/or seed moisture content and temperature the greater the longevity in store (Justice and Bass, 1978). The relationship between seed moisture content, temperature and seed longevity was recognised by Harrington in two rules of thumb (Harrington, 1972). These are that:

a. for seed stored between 5 and 14% seed moisture content, for every 1% reduction in seed moisture content storage life is doubled and

b. for each 5°C reduction in storage temperature storage life is doubled.

The effect of reduced storage temperature and seed moisture content (relative humidity)

can be seen in *Cordia goeldiana* where there was around a 20% loss in viability after seven months storage at 8 °C at 50% relative humidity but complete loss of viability during one month open storage at 26 °C and 80% relative humidity (Vianna, 1983, cited in Hong *et al.*, 1998). In *Cordia alliodora* viability is halved after 12 months storage at 5 °C with 12-18% seed moisture (Tschinkel, 1967 cited in Hong *et al.*, 1998) but viability is maintained at 2-5 °C with 6.9% seed moisture content (Trivino *et al.*, 1990, cited in Hong *et al.*, 1998).

However, interactions between moisture content and temperature are important, for example at higher moisture content freezing damage may occur in seed (Priestley, 1986). There is also some evidence that seed, if kept dormant and stored in a fully hydrated state, will retain viability. Villiers (1974) found that fully-hydrated seed of *Lactuca sativa* (lettuce) cv. Arctic King and Grand Rapids and *Fraxinus americana* stored a 30 °C, 25 °C and 22 °C respectively retained viability in storage for at least 12 months whereas seed stored at the same temperature but lower seed moisture content lost viability. For seed stored at a range of moisture contents from 5-19% as seed moisture content increased the rate of viability loss and accumulation of nuclear damage also increased. Villiers (1974) suggests that at these moisture contents "organelle turnover" does not occur nor do "systems" that can repair damage to DNA operate. This leads the accumulation of damage. In contrast, fully hydrated seed showed a very low level of nuclear damage suggesting these systems are functioning. Villiers (1974) draws the parallel with seed survival in the soil seed bank where some seed, also at high seed moisture content, is able to remain viable for long periods.

2.5.1.2. Ultra-dry storage

In contrast to storage of seed fully hydrated, Ellis *et al.* (1986) reported that for *Sesamum indicum* (sesame) seed stored at 50 °C in laminated aluminium foil packets a reduction in seed moisture content from 5% to 2% increased longevity about 40-fold. They suggest that reducing storage temperature from 20 °C to -20 °C would also increase longevity about 40-fold. This led to the suggestion that when -18 °C storage is not available for genebank storage, the longevity of orthodox seeds can be maximised, if seeds are dried to a moisture content in equilibrium with 10-12% relative humidity at 20 °C and then stored hermetically at ambient or cooler (Ellis *et al.*, 1989). Ellis *et al.* (1989) also suggest that "ultra-dry" storage could be more appropriate for species with oily seeds which store comparatively poorly compared to starchy seeds. At 20 °C and 10-13% relative humidity

many starchy seeds equilibrate to around the 5% moisture content recommended for long term germplasm storage of seed. However, oily seeds at 10-13% relative humidity at 20°C will equilibrate to much lower moisture contents (Ellis *et al.*, 1995). For example, *Brassica napus* (cv. Bienvenu) at 10.6% relative humidity at 20°C equilibrated to 2.8% seed moisture and *Allium cepa* (onion), (cv. White Lisbon) at 9.8% relative humidity at 20°C reached 3.6% seed moisture (Ellis *et al.*, 1996). These authors found that after five years hermetic storage at two temperatures (-20°C and 20°C) there was greater loss of viability at 20°C in *Brassica napus*, *Arachis hypogaea* (groundnut), *Daucus carota* (carrot), *Lactuca sativa* and *Allium cepa* seed stored dry (5.5-6.8% seed moisture) compared to ultra-dry seed (2.0-3.7% seed moisture). No loss of viability was found at either moisture content following storage at -20°C. However, Hong *et al.* (1998) note the technique is controversial. One group of researchers (Vertucci and Roos, 1990) suggest the technique is potentially damaging and that seed moisture contents in equilibrium with 19-27% relative humidity are optimum for seed longevity. The data presented by Ellis *et al.* (1996) argues against the suggestion that drying seeds at 20°C from 5±1% seed moisture to a seed moisture content in equilibrium with around 10% relative humidity is deleterious to the seeds survival in subsequent hermetic storage at 20°C. On the contrary, the data suggests that desiccation to ultra-dry moisture contents increases longevity. The findings of Steiner and Ruckenbauer (1995), where *Avena sativa* and *Hordeum vulgare* stored at low seed moisture (< 5%) and 10-15°C, showed 81% and 90% germination, respectively, after 110 years storage also support the idea of ultra-dry storage resulting in longevity in store at ambient temperatures.

Some early reports (Nakamura, 1975) indicated poorer seed storage life at moisture contents in equilibrium with 10% relative humidity than 30% relative humidity (Nakamura, 1975; Ellis *et al.*, 1996). The reason for this may be that rapid uptake of water by dry seed can result in imbibition injury (Powell and Matthews, 1978). The lower the moisture content the more likely seeds will be damaged (Ellis *et al.*, 1990). To reduce the possibility of imbibition damage Hong *et al.* (1998) suggest that seed at 8% moisture content and below be humidified in a moist atmosphere before germination testing or germinated on 1% water agar.

2.5.1.3 Cryogenic storage

Where long term storage of a decade or more is required for genetic conservation seed

can be stored in liquid nitrogen at -196°C (cryopreservation). Seed moisture is critical for successful cryopreservation (Stanwood and Bass, 1981). Too much moisture can result in "freezable water"; when seeds are frozen this results in seed death because of ice crystallisation (Hong *et al.*, 1998). It is possible to freeze seeds at relatively high moisture contents without freezing injury provided the water in the cells vitrifies (enters the glassy state) rather than freezes (Hong *et al.*, 1998). Traditional cryopreservation techniques involve freeze-induced dehydration, whereas new techniques are vitrification based (Engelmann, 1998).

Stanwood and Bass (1981) suggest that the "upper safe moisture limit" varies with species and cultivar. Stanwood (1983), (cited in Fu *et al.*, 1993) lists a range of high moisture freezing limits (the seed moisture threshold at which seed cooled in liquid nitrogen will decrease in viability) for seed from a low of 9.6% for sesame to high of 28.5% for bean. Prior to cryopreservation not only do the safe seed moisture contents for individual species, in particular those with oily seeds, need to be determined (Hong *et al.*, 1998) but also cooling (Stanwood, 1987; Hong *et al.*, 1998) and thawing (warming) rates (Hong *et al.*, 1998). For example, Stanwood (1987) suggested that for routine cryopreservation of sesame (*Sesamum indicum*) seeds a cooling rate of less than 30°C min^{-1} is preferred and seed moisture content should be kept between 6 and 9%.

2.5.2 Recalcitrant Seed

Recalcitrant seeds are desiccation intolerant and lose viability if dried below a certain moisture content. This can vary between species, for example *Dryobalamops aromatica* (Borneo camphor) will lose viability a below 30-35% seed moisture content (Tang and Tamari, 1973; Tamari, 1976, cited in King and Roberts, 1980a), compared to 23% for *Theobroma cacao* (cocoa), (Hunter,1958, cited in King and Roberts, 1980a). King and Roberts (1980b) used the term critical moisture content to refer to the moisture content at which seed death occurs rapidly. However, Pammenter and Berjak (1999) suggest that it is not possible to identify a "critical moisture content" for a particular species because desiccation tolerance is not dependent only on species characteristics but also the developmental status of the seeds and the conditions under which the seeds are dried, in particular, the rate of dehydration. Vertucci *et al.* (1995) also suggest that this critical moisture content varies with seed maturity and drying temperature. In *Zizania* (wild rice) embryos they found that the more immature the embryo the greater the critical moisture

content and that the critical moisture content increased with decreasing temperature. Finch-Savage (1992) also found that in *Quercus robur* (English or pedunculate oak) seed desiccation tolerance increased during development up to shedding but that seeds did not pass through a fully desiccation-tolerant phase. In contrast, Fu *et al.* (1994) found that in *Clausena lansium* (wampee) seed desiccation tolerance was maximum at 67 days after anthesis and had declined by physiological maturity (74 days after anthesis).

Farrant *et al.* (1988) comment that the essential characteristic of recalcitrant seeds is their lack of, or the non-functioning of, a mechanism which enables orthodox seeds to lose moisture without loss of viability. They further suggest that there is a continuum of recalcitrant seed-types reflecting the geographic origin of a particular species. Species found in tropical forests or wetland areas, where conditions are suitable for germination all of the year, are typically highly recalcitrant. These species tolerate little or any water loss, are sensitive to low temperatures and germinate rapidly after shedding. They quickly lose viability in storage. In temperate or sub-tropical areas conditions may not be suitable for germination at all times of the year, for these species, in the absence of additional water, germination proceeds slowly. These species may tolerate some moisture loss and lower temperatures and can be described as minimally recalcitrant. They may be dehydrated to a limited extent without loss of viability and be stored for relatively long periods (up to eight months for *Quercus alba* (white oak), (Farrant *et al.*, 1988)). The majority of New Zealand native plants are considered to belong to a warm-temperate to mildly sub-tropical climate (Metcalf, 1975), but some have subantarctic affinities (Martin, 1961). This suggests that any recalcitrant New Zealand natives species would lie at the latter, minimally, recalcitrant end of the continuum.

The morphology of the seed may also provide evidence of recalcitrant behaviour. Garwood (1989) has suggested a link between recalcitrance and warm, moist conditions and the development of seeds within a large fleshy fruit.

2.5.2.1 Identification of recalcitrant seed

Some species may appear to be recalcitrant when in fact they are able to tolerate desiccation to relatively low seed moisture contents but drying conditions are critical. For example Kovach and Bradford (1992) report that *Zizania palustris* var. *interior* (North American wild rice), which had been categorised as recalcitrant, will remain viable if dried

to a seed moisture content of approximately 7% at 25°C. However germination declines rapidly if the seed (caryopsis) is desiccated at temperatures below 20°C.

In the past some seeds have been described as recalcitrant simply because drying to low seed moisture was accompanied by a subsequent failure to germinate. This lead to the seeds of some species being misidentified as recalcitrant. For example, *Citrus* spp., once thought to be damaged by drying, are able to be dried to low seed moisture content (5%). However, drying results in an increase in the time taken for germination of viable seed from approximately three to six weeks at 30°C (Soetisna *et al.*, 1985).

Frequently the failure to germinate is due not to loss of viability, but to the induction of dormancy as a result of the drying process. Roberts *et al.* (1984) suggest that the failure to break dormancy was the reason that *Fagus sylvatica* (beech),*Acer saccharum* (sugar maple) and several species of *Prunus* and *Taxus* were identified as damaged by drying.

King and Roberts (1980b) suggest that some seeds described as recalcitrant may in fact lose viability when desiccated because of slow drying rather than an inability of the seed to tolerate desiccation. There is also the possibility that imbibition damage on re-hydration rather than drying *per se* may be the cause of the loss of viability (King and Roberts, 1980b).

There is therefore a need to consider not only seed moisture content but seed maturity and environmental conditions such as storage temperature, drying temperature and drying rate and the effects of drying on optimum germination temperatures, germination rate and dormancy before categorising seed behaviour in storage.

2.5.2.2 Storage of recalcitrant seed

Storage of recalcitrant seed is much more difficult than that of orthodox seed. Recalcitrant seeds are not desiccation tolerant and therefore cannot be stored at low moisture content. Nor can recalcitrant seed be stored at temperatures much below 0°C because ice crystallisation will damage the seed. In fact many tropical recalcitrant species are sensitive to chilling and cannot be cooled below 10-15°C (Roberts and Ellis, 1989). However, within these limits, storage at reduced temperature has been found to increase the longevity in store of some recalcitrant seed. Fu *at al.* (1990) found that in wet stored *Mangifera indica*

(mango) seed longevity was greatest in seed stored at 15°C, but that seed stored at 5-10°C retained viability longer than seed stored at 30°C. Differences in storage longevity between the two cultivars of *M. indica* tested were also found with cv. Guo-zhou Ya losing viability after 120 days moist (44.5% seed moisture) storage at 15°C but cv."Local" retaining 65% viability after 7 months moist (50% seed moisture) storage at 15°C. The authors suggest that this may be a function of the different initial viability (90% for cv.Guo-zhou Ya and 100% for cv. "Local") and vigour of the lots. The possibility that genetic differences between cultivars are also a factor is not discussed.

Hong *et al.* (1998) suggest that the viability of recalcitrant seed can be maintained, at least for a limited period, in aerated conditions provided seed moisture content and temperature are carefully controlled. The seed moisture content should be maintained at or just below that when shed from the parent plant, or in equilibrium with 98-99% relative humidity. The storage temperature is dependent on the geographical origin of the species being stored. For species of tropical origin the storage temperature can vary from 7°C to 17°C but -3°C to 5°C is more appropriate for species of temperate origin. For example, Hong *et al.* (1998) cite the work of Suszka (1971-1974) where seeds of *Quercus* spp. were moist stored for more than three years at -3°C.

The main difficulty in maintaining recalcitrant seeds in wet storage at reduced temperatures is the need for continuous aeration at the same time as preventing germination and fungal contamination (Hong *et al.*, 1998). These authors suggest that the storage medium chosen should maintain the seed at high moisture content and allow diffusion of oxygen to the seed. Information on seed dormancy characteristics can be used to prevent germination of wet-stored seed. Pritchard *et al.* (1996) found no germination occurred in *Aesculus hippocastanum* (horse chestnut) seed stored at 16°C for 330 days but storage at lower temperatures resulted in germination as a result of alleviation of dormancy. Seed stored on agar-water at 16°C for three and a quarter years gave about 40% germination when transferred to 6°C.

Storing recalcitrant seeds under wet storage at reduced temperatures will still not provide longevity in storage required for germplasm storage (Pammenter and Berjak, 1999). Cryopreservation of recalcitrant seed may offer the most promise for longer term storage (Stanwood and Bass, 1981; Roberts *et al.*, 1984; Pammenter and Berjak, 1999). A problem with recalcitrant seed is that, unlike orthodox seed, desiccation to moisture

contents below which there is no freezable moisture for ice crystal formation is not always possible without damaging the seed. One vitrification-based cryopreservation approach is "desiccation" where tissue is partially dehydrated and then frozen by direct immersion in liquid nitrogen (Engelmann, 1998). There is evidence in the literature to suggest that excised embryo axes of some recalcitrant seed can survive rapid desiccation to moisture contents at which ice crystallisation will not occur and then immersion in liquid nitrogen. Fu *et al.* (1990) found that 80% of excised *Mangifera indica* embryo axes survived rapid desiccation (eight hours at 30°C) with silica gel under aseptic conditions to 11.8% moisture and that excised embryonic axes of *Euphoria longan* (longan) could be rapidly dried to around 19% seed moisture in 2.5-3 hours and stored in liquid nitrogen for 12 hours. After thawing at around 30°C the survival percentage of the frozen embryonic axes was 80%. However, drying rate and/or method is important. Fu *et al.* (1993) found that desiccation of excised embryonic axes of *Artocarpus heterophyllus* (jackfruit) seeds using a vacuum pump was most rapid (1 hour to reach 26% moisture) but no embryonic axes survived dessication below 44%, the drying rate in an aseptic air current was intermediate (3 hours to 28% moisture) but 30% of embryonic axes survived desiccation to 26% seed moisture. Silica gel was slowest (6 hours to reach 24% moisture) but 25% of embryonic axes survived desiccation to 16% moisture. These authors also suggest that research is needed into the MS (Murashige and Skoog) medium used to improve the regeneration of the embryonic axes and growth of normal seedlings.

However, there are still many recalcitrant seeds for which it is not possible to desiccate either the seed or seed tissue to moisture contents below the freezable water threshold. Cryoprotectants may allow freezing of hydrated seed tissue (Stanwood and Bass, 1981; Pammenter and Berjak, 1999). *Cocos nucifera* (coconut, a one seeded fruit) is both recalcitrant and large (Chin, 1980). Cryopreservation techniques have been applied to *C. nucifera* embryos. Mature *C. nucifera* embryos, first desiccated in the air flow of a laminar flow cabinet and then pre-grown for 11-20 hours on a medium with 600 g l^{-1} glucose and 15% glycerol were able to survive freezing in liquid nitrogen whatever the pre-growth conditions (Engelmann and Assy-Bah, 1992).

2.6 Dormancy

2.6.1 Definitions of Dormancy

The germination process begins with uptake of water and culminates with elongation of the embryo axis, usually the radicle (Bewley and Black, 1994). However, although conditions (adequate water and oxygen and an appropriate temperature) may be favourable for germination frequently embryo growth does not proceed. If the seed is viable it is likely that the failure to grow is a result of one or more dormancy mechanisms being present within the seed.

The are numerous classifications of dormancy used throughout the literature. These have been based on three criteria (Simpson, 1990):

a. the apparent origin of the control of dormancy, e.g. genetic, structural or environmental

b. the depth of the dormancy, e.g. shallow, deep or relative

c. the timing of the dormancy, e.g. primary, secondary

There are examples of these approaches in the literature. Bewley and Black (1994) define *primary dormancy* as dormancy that develops in the seed during seed development and remains when the seed is shed from the parent plant. Mature non-dormant seed may also enter dormancy in response to certain environmental conditions, for example, anoxia will induce dormancy in *Avena fatua* (wild oat) (Hilhorst, 1998). This dormancy is referred to as *secondary dormancy* (Bewley and Black, 1994; Mayer and Poljakoff-Mayber, 1989).

Hilhorst (1998) distinguishes between active inhibition of germination where compounds such as phenols or other secondary metabolities prevent germination and passive inhibition where conditions for germination are sub-optimal. Hilhorst (1998) uses the terms *enforced dormancy* and *conditional dormancy* to distinguish between passive and active inhibition of germination leading to dormancy.

Frequently, the intact seed will fail to germinate. However if the tissues surrounding the embryo, for example the lemma and palea, caryopsis, pericarp or seed coat, are removed germination of the embryo proceeds. This type of dormancy is referred to as *coat-imposed* dormancy (Corbineau and Côme, 1995; Bewley and Black, 1994), although Bewley and Black (1994) suggest a more appropriate term is *coat-enhanced dormancy*. In other seeds removal of the structures surrounding the embryo does not allow germination to proceed and the inhibition of germination is a function of the embryo. This embryo-based dormancy is termed *embryo dormancy* (Corbineau and Côme, 1995; Bewley and Black, 1994), but, Baskin and Baskin (1982) use the term *innate dormancy* when referring to the stratification requirements of *Cyperus inflexus*.

A confounding factor in classifying the dormancy mechanisms found within seed is that there maybe multiple dormancy mechanisms with a single seed. For example, Ellery and Chapman (2000) found that dormancy in *Arctotheca calendula* (capeweed) was a function of both the seed coat and embryo.

2.6.2 Coat-imposed dormancy

The tissues involved in the imposition of coat-imposed dormancy vary between species and include the endosperm (for example in *Lactuca sativa)*, seed coat (for example in *Sinapis arvensis* (charlock)), pericarp (for example in *Betula pubescens* (birch)), and the lemma and palea (for example in *Avena fatua*) (Bewley and Black, 1994). Dormancy may be imposed by a single tissue or by a combination of one or more tissues, for example in *Avena fatua* dormancy is imposed by both the pericarp and the lemma and palea (Bewley and Black, 1994).

These tissues can impose dormancy via several mechanisms. The seed coat may act as a barrier preventing water uptake by the embryo. This is common in many species, in particular members of the Leguminosae family, Saha and Takahashi (1981) found that dormancy in the leguminous seed, *Crotalaria sericea* was a function of the impermeability of the seed coat to water but that the depth of dormancy within the seed population depended on the physical and morphological characteristics of the seed. Similarly, Bhattacharya and Saha (1990) observed species variation in coat imposed dormancy in *Cassia* spp. They found that the coat impeded water uptake in *Cassia fistula*, but not in *C. occidentalis* or *C. spophera*. In all three species the seed coat was hard but the

structure of the hilum and micropyle differed between species. In *C. spophera* the micropyle was open; in *C. occidentalis* it was partly open but was plugged in the dormant *C. fistula*.

The coat may also restrict gas exchange between the embryo and atmosphere. In particular, the coat may be impermeable to oxygen. Hatterman-Valenti *et al.* (1996) found the seed coat of woolly *Eriochloa villosa* (cupgrass) impeded germination by restricting oxygen availability to the embryo. Edelstein *et al.* (1995) found that, at low temperatures, the seed coat prevented germination in the cold sensitive *Cucumis melo* (melon) cultivar Noy Yizre'el but not in the cold tolerant cultivar Persia-202. The difference in germination at low temperature was attributed to restricted oxygen diffusion through the seed coat (and increased sensitivity of the embryo to hypoxia). The restricted oxygen diffusion was correlated with differences in the seed coat structure of the two cultivars. In the outer layer of the seed coat of Persia-202 intercellular spaces were clearly visible under a scanning electron microscope but the outer coat of Noy Yizer'el was completely sealed.

Oxygen supply to the embryo may also be impeded by chemicals, for example phenolic compounds, such as coumarin, in the seed coat. Oxidation of these compounds by polyphenoloxidases reduces oxygen supply to the embryo (Côme, 1982 cited in Corbineau and Côme, 1995). Qi *et al.* (1993) suggest that restriction of oxygen uptake is also responsible for the coat-imposed dormancy in the Boraginaceae *Cynoglossum officinale* (houndstongue). However while *C. officinale* seeds contain high levels of phenolic compounds, no relationship between methanol extractable compounds and germination could be established.

In a number of species the tissues surrounding the embryo may allow uptake of water but prevent embryo growth by acting as a physical barrier to, for example, radicle expansion. Whiteman and Mendra (1982) suggest that the seed coat of *Brachiaria decumbens* (signal grass) inhibits germination by preventing radicle expansion, although restriction of oxygen uptake may also be involved. In some *Lactuca sativa* cultivars the dormancy is a function of the endosperm surrounding the embryo. Although the endosperm is only two cells wide, the cell walls are relatively thick and composed of mannans. This is sufficient to prevent germination (Bewley and Black, 1994). Weston *et al.* (1992) found that the seed coat of *Cucumis sativus* var. *hardwickii* inhibited germination by constraining radicle growth and that a period of dry storage for up to 180 days was required to alleviate this dormancy.

They suggest this period of storage is necessary to increase the growth potential of the radicle so it can break through the seed coat.

Often coat-imposed dormancy is a function of more than one of these mechanisms, for example, restricted oxygen supply to the embryo and mechanical constraint of embryo growth. Stabell *et al.* (1998) suggest that both play a role in inhibiting germination in *Cynoglossum officinale*. Similarly, Hamilton and Carpenter (1976a) suggest that while the pericarp may physically constrain embryo growth in *Elaeagnus umbellata* (autumn olive) the endocarp and seed coat may also restrict gas exchange by the embryo. Hamilton and Carpenter (1976a) also suggest an inhibitor within the seed may have a role in preventing germination in this species. Mitrakos and Diamantoglou (1984) also comment that while the endocarp restricts hydration and oxygen uptake in fruits of *Olea europaea* (common olive) the endosperm may also inhibit germination.

Germination inhibitors in the tissue surrounding the embryo may prevent germination. These inhibitors include abscisic acid (ABA), coumarin and short chain fatty acids (Bewley and Black, 1994). Hamilton and Carpenter (1976b) report that a coumarin-like inhibitor was found in the endocarp and testa (and embryo) of dormant *Elaeagnus augustifolia* (Russian olive) seed.

2.6.3 Embryo Dormancy

In many cases embryo growth is not prevented by the tissues surrounding the embryo but by factors within the embryo itself. There is evidence to suggest that the cotyledons have a role in inhibiting growth of the embryo axis. Bewley and Black (1994) report that removal of one cotyledon in *Corylus avellana* (common hazel) or in *Euonymus europeus* (European spindle tree), both cotyledons in *Fraxinus excelsior* (European ash) or the scutellum (a modified cotyledon) in *Hordeum vulgare*, partially or completely alleviates the dormancy in the embryonic axis of these species. Similarly, Ellery and Chapman (2000) found that in dormant seed of *Arctotheca calendula* removal of cotyledons promoted germination of embryonic axes compared to de-coated embryos and intact seeds. Ellery and Chapman (2000) also found that leaching of de-coated seed (embryos) increased germination leading them to suggest that a water soluble inhibitor located in the cotyledons may have imposed dormancy. Bewley and Black (1994) report that removal of free and bound ABA from the embryo of *Taxus baccata* (English yew) allows

germination to proceed and suggest that embryo dormancy may be the result of the presence of ABA either in the cotyledons or embryonic axes or both. In contrast Leather *et al.* (1992) (cited in Ellery and Chapman, 2000) suggest that germination may have been a result of the wounding stimulating evolution of ethylene or carbon dioxide rather than ABA removal. Jullien et al. (2000) analysed ABA levels and the effects of exogenous gibberellic acid (GA), ABA and fluridone on germination in dormant and after-ripened (non-dormant) seed of *Nicotiana plumbaginifolia* and *Arabidopsis thaliana* (Arabidopsis) seed. They suggest that the inhibition of ABA synthesis capacity or degradation of ABA or both are keys in allowing germination to proceed and that this occurs during after-ripening. They further suggest that the earliest role of GA in dormancy release is by a "negative effect on accumulation of ABA" in the seed. This is based on their observation that in *Nicotiana plumbaginifolia* exogenous GA inhibited ABA accumulation in dormant seeds. In contrast, in *A. thaliana* seed GA was almost ineffective in breaking dormancy. To account for this the authors suggest that some factor(s) other than GA, are activated during after-ripening or stratification.

In assessing the role of ABA in the regulation of dormancy in seed it is important to consider not only levels of ABA in the seed but also tissue sensitivity to the hormone. In cereals, for example *Triticum aestivum* (Walker-Simmons, 1987), the regulation of dormancy and germination by ABA is dependent not only on the ABA concentration but also the sensitivity of the embryo tissue to ABA (Corbineau and Côme, 2000). Changes in sensitivity of embryo tissue of *Helianthus annuus* seed to both ABA and GA during dormancy release have also been reported by Le Page-Degivry *et al.* (1995).

There are cultivar differences in the sensitivity of the embryo to ABA, for example Walker-Simmons (1987) found the embryo tissue of sprouting resistant *Triticum aestivum* cultivar Brevor was more sensitive to ABA than the sprouting susceptible cultivar Greer. Freshly harvested cereal seeds are considered dormant because they are difficult to germinate at temperatures above 10-20°C (Corbineau and Côme, 2000). Sensitivity of the embryo to ABA is affected by temperature, for example, Corbineau *et al.* (1993) found that in primary dormant *Avena sativa* seed sensitivity to ABA is higher at 30°C than at 20°C. During after-ripening the temperature range at which seed will germinate widens and this is accompanied by a decrease in embryos sensitivity to ABA (Corbineau and Côme, 2000).

2.6.4. After-ripening

Often the dormant seed requires a period of dry storage ("after-ripening") before germination is possible or the germination percentage increases (Mayer and Poljakoff-Mayber, 1989). The period of after-ripening required can vary form several weeks to several years. Matus-Cadiź *et al.* (2001) found that only two (1998 seed) or four (1999 seed) weeks at 24°C was required to alleviate dormancy in annual *Phalaris canariensis*. De Villiers *et al.* (2002) found that the germination of air-dried (two weeks at 20°C) seed of *Albuca exuviata* increased from 38% at the end of the two week air-drying period to 96% after six weeks of dry storage. In contrast a much longer after-ripening period was needed for seed of *Senecio arenarius*; after six weeks dry storage germination was nil but increased to 50.5% after 28 weeks dry storage.

In some species a period of after-ripening alone is not sufficient to alleviate dormancy and other environmental stimuli are required before germination can proceed. Widrlechner and Kovach (2000) report that during four years dry storage (around 23°C and 50% relative humidity) germination of *Cuphea viscosissima* seed increased, but, only for seed germinated in light at 30/20°C. This indicates light is still required for germination. After four years' dry storage germination reached 76%, however, the "viability of the seed lot" was 92% suggesting that in 16% of the seed lot dormancy had still not been alleviated by dry storage. These results again illustrate that dormancy-break in many species frequently involves more than one factor.

2.6.5 Temperature Requirements

In many species dormancy can be alleviated by exposure of hydrated seed to relatively low temperature, in most cases in the range of 1-10°C but in some instances up to 15°C (Bewley and Black, 1994). This exposure of the hydrated seed to low temperature is sometimes referred to as *chilling* (Bewley and Black, 1994) or *stratification* (Mayer and Poljakoff-Mayber, 1989). The length of stratification required can vary, with woody species generally requiring longer stratification times than herbaceous species (Bewley and Black, 1994).

Stratification responses can also vary within a seed population. Finneseth *et al.* (1998) found that *Asimina triloba* (North American pawpaw), a deciduous woody tree, reached

over 50% germination after 7 weeks stratification at 5°C but 10 weeks stratification was required before maximum germination (78%) was reached. Light was not required for germination in this species, however, Bewley and Black (1994) note that sensitivity to light can be enhanced by chilling. The results of Widrlechner and Kovach (2000) support this observation. These authors found that in seed of *Cuphea viscosissima*, as the period of stratification increased, the sensitivity of the seeds to light also increased. In addition, after 16-24 weeks stratification at 4°C, germination reached 80-90% germination in the light compared to only 10-15% germination in the dark. However, germination in the dark after four weeks stratification was only around 2%. No statistical data is presented but if these differences in germination percentage are significant it may suggest that longer stratification times are able to substitute for the light requirement in some seed in the population.

Other species respond to a period of high temperature followed by exposure to low temperature. Ren and Kermode (1999) found that a three day running water soak (20-21°C), followed by four weeks moist storage at 20°C and then eight weeks moist storage at 2-4°C was able to break dormancy in *Chamaecyparis nootkatensis* (yellow cedar). However, interactions between chilling and plant growth regulators and osmotic priming have been observed. Schmitz *et al.* (2001) found in *Chamaecyparis nootkatensis* seed a three day soak in running water, followed by incubation in GA_3 and GA_{4+7} or GA_3 and benzylaminopurine or vacuum infiltration with GA_3 and then 60 days moist stratification at 4°C was effective in promoting germination. A three day soak in running water, followed by a four day incubation with polyethylene glycol (-0.148 and -1.027 MPa), then a one day drying period and two day GA_3 treatment prior to 30 days moist stratification gave 60% germination. Plant growth regulator and osmotic priming treatments were able to remove the warm moist stratification requirement but not the cold moist stratification requirement. However, stratification times were reduced.

Alternating temperatures are also able to alleviate coat-imposed dormancy in ,for example in *Bidens tripartitus*, *Nicotiana tabacum* (tobacco) and *Rumex* spp. (Bewley and Black, 1984). Joley *et al.* (1997) found that fresh achenes of *Centaurea solstitialis* (yellow starthistle) collected in July and August generally showed higher germination at alternating temperatures compared to constant temperature. In particular, achenes harvested in August reached approximately 77% germination when germinated at 25/15°C compared to around 45% at 15°C. However, germination was still less than that observed at 25/15°C

or 15°C in the light (8 hours light, using cool white fluorescent light (60 μmol m^{-2} s^{-1}), 16 hours dark)). Germination at these temperatures, in the light, was nearly 100%, indicating that alternating temperature was only able to substitute for the light requirement of a portion of the seed population. The importance of interactions between light and temperature was demonstrated in this study. Germination of freshly harvested achenes of yellow starthistle, in the light, was nearly 100% at 10, 15 and 20°C but declined to around 60% at 25°C. Interestingly, in achenes harvested in July and stored dry for 21 months at around 21°C, showed higher germination at 25°C than freshly harvested seed in both light and darkness possibly suggesting an after-ripening effect during storage. *Centaurea solstitialis* is an example of the complex interaction of multiple factors required for the alleviation of dormancy in some species. In this species these include harvest date, storage time and regime, light, germination temperature, alternating temperature and achene type (Joley *et al.*, 1997).

In some species exposure to high temperature will also alleviate dormancy. Baskin *et al.* (1998) found that in *Senna obtusifolia* seed dormancy was alleviated by incubation at alternating temperatures that included a high temperature (30/15°C, 35/20°C and 40/25°C with germination significantly higher at each respective temperature regime). Germination in scarified seed occurred at much lower alternating temperatures (15/6°C, 20/10°C and 25/15°C).

2.6.6 Light Requirements

Many seeds are released from dormancy after exposure to light. Freshly harvested seed of *Lapsana communis* and *Matricaria perforata* required light (14 hours per day) for germination (Milberg and Andersson,1997). Seeds of the New Zealand woody species *Alseuosmia macrophylla* (toropapa) did not germinate in the dark but exposure to light allowed germination to proceed indicating seed of this species has a light requirement for germination (Burrows, 1999). The light requirements of *Centaurea solstitialis* have already been discussed (Joley *et al.*, 1997).

The length of time exposure to white light required can vary from a few minutes or seconds to intermittent exposure (Bewley and Black, 1994). Milberg and Andersson (1997) found that seeds of *Capsella bursa-pastoris* required light for germination and that germination was greater after short exposure (5 seconds) to light (R:FR of 0.85)

compared to exposure to full light for 12 hours per day. However, the authors note that because a flourescent tube was used as the full light source and a xenon bulb for the short light exposure the two treatments are not directly comparable. They conclude it is difficult to determine if full-light treatment inhibited germination or if the light quality from short exposure was better for germination. Germination reached nearly 100% in *Centaurea solstitialis* after two days following exposure to 8 hours light. However, even brief exposure to light (ten minutes exposure to flourescent light) was sufficient to improve germination to 93% and 83% for plumed and non-plumed achenes, respectively, compared to that in the dark (Joley *et al.*, 1997).

White light (i.e. sunlight) will break dormancy. Borthwick *at al.* (1952) demonstrated that in the *Lactuca sativa* cultivar Grand Rapids the major dormancy breaking activity is at wavelengths of around 660 nanometres (red region of the spectrum) and the maximum dormancy imposing activity is activity at 730 nanometres. The light signal is perceived by phytochrome (Bewley and Black, 1994; Smith, 2000). Phytochromes exist in two forms, the red-light-absorbing Pr form and the far-red-light-absorbing Pfr form and can be photo-converted between the two forms (Casal and Sánchez, 1998).

Phytochromes are a small family of photoreceptors whose apoproteins are encoded by different genes. Five phytochrome genes (PHYA, PHYB, PHYC, PHYD and PHYE) have been identified in *Arabidopsis thaliana*. These encode for five photoreceptors, phytochrome A (phyA) to phytochrome E (phyE), (Casal and Sánchez, 1998; Smith, 2000). There are also five genes (PHYA, PHYB1, PHYB2, PHYE and PHYF) present in *Lycopersicon esculentum* (tomato) (Casal and Sánchez, 1998). It is likely that in angiosperms the maximum family size is five, although monocotyledonous plants may not have the phyE homologues (Smith, 2000). The stability of the different phytochromes can vary. Phytochrome A in the Pfr form breaks down rapidly in light. In contrast, phytochrome B and phytochrome C are synthesised at lower rates than phytochrome A, but are more stable in the Pfr form (Somers *et al.*, 1991 cited in Casal and Sánchez, 1998). The amount of each of these phytochromes is a result of synthesis and destruction (Casal and Sánchez, 1998). Phytochrome B is found in dry seeds and influences germination in seeds imbibed in the dark. If sufficient phytochrome B is in the Pfr form germination of light requiring seeds can occur in the dark. In contrast to Phytochrome B, Phytochrome A does not appear until several hours after the start of imbibition (Casal and Sánchez, 1998).

Three phytochrome response modes are observed (Casal and Sánchez, 1998). The first is the classical phytochrome response where the germination promoting effect of a pulse of red light on dark-imbibed seeds is cancelled by a subsequent pulse of far red light. This is termed a low fluence response (LFR). The LFR is mediated by phytochrome B but Casal and Sánchez (1998) suggest phytochrome C, phytochrome D and/or phytochrome E may also be involved. The involvement of phytochrome E, in addition to phytochrome B, in the red/far red reversible low fluence response in *Arabidopsis* seeds has been recently confirmed by Hennig *et al.* (2002). The second response identified is the very low fluence response (VLFR). In some circumstances, a pulse of far-red light is able to promote germination above that observed in dark imbibed seeds, for example, if the seeds are pre-incubated at low or high temperatures. This is a VLFR and is likely to be mediated through phytochrome A (Casal and Sánchez ,1998). Herron *et al.* (2000) suggest both the LFR and VLFR are important in seed germination of *Leptospermum scoparium* (manuka) seed. The third response type is the high-irradiance response (HIR) where continuous exposure to far-red light inhibits germination. Casal and Sánchez (1998) report that although phytochrome A is known to mediate HIR's in seedlings, so far, *Arabidopsis thaliana* seeds have not exhibited a HIR response. However, Shichijo *et al.* (2001), using phytochrome (phy)-hypersensitive mutants, have identified phytochrome A as mediating the HIR inhibition of seed germination in tomato.

In addition to the red/far red activity, which is "exclusively mediated" by the phytochromes, activity is also observed by in the blue region of the spectrum (Weller *et al.*, 2001). Phytochrome A and phytochrome B both absorb blue light and are involved in the blue light response, but another group of photoreceptors, the cryptochromes, have been shown to be involved in the blue light responses in *Arabidopsis* and tomato seedlings (Weller *et al.*, 2001).

2.6.7 Dormancy in Boraginaceae

There are a limited number of reports of seed dormancy in members of the Boraginaceae. In *Cynoglossum officinale* restriction of oxygen uptake and physical constraint of embryo growth by the seed coat are suggested as the likely dormancy mechanisms in seed of this species (Qi *et al.*, 1993; Stabell *et al.*, 1996; Stabell *et al.*,1998). Piggin (1976) found that *Echium plantagineum* seed exhibited "considerable" dormancy and cited the work of Ballard (1970) who suggested that dormancy in this species is a function of physical

constraint of embryo growth by the seed coat. However, Piggin (1976) found that storage of seed at alternating temperatures, which the author suggests may be expected to weaken the seed coat, did not increase germination. It is difficult to draw conclusions on the likely dormancy mechanisms in *E. plantagineum* based on this limited study but it interesting to note that germination in light had no effect on the germination percentage. Van Breemen (1984), investigating the germination ecology of *Cynoglossum officinale*, *Echium vulgare* and *Anchusa arvensis*, also found that light did not improve the germination percentage in any species. On the contrary, seed of *C. officinale* collected from either an "exposed" or "sheltered" (in and around scrub) habitat had a higher germination percentage when germinated in the dark compared to germination in 12 hours light/12 hours dark, but there was no difference in the germination in light or darkness of seed collected from an "intermediately sheltered" habitat. Similarly the germination percentage of seed of *A.arvensis* collected from a roadside was higher in darkness but not for seed collected from a "shaded" (*Populus* grove) habitat. The reason for this different germination response to darkness depending on habitat is unclear but both the Piggin (1976) and van Breemen (1984) studies indicate that in these Boraginaceae light does not promote germination. In contrast the Qi *et al.* (1993); Stabell *et al.* (1996); Stabell *et al.* (1998) studies suggest that dormancy in these Boraginaceae is at least partly a function of the seed coat.

CHAPTER 3 SEED STRUCTURE AND SEED STORAGE RESERVES

3.1 Introduction

One of the aims of this study was to determine the nature of the seed storage reserves in seed of *M. hortensia*. The storage reserves found in seed, in particular, the percentage of reserves stored as lipid, can influence the storage life and storage requirements (Copeland and McDonald, 1995; Vertucci and Roos, 1990). Moreover, there is a paucity of fundamental information on the seed biology of *M. hortensia*. This chapter describes determination of both the composition of the seed structure and seed storage reserves of *M. hortensia*.

3.2 Materials and Methods

3.2.1 Seed Material

Seed used in all experiments was obtained from *M. hortensia* "Alba" plants planted in a 6.5 x 7.5 m plot at the rear of the Seed Technology Centre (175° 37'E, 40° 24' S) on the Turitea Campus, Massey University, Palmerston North. The plants were transplanted into the site in July 1996 at a distance of 0.7 m apart. At the time of transplanting plants were approximately 16 months old. The plants were grown in a raised bed approximately 0.30 m high. The bed had a sand base, approximately 0.12 m deep, on top of which was placed a 0.18 m deep bark-based (700 kg/m^3) medium. The bark originally had agricultural lime 3 kg/m^3, dolomite 3 kg/m^3, iron sulphate 0.5 kg/m^3, osmocote® (16:3.5:10:1.2 (N:P:K:Mg) plus trace elements) 4 kg/m^3 and ammonium phosphate 2 kg/m^3 added to it but had been used for eight months for growing Ruzi grass (*Brachiaria ruziziensis*) prior to being placed in the site (Wongsuwan, 1999). Therefore the precise nutrient status of the medium is unknown. Maxicrop liquid fertiliser (Nitrogen 0.65 g/l, phosphorus 0.75 g/l and potassium 2.1 g/l) was applied at a rate of 3 ml/l as a foliar feed at approximately three-four weekly intervals between flowering and seed harvest. In 1997, 1998 and 1999, approximately 10-15g of osmocote per plant and 300g/60m^2 of urea was

applied. In 1998 and 1999 compost and compost containing fish fines, respectively, was applied to individual plants as a basal dressing 2-3 cm deep.

3.2.2 Seed Harvest and Sub-sampling

Flowering began in mid to late September and seed was ready for harvest the following January. Seed heads were harvested by cutting the inflorescence at the mid-point of the stem. Seed was harvested in 1997, 1998, 1999 and 2000, however the bulk of the experimental work was carried out on seed harvested in 1997 (Table 3.1).

Once in the laboratory, nutlets were separated from the stem material by hand and, where appropriate, separated according to colour as either green or black nutlets. Within each colour classification nutlets were mixed and separated into four replicates. To separate the nutlets into four replicates nutlets were placed in a sample container and split in two by pouring them through a riffle divider. The nutlets were then recombined by simultaneously pouring them back through the divider. This was repeated twice to thoroughly mix the nutlets. After the third (final) mixing the nutlets were poured through the divider and split into two lots. Each lot was then split again by pouring it through the divider to create a total of four replicate lots. For experimental work nutlets were randomly sub-sampled from each replicate lot.

Table 3.1: Nutlet harvest date and start time of experimental work. Nutlets or seed were stored in laminated polyester/aluminium foil/polythene packets at 7- 8% moisture and 5°C until required for experimentation.

Experiment	Seed Harvest Date	Experiment Start Date
Seed Histology	January 1997	August 1998
Scanning and Transmission Electron Microscopy	January 1997	September 1999
Lipid Analysis	February 2000	October 2000 and November 2001
Seed Desiccation	January 1997	January 1997
Seed Storage	January 1997	February 1997
Temperature Gradient Plate	January 1997	March 1997
Seed Dormancy I	January 1997	January 1998
Seed Dormancy II	January 1997/January 1998	March 1998

3.2.3 Seed Histology

The killing and fixation and staining procedures described below were adapted from Johansen (1940) unless otherwise stated.

3.2.3.1 Killing and fixing seed material

Twelve seeds, harvested January 1997, were preconditioned by rolling them in moistened 38 lb Regular Weight Seed Germination Paper (Anchor Paper Company, St. Paul Minnesota). The roll was placed in an 500 ml glass jar with approximately 100 ml of water in the bottom and both were placed in a plastic bag and the seed allowed to imbibe at 20°C for 18-20 hours.

After imbibition seeds were cut transversely in half or in thirds and placed in sample holders (Histosette I, Simport Plastics Ltd., Canada), then, immediately immersed in a formalin acetic acid (FAA) fixative. The FAA was prepared by adding 5 ml of glacial acetic

acid and 5 ml of formalin to 90 ml of 70% ethanol. The seed samples were left for 96 hours in a vacuum desiccator to fix.

3.2.3.2 Dehydration

The seed samples were dehydrated through the following 2-methyl-propan-2-ol (tertiary butyl alcohol, TBA) series:

1. TBA (20% v/v) in 60% ethanol (50% v/v): half-an-hour
2. TBA (35% v/v) in 70% ethanol (50% v/v): two hours
3. TBA (55% v/v) in 95% ethanol (45% v/v): two hours
4. TBA (75% v/v) in 100% ethanol (25% v/v): two hours
5. TBA (100% v/v) and erythrosin dye: sixteen hours
6. TBA (100% v/v): three and a half hours
7. TBA (100% v/v): three and a half hours
8. TBA (100% v/v): eighteen hours.

All steps were carried out at room temperature except 5-8 where, because 100% TBA solidifies below 25.5°C, a fan heater set at 35-40°C was used to keep the TBA liquified.

3.2.3.3 Infiltration

After dehydration the seed samples were transferred from TBA to TBA/paraffin and finally to wax using the following protocol:

1. 100% TBA (50% v/v) and paraffin (50% v/v): one hour
2. a beaker of pure melted wax was allowed to solidify. The bulk of the TBA/paraffin solution was decanted and the remainder, with seed material poured into the beaker of wax and placed in a 60°C oven: one hour
3. into fresh wax: three and a half hours
4. into fresh wax: eighteen hours
5. into fresh wax: four and a half hours
6. into fresh wax: three hours
7. into fresh wax: sixty-eight hours (weekend)
8. into fresh wax: four hours (all traces of TBA gone).

3.2.3.4 Embedding

Seed samples were embedded using a Leica EG1160 Histoembedder. An aluminum mould was half filled with molten wax and the mould placed on the cold surface of the embedder. The wax was allowed to partially solidify to prevent the sample resting on the base of the mould when it is embedded. A seed sample was positioned in the still molten inner wax so the surface of interest faced, but did not touch, the base of the mould. This left a wax face on the sample to begin sectioning into. A cassette (Shandon Scientific Ltd., New Zealand), was placed halfway down into the mould to the fill line of the wax and the mould completely filled with wax. The mould was again placed on the cold surface of the embedder and the wax allowed to solidify.

3.2.3.5 Microtoming

The wax, with sample embedded, and cassette were removed from the metal mould. The wax around the sample was trimmed to a trapezoid shape making sure the upper and lower (horizontal) edges were parallel to each other. A Leica RM2145 Microtome was used to section the samples. The sample was positioned in the microtome holder using the cassette to hold the block so the upper and lower edges, and the face of the sample, were parallel to the blade. Both transverse and longitudinal sections were taken. 10 µm thick sections of 4-5 segments were cut and immediately placed in a Leica HI1210 floating bath running at 42°C. Once the ribbon of sections had flattened they were placed on a microscope slide. The microscope slide had been coated with dilute PVA glue (10% v/v), and dried overnight at 40°C on a slide drier.

3.2.3.6 Staining

The sections were stained for cellulose (safranin-fast green), fat ((Sudan IV), protein (Coomassie Brilliant Blue R250), starch (iodine) and chromatin (Feulgens/Schiff's Reagent).

3.2.3.6.1 Dewaxing and ethanol series

For the safranin-fast green, Coomassie Brilliant Blue, iodine and Schiff's staining the seed

sections (on microscope slides) were dewaxed with a histological clearing agent, histo-clear II™ (National Diagnostics, Atlanta, Georgia, USA) and taken down to 70% ethanol using the following protocol:

1. histo-clear II™: ten minutes
2. histo-clear II™(50% v/v) and absolute ethanol (50% v/v): five minutes
3. 100% ethanol: five minutes
4. 100% ethanol: five minutes
5. 95% ethanol: five minutes
6. 85% ethanol: five minutes
7. 70% ethanol: five minutes.

3.2.3.6.2 Safranin-fast green

Slides containing the sections were simultaneously stained in safranin (1% safranin (w/v) in 70% ethanol) and fast green. The fast green stain was prepared by dissolving 0.2 g of fast green in 15 ml of methyl cellosolve and 15 ml of oil of clove leaf (Bronson & Jacobs Pty Ltd., New Zealand Division). Once dissolved, additional fast green was added until a saturated solution was obtained, but keeping the total amount of fast green in the solution to less than 0.3 g to prevent over-staining of the sample sections. Once dissolved, the solution was mixed with 90 ml of 95% ethanol and 30 ml of glacial acetic acid.

The staining protocol was as following:

1. dewax and take down to 70% ethanol (refer to ***3.2.3.6.1***)
2. safranin: twenty-four hours
3. wash off excess stain: thirty seconds
4. 70% ethanol: five minutes
5. 0.5% picric acid: ten seconds
6. 95% ethanol: one minute fifty seconds
7. 95% ethanol: ten seconds
7. fast green: twelve - fourteen seconds
8. oil of clove leaf: rinse
9. oil of clove leaf (50% v/v), histo-clear II™ (25% v/v) and absolute ethanol (25% v/v): five - ten seconds

10. histo-clear II™ (50% v/v) and absolute ethanol (25% v/v): five minutes
11. histo-clear II™: five minutes
12. histo-clear II™: five minutes.

Cover slips were then mounted onto the slides using DPX mountant (containing 80% xylene).

3.2.3.6.3 Sudan IV

A stock solution of Sudan IV was prepared by adding 2.0 g of Sudan IV to one litre of absolute ethanol at room temperature. The solution was boiled gently until the Sudan IV dissolved completely.

The staining solution, a saturated solution of Sudan IV in 70% ethanol, was prepared immediately before use by adding, over five minutes, nine volumes of 45% ethanol to seven volumes of the Sudan IV stock solution, with mixing. The solution was allowed to stand for one hour before being filtered through Whatman No. 1 filter paper (Lee, 1937). The staining solution has a shelf life of four hours, therefore, samples were stained immediately using the following protocol.

Slides containing sections were immersed in the staining solution for ten minutes and then rinsed by immersing the slide in 70% ethanol twice for two seconds each time. Cover slips were mounted onto the slides using glycerol jelly. The glycerol jelly was prepared by dissolving 10 g of gelatin in 60 ml of distilled water. Once dissolved, 70 ml of glycerol and 0.25 g of phenol were added. The solution was warmed with continual stirring until the phenol dissolved. The warm solution was filtered through two thicknesses of cheesecloth into a brown glass jar and allowed to cool (Johansen, 1940). The glycerol jelly was stored at 4 °C when not in use.

To mount the cover slips a small piece of solid glycerol jelly was melted by immersing it in a beaker in warm water. A glass Pasteur pipette was warmed in a 60 °C oven and used to place a few drops of the jelly on the slide and the cover slip was mounted while the jelly remained liquid.

To verify the Sudan IV staining solution would stain fats, *Limnanthes alba* (meadowfoam)

seed, a high oil-containing seed (Pierce and Jain, 1977), was preconditioned as described for *M. hortensia* in **3.2.3.1**. Hand sections of meadowfoam and, preconditioned, *M. hortensia* seed were stained directly with a few drops of the Sudan IV stain solution.

3.2.3.6.4 Coomassie Brilliant Blue R250

A staining solution of 0.02% Coomassie Brilliant Blue R250 solution was prepared by dissolving 0.1 g of Coomassie Brilliant Blue R250 in 500 ml of 1:3 glacial acetic acid:absolute ethanol.

The sections were dewaxed and taken down to 70% ethanol (as described in ***3.2.3.6.1***) and then immersed in 0.02% Coomassie Brilliant Blue R250 for 24 hours. The slides were destained in 1:3 glacial acetic acid:absolute ethanol for two hours before being dehydrated in 98% ethanol for five minutes and then and 100% ethanol for five minutes. Cover slips were mounted on the slides using DPX mountant.

To verify the Coomassie Brilliant Blue solution was staining, a *Pisum sativum* seed was preconditioned as described in **3.2.3.1** and a hand section stained directly with a few drops of the stain.

3.2.3.6.5 Potassium iodide/iodine

A KI/I_2 solution was prepared by dissolving 15 g of potassium iodide and 1.5 g of iodine in 250 ml of distilled water (Jones and Varner, 1967).

Microscope slides were hydrated through the following ethanol series to water.

1. dewax and take down to 70% ethanol (refer to ***3.2.3.6.1***)
2. 60% ethanol: five minutes
3. 50% ethanol: five minutes
4. 40% ethanol: five minutes
5. 30% ethanol: five minutes
6. 20% ethanol: five minutes
7. 10% ethanol: five minutes

8. tap water: fifteen minutes.

The sections were immersed in the KI/I_2 solution for ten minutes and then destained in tap water for 3 minutes. Cover slips were mounted on the slides using water and the stained sections observed immediately.

To verify the KI/I_2 was staining, a hand-section of a stem tuber of *Solanum tuberosum* (potato) was also stained with a few drops of the KI/I_2 stock solution.

3.2.3.6.6 Schiff's Reagent

Seed sections were stained for chromatin material using the standard Feulgens protocol (R.E. Rowland, *pers comm.*) The sections were first dewaxed and taken down to 70% ethanol (refer to ***3.2.3.6.1***). One M HCl was equilibrated to 60°C and the sections immersed in it for 8 minutes. The sections were stained in Schiff's Reagent (BDH Ltd., Dorset, England) for 40 minutes, then rinsed twice by being dipped in 70% ethanol for a few seconds before being allowed to air dry. Cover slips were mounted with DPX mountant.

To confirm the Schiff's Reagent was staining, a *Pisum sativum* and *Limnanthes alba* seed were preconditioned as described in **3.2.3.1** and a hand sections of each stained directly with a few drops of the stain.

3.2.3.6.7 Evaluation

Stained microscope sections were evaluated at a range of magnifications using a Zeiss Axiophot compound microscope with a x 10 eyepiece. Section images were captured with a JVC 3CCD Video Colour Camera (Model Number KY-F55B) connected to the microscope with a x 0.5 attachment.

3.2.4 Transmission Electron Microscopy

Seed harvested in January 1997 was preconditioned as described in **3.2.3.1**. Seed material was then prepared for transmission electron microscopy at the Keith Williamson

Electron Microscope Unit, HortResearch Ltd., Palmerston North.

3.2.4.1 Fixation and evaluation of seed material

The preconditioned seed was dissected into small (1-2 mm) squares and fixed in a primary fixative (3% glutaraldehyde (v/v) and 2% formaldehyde (v/v) buffered in 0.1 M phosphate buffer at pH 7.2) for two hours at room temperature. The phosphate buffer was prepared by dissolving 2.51 g of Na_2HPO_4 and 0.41 g of KH_2PO_4 in 100 ml of water adjusted to a pH of 7.2. The sections were washed, at room temperature, by immersing them twice in fresh 0.1 M phosphate buffer for ten minutes followed by a third immersion of 20 minutes. The samples were then fixed in the secondary fixative (1% osmium tetroxide (OsO_4) (w/v) in 0.1 M phosphate buffer) for one hour at room temperature.

The samples were again washed three times as above in 0.1 M phosphate buffer and then dehydrated in a graded acetone series:

1. 25% acetone: fifteen minutes
2. 50% acetone: fifteen minutes
3. 75% acetone: fifteen minutes
4. 95% acetone: fifteen minutes
5. 100% acetone: fifteen minutes
6. 100% acetone: one hour.

Once in 100% acetone, samples were infiltrated in an acetone (50% v/v) and resin (50% v/v) (Procurc™ 812 epoxy resin) mixture. The seed material and mixture were stirred overnight at room temperature in a sealed container. The container was opened and the acetone allowed to evaporate, leaving the samples infiltrated with resin. The samples were then transferred into 100% resin and stirred for another seven hours at room temperature. After seven hours the samples were embedded in fresh resin in a silicone rubber mould and cured for 48 hours at 60°C.

The samples were removed from the mould. The cured blocks were trimmed to give a parallel cutting surface. One µm sections were cut from the blocks and heat mounted on glass microscope slides. The slides were stained with 0.1 M phosphate buffered 0.05% Toluidine Blue. The slides were then assessed using the Zeiss Axiophot compound microscope used for the histology work and photographed using the camera built into the

microscope. Cured blocks containing areas of interest were retrimmed for cutting electron microscopy sections and 90 nm sections cut using a diamond knife and a Reichert Ultracut E Ultramicrotome. The sections were mounted on a grid and double stained with saturated uranyl acetate in 50% ethanol (v/v) and then lead citrate. Sections were examined and photographed using a Philips Z01C Transmission Electron Microscope. The number and size of the lipid and protein bodies and number of inclusions were counted and measured from the photographs taken of the cotyledon tissue where 0.001 mm = 1μm at zero times magnification. Measurements were corrected for the magnification used. Protein and oil body numbers were only counted in cells that were completely visible in the photograph. Individual sections (photographs) were treated as replicates to calculate standard errors.

3.2.5 Lipid Analysis

The fatty acid and/or lipid content of the *M. hortensia* seed was analysed using three techniques:

1. gas chromatography of fatty acid methyl esters derived from the seed fatty acids
2. extraction of seed lipid using petroleum spirit as the solvent (Soxhlet extraction)
3. extraction of seed lipid using methanol-chloroform as the solvent.

All solvents used in this series of experiments were either Analar® or HPLC grade unless otherwise stated.

3.2.5.1 Gas chromatography

3.2.5.1.1 Derivation of fatty acids

Total fatty acids were extracted and transesterified (methylated) using the following protocol, adapted from Sukhija and Palmquist (1988). A methanolic-hydrochloric acid solution was prepared by adding one volume (3 ml in this experiment) of acetyl chloride (cooled over ice) drop wise to nine volumes (27 ml) of anhydrous methanol, on ice, with shaking.

Initially, in October 2000, duplicate samples of fatty acid methyl esters (FAMES) were prepared by grinding approximately 4 g of 2000-harvest *M. hortensia* seed in a coffee grinder (Braun Type 4 0 41) and 0.15g (sample A) and 0.36 g (sample B) of seed flour sub-sampled. The remaining seed flour was stored in at 4 °C in a refrigerator until required for thin layer chromatography. Subsequently, in November 2001, a second group (samples C-F) of FAMES were prepared by grinding approximately 0.44 g of seed from each replicate (refer to **3.2.2**) of *M. hortensia* seed and by sub-sampling a known amount (0.2-0.25 g) of seed flour.

Seed flour was placed in 20 mm X 100mm Kimax® screw top centrifuge tubes. To each Kimax® tube was added 2 ml of toluene (redistilled over a molecular sieve, nominal pore size 4Å (Sigma Chemical Company, St. Louis, Missouri), 0.5 ml of internal standard (10 mg/ml or 41.2 µmoles/ml of C_{15} (pentadecanoic acid) in toluene) and 3 ml of the freshly prepared methanolic-hydrochloric acid solution. The tubes were tightly stoppered and placed in a water bath running at 70 °C for 2 hours. After two-three minutes the stoppers were checked for tightness and tightened if necessary. The tubes were agitated every twenty minutes. At the end of the two hour incubation the tubes were cooled in ice water and 5 ml of 6% potassium carbonate (K_2CO_3) added. The potassium carbonate was added to remove any residual acetic acid and perform a partial clean-up by forming a salt with any underivatised product. Toluene (2 ml) was added and the mixture inverted two times, the lid was loosened to release the pressure and the tube was inverted another two to three times. The samples were then centrifuged at 2600 rpm for six minutes in a IEC Centra GP8R centrifuge and the top (toluene) fatty acid containing-layer removed. Anhydrous sodium sulphate was added to remove water followed by florisil (magnesium silicate), which is inactivated in water, to remove pigments and triacylglycerols not esterified or fatty acids. The toluene solution was centrifuged at 2600 rpm for six minutes and the supernatant removed and stored in 20 mm X 100 mm Kimax® screw top centrifuge tube in a refrigerator (at 4 °C) until required.

3.2.5.1.2 Hydrogenation of fatty acid derivatives

Approximately 3 ml of sample B methylated fatty acids were placed in a 20 mm X 100mm Kimax® screw top centrifuge tube and an excess (approximately 0.5g) of palladium charcoal added. The unstoppered tube was surrounded with foam and packed in a thick walled flask. The flask was sealed by a rubber bung wrapped in Parafilm® and the

atmosphere in the flask was saturated with gaseous hydrogen via a glass tube in the bung. The methylated fatty acids were continuously agitated in this hydrogen atmosphere at room temperature for 24 hours. At the end of the 24 hour period the hydrogenated fatty acids were re-extracted in 2-4 ml of toluene and the resulting toluene/palladium charcoal suspension centrifuged at 2600 rpm for six minutes. The supernatant containing the methylated fatty acids was removed and stored in a 20 mm X 100mm Kimax® screw top centrifuge tube in a refrigerator until required.

3.2.5.1.3 Analysis of fatty acids

The methylated fatty acids samples A (unsaturated) and B (both saturated and unsaturated) and standards were analysed by injecting 3 or 4 µl (depending on sample concentration) of each sample into a Shimadzu GC-8A Gas Chromatograph. A 2.6 metre packed column (15% ethylene glycol succinate (EGSS-X) on Chromosorb W A/W, (Waters Associates Inc., USA)) was used with nitrogen as the carrier gas at 90 kPa pressure and an oven temperature of 185°C. The temperature of the injection port and detector port were maintained at 200°C. A flame ionisation detector was used to detected fatty acids in the carrier gas.

The gas chromatograph was attached to a Sekonic SS-250F chart recorder. The quantity of individual fatty acids present, in mg/g of seed on a dry weight basis, was determined from the chromatograms (Appendix 1) using the following formula:

$$\frac{\text{height (H) (mm)} \times \text{width(mm)(at half H) of unknown fatty acid}}{\text{height(mm)} \times \text{width(mm)(at half H) of } C_{15}} \div \text{seed dry weight(g)} \times \frac{15}{\text{carbon number}} \times 20.6 \times \frac{\text{mol. weight}}{1000}$$

where 20.6 is the number of µmoles of internal standard present. The chart speed (150 mm/h) remained constant, therefore no correction was required, but where necessary corrections were made for changes in attenuation.

Fatty acids were identified by comparison of peak retention times with retention times of known fatty acids injected (2 µl) immediately after running samples. The standards were prepared by derivation of individual fatty acids purchased from Sigma Chemical Company (St Louis, Missouri) using the protocol described in ***3.2.5.1.1***. Some, but not all, of the "standard" fatty acids were combined and injected as a single "total standard mixture".

To achieve better separation of individual fatty acid methyl esters, 1 µl of samples of sample A (no unsaturated sample B was available) and sample B (hydrogenated), samples C - F and standards were analysed using a Hewlett-Packard 5890 Series II Plus Gas Chromatograph. Samples were injected via a Hewlett-Packard 7673 GC/SFC injector. A BPX-70 capillary column (SGE International Pty Ltd, Ringwood, Victoria, Australia, Appendix 2) was used with a hydrogen gas flow rate of 6.92 ml/minute. The oven temperature was initially set to 120°C and was raised in 5°C/minute increments to 175°C where it remained for the remainder of the run. The injector and detector temperatures were both maintained at 190°C. A flame ionisation detector was used to detect fatty acids in the carrier gas. All gas chromatograph settings and the run sequence were controlled using Hewlett-Packard Series II ChemStation software. Data was outputted to a text file and chromatograms were generated by the ChemStation software (Appendix 3 and Appendix 4). Both were printed using a Hewlett-Packard Desk Jet 500 printer. Areas under individual peak were automatically integrated by the ChemStation software. The quantity of individual fatty acids present, mg/g of seed, were determined using the formula used for the Shimadzu GC-8A chromatograms with the exception that peak areas were read directly from the text file.

3.2.5.1.4 Correction for loss of glycerol residues

Fatty acid weights calculated in ***3.2.5.1.3*** were corrected for loss of the glycerol residue of the triacyglyceride during the transesterification and methylation using the following formula:

$$\frac{\text{amount of fatty acid (mg g}^{-1})}{\text{molecular weight of fatty acid (mol g}^{-1})} \div 3 \times 41.1 \text{ mol g}^{-1}$$

where 3 is the number of acyl groups in the triacylglyceride and 41.1 the molecular weight of the glyceride residue less the three hydroxyl groups lost in the formation of the ester bond.

Mean and standard errors were calculated, where appropriate, for retention times, fatty acid weights and percentages.

3.2.5.2 Soxhlet extraction

Two 28 mm (internal diameter) x 80 mm Whatman single thickness cellulose extraction thimbles were dried overnight at 60°C and then allowed to cool in a desiccator for twenty minutes before being weighed. Approximately 2.7 g of *M. hortensia* seed was ground in a coffee grinder. The sample was split in two and each sub-sample placed in a pre-weighed extraction thimble and then re-dried at 60°C for half-an-hour. The thimble and seed was allowed to cool in a desiccator for twenty minutes before being re-weighed. A metal beaker, one for each sample, was also pre-dried at 60°C, allowed to cool and then weighed.

Lipids were extracted using a Tecator HT 1043 extraction unit (soxhlet system). Fifty ml of petroleum spirit (60-80°C fraction) was placed in each metal beaker. A piece of cotton wool was placed in the top of each thimble to prevent splashing and the thimble inserted in a metal ring. The thimbles were positioned in the extraction unit via magnets and the metal ring and a beaker of known weight placed under each thimble. The temperature was raised to 125°C by pumping heated Dow Corning silicone fluid (200 Fluid 50 c.s.) through the system. Once the temperature had reached 125°C, the petroleum spirit was allowed to reflux through each thimble for half-an-hour. At the end of the half-hour the thimble was immersed in the petroleum spirit and the petroleum spirit allowed to rinse through the thimble at 125°C for another half-an-hour. The system was then opened and air forced through until the majority of the petroleum spirit had evaporated from each beaker (10-12 minutes). The beakers were removed from the system and the remaining petroleum spirit allowed to evaporate at room temperature. The beakers, and extracted lipid, were placed in a 60°C oven for 10 minutes to ensure no solvent remained and then put into a desiccator for 10 minutes to cool before being re-weighed.

The percentage of lipid present was calculated by dividing the weight of lipid extracted by the seed weight, prior to pre-drying at 60°C, corrected to a dry weight basis. The mean of the duplicate samples and standard error associated with that mean was then calculated.

3.2.5.3 Methanol-chloroform extraction

Total fatty acids were extracted from duplicate samples of a known amount (approximately 2 g) of *M. hortensia* seed (ground in a Braun Type 4 0 41 coffee grinder). Each sample was agitated in a 50 ml Falcon centrifuge tube containing 20 ml of 50:50 methanol/chloroform. Each tube was left to stand for 90 minutes, with shaking every 30 minutes. The tubes were centrifuged (IEC Centra GP8R centrifuge) at 3100 rpm for 7 minutes at 16°C and the methanol/chloroform supernatant from each pipetted into a round-bottomed flask. Another 20 ml of 50:50 methanol/chloroform was added to the centrifuge tubes and the seed flour homogenised in a Kinematica GmbH PT 10.35 Poytron homogeniser. To reduce sample loss the homogeniser head was rinsed with a minimum of methanol into the centrifuge tube and the samples left to extract for a further 15 minutes with agitation every five minutes. The samples were re-centrifuged and the methanol/chloroform supernatant added to the first methanol/chloroform extract. Chloroform (20 ml) was added to the remaining seed flour and allowed to extract for a further 15 minutes with agitation every five minutes. While the chloroform was extracting the volume of the methanol/chloroform was reduced to 4-5 ml on a rotary evaporator (Büchii Rotavapor-Re) using a water bath temperature of 45°C. For each sample the chloroform only extract was centrifuged (3100 rpm for 7 minutes at 16°C) and the supernatant added to the round-bottomed flask containing the reduced methanol/chloroform extract. The total volume was again reduced to 4-5 ml on the rotary evaporator. The concentrate was transferred to a 50 ml Falcon centrifuge tube and the round bottom flask rinsed with an equal volume of water to remove any polar compounds from the concentrate. Each flask was rinsed with a 10 ml aliquot of chloroform followed by a further two 5 ml aliquots of chloroform. The water layer was aspirated off and anhydrous sodium sulphate (1-2 g) was added to the chloroform to remove any residual water. The lipid-containing-extracts were left overnight in the dark at room temperature.

The following day 1-2 g of anhydrous sodium sulphate was placed in the bottom of a fluted sheets of 125 mm Whatman No. 1 filter paper and the extracts filtered through them into round-bottomed flasks. The centrifuge tubes were rinsed three times with chloroform. The chloroform washings were also passed through the filter paper/anhydrous sodium sulphate into the round-bottomed flask. The chloroform extracts were reduced to 1- 3 ml on the rotary evaporator, again, using a water bath set at 45°C. The reduced extracts were pipetted into individual 10 ml volumetric flasks that had been dried in a 60°C oven

and cooled in a desiccator. The volume in each flask was made up to 10 ml with chloroform washings from the round-bottomed flask. The 10 ml extracts were transferred to 25 ml beakers. From each beaker 5 ml was transferred into a second weighed 25 ml beaker. The volumetric flask was rinsed with a minimum of chloroform and the washings added to the 5 ml of extract in the original beaker. The beakers were left in a fume-hood over a weekend (65 hours) to allow the chloroform to evaporate. To remove any residual chloroform, lipid extracts were dried in a 60°C oven for fifteen minutes and then cooled in a desiccator for a further ten minutes. The lipid and beakers were then weighed and the mean weight of lipid per gram of seed determined on a dry weight basis. The weight of lipid for the two 5 ml volumes of the same sample were combined and the mean of the duplicate samples and the standard error associated with that mean were calculated.

3.2.5.4 Thin layer chromatography

3.2.5.4.1 Lipid extraction

Lipids were extracted from 1-2 g of ground *M. hortensia* seed remaining from the FAME extraction (***3.2.5.1.1***) by agitating the seed flour in a 125 ml volumetric flask containing 20 ml of 50:50 methanol/chloroform. The flask was left to stand for 30 minutes, with shaking every ten minutes. The 50:50 methanol/chloroform mixture was decanted off and a further 20 ml of chloroform added to the flask. The flask was left to stand for a further 30 minutes, with shaking every ten minutes, before the contents were transferred to a 50 ml Falcon centrifuge tube and centrifuged at 3100 rpm for 7 minutes. The 50:50 methanol/chloroform mixture was transferred to a round-bottomed flask and the volume reduced to 4-5 ml on a rotary evaporator (Büchii Rotavapor-Re) using a water bath temperature of 45°C. The chloroform only extract was added and the solution again reduced to 4-5 ml on the rotary evaporator. The concentrate was transferred to a 50 ml Falcon centrifuge tube and the round bottom flask rinsed with an equal volume of water and then with a 10 ml aliquot of chloroform followed by two 5 ml aliquots of chloroform. The water layer was aspirated off and anhydrous sodium sulphate (1-2 g) was added to the chloroform. The mixture was centrifuged at 2600 rpm for six minutes. The fatty acid-containing chloroform was transferred to a round bottom flask and reduced to a small volume (approximately 5 ml) on the rotary evaporator in a water bath running at 45°C. The solution was placed in a 20 mm X 100 mm Kimax® screw top centrifuge tube and stored in a refrigerator (at 4°C) until required.

3.2.5.4.2 Preparation of plates

Thin layer chromatography plates were prepared by washing 200 mm x 200 mm x 2 mm Agee® Agricultural Glass plates twice in acetone to remove all traces of oil. The cleaned plates were mounted on a guide. A slurry was prepared by suspending 40 g Kieselgel $60HF_{254}$ in 110 ml of water. The suspension was agitated twice and then poured in a spreader set for a plate thickness of 0.25 mm. The spreader was attached to the guide and moved along the guide depositing the slurry as a thin (0.25mm) film on top of the plates. The plates were allowed to air dry and then activated by placing them in a 105°C oven overnight.

3.2.5.4.3 Lipid component identification

A 200 µm capillary tube was used to load the following fatty acid samples and standards onto the thin layer plates:

1. *Limnanthes alba* extracted as described for *M. hortensia* in ***3.2.5.2.1***
2. *M. hortensia*
3. lecithin (phospholipid)
4. triacylglycerol (extracted from coconut)
5. cholesterol (sterol)
6. palmitic acid (fatty acid).

Adjacent duplicate spots of each sample were loaded onto the bottom of the plate. Twice as much sample (two spots dispensed from the capillary tube) was loaded at the left position compared to the right. The plate was placed in a glass tank containing 10 ml of a mixture of 70 ml of heptane (69%), 30 ml of diethyl-ether (30%) and 1 ml of glacial acetic acid (1%). A glass lid was placed on the tank to seal the system and the tank placed in a fume hood. Once the solvent front had moved three-quarters of the way up the plate the plate was removed from the tank and placed with the solvent front downwards against the tank to air-dry. The dry plate was sprayed with 2,7-dichloroflourescein (0.1 g in 100 ml of methanol) using a glass flask with a spray head made from glass tubing attached and an air pump. A long wavelength ultraviolet light source (Chromovue) was used to observe and photograph the staining pattern.

3.3 Results

3.3.1 General Characteristics

Seed of *M. hortensia* is contained within a nutlet. The nutlets are flat with an uneven serrated wing all the way around. At high seed moisture content the nutlet body is green and fleshy but as seed moisture is lost the nutlet body turns brown-black to black and becomes papery (Plates 3.1 and 3.1). Each nutlet contains a single seed. At maturity the seed is "free" within the nutlet. The seed is obovate and generally flat to slightly concave in profile. At 7.5% seed moisture a typical seed is approximately 8-9 mm long and 6 mm wide at it widest point and 1 mm thick. The seed coat is olive-green to black in colour and is wrinkled and leathery in appearance (Plate 3.3). The seed is a dicotyledon. The embryo is predominantly cotyledonary tissue with a small embryo axis at the proximal end of the seed (Figure 3.1). Germination in *M. hortensia* is epigeal; the cotyledons are carried above ground by extension of the hypocotyl. The cotyledons expand and become photosynthetic. The first, visible sign, of embryo growth is penetration of the radicle through the seed coat, although in some seed the cotyledon emerges first. In these instances, if the radicle remains trapped in the coat, it can decay.

Plate 3.1: *M. hortensia* fruiting head showing both clusters of white flowers and nutlets.

Plate 3.2: *M. hortensia* fruiting head showing both green (➔) and black (→) nutlets.

Plate 3.3: A single seed of *M. hortensia* showing the wrinkled and leathery appearance of the seed coat (seed length approximately 9 mm, moisture content approximately 7.5%).

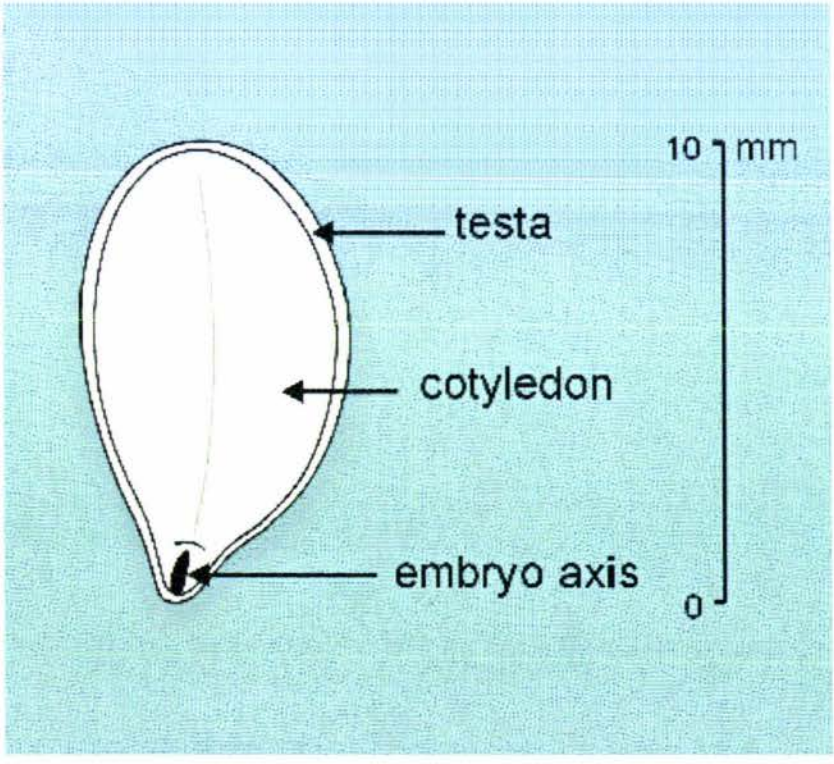


Figure 3.1: Stylised diagram of a *M. hortensia* seed, bisecting the embryo axis longitudinally, showing the approximate size and position of the embryo axis, cotyledon and testa (seed coat).

3.3.2 Histology

Histochemical evaluation of the embryo following staining with Coomassie Brilliant Blue indicated the presence of protein and cellulose but an absence of starch. Blue-stained spherical protein bodies were seen to be distributed throughout the cotyledons, vascular tissue and epidermis (Plates 3.4 and 3.5). A single cell layer between the embryo and testa, is identified as endosperm. The endosperm cells also appear to contain protein bodies (Plate 3.6). Sections stained with safranin and fast green showed positive staining for cell wall cellulose and cytoplasm (both green) and for nuclei (brilliant red) (Plate 3.7). Confirmation of the presence of nuclear material was obtained with positive staining of chromatin with Schiff's Reagent (Plate 3.8) although some non-specific staining was observed in the epidermal region. In contrast to the cotyledon cells the endosperm cells are oblong and cell walls are thicker (Plates 3.9 and 3.10). No nuclei were clearly visible in the endosperm cells.

Staining of sections for fat with Sudan IV was not definitive with sections showing some weak positive staining but this appeared to be non-specific. Staining was insufficient for any contrast between stained and non-stained areas in the section to be visible in the photographs taken. However, in hand sectioned tissue stained with Sudan IV red-stained spherical oil droplets, presumably released during sectioning of the seed, were observed (Plates 3.11 and 3.12).

Positive staining of hand-sectioned meadowfoam seed and potato tissue confirmed that both the Sudan IV and potassium iodide/iodine stains were functioning. Similarly Schiff's Reagent stained positively for chromatin material in hand-sectioned *Pisum sativum* and *Limnanthes alba* seed tissue confirming that this stain was also functioning. Non-specific staining with Schiff's Reagent was also observed in these hand-sections (Plates 3.13 and 3.14).

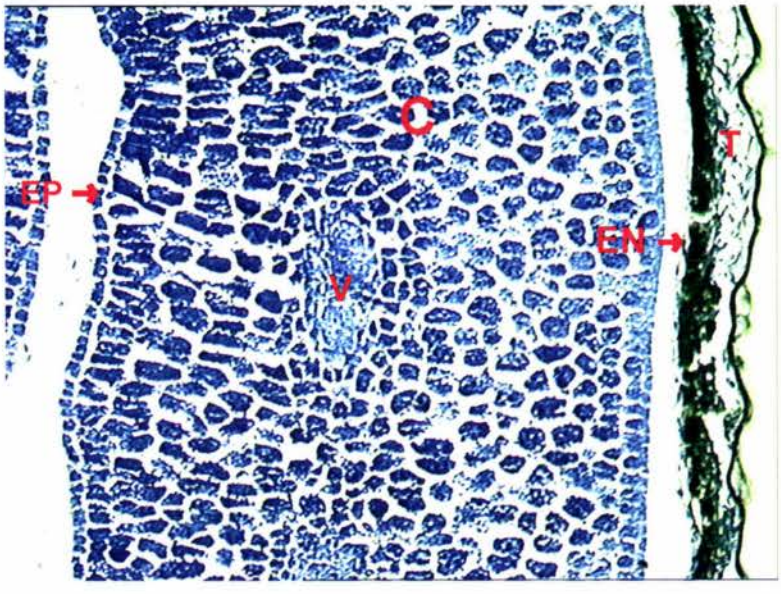


Plate 3.4: Transverse section through a *M. hortensia* cotyledon stained with Coomassie Brilliant Blue R250 showing staining to the cotyledon (C), vascular tissue (V) and epidermis (EP). Also visible is the single cell layer of remnant endosperm (EN) attached to the testa (T). Magnification 100x.

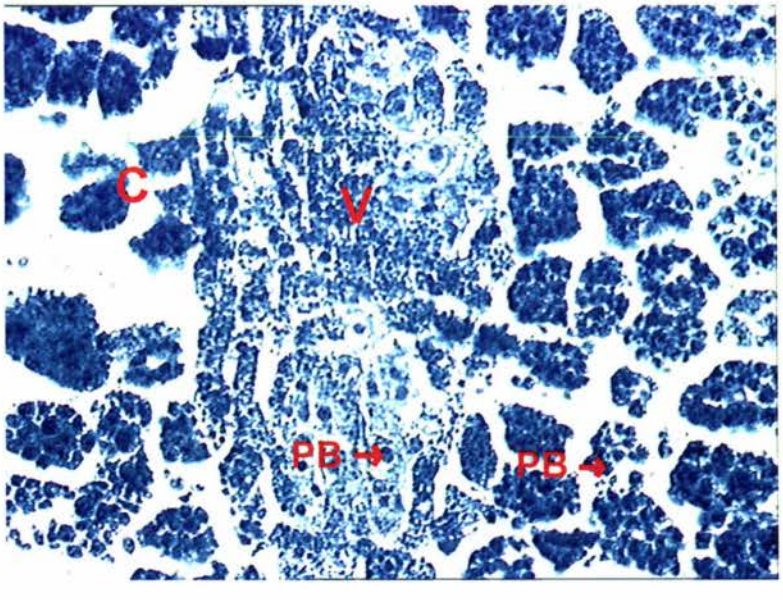


Plate 3.5: Transverse section through a *M. hortensia* cotyledon stained with Coomassie Brilliant Blue R250 showing staining to protein bodies (PB) in the cotyledon (C) and vascular tissue (V). Magnification 400x.

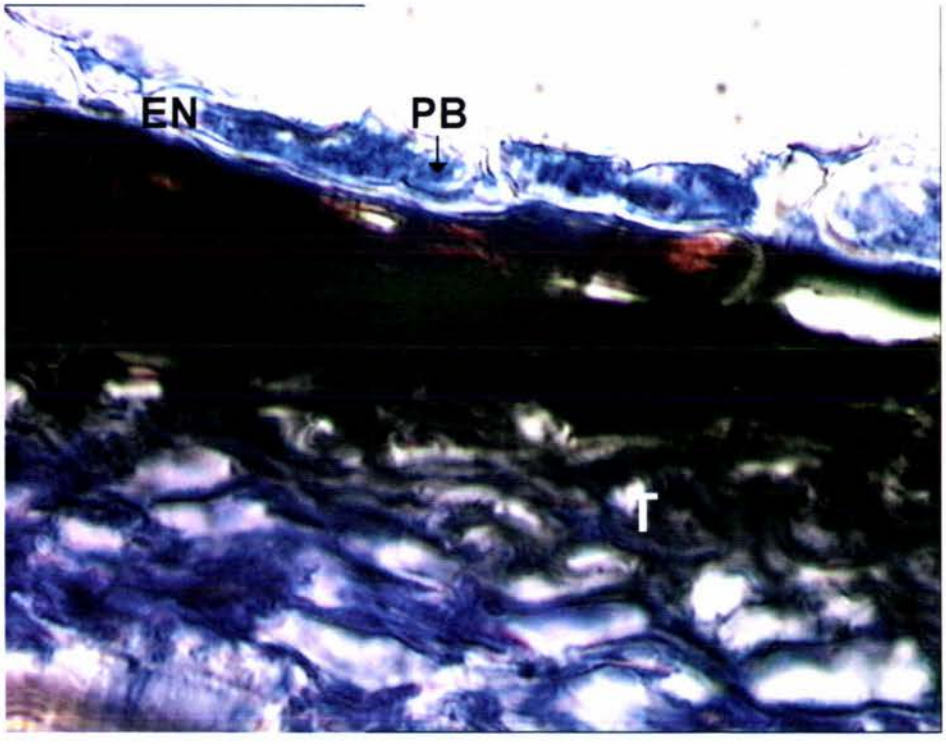


Plate 3.6: Transverse section through the testa (T) and endosperm (EN) of a *M. hortensia* seed stained with Coomassie Brilliant Blue R250 showing staining to protein bodies (PB) in the endosperm layer. Magnification 1000x.

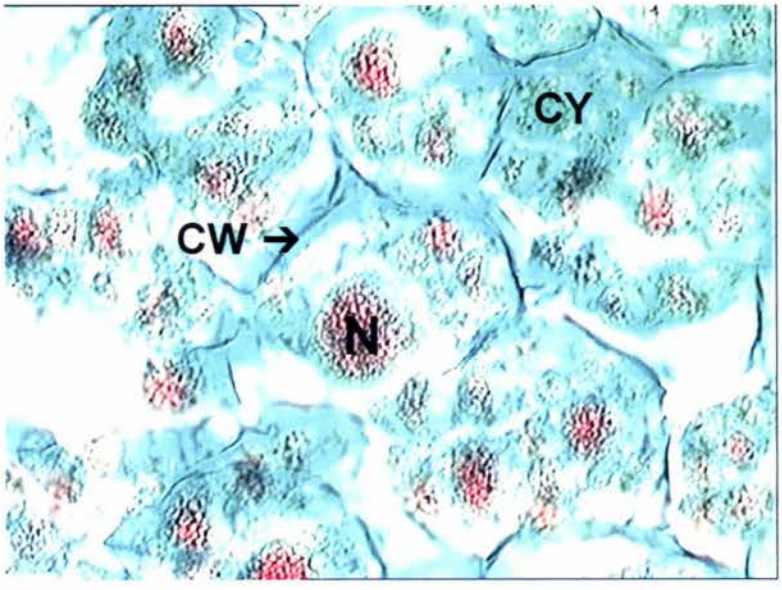


Plate 3.7: Transverse section through a *M. hortensia* embryo stained with safranin and fast green showing staining to cell wall cellulose (CW), cytoplasm (CY) and nuclei (N). Magnification 1000x.

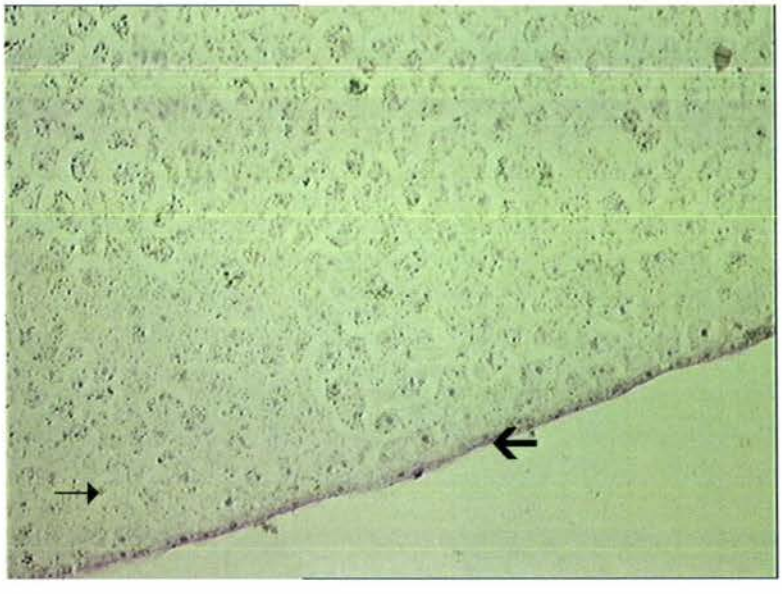


Plate 3.8: Longitudinal section through a *M. hortensia* embryo stained for one hour with Schiff's Reagent showing staining to cell nuclei (→) and non-specific staining in the epidermal region (➔). Magnification 200x.

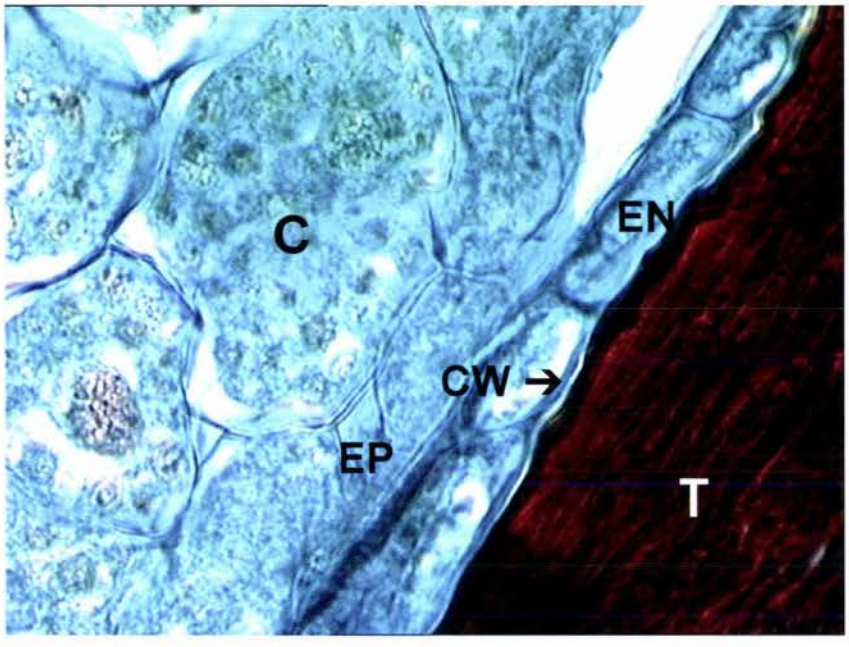


Plate 3.9: Transverse section through a *M. hortensia* seed stained with safranin and fast green showing the thickened cell walls (CW) of the endosperm (EN) compared to the cotyledon (C) and epidermis (EP) cell walls. The dark mass to the right of the endosperm is testa (T). Magnification 1000x.

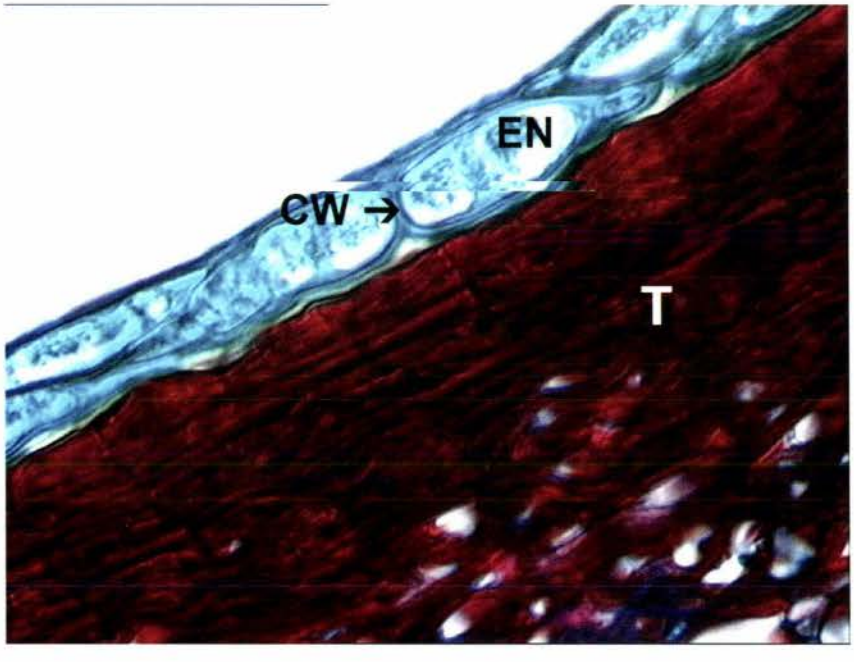
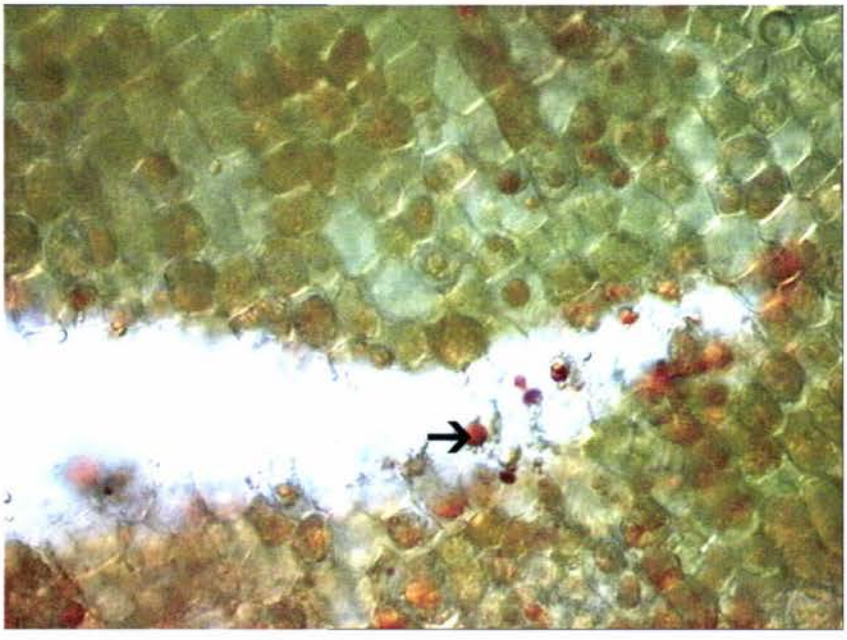
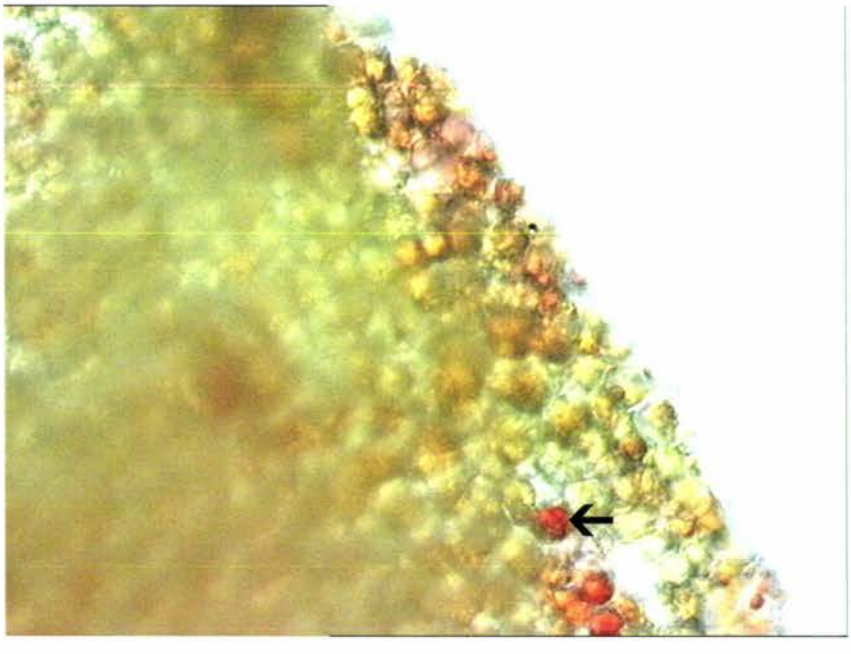


Plate 3.10: Transverse section through a *M. hortensia* seed stained with safranin and fast green showing the thickened cell walls (CW) of the endosperm (EN). The dark mass to the right of the endosperm is testa (T). Magnification 1000x.





Plates 3.11 (top) and 3.12 (bottom): Hand sections through a *M. hortensia* seed stained with Sudan IV showing oil droplets (→) from disrupted cells. Magnification 200x.

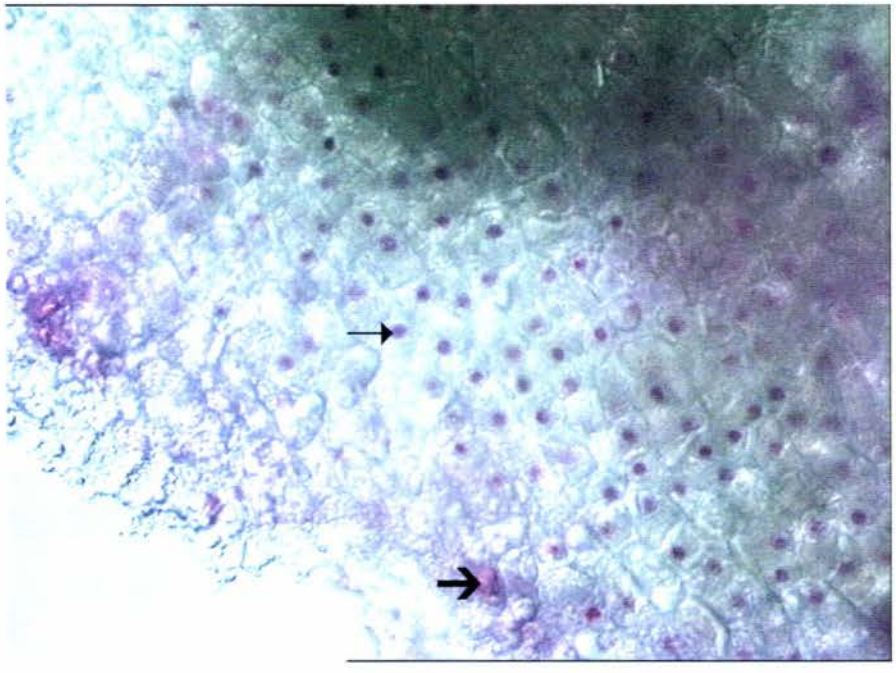


Plate 3.13: Hand section through a *Limnanthes alba* seed stained with Schiff's Reagent for one hour showing staining to nuclei (→) and non-specific staining in some cells (➔). Magnification 400x.

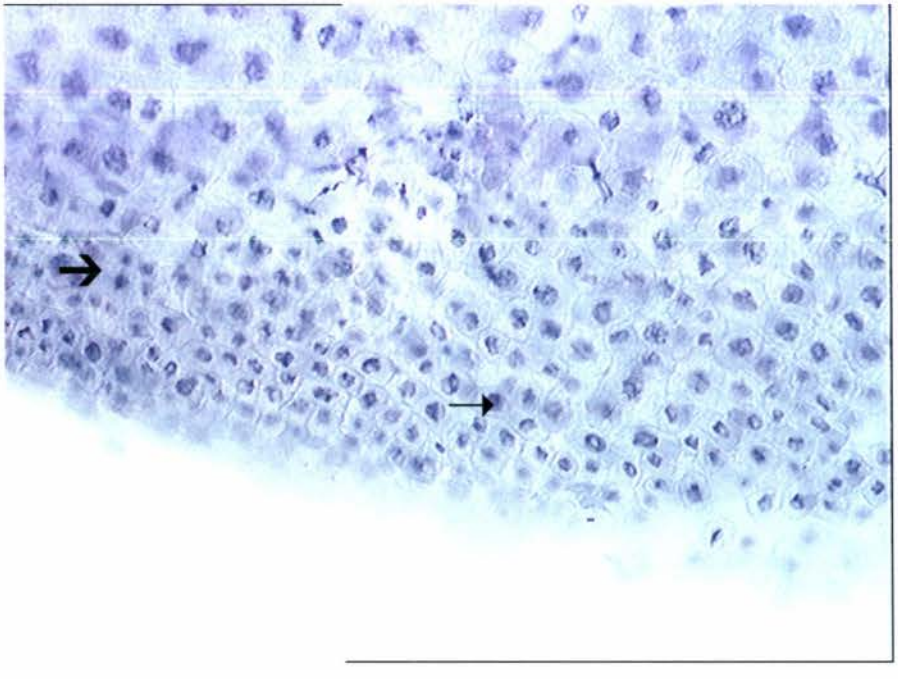


Plate 3.14: Hand section through a *Pisum sativum* radicle stained with Schiff's Reagent for one hour showing staining to nuclei (→) and non-specific staining in cells (➔). Magnification 200x.

3.3.3 Transmission Electron Microscopy

Light microscopy confirmed the presence of vascular tissue within the cotyledons of *M. hortensia* and a single-cell thick layer of epidermal cells surrounding the cotyledons (Plate 3.15). The single cell layer of remnant endosperm, is clearly visible in sections through the testa (Plate 3.16).

Transmission electron microscopy showed that the cell cytoplasm in the embryo is heavily packed with lipid bodies (Plate 3.17). The bodies ranged in shape from round to oval in appearance. Stored within the cytoplasm are also protein bodies. These are much fewer in number than the lipid bodies but are significantly larger (Table 3.2). Inclusions are found within the dark proteinaceous matrix of some of the protein bodies. These inclusions are white or opaque in colour. The majority are round in shape, although some are oblong or have an irregular appearance. The lipid and protein bodies occupy the majority of the cellular space with the remaining area appearing to be cytoplasm. In some cells a single nucleus is visible (Plate 3.18).

Table 3.2 The mean number and diameter of oil or protein bodies and protein inclusions in the cotyledon cells of *M. hortensia*.

Structure	Number (± standard error, n)	Diameter (± standard error, n), (µm)
Oil Bodies	255.8 (±44.18, 8)	0.86 (±0.046, 5)
Protein Bodies	9.6 (±2.78, 15)	3.02 (±0.243, 13)
Inclusions	1.2 (±0.02, 13)	0.81[a] (±0.146, 7)

n = the number of replicates analysed

[a]For oblong or irregular inclusions the longest axis was measured

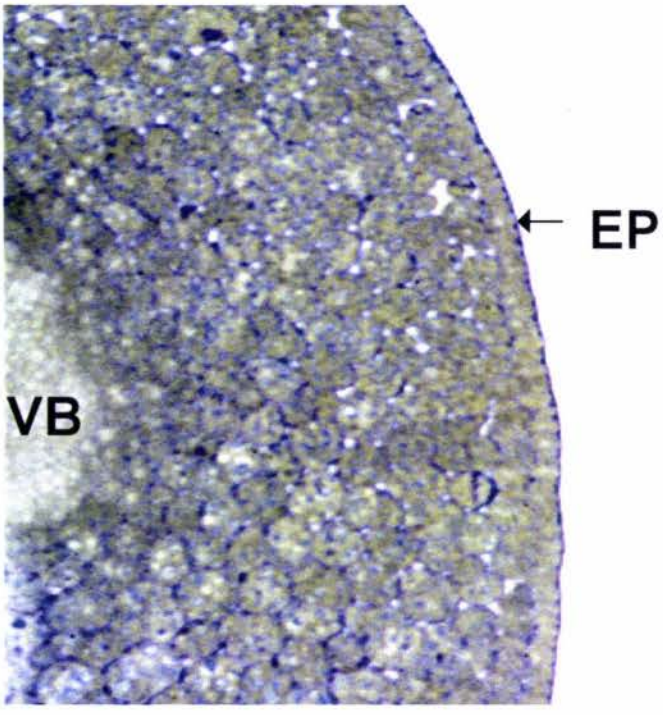


Plate 3.15: Transverse section through a *M. hortensia* cotyledon stained with Toluidine Blue showing the vascular bundle (VB) within the cotyledon and single-cell outer epidermis (EP). Magnification 100x.

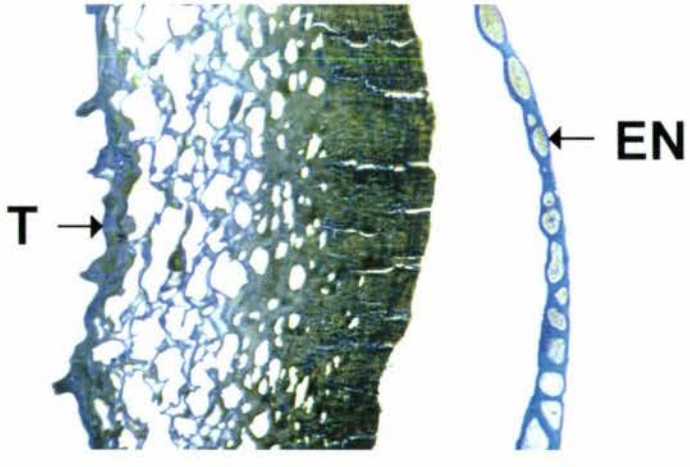


Plate 3.16: Section through the seed coat of *M. hortensia* stained with Toluidine Blue showing the testa (T) and single cell layer of remnant endosperm (EN). Magnification 400x.

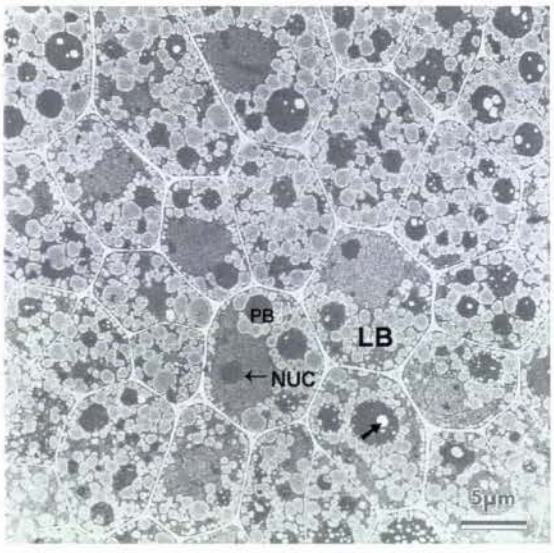


Plate 3.17: Transverse section through a *M. hortensia* embryo showing the cells heavily packed with lipid bodies (LB) and a smaller number of protein bodies (PB). Many of the protein bodies contain inclusions or tears where inclusions have been lost during sectioning (↗). A single nucleus (NUC) is visible in some cells.

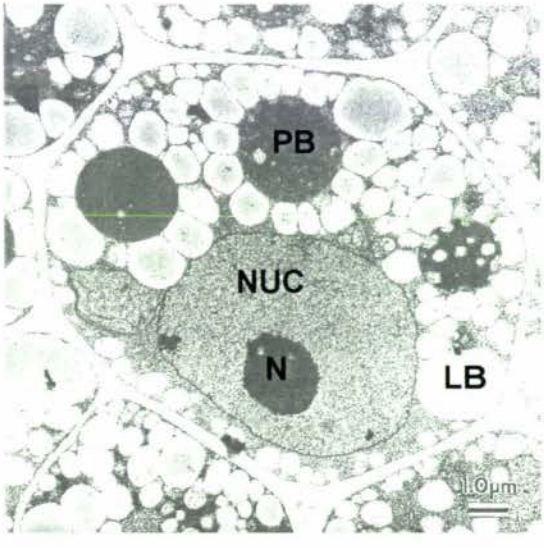


Plate 3.18: Transverse section across a *M. hortensia* embryo cell showing a single nucleus (NUC), (containing a nucleolus (N)), surrounded by lipid (LB) and protein (PB) bodies.

3.3.4 Lipid Analysis

3.3.4.1 Gas chromatography

The fatty acids present in *M. hortensia* were identified by comparing retention times of sample fatty acid peaks with those in the total standard mixture. The loss of peaks assigned to unsaturated fatty acids and the increase in peaks assigned to saturated fatty acids in the saturated (hydrogenated) sample (Appendix 1) confirmed many of the identifications made from comparison of retention times.

In the chromatogram from the packed column three fatty acid peaks in the unsaturated *M. hortensia* sample did not have corresponding peaks in the standard mixture. These were peaks with retention times of 16.8 minutes, 44.0 minutes and 57.2 minutes respectively. These three unidentified peaks were not present in the hydrogenated *M. hortensia* sample suggesting they represent unsaturated fatty acids (Table 3.3).

The close proximity of the 16.8 minute peak to the palmitic acid ($C_{16:0}$) peak (14.4 minutes) suggests that this unknown peak may represent palmitoleic acid ($C_{16:1}$).

The second, unidentified, peak (retention time 44.0 minutes) is too early to be the α-isomer of linolenic acid ($C_{18:3}$, $\Delta^{9,12,15}$), which has a standard retention time of 48.6 minutes. It is possible that this peak represents the γ-isomer of linolenic acid ($C_{18:3}$, $\Delta^{6,9,12}$). The *M. hortensia* sample does have a peak with a retention time (48.8 minutes) close to that of the α-linolenic acid standard. In the hydrogenated sample the peaks at 44.0 minutes and 48.8 minutes disappear indicating they represent unsaturated fatty acids. The loss of these two peaks was accompanied by a commensurate increase in the peak representing stearic acid (77.6%). Two substantially reduced peaks representing oleic (1.1%) and linoleic (0.9%) acid remain indicating incomplete hydrogenation has occurred. In the hydrogenated sample the C_{18} peaks comprise 79.6% of the fatty acid complement compared to 80.8 (±0.45)% in the saturated sample. This latter figure assumes the peak with the 48.8 minute retention time is α-linolenic acid, in the unsaturated sample (Table 3.3). This difference can mostly be accounted for by sampling variation. However, in the hydrogenated sample a peak with a similar retention time (49.6 minutes) not only remains but shows an increase from 2.0 (±0.03)% fatty acid in the unsaturated sample to 5.8% in the hydrogenated sample. The peak with a 48.8 minute retention time may therefore be

arachidic acid ($C_{20:0}$) rather than α-linolenic acid or, more likely, a combination of both.

The increase in area of the peak with a retention time of 49.6 minutes and disappearance of the third unknown peak (retention time 57.2 minutes) after hydrogenation is consistent with this third unknown peak representing an unsaturated C_{20} fatty acid, possibly *cis*-11-eicosenoic acid ($C_{20:1}$). The combined fatty acid percentage of the peaks with retention times 48.8 minutes and 57.2 minutes is 6.5 (± 0.70) %. The peak with a retention time of 49.6 minutes contributed 5.8% to the fatty acid total in the hydrogenated sample. This is lower than the 6.5% of the combined peaks but this difference can also be accounted by the sampling variation in the unsaturated sample alone. The identity of the 48.8 minute peak therefore remains unclear.

In contrast to the packed column separation of unsaturated and hydrogenated *M. hortensia* samples on the BPX-70 capillary column resulted in greater differentiation of the component fatty acids (Appendix 3). This allowed the presence of both α-linolenic acid and arachidic acid to be confirmed (Table 3.4). However, both are present in relatively small amounts, 1.1 (±0.03)% and 1.5 (±0.03)% of total fatty acids respectively. Also identified were two peaks with retention times of 7.889 minutes and 8.534 minutes. These retention times are to short for these peaks to represent arachidonic acid. Neither peak was identified in the hydrogenated sample (Table 3.5). These peaks may represent the unsaturated C_{20} fatty acids (*cis*-11-eicosenoic acid ($C_{20:1}$) and *cis*-11,14-eicosadienoic acid ($C_{20:2}$). If so the monounsaturated *cis*-11-eicosenoic acid contributes 5.4 (±0.03)% to the total amount of fatty acids present in the seed. The much reduced retention times on the capillary column also enabled identification of four longer chain, C_{22} and C_{24}, fatty acids in the seed. Three of these longer chain fatty acids could be identified by comparing their retention times with those of standards separated on the column (Table 3.4). These fatty acids were identified as behenic ($C_{22:0}$), erucic ($C_{22:1}$) and lignoceric acid ($C_{24:0}$). The fourth peak had a retention time of 12.172 minutes. None of the standards used had a similar retention time. In the hydrogenated sample this peak, contributing 2.6% of the fatty acid compliment, disappears (Appendix 4). This 2.6% loss is accounted for by an increase in the percentage of lignoceric acid from 0.3% in the unsaturated sample to 2.8% after hydrogenation (Table 3.5). It is likely this peak represents an unsaturated C_{24} fatty acid. The close proximity of this peak to the lignoceric acid peak (retention time 11.749 minutes) suggests it may be the mono-unsaturated nervonic ($C_{24:1}$) acid.

The percentage of C_{18} carboxylic acids present is 70.9 (±0.34)% (Table 3.4). These are predominantly the unsaturated C_{18} acids; oleic acid (40.7 (±0.25)%), linoleic acid (15.2 (±0.26)%) and γ-linolenic acid (9.0 (±0.14)%). The total percentage of C_{18} fatty acids identified on the packed column is higher than that on the BPX-70 capillary column however this can be accounted for by the contribution of the C_{22} (3.2 (±0.04)%) and C_{24} (3.3 (±0.05)%) fatty acids not identified on the packed column and separation of percentage contributions from α-linolenic (1.1 (±0.03)%) and arachidic (1.5 (±0.03)%) acid.

The peak assigned to erucic acid has a retention time (10.062 minutes) similar to that of the *cis*-5, 8, 11, 14, 17-eicosapentaenoic acid ($C_{20:5}$) standard (10.009 minutes), (Table 3.5). It is possible that the sample peak at 9.991 minutes represents this unsaturated C_{20} fatty acid. However, in the hydrogenated sample this peak, contributing 2.4% of the fatty acid content of the seed, disappears. This 2.4% loss is accounted for by an increase in the percentage of behenic acid from 0.5% prior to hydrogenation to 2.8% after hydrogenation (Table 3.5). The 9.991 minutes peak must represent an unsaturated acid and this unsaturated acid is most likely erucic acid.

The total amount of fatty acids in *M. hortensia* calculated by measuring the area under the chromatogram peaks of the capillary column was 219.7 (± 17.96) mg of fatty acid/gram of seed or 22.0 (±1.80)%, (Table 3.4).

Table 3.3 **Retention times (minutes) and percentage of fatty acid methyl esters extracted from *M. hortensia* (unsaturated samples A and B and hydrogenated sample B) seed and separated on a 2.6 metre (15% ethylene glycol succinate (EGSS-X) on Chromosorb W A/W) packed column.**

Carbon Number	Systematic Name	Common Name	Retention Time (Minutes)			Fatty Acid Percentage (%)	
			Standards	*M. hortensia*	*M. hortensia* after hydrogenation	*M. hortensia*[a]	*M. hortensia* after hydrogenation[b]
c14:0	tetradecanoic	Myristic	7.6	-	-	-	-
c16:0	hexadecanoic	Palmitic	14.2	14.4	14.6	11.2 (±0.77)	14.6
C16:1 9*c*	cis-9-hexadecenoic	Palmitoleic	-	16.8	-	3.5 (±0.50)	-
c18:0	octadecanoic	Stearic	26.4	26.8	27.6	4.1 (±0.42)	77.6
c18:1 9*c*	*cis*-9-octadecenoic	Oleic	30	30.4	30.8	41.3 (±0.29)	1.1
c18:2 9*c*, 12*c*	*cis,cis*-9,12-octadecadienoic	Linoleic	37.2	37.2	-	20.1 (±0.27)	0.9
c18:3 6*c*, 9*c*, 12*c*	*cis*, *cis*, *cis*-6, 9, 12-octadecatrienoic	γ-Linolenic	-	44.0	-	13.3 ((±1.41)	-
c18:3 9c, 12*c*, 15*c*	*cis*, *cis*, *cis*-9, 12, 15-octadecatrienoic	α-Linolenic	48.6	48.8	-	2.0 ((±0.03)	-
c20:0	eicosanoic	Arachidic	-		49.6		5.8
c20:1 11*c*	*cis*-11-eicosenoic	-	-	57.2	-	4.5 (±0.73)	-
Total						100	100

[a]fatty acid percentages in this column are averaged for samples A and B
[b]hydrogenated fatty acid percentages in this column are from sample B only.

Table 3.4 **Retention times (minutes) and weight and percentage of fatty acid methyl esters extracted from *M. hortensia* seed (samples C-F) and separated on a BPX-70 capillary column.**

Carbon Number	Systematic Name	Common Name	Retention Time (Minutes)		Fatty Acid Weight (mg/g seed) (± standard error)	Fatty Acid Percentage (%) (± standard error)
			Standards	*M. hortensia* (± standard error)		
c 10:0	n-decanoic	Capric	0.830	-	-	-
c12:0	dodecanoic	Lauric	1.312	-	-	-
c14:0	tetradecanoic	Myristic	2.214	-	-	-
c16:0	hexadecanoic	Palmitic	3.64	3.651 (±0.0029)	29.1 (±1.77)	13.3 (±0.27)
c16:1 9*c*	cis-9-hexadecenoic	Palmitoleic	-	3.965 (±0.0030)	4.9 (±0.20)	2.3 (±0.19)
c18:0	octadecanoic	Stearic	5.501	5.516 (±0..0024)	10.8 (±1.04)	4.9 (±0.13)
c18:1 9*c*	*cis*-9-octadecenoic	Oleic	5.794	5.863 (±0.0048)	89.4 (±7.56)	40.7 (±0.25)
c18:2 9*c*, 12*c*	*cis,cis*-9,12-octadecadienoic	Linoleic	6.416	6.444 (±0.0023)	33.5 (±3.11)	15.2 (±0.26)
c19:0	nonadecanoic	-	6.549	-	-	-
c18:3 6*c*, 9*c*, 12*c*	*cis*, *cis*, *cis*-6, 9, 12-octadecatrienoic	γ-Linolenic	-	6.829 (±0.0017)	19.9 (±1.74)	9.0 (±0.14)
c18:3 9*c*, 12*c*, 15*c*	*cis*, *cis*, *cis*-9, 12, 15-octadecatrienoic	α-Linolenic	7.202	7.203 (±0.0023)	2.5 (±0.23)	1.1 (±0.03)
c20:0	eicosanoic	Arachidic	7.596	7.598 (±0.0023)	3.4 (±0.33)	1.5 (±0.03)
c20:1 11*c*	*cis*-11-eicosenoic	-	-	7.889 (±0.0018)	11.8 (±1.00)	5.4 (±0.03)
c20:2 11*c*, 14*c*	*cis*-11, 14-eicosadienoic	-		8.534 (±0.0023)	0.3 (±0.10)	0.1 (±0.05)
c20:4 5*c*, 8*c*, 11*c*, 14*c*	*all-cis*-5, 8, 11, 14-eicosatetraenoic	Arachidonic	9.931	-	-	-
c20:5 5*c*, 8*c*, 11*c*, 14*c*, 17*c*	*all-cis*-5, 8, 11, 14, 17-eicosapentaenoic	-	10.009	-	-	-
c22:0	docosanoic	Behenic	9.694	9.661 (±0.0019)	1.3 (±0.15)	0.6 (±0.02)
c22:1 13*c*	*cis*-13-docosenoic	Erucic	10.062	9.991 (±0.0015)	5.6 (±0.50)	2.6 (±0.03)
c24:0	tetracosanoic	Lignoceric	11.805	11.749 (±0.0017)	0.9 (±0.10)	0.4 (±0.01)
c24:1 15*c*	*cis*-15-tetracosenoic	Nervonic	-	12.172 (±0.0021)	6.3 (±0.58)	2.9 (±0.05)
Total Fatty Acid					219.7 (±18.0)	100

Table 3.5 **Retention times (minutes) and percentage of fatty acid methyl esters extracted from *M. hortensia* seed (samples A (unsaturated) and B (hydrogenated)) and separated on a BPX-70 capillary column.**

Carbon Number	Systematic Name	Common Name	Retention Time (Minutes)			Fatty Acid Percentage (%)	
			Standards	*M. hortensia*	*M. hortensia* after hydrogenation	*M. hortensia*[a]	*M. hortensia* after hydrogenation[b]
c10:0	n-decanoic	Capric	0.830	-	-	-	-
c12:0	dodecanoic	Lauric	1.312	-	-	-	-
c14:0	tetradecanoic	Myristic	2.214	-	-	-	-
c16:0	hexadecanoic	Palmitic	3.640	3.658	3.65	14.7	18.7
c16:1	9-hexadecenoic	Palmitoleic	-	3.970	-	2.0	-
c18:0	octadecanoic	Stearic	5.501	5.522	5.592	4.7	70
c18:1 9*c*	*cis*-9-octadecenoic	Oleic	5.794	5.866	-	37.0	-
c18:2 9*c*, 12*c*	*cis,cis*-9,12-octadecadienoic	Linoleic	6.416	6.457	-	17.3	-
c19:0	nonadecanoic	-	6.549	-	-	-	-
c18:3 6*c*, 9*c*, 12*c*	*cis, cis, cis*-6, 9, 12-octadecatrienoic	γ-Linolenic	-	6.837	-	10.0	-
c18:3 9*c*, 12*c*, 15*c*	*cis, cis, cis*-9, 12, 15-octadecatrienoic	α-Linolenic	7.202	7.209	-	1.3	-
c20:0	eicosanoic	Arachidic	7.596	7.610	7.576	1.6	5.8
c20:1 11*c*	*cis*-11-eicosenoic	-	-	7.897	-	5.3	-
c20:2 11*c*, 14*c*	*cis*-11, 14-eicosadienoic	-	-	8.541	-	0.1	-
c20:4 5*c*, 8*c*, 11*c*, 14*c*	*all-cis*-5, 8, 11, 14-eicosatetraenoic	Arachidonic	9.931	-	-	-	-
c20:5 5*c*, 8*c*, 11*c*, 14*c*, 17*c*	*all-cis*-5, 8, 11, 14, 17-eicosapentaenoic	-	10.009	-	-	-	-
c22:0	docosanoic	Behenic	9.694	9.665	9.666	0.5	2.7
c22:1 13*c*	*cis*-13-docosenoic	Erucic	10.062	9.995	-	2.4	-
c24:0	tetracosanoic	Lignoceric	11.805	11.754	11.757	0.3	2.8
c24:1 15*c*	*cis*-15-tetracosenoic	Nervonic	-	12.178	-	2.6	-
Total Fatty Acid						100	100

3.3.4.2 Soxhlet and methanol-chloroform extractions

The percentage of lipid in *M. hortensia* seed determined by the soxhlet (petroleum spirit) extraction was 23.3 (± 0.85)%. This compares to 24.4 (± 0.25)% determined by the methanol-chloroform extraction. There was no significant difference in the percentage of lipid extracted from *M. hortensia* seed between assays. Nor were these percentages significantly different from the 22.0 (±1.80)% of fatty acid calculated by measuring the area under the chromatogram peaks of the capillary column.

3.3.4.3 Thin layer chromatography

Fractionation of lipid extracted from *M. hortensia* seed indicated the lipid present was predominantly triacylglycerols with some phospholipid present and a small amount of sterols. However, no free fatty acids were detected (Plate 3.19).

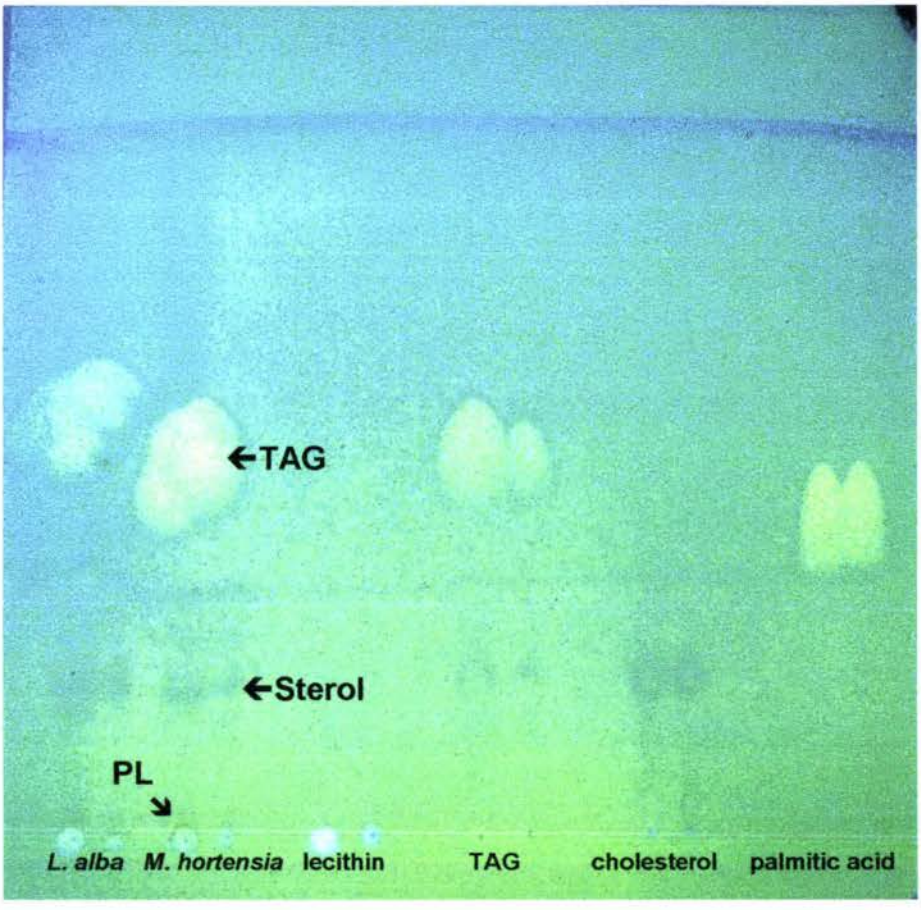


Plate 3.19: Thin layer chromatography (TLC) plate showing fractionation of lipid extracted from *M. hortensia* seed into the triacylglycerol (TAG), sterol and phospholipid (PL) components. Note on the TLC plate the phospholipid fraction remains at the origin. The TLC plate was photographed over a long wave ultraviolet light source.

3.4 Discussion

3.4.1 Histology

No histological or ultrastructural studies have been reported in the literature for *M. hortensia* seed, therefore, comparisons are made with other members of the Boraginaceae family.

The presence of vascular tissue within the cotyledons has been reported in other Boraginaceae, for example, Quinn *et al.* (1989) report by day 6 after fertilisation vascular traces were present in the expanded cotyledons of developing embryos of *Borago officinalis*. The single layer of cells observed between the epidermis and testa is likely to be remnant endosperm. Webb and Simpson (2001) suggest endosperm is usually absent in Boraginaceae but when present is "fleshy". Corner (1976) in his description of seed of Boraginaceae comments that the endosperm comprises several cell layers or is reduced to a single layer. Quinn *et al.* (1989) identified a free nuclear endosperm layer inside the ovule wall of a developing *B. officinalis* embryo and reports that the endosperm remains free nuclear until maturity.

West and Lott (1991) report that in *Begonia semperflorens* (a member of the Begoniaceae) the endosperm persists as a single cell layer that surrounds the embryo. The endosperm cells observed in *B. semperflorens* are oblong and have thickened cell walls; similar to the appearance of those in the single cell layer identified in *M.hortensia*. Although in an unrelated taxon the similarity between the single cell layer in *B. semperflorens* and *M. hortensia* may further suggest that this tissue is endosperm in *M. hortensia*.

The histology study indicated that the principal storage reserves in *M. hortensia* seed are protein with no starch reserves apparent, but the results for lipid were unclear. The presence of proteinaceous storage reserves was confirmed by the transmission electron microscopy which showed the cytoplasm within the embryo cells contain a number of protein bodies. The transmission electron microscopy also indicated the cytoplasm contained a large number of smaller lipid bodies confirming the observation of oil droplets "leaking" from hand sectioned *M. hortensia* seed. The reason for the poor and/or non-

specific staining for fat in the sectioned samples is difficult to explain. Johansen (1940) comments that because alcohol is a fat solvent exposure of the samples to alcohol should be kept to a minimum. It may be that lipid was lost during preparation of the sections for staining, however, it was certainly retained in the hand sectioned samples.

Huang *et al.* (1993) give a range of 0.65-2.0 μm for the diameter of oil bodies found in seed. Although in this study the oil bodies were not measured from serial sections, and therefore only giving an approximate indication of size, the 0.86 μm "diameter" of the oil bodies identified in *M. hortensia* does fall within the range identified by Huang *et al.* (1993).

For the same reason the numbers of oil bodies, and to a lesser extent the protein bodies, given should be regarded as indicative only. Moreover, these were assessed from photographs of EM sections and only for cells that were completely visible in the photograph. At higher magnifications only smaller cells could be evaluated and at lower magnifications some smaller oil bodies were not clearly distinguished. The numbers given may therefore be an underestimate of the number present. However a comparison of the mean "diameter" and number of protein bodies with those of the oil bodies does illustrate that while the protein bodies are significantly larger than the oil bodies, the oil bodies are far more numerous. A number of globoid-like inclusions are contained within these protein bodies. Although these inclusions differ in colour Otegui *et al.* (1998) suggest globoid inclusions are frequently removed during dehydration leaving a hole in the section that is white in appearance. It is likely, therefore, that the inclusions are of a single type and contain phytin.

3.4.2 Lipid Analysis

3.4.2.1 Lipid quantification

The soxhlet and methanol-chloroform extractions both indicated that seed of *M. hortensia* contains around 23-24% lipid. The two methods do not extract the same lipid types. In the soxhlet method used in this work no acid digestion step was included, and the solvent used (petroleum spirit) is non-polar. Only triacylglycerols and other simple (non-polar) lipids would have been extracted. Lipid physically "entrained" within the sample matrix and

complex lipids physically or chemically bound to proteins, polysaccharides and other cellular components are left behind (Lumley and Colwell, 1991). Nor would any polar lipids be extracted. In contrast, with a methanol-chloroform extraction very polar lipids are soluble. The fat percentage determined by this method did not differ significantly from that of the soxhlet extraction, suggesting the majority of lipids in *M. hortensia* seed are non-polar.

The fatty acid percentage (22.0%) calculated by measurement of fatty acid peak areas on the capillary column chromatogram was not significantly different to that identified by either the soxhlet or methanol-chloroform methods. The calculation of lipid percentage by measurement of peak areas may have been expected to yield a lower percentage than the other methods since only the triacylglycerol fraction is assessed. The similar percentages assessed by the three methods used suggests lipids present in the seed are predominantly triacylglycerols. This observation is consistent that of Senanayake and Shahidi (2000) who report that neutral lipids comprised 95.7% of total lipids in *Borago officinalis* and that 99.1% of the neutral lipids were triacylglycerols with free fatty acids and sterols comprising 0.91% and 0.02% respectively of the remainder. The thin layer chromatography study also indicated lipids in *M. hortensia* seed are predominately triacylglycerols with a small amount of sterols and phospholipids present, although no percentages were calculated.

The percentage of lipid identified in *M. hortensia* is similar to that reported for other Boraginaceae. Tsevegsüren and Aitzetmüller (1996) found that "air-dried" seed of three Mongolian *Myosotis* species had oil contents of 28.3% (*Myosotis sylvatica*)), 27.4% (*Myosotis caespitosa*) and 24.3% (*Myosotis suaveolens*). There is, however, considerable variation in the percentage of lipid reserves found in members of the Boraginaceae family. For example Senanayake and Shahidi (2000), found that total lipids constituted 34% of the dry matter of *Borago officinalis* seed. Quinn *et al.* (1989) report similar levels (28-38%) of lipid in *B. officinalis* seed. In contrast, Tsevegsüren and Aitzetmüller (1996) report an oil content of 4.6% in *Lappula intermedia*, another Mongolian Boraginaceae. Ahmad *et al.* (1978) found that seeds of *Heliotropium eichwaldi* contained 26% oil and those of *Heliotropium indicum* 12% oil. There appears to be some variation in oil content within species. In the Boraginaceae, *Trichodesma zeylanicum*, Sundar Rao and Sino (1992) report a relatively high oil content of 37.7% on a dry weight basis in seed sourced in Australia. This compares to 30% found by Hosamani (1994) in seed sourced in India. In

the Hosamani (1994) study no information on the moisture status of the seed is given, nor is any cultivar information provided in either study. Calculation of oil contents on a different moisture content basis or use of different cultivars may explain the difference. Caviness (1974) also reports variation in soybean oil content with variety and location, suggesting some phenotypic variation in oil content, however the variation reported was only several percent. This study looked at only one harvest year for seed grown in a single location using the "Alba" form of *M. hortensia*. Determination of oil content from seed produced in another growing season and location would determine the extent, if any, of phenotypic variation in oil content of *M. hortensia*. A comparison between the oil content of the "Alba" form with that of the more common blue-flowered *M. hortensia* would indicate if there was any variation between the two.

The oil content of *M. hortensia*, at around 23-24%, is much higher than that found in species considered to be "non-oily", for example, *Pisum sativum* , 6%, *Triticum aestivum*, 2% or *Hordeum vulgare*, 3% (Bewley and Black, 1994). The oil content is much closer to that reported for some cultivars of *Glycine max*, for example 23.9% (variety Dare) and 22.1% (variety Bragg), (Caviness, 1974), 20% (variety Williams 82, Dewine Seed Co.), (Vertucci and Roos,1990); *Sinapis arvensis* or *Brassica kaber* var. pinnatifada (wild rape) has an oil content of 25% (Daun, 1983) and *Gossypium hirsutum*, 19% (Wallis *et al.*, 2000). All these species are considered to be oilseeds (Pritchard, 1991). *M. hortensia* can therefore be considered to be an "oilseed". The relatively high levels of lipid reserves in *M. hortensia* are likely to have a strong influence on the storage requirements of seed of this species. This will be discussed in Chapter 4.

3.4.2.2 Fatty acid composition

The longer BPX-70 capillary column gave much greater resolution and a more efficient separation than the packed column. This enabled better identification of fatty acids. For this reason only the data obtained from the BPX-70 capillary column will be used to discuss the fatty acid composition of *M. hortensia*.

However, before discussing the individual fatty acids present in *M. hortensia* several limitations of the gas chromatography/FAMES technique used should be noted. The methyl esters of the short chain fatty acids (C_4-C_{10}) are volatile and losses can occur during the preparation of the fatty acid methyl esters (Rossell, 1991). No fatty acids of

carbon chain length less than sixteen were identified in this study and the assumption is made that no shorter chain fatty acids were present. A C_{10} fatty acid was included as a standard and appeared on the chromatogram indicating that during the derivation of the standards at least, using the same protocol as that of the samples, not all, if any of the C_{10} fatty acid methyl esters were lost. This and the fact that the percentage of lipid present calculated by measuring peak areas on the chromatogram did not differ significantly from that determined using either the soxhlet or methanol-chloroform methods suggests the assumption that no short chain fatty acids were present is valid. The major sources of error in quantitative analysis of free fatty acids by gas chromatography are errors in sample preparation (McNair and Miller, 1998), for example grinding or extraction losses, leading to an underestimation of fatty acids levels. In this work an internal standard was added at the beginning of the extraction to correct for any extraction losses. Again the close agreement between the three methods used to determine the lipid content, each grinding a new seed sample and using a different extraction procedure, suggests that grinding and extraction losses were minimal or, less likely, constant between methods.

The identification of fatty acids present in *M. hortensia* seed was principally achieved through the comparison of retention times of known fatty acid standards with unknown fatty acids in the FAMES sample. These identifications have not been confirmed by structural analysis using, for example, mass spectrometry and may be argued as being tentative until this is done. However, comparison of the changes in fatty acid percentages before and after hydrogenation did support the identification of fatty acids made from standard fatty acid retention times. In addition, where more than one standard FAME had a retention time near those of sample FAMES again changes in fatty acid percentages before and after hydrogenation where able to eliminate one of the two possibilities. For example, both erucic and *cis*-5,8,11,14,17-eicosapentaenoic acid had similar retention times but *cis*-5,8,11,14,17-eicosapentaenoic acid was eliminated by comparing fatty acid percentages before and after hydrogenation.

The majority of fatty acids present in *M. hortensia* seed are C_{18} fatty acids, predominately the unsaturated oleic, linoleic and γ-linolenic fatty acids. Palmitic acid is also present. Erucic acid and, possibly, *cis*-11-eicosenoic and nervonic acid are present in smaller amounts. Of these, palmitic, oleic, linoleic and erucic acid are commonly found in storage lipids of higher plants (Wallis *et al.*, 2000). Lauric ($C_{12:0}$) and myristic ($C_{14:0}$) acid are also common in storage lipids, however, neither were identified in *M. hortensia* seed. Linolenic

acid is also commonly found in plants but usually as α-linolenic acid (Wallis *et al.*, 2000). In *M. hortensia* seed there is relatively little α-linolenic acid with most of the $C_{18:3}$ fatty acid present as the γ-isomer. γ-Linolenic acid has been identified in seed of numerous Boraginaceae, most notably *Borago officinalis* (Senanayake and Shahidi, 2000; Quinn *et al.*, 1989; Whipkey *et al.*, 1988), but also *Myosotis sylvatica*, *M. suaveolens* and *M. caespitosa* and *Cynoglossum divaricatum* (Tsevegsüren and Aitzetmüller, 1996), *Onosmodium hispidissimum* (MacKenzie *et al.*, 1993) and *Trichodesma zeylanicum* (Sundar Rao and Sino, 1992).

The fatty acid distribution identified in *M. hortensia* seed is typical of that reported in other Boraginaceae. For example in *Borago officinalis* the majority (80.2%) of fatty acids present in the total lipid fraction are also C_{18} fatty acids (Senanayake and Shahidi, 2000), and, as in seed of *M. hortensia*, these are predominantly the unsaturated oleic (17.8%), linoleic (37.2%) and γ-linolenic (20.5%) acids. There are similarly low levels of α-linolenic acid in *B. officinalis* with Senanayake and Shahidi (2000) reporting none until four days after the beginning of germination, nor was any lauric or myristic acid identified, but palmitic (10.8%), erucic (2.4%) and nervonic (1.6%) acid were present. MacKenzie *et al.* (1993) also found that the majority of fatty acids present in *Onosmodium hispidissimum* were C_{18} fatty acids (81.1%), again predominately oleic (13.5%), linoleic (18.2%) and γ-linolenic acid (26.8%). In contrast to *B. officinalis* a significant percentage (20.1%) of α-linolenic was also found. Palmitic acid (6.5%) and a small amount (0.2%) of erucic acid were also present but no nervonic acid was identified.

3.4.2.3 Commercial importance of γ-linolenic acid

Many of the fatty acids found in seed oils have commercial value. γ-Linolenic acid is important in the biomedical and cosmetic industries (Guil-Guerrero *et al.*, 2000). It is also a potential dietary source for use relieving the symptoms of rheumatoid arthritis, high blood pressure and multiple sclerosis (MacKenzie *et al.*, 1993) and is registered for pharmaceutical use for the treatment of eczema and mastalgia (Sayanova *et al.*, 1999). γ-Linolenic acid is the Δ^6-desaturase product of linoleic acid (Hansen Petrik *et al.*, 2000). A small percentage of the general population have a Δ^6-destaurase deficiency. This is a hereditary or acquired deficiency that occurs particularly in the elderly or those suffering from stress, diabetes or alcoholism (Aitzetmüller and Werner, 1991). γ-Linolenic acid already contains a Δ^6 double bond and can, therefore, be converted into arachidonic acid

without the Δ^6-destaurase step (Aitzetmüller and Werner, 1991). Arachidonic acid is a precursor for the prostaglandins and thromboxanes (Sayanova *et al.*, 1999), and are involved in the "moment-by-moment" regulation of cellular function (Lapinskas, 1993). Of these prostaglandin E_1, in particular, has a range of desirable clinical effects, for example lowering blood pressure and inhibiting cholesterol synthesis (Lapinskas, 1993).

Commercial sources of γ-linolenic acid are almost entirely restricted to seed oils from three plants, *Oenothera biennis* , *Borago officinalis* and *Ribes nigrum*, (Guil-Guerrero *et al.*, 2000). Some fungi, for example *Mucor javanicus*, are also sources of γ-linolenic acid (Lapinskas,1993). *O. biennis*, *B. officinalis* and *R. nigrum* contain 7-14% (Mackenzie *et al.*, 1993), 17-25% (Quinn *et al.*, 1989) and 15-20% (Guil-Guerrero *et al.*, 2000) γ-linolenic acid respectively in their seed oils. The percentage of γ-linolenic acid found in *M. hortensia* compares favourably to that in *O. biennis* oil but is less than that in *B. officinalis* and *R. nigrum*. However, comparisons of γ-linolenic percentages alone are simplistic and biological efficacy and safety of the source must be considered, as well as processing costs and seed yields (Lapinskas, 1993). Although *O. biennis* oil is a relatively dilute source of γ-linolenic acid it is twice as effective at stimulating prostaglandin E_1 production than fungal sources and ten times better than *B. officinalis* or *R. nigrum* at equal doses of γ-linolenic acid (Lapinskas, 1993). There are problems with the supply of both *R. nigrum* and *O. biennis* oil. Lapinskas (1993) comments that *R. nigrum* seed is a byproduct of the juice industry meaning supply is both inflexible and subject to demand levels.

M. hortensia does not produce seed in the first year. This and the difficulty in maintaining the plant, particularly, over summer, in warm environments, may limit the use of seed as a source of γ-linolenic acid. An exception may be in the plants natural environment on the Chatham Islands, where, problems of maintaining the plant should be reduced, if not eliminated. However, if well nourished, the plant is a prolific seeder. This, and the limitations of the other seed sources of γ-linolenic acid, means further investigation of *M. hortensia* as an alternative source of γ-linolenic acid could be considered. The biological efficacy and safety of γ-linolenic acid extracted from *M. hortensia* seed must be established. Seed production systems for the species, for example, planting densities, timing and method of harvest, pest-control (for example weed) strategies would also need to be developed.

Another commercially important seed oil is stearidonic acid ($C_{18:4}$, $\Delta^{6, 9, 12, 15}$). Stearidonic

acid also contains a Δ^6 double bond and therefore does not need the Δ^6-desaturase step for transformation into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), (Aitzetmüller and Werner, 1991). While γ-linolenic acid has been found in a wide range of plant families the simultaneous occurrence of γ-linolenic and stearidonic acids has only been reported in seed oils of Boraginaceae (Guil-Guerrero *et al.*, 2001a) and Primulaceae (Aitzetmüller and Werner, 1991) and *Ribes* spp. (Wolf *et al.*, 1983; Johansson *et al.*, 1997; Ruiz del Castillo *et al.*, 2002). Tsevegsüren and Aitzetmüller (1996) found both γ-linolenic acid and stearidonic acid in all the Boraginaceae seed they investigated. In *Myosotis sylvatica*, *M. suaveolens* and *M. caespitosa* the percentage of γ-linolenic was 7.3, 8.5 and 9.6% compared to 6.5, 8.6 and 10.3% respectively for stearidonic acid. However stearidonic acid is not present in all Boraginaceae, for example, Whipkey *et al.* (1988) found no stearidonic acid in *Borago officinalis*.

In this study a stearidonic acid standard was not included, hence, the retention time of this fatty acid in our system is unknown. Tsevegsüren and Aitzetmüller (1996), using a BPX-70 fused-silica capillary column, identified the peak representing stearidonic acid as appearing between that of α-linolenic acid and *cis*-11-eicosenoic acid. Arachidic acid was not identified in the chromatograms presented. Johansson *et al.* (1997), using a NB-351 fused-silica capillary column, report that stearidonic acid has a retention time between those of α-linolenic and arachidic acid. No unidentified peaks between α-linolenic and arachidic acid were found in this study. The high resolution and narrow peak retention times make it unlikely that the arachidic acid peak is a mixture of both arachidic acid and stearidonic. Moreover, given the relatively low percentage of arachidic acid present, even if stearidonic acid is "concealed" within the arachidic acid peak it is likely to be so in only very small amounts.

3.4.3 Protein Content

This study also found that *M. hortensia* seed also contained protein storage reserves. No attempt was made in this study to identify or quantify these proteins. However, if identification and quantification of these proteins established their nutritional value as stock feed this would provide an economic use for the seed cake remaining after oil extraction. This may improve the commercial viability of *M. hortensia* as an alternative source of γ-linolenic acid.

3.4.4 Conclusions

This work has identified that food reserves in seed of *M. hortensia* are stored as both oil and protein with no starch reserves apparent. *M. hortensia* seed has an oil content of 24% and can therefore be considered an oilseed. The fatty acid profile in the seed oil is similar to that of several other members of the Boraginaceae and includes the commercially important γ-linolenic acid but not stearidonic acid. The percentage of γ-linolenic acid present is lower than that found in other commercial seed sources of γ-linolenic acid, with the exception of *Oenothera biennis* . However, problems with the other commercial sources, suggest an assessment of the biological efficacy and safety of γ-linolenic acid obtained from *M. hortensia* is worthwhile. The results of these assessments should indicate whether *M. hortensia* seed could be used as an alternative source of γ-linolenic acid and if protocols for large scale seed production need to be determined.

CHAPTER 4 SEED DESICCATION AND STORAGE

4.1 Introduction

There are suggestions in the literature (Fountain and Outred, 1991) that seed of *M. hortensia* may be recalcitrant. If so, this would severely limit our ability to store seed of this species. An experiment was therefore designed to determine if seed of *M. hortensia* could be desiccated to a low seed moisture content (less than 10%) without loss of viability. A second experiment was set up to determine the longevity in store of seed at this moisture content and 5°C. An open storage treatment was also included in this second experiment to determine the effect of open storage under refrigerated conditions on seed longevity.

4.2 Material and Methods

4.2.1 Seed Moisture Content, Thousand Seed Weight, Viability and Germination Testing

The following protocols were used in this Chapter and Chapter 5 (Germination and Dormancy) to determine seed moisture content, thousand seed weight, seed viability and germination. Four replicates were for all tests unless otherwise stated.

4.2.1.1 Seed moisture content and thousand seed weight

Seed moisture content was determined using the forced air oven method (ISTA, 1993). Fifteen intact seeds (unless otherwise stated) of known weight per replicate were dried at 103°C for 17 hours. The formulae below where used to calculate the seed moisture content on both a fresh and dry weight basis. Moisture content data presented is on a fresh weight basis unless otherwise stated.

$$\text{seed moisture content (\% fresh weight)} = \frac{W_1 - W_2}{W_1} \times 100$$

$$\text{seed moisture content (\% dry weight)} = \frac{W_1 - W_2}{W_2} \times 100$$

where W_1 = the seed weight before oven drying

W_2 = the seed weight after oven drying

The thousand seed weight was calculated from the seed moisture content data where the dry weight was divided by the number of seeds tested and multiplied by one thousand to give the thousand seed weight.

4.2.1.2 Seed viability

Seed viability was determined using the topographical tetrazolium test (ISTA, 1993). Twenty-five seeds (unless otherwise stated) per replicate were preconditioned by rolling them in moistened 38 lb regular weight seed germination paper (Anchor Paper Company, St. Paul, Minnesota). The roll was placed in a jar with approximately 2 cm of water in the bottom. The jar and roll were covered with a plastic bag and left at 20°C for 24 hours. After 24 hours a scalpel was used to make a 5 -10 mm cut in the cotyledon area of the softened seed coat and the coat peeled off. The embryo was placed in a solution of 1% (w/v) 2,3,5-triphenyl tetrazolium chloride made up in phosphate buffer (3.631g KH_2PO_4 and 5.683g Na_2HPO_4 per litre of distilled water (adapted from Enescu, 1991) at 20°C for a further 24 hours.

Seed was classified as viable if both the radicle and plumule area and more than 50% of both cotyledons (but including that part of the cotyledons near the radicle) showed uniform red staining (adapted from Enescu, 1991) (Plates 4.1 to 4.6).

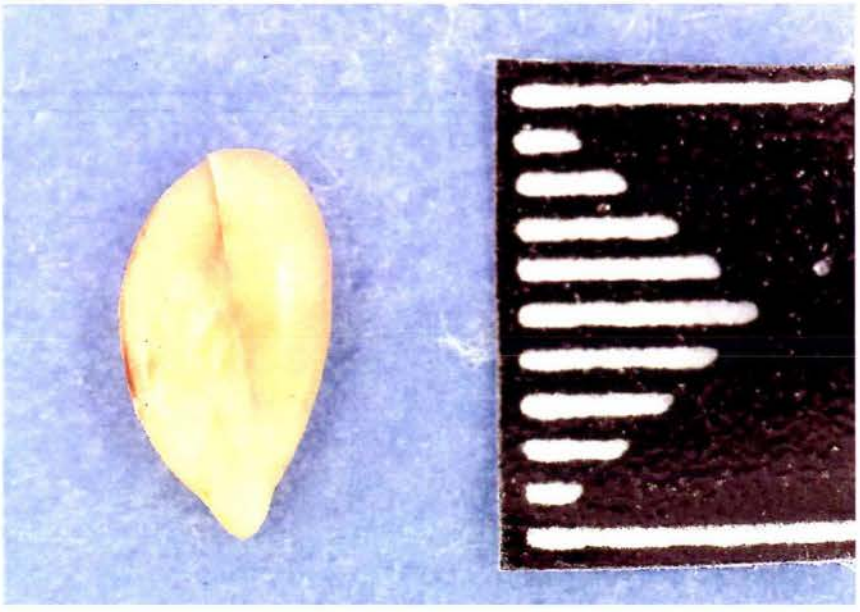


Plate 4.1: *M. hortensia* embryo stained with 2,3,5-triphenyl tetrazolium chloride at 20°C for 24 hours. There is no staining to the embryo. The embryo is classified as non-viable. Scale: 10mm.

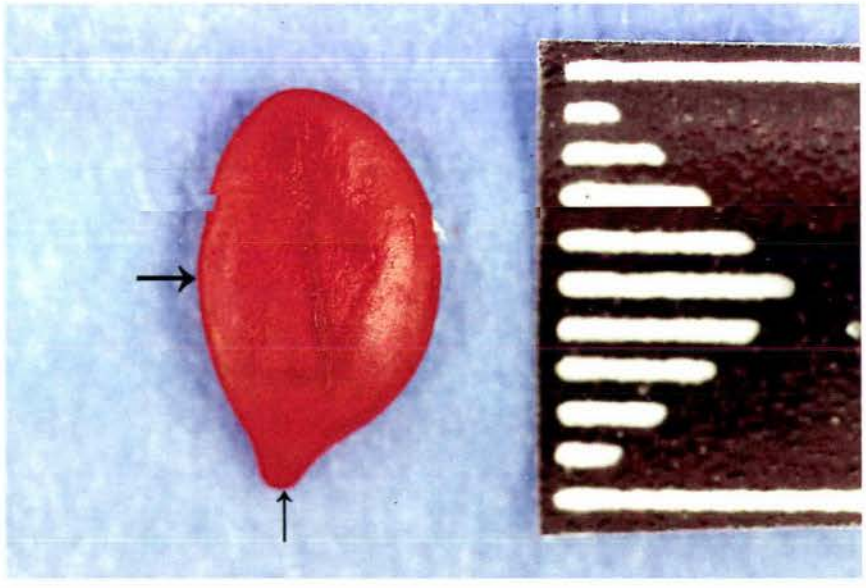


Plate 4.2: *M. hortensia* embryo stained with 2,3,5-triphenyl tetrazolium chloride at 20°C for 24 hours. There is uniform staining to both the radicle (↑) and cotyledons (→). The embryo is classified as viable. Scale: 10mm.

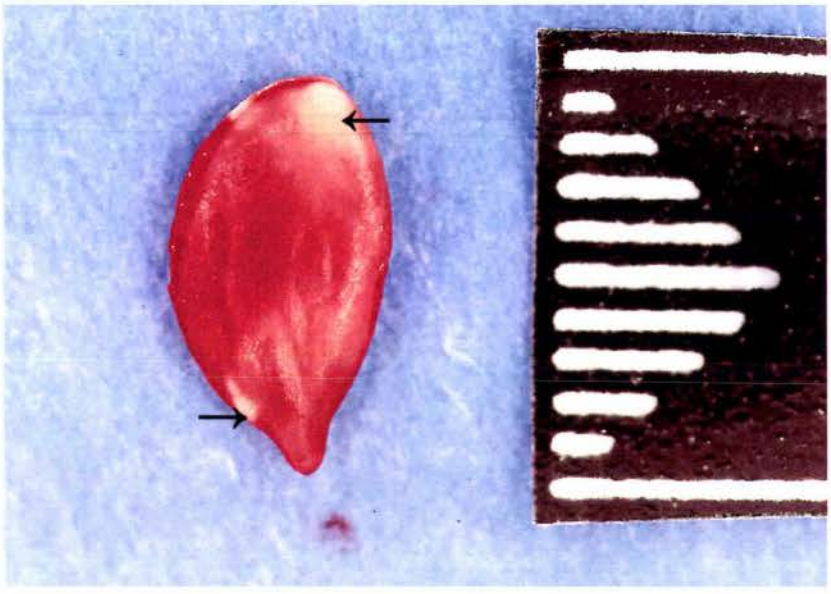


Plate 4.3: *M. hortensia* embryo stained with 2,3,5-triphenyl tetrazolium chloride at 20°C for 24 hours. There is incomplete staining to the cotyledons (→) but more than 50% of the cotyledon tissue is stained. The embryo is classified as viable. Scale: 10mm.

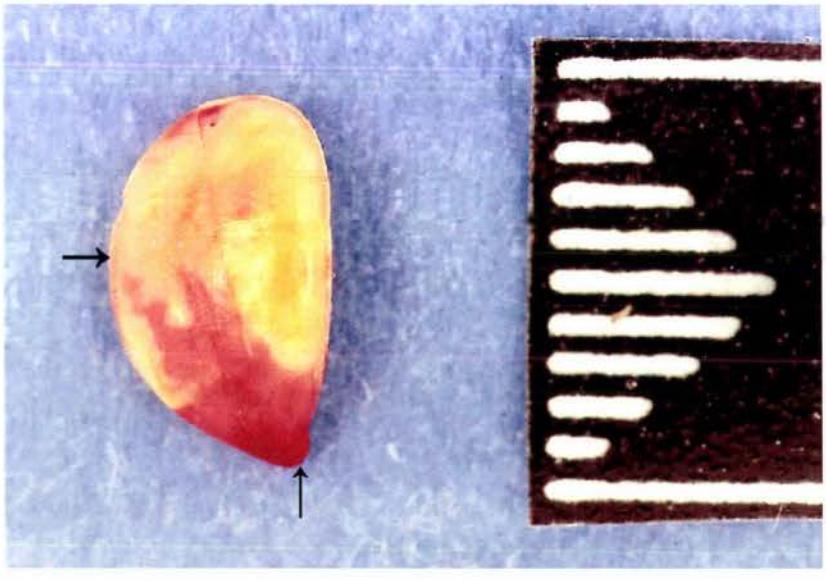


Plate 4.4: *M. hortensia* embryo stained with 2,3,5-triphenyl tetrazolium chloride at 20°C for 24 hours. There is uniform staining to the radicle (↑) but insufficient staining to the cotyledons (→). The embryo is classified as non-viable. Scale: 10mm.

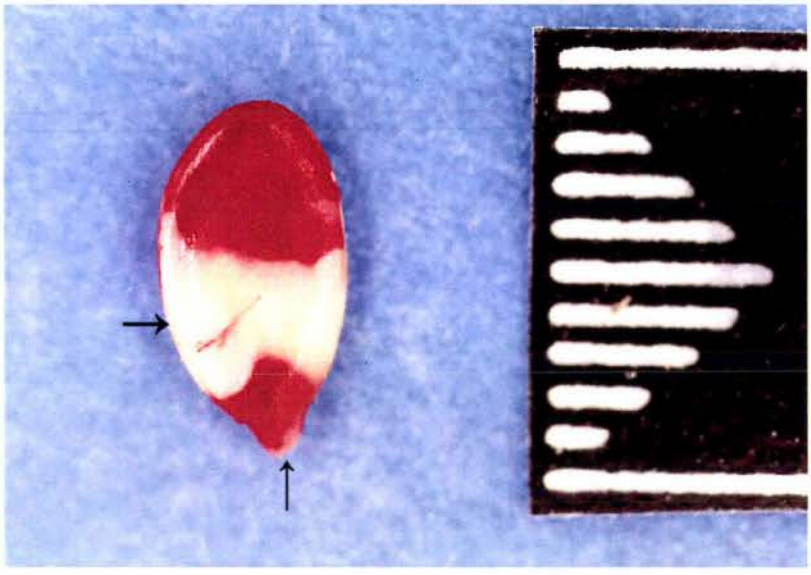


Plate 4.5: *M. hortensia* embryo stained with 2,3,5-triphenyl tetrazolium chloride at 20°C for 24 hours. There is insufficient staining to the cotyledons (→) near the radicle and uneven staining to the radicle (↑). The embryo is classified as non-viable. Scale: 10mm.

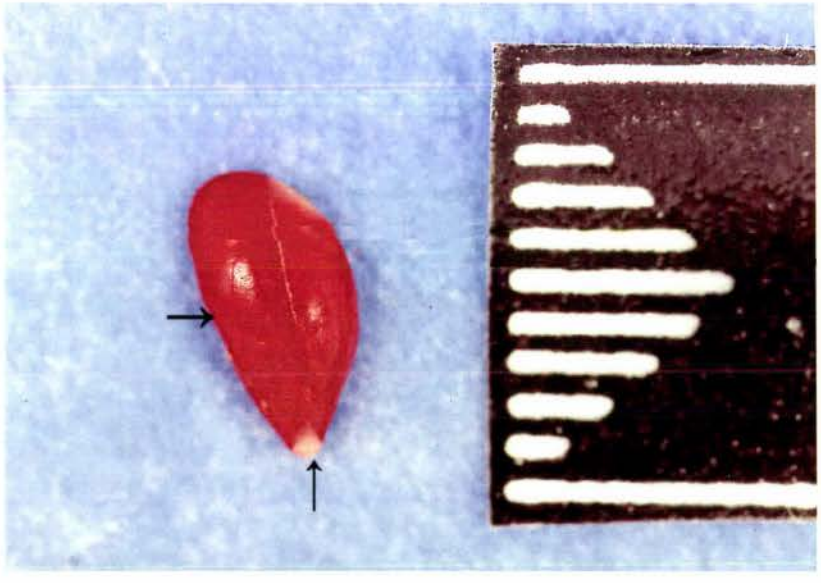


Plate 4.6: *M. hortensia* embryo stained with 2,3,5-triphenyl tetrazolium chloride at 20°C for 24 hours. There is uniform staining to the cotyledons (→) but no staining to the radicle (↑). The embryo is classified as non-viable. Scale: 10mm.

4.2.1.3 Seed germination

Seed germination was determined by germinating 25 (unless otherwise stated) seeds per replicate in plastic tupperware™ boxes on two pieces of steel blue seed germination blotter (Anchor Paper Company, St. Paul, Minnesota) at alternating temperatures of 10°C (±2°C) for 16 hours and 15°C (± 2°C) for 8 hours in the dark (except for setting up and counting germination). These germination temperatures were selected as appropriate because of the range of temperatures experienced at the Waitangi Meteorological Office in the Chatham Islands (1956-1980) in January. At this station the mean daily maximum was 17.6°C and the mean daily minimum 11.4°C (Thompson, 1983).

Prior to being placed on the germination blotter seeds were dusted with the fungicide Thiram 80W (800g/kg thiram) as a powder. Twenty-five seeds were placed in a conical flask and a volume of Thiram 80W in the ratio of half a seed volume of Thiram 80W to every 25 seeds was added. The flask was swirled until the seeds were evenly coated with Thiram 80W. Excess Thiram 80W was removed by sieving the seed. (A preliminary experiment of germinating seed with and without Thiram 80W demonstrated that Thiram 80W was not toxic to the seed (Appendix 5)).

In all germination trials each replicate was treated as a block and treatments were randomised within that block. The four blocks were placed on the same shelf in the 10/15°C germination cabinet.

Seed germination was monitored weekly. Germination was scored when the radicle or cotyledon had emerged from the seed coat. The seedlings were classified as normal and abnormal or dead using the following criteria (adapted from Bekendam and Grob, 1979).

A seedling was classified as normal if it had:

1. a well developed primary root with no or only slight defects (slight discolouration or necrotic spots or minor cracks or splits). Secondary roots were not taken into account if the primary root was defective
2. an intact hypocotyl with no or only slight defects as described for the root system development
3. cotyledons which were intact or, if damaged, showed less than 50% damage to the cotyledon tissue.

Seedlings were classified as abnormal if they had:

1. a missing or defective primary root. A defective primary root was one that was stunted or stubby, spindly or glassy/opaque, trapped in the seed coat or decayed as a result of primary infection.
2. a defective hypocotyl. A defective hypocotyl was one that was short and thick, split, spindly or glassy or decayed as a result of primary infection.
3. defective or missing cotyledons, defective to such an extent that less that 50% of the tissue was functioning normally. Damage may be due to necrosis or decay as a result of primary infection. Note if one cotyledon was missing but the other was healthy and intact, the seedling was classified as normal.

Seed was classified as dead when the embryo was soft when pressed. At times the embryo was also discoloured, appearing brown in colour. Fresh ungerminated seed remained turgid and the embryo appeared white or off-white in colour.

4.2.1.4 Calculation of the rate of germination (T_{50})

The rate of germination (T_{50}) was calculated as described by Coolbear *et al.* (1984) where T_{50} is defined as the time taken from sowing for the median seed to germinate. Germination was scored as radicle or cotyledon emergence from the seed coat, the latter assumed that cotyledon emergence was a result of the growing radicle forcing the cotyledons through the seed coat.

Mathematically T_{50} is defined as:

$$T_{50} = t_i + \left[\frac{\frac{N+1}{2} - n_i}{(n_j - n_i)} \right] \times (t_j - t_i)$$

n_i and n_j are cumulative germination counts at adjacent counting times t_i and t_j, where $n_i < (N + 1)/2 < n_j$ and N is the total number of seeds that germinated.

This method only uses the two germination times bracketing the median germination time to calculate T_{50} values. An alternative method for determining the median germination time is to fit a germination curve to the data. This germination curve is usually sigmoidal (Bewley and Black, 1994). The advantage of fitting a germination curve is that all germination data points are used to determine the median germination time rather than just the two bracketing the median germination time. However, because of the slow and erratic germination of *M. hortensia* seed there was little advantage to be gained by attempting to fit germination curves rather than calculating T_{50}'s. Similarly, uniformity of germination, defined as the difference between the time taken to reach 10% and 90% germination, was not calculated as the slow germination rate made comparisons meaningless.

4.2.2 Desiccation Experiment

Seed heads were randomly harvested from the Turitea Campus plot on 21 January 1997. Nutlets were removed from the seed head and sorted according to colour into green and black. Nutlets within each colour classification were split into four replicates. Seed moisture content, seed viability and germination were determined immediately. Remaining nutlets in each replicate were placed as a single layer in an aluminum pan (internal diameter 370 mm). The pan and nutlets were weighed and placed in a controlled environment room running at 20°C. An indication of moisture loss from the nutlets was obtained by monitoring weight loss from the pan over time. At moisture content intervals of approximately 10-15%, nutlets were sub-sampled and the seed removed from the nutlet. Seed moisture content, thousand seed weight and viability were determined

immediately and seed from each replicate set to germinate. For green seed, to conserve seeds, only ten seeds were used for moisture content determination and 15 seeds for seed viability and germination, except for the last sampling where 15 seeds were used for moisture content determination and 25 seeds for seed viability and germination.

Seed moisture content was monitored until it remained constant and the remaining nutlets placed into storage using a variety of storage regimes. Again, to conserve seeds, the same seed was used to determine both the germination percentage and rate (T_{50}) when a constant seed moisture content had been reached and prior to the seed being placed in storage ("zero months storage"). "Black" seed had been split into four storage treatments (see **4.2.3**). However, since there is no effect of storage at "zero months storage", the germination percentage for seed at the final (constant) seed moisture in the desiccation experiment was calculated for each replicate by averaging the germination data for each storage treatment within the same replicate. For "green" seed there was only one storage treatment (seed: closed storage), hence this data is common to both experiments.

After 92 weeks 26 seeds remained ungerminated, these were pricked in the cotyledon area with a Type 00 (0.30 mm) insect pin to promote germination, The point where the seed coat ruptured for those seed that germinated was recorded.

The rate of germination was calculated as described in **4.2.1.4.**

4.2.3 Seed Storage Experiment

Nutlets within each replicate were split into four sub-samples using the riffle divider as described in **3.2.2**. Seed was removed from the nutlets for two sub-samples. One sub-sample of seeds and one sub-sample of nutlets were stored in heat sealed 116 x 188 mm 12/20/50 micron laminated polyester/aluminium foil/polythene packets ("closed storage"). These packets are impermeable to both water and oxygen (Tungjaroenchai, 1990). The other sub-sample of seeds and the other sub-sample of nutlets were stored in 114 x 225 mm Kraft™ self sealing envelopes ("open storage"). This type of packaging is permeable to both water and oxygen (Justice and Bass, 1978).

This gave four storage treatments:

1. nutlet: closed storage
2. nutlet open storage
3. seed: closed storage
4. seed: open storage.

Each replicate was treated as a block and treatments were randomised within that block. The four blocks were placed on the same shelf in a controlled environment room at 5°C (± 2°C).

At the beginning of storage period and after nine, fourteen and twenty-one months storage nutlets or seed were sub-sampled from the packages. The polyester/aluminium foil/polythene packets were resealed using the heat sealer and the envelopes with tape. The seed moisture content, normal and abnormal germination percentage and the percentage of fresh ungerminated and dead seed and rate of germination (T_{50}) were calculated as described in **4.2.1.1**, **4.2.1.3** and **4.2.1.4**. At the 21 month sampling only seed from two replicates for the closed storage treatments remained, hence only two replicates were used to assess seed moisture content and germination and for some replicates only 5 seeds were available for the moisture content determination and 15 seeds for the seed germination.

4.2.4 Relative Humidity

The relative humidity of both the desiccation (20°C controlled environment room) and storage (5°C controlled environment room) environments was measured using a wet and dry bulb thermometer.

4.2.5 Data Analysis

The general linear models procedure (Proc GLM) in SAS for Windows (Release 8.02 TS Level 02M0, SAS Institute, Cary, North Carolina) was used to perform an the analysis of variance (ANOVA) on the data. The T_{50} data was log transformed in an attempt to normalise the data. The log transformation improved the Shapiro-Wilk (W) statistic in the test for normality from 0.823816 to 0.877741 but did not completely normalise the data

(P<W 0.0006).

The LSD procedure was used to compare means, but, only where the ANOVA F-test identified a significant difference between treatments i.e. a protected LSD (Ott, 1988). However, because of the large number of pair-wise comparisons, particularly in the storage experiment, there is a chance of falsely declaring a significant difference when using the LSD. Consequently, the more conservative (Ott, 1988), Tukey procedure was also used to compare means. For interaction effects the probability values for the hypothesis that two means being compared were equal were also determined using the LSD and the LSD adjusted for the Tukey. Results are presented for comparisons using the LSD procedure, but, where major differences exist between the two procedures both results are noted in the text.

The LSD values presented in tables were calculated from the mean sum squares of the error (MSE) in the ANOVA table for the comparisons and assuming a sample size (number of replicates) of four, despite less than four replicates being used in some treatments. However, the probability values for the hypothesis that two means being compared were equal using the LSD were used to verify the comparisons made by the LSD calculated from the mean sum squares of the error.

Where an individual mean is presented in the text numbers following in brackets are the standard error of that mean.

4.3 Results

4.3.1 Desiccation Experiment

The moisture content at harvest was 35.5 (± 2.61)% for seed extracted from black nutlets and 47.4 (± 1.0)% for seed extracted from green nutlets. At the end of the desiccation period "black" and "green" seed reached constant moisture contents of 7.6 (±0.07)% and 7.4 (±0.28)%, respectively. There was no significant difference in the seed moisture content of "black" and "green" seed at the end of the desiccation period. The nutlet colour at harvest was also indicative of the seed coat colour i.e. the seed coat of seed extracted from green nutlets was green and that from black nutlets black. As seed moisture content declined the seed coat colour of seed harvested green changed to black.

The viability of "green" seed at the harvest moisture content (47.4%) and 45.1% was significantly higher ($P<0.05$) than seed harvested at 35.5% ("black") at any desiccation moisture content, except (for the Tukey only), between "green" seed at 45.1% and "black seed" at 33.5%. The LSD test, but not the Tukey, also indicated the viability of "green" seed at 47.4% and 45.1% was significantly higher ($P<0.05$) than "green" seed at all other moisture contents. There was no significant difference in the viability of "green" seed at the lower seed moisture contents (38.6%, 16.1% and 7.4%) and "black" seed except for "black" seed at 10.2% (Table 4.1).

There was no significant change in the viability of "black" seed as seed moisture content declined, except at 10.2% seed moisture content which had a significantly lower ($P<0.05$) viability than "black" seed at all other moisture contents (LSD) or 33.5% seed moisture content (Tukey).

The germination data was not consistent with the viability results. Within each colour classification at the end of the dessication there was no significant difference in normal germination percentage compared to that at harvest. Nor was there any change in normal germination percentage as seed moisture declined, except, in "black" seed at 33.5% seed moisture compared to the same seed at 10.2% seed moisture. Here the LSD test, but not the Tukey, indicated a significant difference ($P<0.05$) in the normal germination percentages.

There was no significant difference in the normal germination percentage between "green" and "black" seed at harvest. However, at the end of the desiccation the LSD test, but not the Tukey, indicated "green" seed had a significantly higher ($P<0.05$) normal germination percentage(91%) than "black"seed (78%). This apparently lower normal germination percentage in "black" seed was matched by a significantly higher ($P<0.05$) percentage of dead seed in "black" seed (both LSD and Tukey tests).

The germination of seed at all moisture contents was slow and erratic. The time taken to reach 50% germination (T_{50}) ranged from 17 to 23 weeks, depending on seed moisture content. The rate of germination differed significantly ($P<0.05$) between moisture contents. At some of the intermediate moisture contents seed of both harvests had a slower rate of germination than freshly harvested seed and seed desiccated to 7.5% seed moisture.

However, there was no significant difference in the rate of germination of freshly harvested seed and seed at the end of desiccation, nor did germination rate differ between seed harvested at 47.4% and 35.5% seed moisture content.

There was no difference in the thousand seed dry weight between seed harvested at 47.4% seed moisture content and that harvested at 35.5%, nor did the thousand seed weight change as seed moisture content declined.

Table 4.1 Changes in moisture content, viability, germination and time to 50% germination (T_{50})of seed dried as a single layer of nutlets in a controlled environment room at 20°C.

Nutlet/Seed Colour and Seed Moisture Content (%) at Harvest	Seed Moisture Content (%)	Tetrazolium Test				Germination Test			T_{50} (days)	Thousand Seed Dry Weight (g)
		Viability (%)	Doubtful (%)	Non-viable (%)	Dead (%)	Normal (%)	Abnormal (%)	Dead (%)		
Green (47.4 %)	47.4	98	0	2	0	90	8	2	137	34.4
	45.1	97	1	2	0	83	9	8	120	36.8
	38.6	88	2	10	0	93	5	2	146	37.3
	16.1	88	3	5	4	92	8	0	146	36.4
	7.4	89	2	6	3	91	6	3	133	38.0
Black (35.5 %)	35.5	84	3	3	10	79	4	17	122	33.2
	33.5	85	0	2	13	88	2	10	150	38.6
	15.1	84	0	6	10	81	5	14	154	39.8
	10.2	73	1	4	22	72	2	26	145	37.8
	7.6	82	1	5	12	78	4	18	130	35.2
$LSD_{(0.05)}$		7.1	NS	NS	5.7	11.1	NS	7.3	19.1	NS

4.3.2 Storage Experiment

The normal germination percentage of seed when placed in store was 77 (±2.9)% (seed harvested at 35.5% seed moisture content, pooled for all four storage treatments) and 91 (±2.5)% (seed harvested at 47.4% seed moisture content). The moisture content of seed when placed in store (pooled for both harvest moisture contents and all storage treatments) was 7.5 (±0.08)% (8.2 (±0.09)% on a dry weight basis).

4.3.2.1 Open storage

In open storage (seed harvested at 35.5% seed moisture content only), at the nine month sampling the seed moisture content had increased significantly ($P<0.05$) from 7.5 (±0.08)% to 9.5 (±0.21)% (10.6 (±0.26)% on a dry weight basis) (pooled for both seed and nutlets). The moisture content remained at this elevated level at all subsequent sampling. The seed moisture content pooled for open storage treatments at nine, 14 and 21 months was 9.7 (±0.14)% (10.8 (±0.17)% on a dry weight basis). There was no significant difference in the seed moisture content of seed stored as seed compared to seed stored as nutlets.

This relatively small, but significant, increase in seed moisture content was sufficient to cause a loss in normal germination capacity by the nine month sampling (Table 4.2), although the Tukey suggested there was no significant decline in the normal germination percentage of seed in the nutlets until 14 months. There was no significant difference in normal germination between 9 and 14 months, but after 21 months of open storage germination had declined to 1%. This loss of germination capacity was accompanied by an increase in both the number of seedlings showing no or abnormal root development (stunted and/or opaque roots) and the number of dead seeds. Within each storage treatment (seed or nutlets), at nine months storage the LSD test indicated a significantly higher ($P<0.05$) number of abnormal seedlings in seed stored both as seed and nutlets. In contrast, the Tukey test indicated no significant increase in the number of abnormal seedlings in seed stored as nutlets until the 14 month sampling and no difference in seed stored as seed at any sampling date. There was a significant increase ($P<0.05$) in the number of dead seed in seed stored as seed at nine months (LSD) or 14 months (Tukey), but not in nutlets until 21 months storage. Also within each open storage treatment (nutlet and seed) the LSD test, but not the Tukey, indicated at 14 months storage there was a

significantly higher ($P<0.05$) number of abnormal seedlings than at 21 months storage. The decline in abnormal germination percentage at 21 months was accompanied by a significant increase in the number of dead seed.

Comparing seed stored as nutlets with those stored as seed at 14 and 21 months storage, the LSD test, but not the Tukey, indicated seed stored in the nutlet had a higher level of abnormal seedling development and fewer dead seeds compared to seed that had been shelled prior to storage. However, there was no significant difference in the number of normal seedlings in seed stored in the nutlet or shelled at either sampling date.

4.3.2.2 Closed storage

The moisture content of seed in laminated polyester/aluminium foil/polythene packets did not change throughout the storage trial. The seed moisture content, pooled for all closed storage treatments at nine, 14 and 21 months, was 7.4 (±0.12)% (8.0 (±0.14)% dry weight). Seed harvested at 35.5% moisture content and stored in laminated polyester/aluminium foil/polythene packets showed no loss of germination capacity after 21 months storage, irrespective of whether the seed was stored in the nutlet or shelled. Seed harvested at 47.4% seed moisture content showed no significant decline in normal germination capacity after 14 months storage (Table 4.2). Insufficient seed was available to continue the storage trial for seed harvested at 47.4% beyond 14 months.

A comparison of the pooled normal germination percentages for all sampling times for seed harvested at 47.4% seed moisture content with that for seed harvested at 35.5% seed moisture and stored as nutlets or seed in laminated polyester/aluminium foil/polythene packets showed that seed harvested at 47.4% had a significantly higher ($P<0.05$) normal germination percentage than seed harvested at 35.5% seed moisture. This difference was a result of a significantly higher ($P<0.05$) percentage of dead seed in seed harvested at 35.5% seed moisture content (Table 4.3).

Table 4.2 Changes in germination of seed and nutlets of *Myosotidium hortensia* in open and closed storage at 5°C.

Storage Time (Months)	Normal Germination (%)					Abnormal Germination (%)					Dead Seed (%)				
	Harvest Moisture Content					Harvest Moisture Content (%)					Harvest Moisture Content (%)				
	35.5 %				47.4 %	35.5 %				47.4 %	35.5 %				47.4%
	Open Seed	Open Nutlet	Closed Seed	Closed Nutlet	Closed Seed	Open Seed	Open Nutlet	Closed Seed	Closed Nutlet	Closed Seed	Open Seed	Open Nutlet	Closed Seed	Closed Nutlet	Closed Seed
0	79	78	78	71	91	4	6	4	3	6	17	16	19	26	3
9	49	52	65	68	81	14	21	10	11	15	37	27	26	20	4
14	39	39	75	76	83	16	32	5	12	9	46	29	20	12	8
21	1	1	67	70	-	7	21	6	4	-	93	78	27	26	-
$LSD_{(0.05)}$			14.9					9.0					14.2		

Table 4.3 Normal and abnormal germination and dead seed percentage of seed and nutlets of *Myosotidium hortensia* in closed storage at 5°C pooled for sampling time (0, 9, 14 and 21 months).

Harvest Moisture Content (%)	Storage Treatment	Normal Germination (%) (±Standard Error)	Abnormal Germination (%) (±Standard Error)	Dead (%) (±Standard Error)
47.4	Seed	85	10	5
35.5	Seed	72	6	22
35.5	Nutlet	71	8	21
	$LSD_{(0.05)}$	8.6	5.2	8.2

The rate of germination (T_{50}) was calculated for all treatments, however, the decline in germination under open storage meant germination rates were calculated on very small seed numbers, particularly for the longer storage periods, hence, only the time to 50% germination for closed storage is presented (Table 4.4). At some intermediate sampling times there were significant differences ($P<0.05$) in the rate of germination, mainly as a result of a T_{50} of 99 days for the 9 month sampling of seed stored as seed and, to a lesser extent, of 109 days for seed harvested at 47.4% seed moisture content result. However, the rate of germination at the end of the storage period (14 months for seed harvested at 47.4% seed moisture content and 21 months for seed harvested at 35.5% seed moisture content) was not significantly different ($P<0.05$) to that at the beginning of the storage trial.

The T_{50} values obtained in the storage experiment were also similar to those obtained in the desiccation trial, accepting the commonality of the 7.4% ("green" seed) and 7.6% ("black" seed) and the 0 month storage data. The time required to reach 50% germination, pooled for all storage treatments and sampling times, was 134 (±5.2) days compared to 138 (±2.6) days in the desiccation trial (T_{50}'s pooled for all seed moisture contents).

Table 4.4 Changes in the time to 50% germination (T_{50}) of seed and nutlets of *M. hortensia* in closed storage at 5°C.

Storage Time (Months)	Time to 50% Germination (days) Harvest Moisture Content		
	35.5 %		47.4 %
	Closed Seed	Closed Nutlet	Closed Seed
0	132 (2.118)	130 (2.111)	133 (2.122)
9	99 (1.989)	150 (2.138)	109 (2.035)
14	165 (2.215)	168 (2.222)	156 (2.194)
21	128 (2.106)	99 (1.993)	-
$LSD_{(0.05)}$		0.1067	

4.4 Discussion

4.4.1 Relative Humidity Measurements

At the end of the desiccation period seed reached an equilibrium moisture content of 7.5% and in open storage this increased to 9.5%. The measured humidity in the 20°C desiccation environment ranged from 75 to 85% and 85 to 95% in the 5°C storage environment. The equilibrium moisture contents reached by the seed at both relative humidity ranges are low compared to those reported in the literature for, for example, *Glycine max* which has a similar lipid content to *M. hortensia*. Vertucci and Roos (1990) found that *G. max* cv. Williams 82 (Dewine Seed Co.), with a lipid content of 20% (dry weight), reached an equilibrium moisture content of 7.5% (dry weight) at a relative humidity of 40-45% and 25°C. Similarly, Pixton and Warburton (1971) found that *G. max* (lipid content 20.5% (dry weight)) reached 7.5% seed moisture at a relative humidity of around 42% and 20°C. Moreover at 10°C (the lowest temperature for which data is presented) *G. max* seed reached an equilibrium moisture content of 9.5% at around 57% relative humidity. These data suggest the relative humidity measurements made in the storage environment may be incorrect and should be treated with caution. Consequently, the relative humidity at which *M. hortensia* seed reached equilibrium moisture contents of 7.5% and 9.5% is uncertain.

4.4.2 Desiccation Experiment

In analysing the data some treatment means were declared significantly different by the LSD test but not the Tukey test. This is not surprising given the more conservative nature of the Tukey test (Ott, 1998). Moreover, limited seed availability meant a relatively small number of seeds (25 or less) was used for the germination and seed moisture content testing. Each seed therefore contributes 4% to the normal, abnormal or dead germination percentage compared to only 2% or 1%, respectively for 50 or 100 seeds. This may have contributed to the high variability of some of the data. This variability may be masking some treatment effects, particularly for comparisons with the more conservative Tukey test.

In most cases where a conflict exists between the LSD and Tukey tests the results in

question are not key results. For example the difference in the normal germination percentage of "black" seed at 10.2% compared to 33.5% seed moisture content indicated by the LSD, but not the Tukey, is less important than the observation that the normal germination percentage at the harvest moisture content was not significantly different from that after desiccation (7.5%). In discussing the data reference is only made to conflicting comparisons when they are relevant to conclusions being drawn.

4.4.2.1 Viability and germination changes during desiccation

The key result from the desiccation experiment is that seed of *M. hortensia* can be desiccated to a seed moisture content of 7.5% without loss of germination. This suggests the seed is not recalcitrant as speculated by Fountain and Outred (1991) and can be desiccated to a seed moisture content in the range considered safe (Copeland and McDonald, 1995; Thomson, 1979) for storage of orthodox seed.

The inconsistency between the viability and germination data where the higher viability at harvest of seed harvested at 47.4% moisture content compared to seed harvested at 35.5% was not reflected in a difference in the germination percentage at harvest may reflect the information the tests (germination versus the tetrazolium test) provide and the differing precision of each. In the germination test, by evaluating seedling development, the percentages of normal and abnormal seedlings can be determined. In contrast, in the tetrazolium test, where staining patterns need to be interpreted, identification of seeds that will develop into abnormal seedlings is more subjective. It is possible that some seed assessed as viable in a tetrazolium test may have developed into abnormal seedlings in a germination test. A comparison of the tetrazolium and germination test results for seed at 47.4% seed moisture gives some support to this suggestion. Here the viable seed percentage was 98%, compared to a normal seedling germination percentage of 90% and abnormal seedling percentage of 8%. In addition the tetrazolium test results, although more subjective in their interpretation, are typically less variable than germination results which can be affected by moisture, temperature and microorganisms such as fungi and bacteria (Grabe, 1970). In this study this was reflected in the higher least significant difference value associated with the germination data.

At the end of the desiccation period, seed harvested at 47.4% had a higher normal germination percentage than seed harvested at 35.5% seed moisture but only with the

LSD comparison. The large difference in the normal germination percentage (13%) of "green" versus "black" seed at 7.5% seed moisture again suggests that this may be a real effect that the more conservative Tukey is not declaring, again, because of the variability of the data. The significantly lower percentage of dead seed in seed harvested at 47.4% moisture compared to that harvested at 35.5% is consistent with the suggestion that seed harvested at the higher seed moisture content has a higher normal germination percentage. It seems likely therefore that the moisture content at harvest influences the percentage of normal and dead seed. The reason for this is unclear. On the germination blotter some of the dead seeds were surrounded by a slime characteristic of bacterial growth. It is possible that the delay in harvesting this (naturally) drier seed may have resulted in a concomitant increase in bacterial loading on the seed. This result may be an important factor in defining the best timing of harvesting in this species.

Before deciding to harvest seed at 47.4% seed moisture verification that mass maturity has been reached at this moisture is necessary. As a seed develops it will reach a maximum dry weight, defined as the point of physiological (or mass) maturity, marking the end of food reserve accumulation; dry weight will then remain constant (Bewley and Black, 1994). Seed should usually be harvested after mass maturity has been reached. Earlier harvest will result in seed with lower food reserves (and consequently poorer vigour) and seed that is more liable to processing damage (Thomson, 1979). In this study seed dry weight did not differ between 47.4% and 35.5% seed moisture suggesting mass maturity had been reached by these moisture contents. This conclusion is supported by data, albeit from different species, of Phetpradap (1992) and Fakava (1992) who found that at maximum dry weight, seed moisture content on a fresh weight basis was 65% in hybrid *Dahlia spp.* cv. Unwins and 75% in *Tropaeolum majus* (nasturtium), respectively.

While food reserve accumulation appears to have been completed at 47.4% seed moisture, harvesting and processing nutlet material at this high moisture content without damage and the cost of artificially drying nutlet material are issues not addressed in this study. Whether any additional problems and/or costs associated with harvest at higher seed moisture are justified by any increase in normal germination percentage would need to be determined. Moreover, the data presented here is from only one harvest year and variation from year to year may occur. For example, Demir and Ellis (1992) in a series of experiments conducted in 1989 and 1990 found that in *Lycopersicon esculentum* cv. Moneymaker seed mass maturity was reached at seed moisture contents of 61-72% in

1989 but in 1990 it was not reached until 53% seed moisture. Data from other harvest years will be needed to both confirm that at 47.4% seed moisture content seed of *M. hortensia* has reached mass maturity and that the higher germination percentage in seed harvested at this moisture content compared to seed harvested at 35.5% moisture content is both a real effect and repeatable for different production seasons.

4.4.2.2 Dormancy in *M. hortensia*

There is strong evidence to conclude that the seed of *M. hortensia* used in this study was dormant. The slow and erratic germination behaviour observed is characteristic of a dormant seed population (Mayer and Poljakoff-Mayber, 1989). Metcalf (1995) also comments on the slow and erratic germination behaviour of *M. hortensia* seed suggesting that dormancy is typical of seed of this species when dispersed from the parent plant. He further comments that some seed shed in late summer will not emerge until the following spring. This period (around 180-210 days) is broadly similar to the T_{50} times found in this study given that the percentage of the seed that have germinated by early spring may not be the same as indicated by the T_{50}. Several authors (Maloy, 1992; Matthews, 1993) note that *M. hortensia* seed will germinate readily if fresh. However a comparison of the T_{50} data for freshly harvested seed with that of seed desiccated to around 7.5% provides no evidence to suggest that fresh seed will germinated any more readily than seed at low moisture content. Maloy (1992) and Matthews (1993) may be drawing their conclusions on the observation of early emergence of a small percentage of the seed population but, as neither provides germination percentages, this is speculative.

There are reports in the literature of a correlation between seed colour and dormancy status. Stabell *et al.* (1996) report that in *Cynoglossum officinale* there is a change in seed colour from light yellow through light green and yellow to brown as the seed develops. In this species these colour changes were accompanied by a increase in coat-imposed dormancy with seeds harvested 44 days after anthesis, and classified as green/yellow remaining non-dormant, but those harvested 72 days after anthesis, and classified as brown, dormant. In the absence of seed moisture content data it is difficult to make comparisons, but "green" seed of *M. hortensia* was already dormant suggesting that dormancy in this species may be imposed earlier. However, until a seed development study is carried out on this species this can not be confirmed.

4.4.3 Storage

As in the analysis of the data from the desiccation experiment differences declared significant by the LSD were not always consistent with those declared significant by the the Tukey. Again the reasons for this are most likely the more conservative nature of the Tukey test coupled with the high variability of some of the data as a result of the small number of individual seeds evaluated. A key disagreement between the two tests is when seed, stored as nutlets, under open storage conditions show a significant decline in normal germination percentage. The LSD indicated that this had occurred by nine months of storage whereas the Tukey indicated no decline at the nine month sampling ($P>0.0712$) but a significant decline at the 14 month sampling. The normal germination percentages at 0 and nine months were 78% and 52% respectively, a difference of 26%. In contrast for seed stored as seed under open storage conditions a decline in normal germination from 79% to 49%, a difference of 30%, was found to be significantly different by both comparison tests (Tukey, $P>0.0148$). The percentage difference in the normal germination at 0 and nine months is therefore similar for both nutlets and seed but the significance of the results is not. Moreover, although a significant decline in normal germination was measured at 14 months no information is available on when in the five months between sampling this significant decline occurred. However, the large decline in the mean normal germination percentage already observed at nine months suggests it may have been closer to nine months than 14 months. It seems reasonable to conclude the significant decline in normal germination for seed stored as nutlets under open storage conditions occurred at nine months, accepting the LSD comparison, or at least near nine months, accepting the Tukey comparison. In practical terms the distinction between nine and 14 months is not important. The reason is loss of vigour precedes loss of germination capacity (Justice and Bass, 1978), therefore, it is likely that by nine months open storage the seed had deteriorated to the point where seed quality was poor and the seed is of limited value.

Similarly, disagreement to the time in storage before the number of abnormals and dead seeds increases, while interesting experimentally, are of little practical importance since the observation of both abnormal and dead seeds occurs well after normal germination, and, in the case of dead seed, seed viability is lost.

4.4.3.1 Storage behaviour of *M. hortensia*

This work found that at low seed moisture content (7.5%) and storage temperature (5°C) *M. hortensia* seed can be stored for at least 21 months without loss of viability and therefore agrees with Metcalf (1995) who suggests that *M. hortensia* seed will store for 12 months or more. The findings of this work are also consistent with the suggestion of Hong *et al.* (1998) that seed storage behaviour in the Boraginaceae appears to be orthodox. However, one of the conditions that Hong *et al.* (1998) require for seed to be defined as orthodox is that the seed is able to survive desiccation to seed moisture contents of at least to 2% to 6%, depending on species. Having established that seed of *M. hortensia* can be desiccated to 7.5% moisture content without loss of germination evaluation of the longevity of seed of *M. hortensia* at these lower seed moisture contents should be undertaken, in particular, given the high lipid content of *M. hortensia* seed and the suggestion by Ellis *et al.* (1989) that "ultra-dry" storage at very low seed moisture could be more appropriate for seed with high lipid contents.

Although seed of *M. hortensia* can be stored for at least 21 months control of seed moisture content appears to be critical to prolonging storage life in this species. In this study an increase in seed moisture content of around two percent to 9.5% had a detrimental affect on seed germination by nine months of storage. There are reports in the literature reporting similar declines in germination as a result of a relatively small increase in seed moisture content. Preest (1979) found that *Agathis australis* (kauri) seed stored at 5°C and 10% seed moisture content showed a decline in germination from 88% to 74% after 14 months and 56% after 26 months storage respectively. In contrast seed stored at 5°C and 6% seed moisture retained 83.5% and 77.5% germination, respectively, after the same periods of storage. Marques and de Barros (1999) also report that a relatively small increase in seed moisture content from 10.8% to 12.5% and 13.3% during storage of *Achyrocline satureioides* seed (a member of the Asteraceae) resulted in a decline in germination after six months storage. Seed stored at 10.8% and 19-23°C showed no decline in germination over the six months storage.

The storage conditions required to maintain orthodox seed in store for more than 12 months at least (and frequently longer), are well understood. For non-germplasm storage essentially these are to reduce and maintain temperature at 5-10°C and store the seed at low moisture content (Thomson, 1979; Copeland and McDonald, 1995). However, the

"safe" storage moisture content can vary depending on the composition of the seed. Vertucci and Roos (1990) found that the moisture content at which maximum vigour after storage was obtained correlated with the lipid content of the seed. They suggest that seeds with higher lipid content have lower moisture contents for optimum storage. Seed with high lipid contents should be stored at no more than 8% moisture and starchy seeds at no more than 14%. These are the approximate equilibrium moisture contents high lipid containing seed and starchy seed will reach, respectively, at a relative humidity of 65-70% (Thomson, 1979). The different seed moisture contents at the same relative humidity are reached because seeds with high lipid contents sorb less water (Vertucci and Roos, 1990). This means that at the same relative humidity the seed moisture content decreases as the lipid content of the seed increases (Walters, 1998).

This work (Chapter 3) has established that seed of *M. hortensia* has a high lipid content (24% of the dry weight). The high lipid content of seed of *M. hortensia* is therefore likely to influence its moisture content for safe storage. In agreement with Thomson (1979), at a moisture content of less than 8% (7.5%) and at low temperature (5°C) *M. hortensia* seed will maintain germination in store. Similarly, Ramiro *et al.* (1995) found that seed of *Brassica cretica* and *B. montana* could be stored for up 8 years at 5°C and 8% seed moisture content without a significant loss in germination. Although no comparisons are made with storage at higher seed moisture content these results illustrate the potential for longer term storage at a moisture content and temperature similar to that used to store *M. hortensia* (7.5%) for 21 months without loss of germination.

Although the storage moisture content is important in determining the longevity of seed in store, the storage temperature is also important (Harrington, 1972; Justice and Bass, 1978; Priestley, 1986). Seed deterioration is usually slow at low temperature (Vertucci *et al.* 1994). The *M. hortensia* seed was stored at low temperature (5°C) and the relatively rapid loss of viability at this temperature even at moisture contents likely to be above those optimum for storage of seed of this species seems surprising. For example, Chai *et al.* (1998) found that *Glycine max* seed (cv. No. 2132) stored at 7.2% seed moisture content retained 97% germination after 12 months but at 9.8% seed moisture the same seed showed a decline in germination to 72%. However the decline in germination at 9.8% seed moisture occurred at temperatures (averaging 18°C with a range from 0 to 35°C) much higher than those used in this study. Although Chai *et al.* (1998) do not give the lipid content of the *G. max* cultivar used, reports in the literature (Pixton and Warburton, 1971;

Vertucci and Roos, 1990) indicate the lipid content of *G. max* seed is usually around 20% and therefore similar to that of *M. hortensia*.

There are reports of loss of germination at low temperature within a short period. *Cordia goeldiana* lost around 20% viability after seven months storage at 8°C and 50% relative humidity; viability was completely lost during one month open storage at 26°C and 80% relative humidity (Vianna, 1983, cited in Hong *et al.*, 1998). Similarly *Cordia alliodora* viability is halved after 12 months storage at 5°C, but, at a much higher 12-18% seed moisture (Tschinkel, 1967 cited in Hong *et al.*, 1998). In this species viability is maintained at 2-5°C with 6.9% seed moisture content (Trivino *et al.*, 1990, cited in Hong *et al.*, 1998). *Agathis australis* seed stored under similar conditions (5°C and 10% seed moisture) showed a germination decline within 14 months (Preest, 1979). However, in the absence of any information on the level of lipid storage reserves in seed of these species, it is difficult to draw direct comparisons with storage behaviour observed in *M. hortensia*.

It seems likely that because of the high lipid content a seed moisture content of 9.5% is above the optimum for storage of *M. hortensia* seed but whether this alone accounts for the loss of germination at 5°C is not clear. Other factors may be influencing that rate of deterioration. Loss of seed vigour precedes loss of viability (Justice and Bass, 1978).The relatively low normal germination percentage (77%) of seed harvested at 35.5% moisture content suggests that the vigour of this seed when placed in store may have been low. If the seed was of low vigour this may have accelerated the loss of germination capacity under the less ideal open storage conditions. Storage of seed with varying levels of vigour at a range of temperatures and moisture contents may answer this question.

This work has established that seed of *M. hortensia* is desiccation tolerant and can be safely stored at low temperature and seed moisture. Clearly if seed of *M. hortensia* is stored under refrigerated conditions the reduced moisture holding capacity of the air (Copeland and McDonald, 1995) and consequent higher relative humidity means it is likely that seed moisture content will increase above that safe for storage. Therefore, either dehumidification systems or water impermeable packaging will be required. Storage at constant higher temperatures and under ambient conditions was not evaluated in this work. An evaluation of the storage potential of the seed at ambient may be useful as a cheaper alternative to refrigerated storage in water impermeable packaging, although given the high value of the seed ($ 330.00/1000 seed (Anonymous, 2000)) the additional

expense of closed low temperature storage may be justified.

Storage of *M. hortensia* as seed instead of nutlets had no effect on storage potential under the same storage conditions except in open storage. In open storage, storage within the nutlet delayed seed death, but given that the seed that remained viable showed abnormal seedling (root) development the distinction has no practical relevance. It does however demonstrate that loss of viability can be regarded as a single continuum with different components of the embryo exhibiting ageing damage independent of others.

The higher germination percentage observed in seed harvested at 47.4% seed moisture content compared to seed harvested at 35.5% appears to be maintained during the 14 month storage period for which comparisons are available, again indicating, that timing of harvest may be an important factor in maximising storage life in this species.

4.4.3.2 The effect of storage on dormancy

Storage of seed for 21 months at 7.5% seed moisture content did not reduce the time to 50% germination (134 days) indicating storage did not alleviate the dormancy observed in *M. hortensia* seed. It is unlikely, therefore, that the seed has an after-ripening requirement. The apparently shorter time required to reach 50% germination in two of the nine month treatments is difficult to explain. It seems unlikely that dormancy had been alleviated and then reimposed since the effect was not observed in seed stored as nutlets stored under the same conditions. Moreover, the T_{50} times still remained at around 100 days suggesting the seed still maintained a high level of dormancy.

4.4.4 Conclusions

Seed of *M. hortensia* appears to be orthodox in its seed storage behaviour. It can be desiccated to 7.5% seed moisture and stored at that moisture and 5°C for at least 21 months without loss of germination. However, control of seed moisture appears to be critical in maintaining germination in store. An increase in seed moisture content to 9.5% resulted in a complete loss of germination within 21 months. It seems likely that this moisture content is above that optimal for storage of the high lipid containing *M. hortensia* seed, but whether this alone can account for the loss of germination at 5°C is not certain. Storage of seed under a wider range of storage conditions and an assessment of the

vigour of the seed going into store may help answer these questions.

CHAPTER 5 SEED GERMINATION AND DORMANCY

5.1 Introduction

Although there are suggestions of a range of temperatures (10-15°C) suitable for germination of *M. hortensia* (Metcalf, 1995), the optimum germination temperature for this species has not been reported in the scientific literature. This experiment was designed to determine the temperature or temperature range at which germination (up to and including normal seedling development) of *M. hortensia* is most rapid. However, the failure of seed to show high germination at any temperature on a temperature gradient plate suggested a dormancy mechanism rather than temperature was limiting germination. A series of experiments was designed to both confirm the presence of dormancy in seed of *M. hortensia* and determine the mechanism(s) by which any dormancy is imposed.

5.2 Materials and Methods

5.2.1 Seed Material, Harvest and Sub-sampling

Seed heads from the 1996/1997 production year were harvested on 23 January 1997 and 31 January 1997. Nutlets within each sampling date were separated from the stem material, separated into green and black nutlets and within each colour classification split into four replicates as described in **3.2.2**. Nutlets were air-dried in a controlled environment room at 20°C until the seed reached a constant seed moisture content (approximately 7.5%). Nutlets from the two sampling dates were combined by mixing the same replicate from the two samplings together through a riffle divider. The seed was extracted from the nutlets and placed in sealed storage (12/20/50 micron laminated polyester/aluminium foil/polythene packets) at 5°C until required for the temperature gradient plate and, subsequent, dormancy-breaking experiments.

A second lot of seed heads from the 1997/1998 production year were harvested on 7 January 1998 and split into four replicates as described in **3.2.2**. Nutlets were air-dried in the same 20°C controlled environment room and reached an equilibrium moisture content of approximately 7.5%. This seed was also extracted from the nutlets and placed in sealed

storage at 5°C until required.

Seed extracted from black nutlets only was used for both the temperature gradient plate and dormancy-breaking experiments.

5.2.2 Temperature Gradient Plate Experiment

A Grant Temperature Gradient Plate (Grant Instruments Limited, Cambridge, United Kingdom) was used to determine the optimum germination temperature(s). The temperature gradient plate was set to run a one-way gradient, using a temperature range of 5°C to 24°C. The plate was located in a controlled environment room operating at 20°C with continuous lighting from two fluorescent tubes (Philips 65 W, cool white). The intensity of light received by the seed (through the two layers of clear glass on the plate) was approximately 4.7 $\mu mol\ m^{-2}\ s^{-1}$ (Logan, 1999). To reduce any shading effects the plate was positioned so that lighting was directly overhead. The plate was left 24 hours to stabilise at the set temperatures before any seed was sown.

The plate was "split" in half. On each half a single layer of K-22 Kimpak® (Anchor Paper Company, St. Paul, Minnesota) was placed on the plate surface and three layers of steel blue seed germination blotters (Anchor Paper Company, St. Paul, Minnesota) placed on top. A 20 mm corridor (no Kimpak or germination blotters) was left through the middle of the plate between the Kimpak/germination blotters.

Prior to sowing the replicate was split in two using a riffle divider. Half of the seed was dusted with thiram, and set to germinate on half of the plate. Twenty-five seeds per temperature were placed of the plate at 1°C increments (37 mm) up the plate.

To potentially remove any germination inhibitors in the seed coat the remaining half of the *M. hortensia* seed was washed in running water. A washing time of 2.5 hours was used (K.A.Hill, *pers. comm.*) and seed washed at room temperature (17°C). ISTA (1993) recommends washing seed in running water at 25°C to remove germination inhibitors from the pericarp or seed coat. In this experiment a lower washing temperature (17°C) was used to more closely reflect temperatures experienced on the Chatham Islands. After washing seed was surface dried then between two sheets of paper toweling and then air-dried at 20°C for one hour. This seed was dusted with thiram and set to germinate as

above on the other half of the plate. It was assumed that the corridor up the plate would prevent contact of any inhibitors leached from the unwashed seed with washed seed.

The relatively large size of the *M. hortensia* seed meant that the two treatments (unwashed and washed seed) of 25 seeds each occupied the full width of the plate. The decision was therefore made to replicate the treatments in time i.e. only one replicate was placed on the plate at any one time. It was assumed that there would be little or no physiological changes in the seed of the other replicates while in store at low temperature and seed moisture content.

5.2.3 Preliminary Dormancy-breaking Experiment

After 11 weeks on the plate the majority of the seed remained ungerminated. Ungerminated seed was removed from the plate and the still imbibed seed for each treatment combination (washed/unwashed seed x germination temperature) split in two by hand. The seed coat was removed from half the seed by making a 5-10mm scalpel cut across the seed coat in the cotyledon area and peeling the coat off. This peeled seed and unpeeled seed was set to germinate as described in **4.2.1.3** (i.e. at at alternating temperatures of 10°C and 15°C in the dark), except the seed was not dusted with thiram. In seed retaining its seed coat, germination was scored as either radicle or cotyledon emergence. In peeled seed germination was scored as radicle growth. Radicle growth was defined as occurring when the radicle was longer than 2 mm.

5.2.4 Main Dormancy-breaking Experiments

A fully replicated dormancy-breaking experiment, including additional treatments, using now nearly twelve-month old seed was set up in January 1998. Seed remaining from the replicate used for the temperature gradient plate work and seed, in store for evaluation on the temperature gradient plate, from the other three replicates (described in **5.2.1**) was mixed and re-combined using a riffle divider and then split into four new replicates (as described in **3.2.2.**) containing approximately the same number of seed. Seed was pre-conditioned as described in **4.2.1.2**, then surface dried by pressing it between two sheets of Whatman Number One filter paper and manipulated in the following ways:

1. seed coat cut completely around the circumference of the seed, with a scalpel, at a point 2-3 mm above the embryo axis ("cut seed")
2. a scalpel used to remove (chip) a 2-3 mm length of seed coat from the distal end of the seed to expose the cotyledon ("chipped seed")
3. a scalpel used to make a 5-10 mm cut in the cotyledon area of softened seed coat and the coat peeled off ("peeled seed")
4. seed coat removed as in 3. above and the peeled seed germinated on seed leachate ("peeled on leachate"). The leachate was obtained by soaking 80 seeds in 25 ml of distilled water in a 125 ml conical flask ("soaked seed)" at 20°C for 24 hours. The flask was wrapped in aluminium foil and agitated on an orbital shaker
5. seed coat removed as in 3. above and peeled seed set to germinate on distilled water with seed coats from all the peeled seed placed under the first blotter ("peeled on coats")
6. soaked seeds from 4. above where peeled as in 3. ("ex-soak peeled").

Intact seed was treated in the following ways:

1. seed preconditioned overnight as described in section **4.2.1.2.** ("preconditioned seed")
2. seed placed in a Buchner funnel (size 2) and washed for 24 hours in running tap water at 20-22°C. The funnel was inclined on an angle in a tripod and the tripod positioned so the tap water continually agitated the seeds ("washed seed")
3. soaked seed, from 5. above ("soaked seed")
4. seed without pre-conditioning ("control seed").

Seed was placed for germination as described in **4.2.1.3** (i.e. at alternating temperatures of 10°C and 15°C in the dark), except, peeled seed was not dusted with thiram. The cut and chipped seed dusted with thiram prior to the cutting treatments to reduce the likelihood of the scalpel blade carrying thiram through to the embryo. Unless noted above seed was set to germinate on water.

The seed moisture content was determined at the end of the 24 hour preconditioning, soaking or washing period using the method described in **4.2.1.1**. In addition the seed moisture content of seed that had been preconditioned for one-and-a-half hours was determined.

A second experiment was set up in March 1998 using seed remaining from the 23 January and 31 January 1997 harvests and seed harvested on 7 January 1998. The peeling (germinated on water only), cutting and chipping treatments described above were repeated. An additional treatment of pricking the seed coat seed was included as a less severe manipulation of the seed. Seed was pre-conditioned as detailed in **4.2.1.2.** and the coat pricked in the middle (cotyledon) area with a dissecting needle (diameter 0.6-0.8 mm). The supply of 1998 harvest seed was limited therefore the pricking treatment was applied to 1997 harvest seed only. Seed was placed on for germination as for the first dormancy-breaking experiment but seed moisture content was not assessed in this experiment.

In both experiments germination was scored as either radicle or cotyledon emergence in seed retaining its seed coat (including chipped seed). In peeled seed germination was scored as radicle growth. Radicle growth was defined as occurring when the radicle was longer than 2 mm. For seed pricked with a dissecting needle the rupture point of the seed coat for those seed that germinated was recorded.

For both experiments normal germination was assessed as described in **4.2.1.3**, unless otherwise stated. A measure of the rate of germination (T_{50}) was calculated as in **4.2.1.4**.

After 142 weeks only 15 seeds out of the 1900 seeds set up remained ungerminated from the two dormancy-breaking experiments. This seed was pricked in the cotyledon area with a Type 00 (0.30 mm) insect pin to promote germination. Again the rupture point of the seed coat for those seed that germinated was recorded.

5.2.5 Scanning Electron Microscopy of the Seed Coat

Seed harvested in January 1997 was preconditioned by rolling it in moistened 38 lb Regular Weight Seed Germination Paper (Anchor Paper Company, St. Paul, Minnesota). The roll was placed in a 500 ml glass jar with approximately 100 ml of water in the bottom and both were placed in a plastic bag and the seed allowed to imbibe at 20°C for 17 hours.

Seed material was taken to the Keith Williamson Electron Microscope Unit, HortResearch

Ltd., Palmerston North for preparation for scanning electron microscopy.

5.2.5.1 Fixation and scanning of the seed coat

Seed was dissected into small, 1-2 mm square, samples and fixed in a primary fixative (3% glutaraldehyde (v/v) and 2% formaldehyde (v/v) buffered in 0.1 M phosphate buffer at pH 7.2) for two hours at room temperature. The phosphate buffer was prepared by dissolving 2.51 g of Na_2HPO_4 and 0.41 g of KH_2PO_4 in 100 ml of water and adjusting the pH to 7.2. The sections were washed, at room temperature, by immersing them twice in fresh 0.1 M phosphate buffer for ten minutes followed by a third immersion of 20 minutes. The samples were then fixed in the secondary fixative (1% osmium tetroxide (OsO_4) (w/v) in 0.1 M phosphate buffer) for one hour at room temperature.

The samples were again washed three times as above in 0.1 M phosphate buffer and then dehydrated in a graded acetone series:

1. 25% acetone: fifteen minutes
2. 50% acetone: fifteen minutes
3. 75% acetone: fifteen minutes
4. 95% acetone: fifteen minutes
5. 100% acetone: fifteen minutes
6. 100% acetone: one hour

The samples were transferred in acetone to a Polaron E3000 Critical Point Drier and dried using CO_2 as the critical point fluid. After critical point drying samples were mounted on an aluminium scanning electron microscopy stub using double sided tape and gold sputter-coated. Samples were examined using a Cambridge 250 MkIII Scanning Electron Microscope.

5.2.6 Data Analysis

The general linear models procedure (Proc GLM) in SAS for Windows (Release 8.02 TS Level 02M0, SAS Institute, Cary, North Carolina) was used to perform an analysis of variance (ANOVA) on the data. The least significant difference (LSD) procedure was used to compare means, but, only where the ANOVA F-test identified a significant difference

between treatments i.e. a protected LSD (Ott, 1988). However, because of the large number of pair-wise comparisons, particularly in the first dormancy-breaking experiment, there is a chance of falsely declaring a significant difference when using the LSD. Consequently, the more conservative (Ott, 1988) Tukey procedure was also used to compare means. For interaction effects the probability values for the hypothesis that two means being compared were equal were also determined using the LSD and the LSD adjusted for the Tukey. Results are presented for comparisons using the LSD , but, where major differences exist between the two procedures both results are noted in the text.

The T_{50} data were log transformed and the square root of the all abnormal germination percentage data taken, except, for the combined abnormal germination data. These transformations were to normalise the data and/or remove heterogeneous variance within the data. The seed moisture content percentages were also log transformed to reduce skewness and heterogeneous variance in this data.

In the second dormancy-breaking experiment not all treatments included in the first were repeated. To avoid biasing the analysis where some treatments were repeated three times and others only once, the two dormancy-breaking experiments were analysed separately.

The LSD values presented in tables were calculated from the mean sum squares of the error (MSE) in the ANOVA table for the comparisons and using a sample size (number of replicates) of four. The probability values for the hypothesis that two means being compared were equal using the LSD were used to verify the differences significant declared by the LSD calculated from the mean sum squares.

Where an individual mean is presented in the text numbers following in brackets are the standard error of that mean.

5.3 Results

5.3.1 Temperature Gradient Plate

After 11 weeks on the temperature gradient plate little (< 5%) or no germination was observed in washed or unwashed seed at any temperature between 5°C and 24°C, indicating that a factor other than temperature was limiting germination.

5.3.2 Preliminary Dormancy-breaking Experiment

In contrast to the poor germination observed on the plate, removal of the seed coat accelerated germination. In peeled seed 88% radicle growth was reached within two weeks of removal of the seed coat compared to only 1% in unpeeled seed. In unpeeled seed radicle emergence did not reach a similar level (93%) until five weeks after seed was removed from the plate (Figure 5.1).

There appeared to be no effect of washing the seed for 2.5 hours or germination temperature experienced by the seed on the plate on the subsequent rate of radicle growth in peeled seed or emergence in unpeeled seed. In addition, there was no difference in the final normal germination percentage for washed and unwashed seed from any germination temperature on the plate (Appendix 6).

5.3.3 Main Dormancy-breaking Experiments

5.3.3.1 Rate of germination

In both dormancy-breaking experiments germination of control seed followed a similar pattern to that observed in the desiccation and storage experiments i.e. it was erratic and slow. Neither soaking, pre-conditioning nor washing seed for 24 hours improved the rate of germination compared to control seed nor was there any difference in the rate of germination between "peeled seed", "peeled on leachate", "peeled on coats" or "ex-soak peeled" (Table 5.1). However, weakening or removing the seed coat allowed a dramatic improvement in the germination rate (Table 5.1 and 5.2). Germination was most rapid when the seed coat was completely removed or the seed coat was cut above the embryo

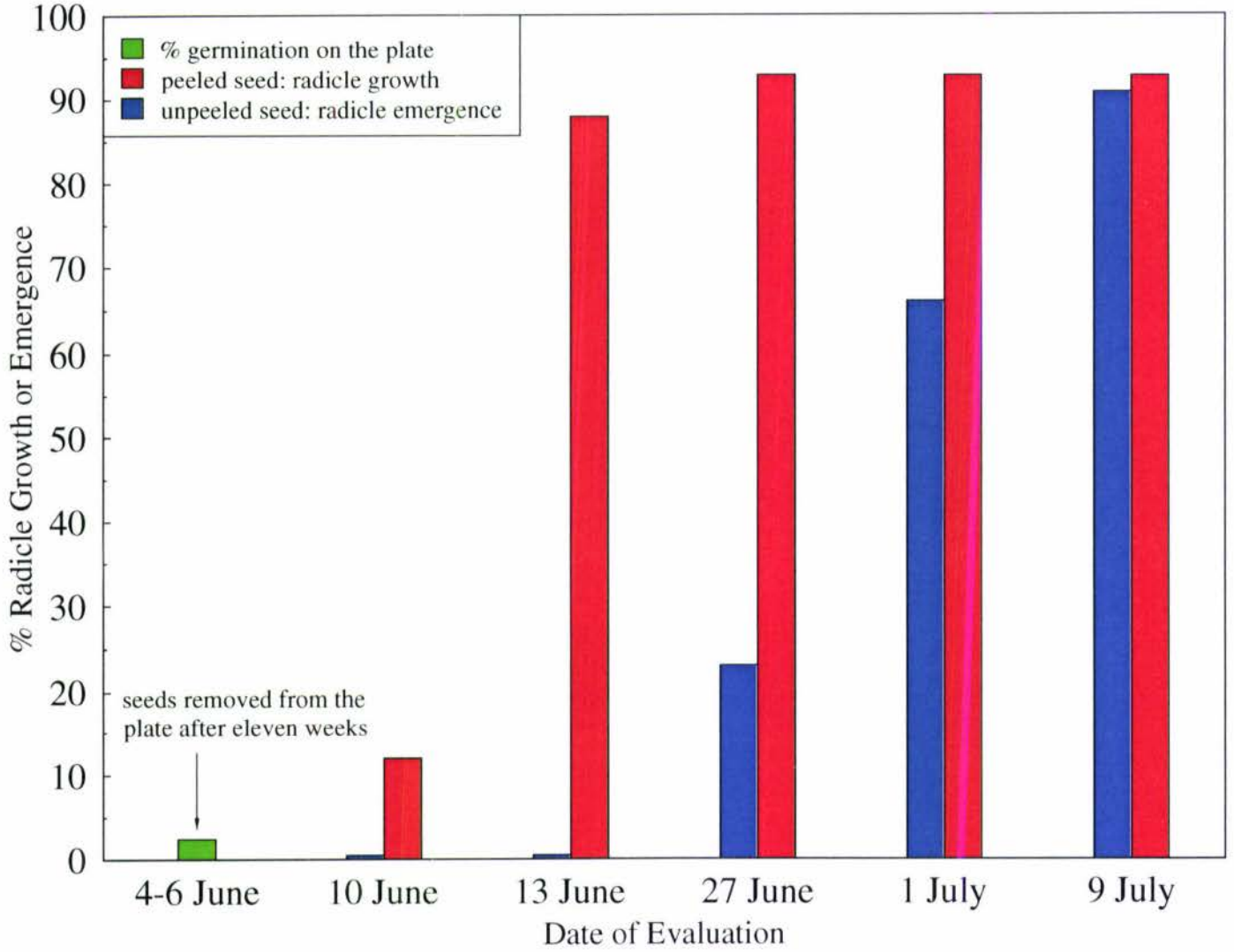


Figure 5.1: Comparison between the rate of radicle growth in peeled seed and radicle emergence in unpeeled seed. Percentages are for the pooled emergence of seed removed from different temperatures on the temperature gradient plate.

axis. In chipped seed the rate of germination was significantly slower ($P<0.05$) than the peeling and cutting (LSD procedure only) treatments. Chipping allowed the cotyledonary tissue to emerge from the coat at the position of the chip. However, it was unclear if this occurred as a result of direct expansion of the cotyledons or radicle growth pushing the cotyledons out of the coat or a combination of both.

Pricking the seed coat also allowed germination to proceed at a significantly faster rate ($P<0.05$) than in control seed or treatments that left the coat intact, but at a significantly slower rate than for treatments where the seed coat was removed, cut, or chipped (Table 5.2). In 84% of the pricked seed that germinated the coat ruptured at the entry point of the dissecting needle. In contrast when ungerminated seed in both the dormancy and storage experiment was pricked with a finer insect pin only 37% of the seed that germinated ruptured at the point of the prick. The time taken to reach 50% germination from the time of pricking with the insect needle was 27 days compared to 35 days in seed pricked with a dissecting needle.

There was no effect of year of harvest on the rate of germination for the respective dormancy-breaking treatments.

5.3.3.2 Radicle emergence, normal and abnormal germination and dead seed

In this section of the results the two main dormancy-breaking experiments are discussed separately.

In the first main dormancy-breaking experiment (1997 harvested seed only) final radicle emergence percentage did not differ between treatments. In contrast the normal germination percentage of peeled seed was significantly lower ($P<0.05$) than that of control seed (Table 5.1). The reduced normal germination in peeled seed was a result of a significant increase ($P<0.05$) in the number of abnormal seedlings, in particular, seedlings with infected radicles or cotyledons. The LSD, but not the Tukey, also indicated a significantly higher ($P<0.05$) number of seedlings with glassy root development in peeled seed compared to control seed. There was a significant difference ($P<0.05$) in the normal germination percentage of control seed compared to cut (LSD, but not Tukey) but not between control and chipped seed.

Table 5.1 Germination rate, radicle emergence, normal and abnormal germination and dead seed percentage of *M. hortensia* seed harvested in 1997 after dormancy-breaking treatment.

Dormancy-breaking treatment	Rate of Germination (T-50, days)	Final Radicle Emergence (%)	Normal Germination (%)	Abnormal Germination				Dead (%)
				Glassy Roots (%)	Infected Seedlings (%)	Other (%)	Combined[a] (%)	
control	155.2 (2.19)	87	79	4 (1.66)	3 (1.50)	2 (0.93)	9	12
preconditioned only	175.8 (2.25)	92	79	7 (1.83)	1 (0.50)	5 (1.87)	13	8
24 hour washing	167.2 (2.22)	90	84	4 (1.71)	1 (0.50)	2 (1.00)	7	9
seed soaked for 24 hours	161.2 (2.21)	81	67	7 (2.20)	2 (0..70)	5 (1.87)	14	19
seed coat chipped at the distal end	16.5 (1.22)	91	62	18 (4.13)	7 (2.24)	3 (0.83)	28	10
seed coat cut above the embryo axis	13.3 (1.12)	88	55	18 (4.13)	7 (2.23)	6 (2.07)	31	14
peeled seed germinated on water	12.3 (1.09)	87	46	26 (4.90)	19 (4.14)	1 (0.43)	46	8
seed soaked for 24 hours, peeled and germinated on water	11.9 (1.07)	85	45	20 (4.46)	16 (3.93)	2 (0.70)	38	17
peeled seed germinated on seed coats	12.0 (1.08)	86	48	20 (4.23)	23 (4.45)	0 (0)	43	9
peeled seed germinated on seed leachate	12.3 (1.09)	88	22	23 (4.62)	41 (6.38)	2 (0.66)	66	12
LSD, P<0.05	0.067	NS	17.7	1.984	1.944	NS	15.4	NS

[a]combined is the total percentage of abnormal seedlings i.e. combined for abnormal germination categories
transformed means are in brackets, for transformed data LSDs are for comparing transformed means.

Table 5.2 Germination rate, radicle emergence, normal and abnormal germination and dead seed percentage of *M. hortensia* seed harvested in 1997 and 1998 after dormancy-breaking treatment.

Dormancy-breaking Treatment	Rate of Germination (T-50, days)		Final Radicle Emergence (%)		Normal Germination (%)		Abnormal Germination								Dead (%)	
							Glassy Roots (%)		Infected Seedlings (%)		Other (%)		Combined[a] (%)			
	1997	1998	1997	1998	1997	1998	1997	1998	1997	1998	1997	1998	1997	1998	1997	1998
control (preconditioned)	196.3 (2.29)	203.7 (2.31)	81	75	75	66	4 (1.71)	3 (1.25)	1 (0.50)	3 (1.56)	0 (0)	3 (1.56)	5	9	20	25
seed coat pricked with a dissecting needle	34.7 (1.54)	-	82	-	79	-	1 (0.50)	-	0 (0)	-	2 (1.00)	-	3	-	18	-
seed coat chipped at the distal end	19.3 (1.29)	19.8 (1.30)	86	69	63	53	10 (2.64)	1 (0.50)	4 (1.71)	7 (2.21)	10 (3.06)	8 (2.21)	24	15	13	31
seed coat cut above the embryo axis	13.6 (1.13)	13.2 (1.12)	81	73	50	53	24 (4.28)	12 (3.44)	1 (0.50)	2 (1.00)	8 (2.44)	7 (2.54)	33	21	17	26
peeled seed germinated on water	12.9 (1.11)	12.5 (1.10)	81	76	47	60	26 (5.04)	8 (2.37)	4 (1.71)	3 (1.21)	11 (3.16)	7 (2.57)	41	18	12	22
LSD, P<0.05	0.078		NS		20.9		2.192		NS		1.705		14.04		18.2	

[a]combined is the total percentage of abnormal seedlings i.e. combined for abnormal germination categories
transformed means are in brackets, for transformed data LSDs are for comparing transformed means
LSDs are for comparing means both down the columns and across the rows.

The normal germination percentage for peeled seed germinated on seed leachate was 22% compared to 46% (normal germination percentage pooled) for the other peeling treatments). Despite this 24% difference in the normal germination percentage only the LSD, but not the Tukey, indicated a significantly lower normal germination percentage ($P<0.05$) on seed leachate compared to the other peeling treatments. There was no significant difference in the number of seedlings that developed glassy roots between the different peeling treatments ("peeled seed", "peeled on leachate", "peeled on coats" or "ex-soak peeled"). However, for peeled seed germinated on seed leachate there was a significantly higher ($P<0.05$) number of seedlings with infected radicles or cotyledons (classified as abnormal seedlings) compared to peeled seed germinated on water but not peeled seed germinated on both water and seed coats ("peeled on coats"). Some normal seedlings rotted but these were classified as normal because it was assumed that the infection came from the germination medium rather than seed (secondary infection). Some seedlings exhibiting glassy root development also became infected, however, as this occurred after abnormal root development was observed these seedlings were classified as abnormal because of the glassy root development. Other abnormal seedling development observed included no radicle development, stunted or weak radicles and deformed cotyledons. These were placed into the category "other".

In the second main dormancy-breaking experiment (1997 and 1998 harvested seed) unbalanced data was analysed. Specifically, the 1997 mean included data for the pricking treatment whereas the 1998 mean did not. The analysis was influenced by this additional treatment. In comparing harvest years, there was no significant effect of year of harvest on the percentage of normal, infected or "other" abnormal seedlings, irrespective of whether data was analysed with or without the pricking treatment included. However, in analysing the data for glassy root development and "combined" abnormal seedling development in both analyses there was no significant difference between harvest years when the data for pricked seed was included but there was a significant difference ($P<0.05$) when this data was excluded. This is because the 1997 mean is drawn down by the low percentage of seedlings showing glassy root development or abnormal seedlings in the pricking treatment whereas the 1998 mean is not. For seedlings showing glassy root development when the pricking treatment was removed the pooled transformed means for the common treatments were 3.4167 (1997 harvest) and 1.8884 (1998 harvest). The LSD and minimum significant difference (Tukey) comparing the pooled means for the common treatments is 1.1408, which is less than the difference between the two means,

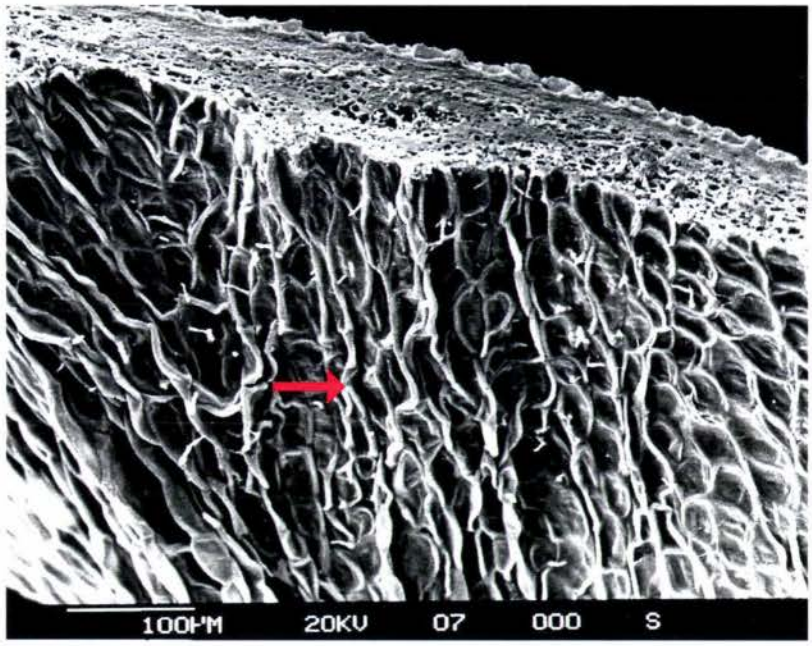


Plate 5.1: Transverse section across the seed coat of *M. hortensia* showing heavy ridging (→) on the outer coat surface and the continuous nature of the coat surface.

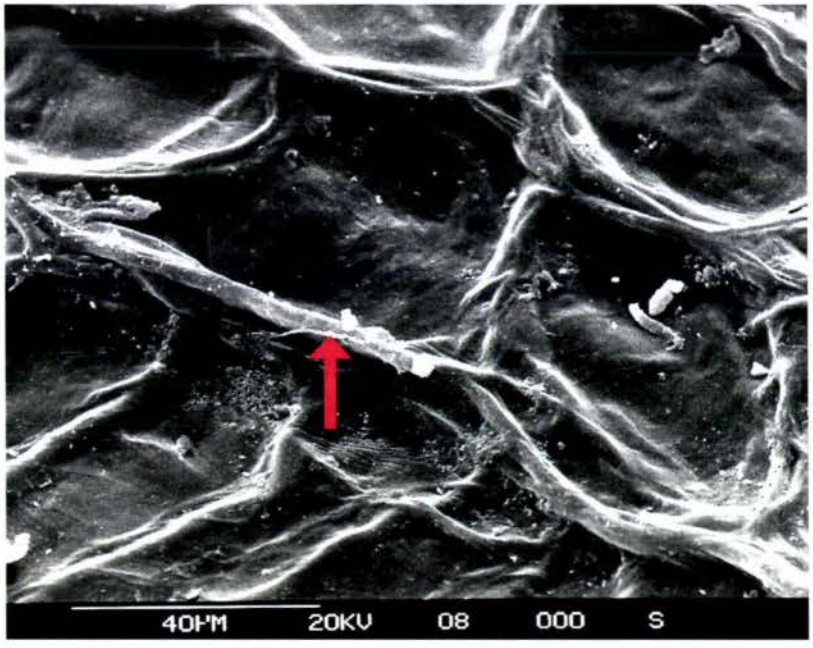


Plate 5.2: Outer seed coat surface of *M. hortensia* seed showing ridges on the coat surface (→) and the lack of spaces or fissures in the coat surface.

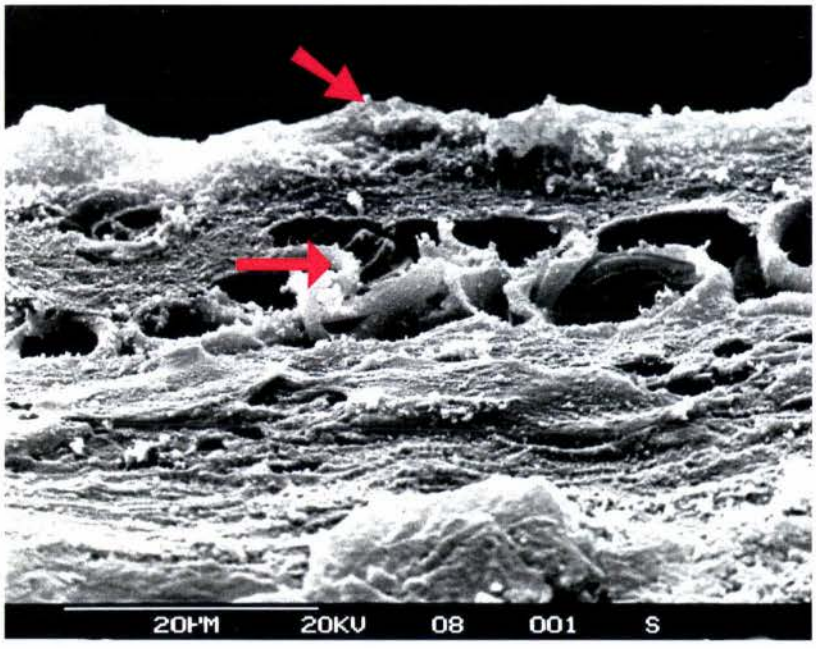


Plate 5.3: Transverse section across the seed coat of *M. hortensia* showing vascular elements (→) below the outer surface of the seed coat (↘).

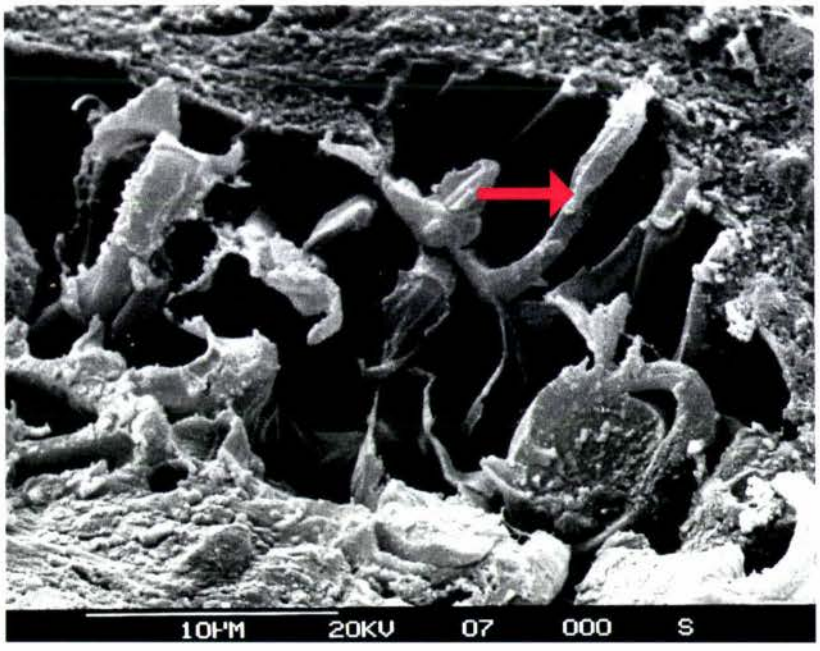


Plate 5.4: Transverse section across the surface of *M. hortensia* seed coat showing details of the secondary thickening (→) in the vascular elements.

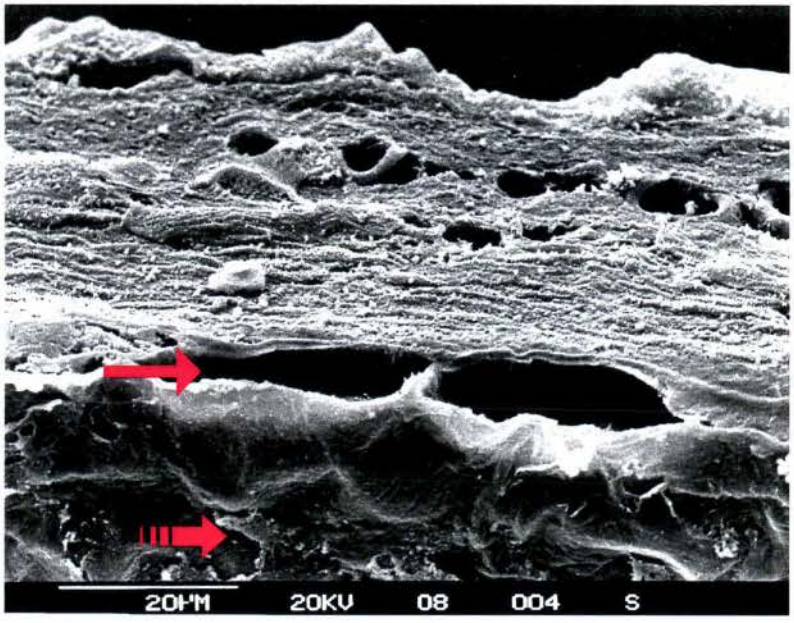


Plate 5.5: Transverse section across the seed coat of *M. hortensia* showing the zone of empty cells (→) immediately above the inner surface of the seed coat (→).

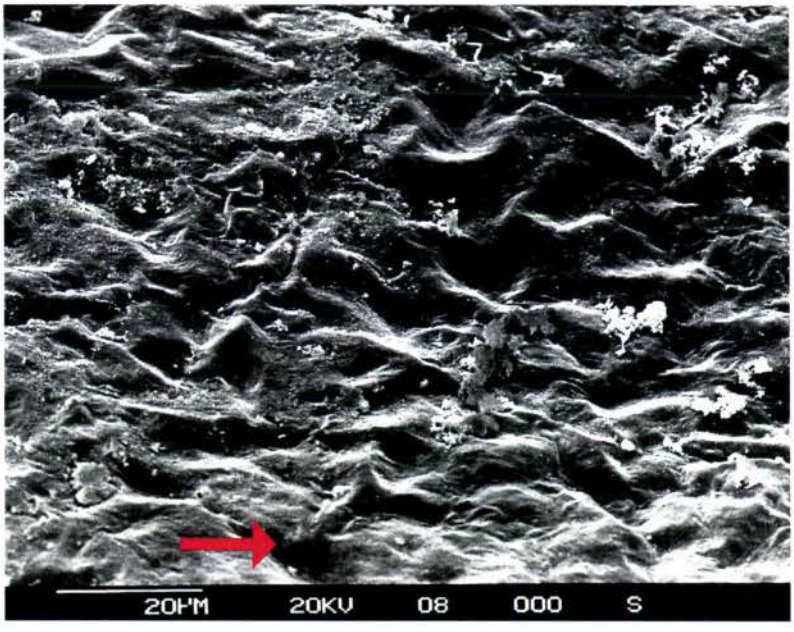


Plate 5.6: Section across the inner surface of *M. hortensia* seed coat showing hummock-like appearance (→) of the inner coat.

indicating seed from the 1997 had a higher percentage of seedling with glassy root development. Comparing specific dormancy-breaking treatments between 1997 and 1998 harvest seed (excluding the pricking treatment), in peeled seed from the 1997 harvest there was a significantly higher ($P<0.05$) percentage of seedlings showing both abnormal seedling development overall and glassy root development in particular (LSD only). There was no significant difference in abnormal seedling development between harvest years between any other dormancy-breaking treatments or control seed. Comparing dormancy-breaking treatments within the 1998 harvest seed there was no significant difference in the number of seedlings exhibiting glassy root development between any of the dormancy-breaking treatments, except, when comparing chipped and cut seed (LSD only). In contrast, as in the first experiment, for 1997 harvest seed peeled and cut seed had a significantly higher ($P<0.05$) percentage of seedlings showing glassy root development than control seed but only with the LSD only. However, both the LSD and Tukey indicated that peeled seed and cut seed had a significantly higher percentage ($P<0.05$) of abnormal seedlings than the control.

The unbalanced design of the experiment also influenced the results obtained when analysing the effect of dormancy-breaking treatment on the normal germination percentage. When the pricking treatment was included, the LSD, but not the Tukey, indicated that the normal germination percentage (pooled for harvest years) for control seed was again significantly higher ($P<0.05$) than in peeled seed. However, when the normal germination data for pricking treatment seed was removed there was no significant effect of any dormancy-breaking treatment on normal germination percentage.

There was no significant difference in the percentage of seed with infected radicles or cotyledons between years or dormancy-breaking treatments.

5.3.3.3 Seed Moisture Content

There was no significant difference in the final seed moisture content after preconditioning, soaking or washing. The pooled seed moisture content for these treatments was 42.7(±0.56)%. In contrast the moisture content of control seed was 7.4(±0.26)% and for seed imbibed for 1.5 hours 20.6(±0.51)%. These three seed moisture contents were all significantly different ($P<0.05$). Seed moisture contents for individual treatments are given in Appendix 7.

5.3.4 Scanning Electron Microscopy of the Seed Coat

Scanning electron microscopy revealed a network of ridges on the surface of the seed coat of *M. hortensia* (Plate 5.1 and 5.2). The seed coat surface is continuous with no individual plates or cracks or fissures visible. Below the surface are structures that appear to be vascular elements (Plate 5.3). These elements show secondary cell wall development (Plate 5.4). Just under the inner surface of the seed coat is a zone of empty cells. These cells do not have the secondary cell wall development of the vascular elements observed immediately below the outer seed coat (Plate 5.5). The inner surface of the seed coat has a hummock-like appearance in contrast to the network of ridges observed on the outer surface (Plate 5.6).

5.4 Discussion

5.4.1 Temperature Gradient Plate

The rationale for placing seed on the temperature gradient plate was the suggestion by Maloy (1992) that seed of *M. hortensia* will germinate readily if fresh. The temperature gradient plate is useful for determining the optimum germination range for seed of most species but assumes that temperature is the factor limiting germination. There was little or no germination at any temperature between 5°C and 24°C on the plate. The temperature range used includes that suggested by Metcalf (1995) for germination of *M. hortensia* seed (10-15°C). This failure of the seed to germinate over a wide range of temperatures indicates temperature is not the factor limiting the rate of germination in *M. hortensia* seed. This is in agreement with Metcalf (1995) who commented that germination could be slow and erratic.

Seed on the plate was set to germinate in full light, but failed to germinate. It therefore seems unlikely that a light stimulus is required for germination in *M. hortensia* seed. This conclusion is consistent with results reported for other Boraginaceae, for example, Piggin (1976) found that light did not promote germination in dormant *Echium plantagineum* seed. Similarly van Breemen (1984) found that light did not improve the germination in *Cynoglossum officinale*, *Echium vulgare* and *Anchusa arvensis*. Moreover, no reports in the literature were found of other Boraginaceae requiring light for germination.

5.4.2 Preliminary Dormancy-breaking Experiment

The almost immediate germination after removal of the seed coat suggests that dormancy is inhibiting germination in *M. hortensia* and that this dormancy is most likely a function of the seed coat.

Although seed in the preliminary dormancy-breaking experiment had been treated differently (eleven weeks incubation at different temperatures on the plate and a washing treatment applied to half the seed) there was not apparent effect of these treatments on the rate of radicle growth after removal of the coat. The apparently uniform radicle growth of seed in response to removal of the seed coat suggests the seed used in the preliminary

experiment had the same dormancy status same despite different earlier treatments.

Interestingly, removal and transfer of intact seed from the temperature gradient plate to new germination blotters, at an alternating germination temperature of 10°C/15°C, allowed germination to increase from less than 5% to around 93% in five weeks. Allowing for the 11 weeks seed was on the plate, the seed took 112 days to reach over 90% germination. This is a more rapid rate of germination than that observed in *M. hortensia* in either the desiccation or storage experiments where seed took around 136 days to reach 50% germination. This suggests that although seed removed from the plate has the same level of dormancy this dormancy level differs from that of seed that had not been on the plate, i.e. eleven weeks on the plate appears to have partially alleviated the dormancy in the seed. The mechanism by which the dormancy has been partly alleviated is unclear but there is some evidence in the literature that wetting and drying cycles are important in increasing germination rates. For example, Baskin and Baskin (1982) found that seeds of *Cyperus inflexus* germinated faster when exposed to wetting and drying cycles. The germination surface of the temperature gradient plate is a 780 mm x 780 mm aluminium plate covered with Kimpak and germination blotters. Although this surface is covered with glass on a plastic grid, the system is not completely sealed and moisture is lost from the blotters. Over extended germination periods water must be added to the blotters. This is achieved by using a mist sprayer to disperse the volume of water added and minimise any temperature shift of the seed and blotter from that of the plate to that of the added water. Nonetheless as a result of water loss and re-addition to the plate, in effect, these seed are being subjected to a series of wetting and drying cycles. This may have weakened the seed coat or provided other conditions (physical or chemical) that affected the dormancy mechanism in the seed. Transfer to newly wetted blotters may have been sufficient to allow radicle penetration of the seed coat.

5.4.3 Main Dormancy-breaking Experiments and Scanning Electron Microscopy

5.4.3.1. Role of the seed coat in the regulation of dormancy

The observation in the preliminary dormancy-breaking experiment that removal of the seed coat accelerates germination of *M. hortensia* seed was confirmed in the main dormancy-breaking experiments for both harvest years. The almost immediate germination after removal of the seed coat also indicates the embryo of *M. hortensia* is

not dormant. These two results confirm that dormancy in *M. hortensia* seed of this species is a function of the seed coat. This dormancy was observed in both production years indicating that coat-imposed dormancy is typical of *M. hortensia* seed at maturity and again supports Metcalf's observation (Metcalf, 1995) that germination is slow and erratic.

The seed coat may impose dormancy in one or more ways; by restricting water uptake (Tomer and Singh, 1993); preventing loss of inhibitors from the "inner seed tissues"; by the presence of inhibitors within the coat; by restricting gas exchange with the embryo (Qi *et al.*, 1993; Edelstein *et al.*, 1995) or by physically constraining embryo growth (Stabell *et al.*, 1998).

The rise in seed moisture content after 1.5 hours imbibition and during pre-conditioning suggests that the seed coat of *M. hortensia* does not restrict water uptake and, therefore seed coat impermeability to water is not a dormancy factor in seed of this species. Neither washing the seed in running water nor soaking the seed for 24 hours improved the rate of germination. Nor did germinating seed on seed leachate (or seed coats) impede the rate of germination. These results suggest it is unlikely that water-soluble inhibitors in the seed coat are delaying germination. It seems unlikely that the seed coat is preventing loss of germination inhibitors from the embryo since the pricking treatment, where the seed coat essentially remained intact, allowed germination to proceed more rapidly than in control seed. It is possible inhibitors may have leached out through the prick point but given that rapid germination also occurred in seed where the prick point was not in contact with the blotter this also seems unlikely.

The peeling, cutting and chipping treatments were effective in allowing germination to proceed. The chipping treatment was less effective than the peeling and cutting treatments in increasing the rate of germination. This may, however, may be an experimental artifact resulting from early radicle growth being masked by the surrounding seed coat.

Taken together these experiments suggest that the dormancy is a function of either physical constraint of embryo growth or restriction of oxygen uptake, or both, but not restriction of water uptake by the seed coat or the presence of water-soluble germination inhibitors in the coat. However, the peeling, cutting and chipping treatments, both weaken the seed coat, alleviating any physical constraint by the coat to embryo growth, and

improve gas exchange to and from the embryo. It, therefore, remains unclear if the dormancy is a function of physical constraint of embryo growth, restriction of gas exchange or a combination of both.

There is evidence for both in another member of the Boraginaceae, *Cynoglossum officinale*. Qi *et al.* (1993) found that the rate of oxygen uptake in intact seed of *C. officinale* was low but increased six-fold when seed was decoated. However, the rate of carbon dioxide evolution increased by only 70%, therefore, as the authors suggest, this higher rate of oxygen consumption can not be required for respiration alone. Qi *et al.* (1993) suggest it may be necessary for oxidation of germination-inhibiting phenolic substances in the seed. However, the level of methanol-soluble phenolics (present in high levels in *C. officinale*) did not change during decoating-induced germination. Stabell *et al.* (1998) in a follow-up study found that pricking the seed coat and germinating the seed in an elevated oxygen atmosphere resulted in 83% germination after 14 weeks compared to only 50% for pricked seed in air and 2.5% for intact seed in air. Oxygen uptake increased only slightly after pricking but the germination percentage in air of seed that had been pricked was around 45% higher than intact seed. These authors suggest that restriction of oxygen uptake by the seed coat is only partially responsible for the regulation of dormancy in *C. officinale* and other factors such a physical constraint of embryo growth by the seed coat may regulate dormancy in *C. officinale*.

As noted in this study pricking the coat also allowed germination to proceed more rapidly than in control seed. In *M. hortensia* seed this treatment was also sufficient to weaken the coat so in the majority of seeds when germination began the coat ruptured at the entry point of the needle rather than the radicle emerging through the coat as in control seed. This treatment was therefore unable to resolve the question of whether germination was allowed to proceed because of weakening of the coat or because of increased oxygen uptake. In the latter case the coat may have ruptured at the prick point simply because it was a weaker area of the coat than that surrounding the radicle. This seems likely given that the percentage rupture at the prick point declined when the coat was pricked with a finer insect mounting pin. However, this latter data is from a very small sample of seed from two separate experiments and therefore should be treated with some caution. Nonetheless, support for this suggestion again comes from Stabell *et al.* (1998) who pricked the seed coat of *C. officinale* with a ~0.1-0.2 mm needle. The diameter of this needle is less than either of the needles used in this study. Stabell *et al.* (1998) found that

the seed coat ruptured at the point of pricking in only 20% of the pricked seed that germinated. The authors conclude that if mechanical weakening alone was sufficient to alleviate dormancy in *C. officinale* the percentage of rupture at the prick point would have been higher and that the seed dormancy is a function of both restriction of oxygen uptake and embryo growth by the seed coat.

Stabell *at al.* (1998) investigated the structure of the seed coat of *C. officinale*. They found a network of ridges on the seed coat surface and vascular elements immediately below the surface, and that both the seed coat and vascular elements contained lignin. They suggest that these ridges and lignification physically strengthen the coat allowing that coat to constrain embryo growth. Corner (1976) noted that lignification is typical of some genera of Boraginaceae, for example *Ehretia* and *Heliotropium*. The presence of similar structures in the seed coat of *M. hortensia* offers the possibility that physical strengthening of the seed coat is also involved in the regulation of dormancy in *M. hortensia*.

The seed coat surface of *M. hortensia* is also continuous with no intercellular spaces visible. Edelstein *et al.* (1995) compared the seed coat structure and germination at low temperature of two varieties of *Cucumis melo* seed, Noy Yizre'el and Persia 202. They found that seed coat resistance to germination was less in Persia 202 than in Noy Yizer'el. There were prominent intercellular spaces in the outer layer of the seed coat of Persia 202. In contrast in Nor Yizer'el they were completely sealed. The authors suggest that the oxygen diffusion is restricted in seed with coats containing densely packed cells lacking intercellular spaces. The absence of intercellular spaces in the seed coat of *M. hortensia* may also impair oxygen uptake in this species, however, this remains speculative.

Before any final conclusions can be drawn on how the seed coat regulates dormancy in seed of *M. hortensia* further research is required into changes in oxygen uptake in *M. hortensia* seed with and without seed coats and the point of seed coat rupture in seed pricked with a finer (< 0.2 mm) needle. Assessment of the level of phenolic compounds in the embryo or coat of *M. hortensia* may also provide an indication of the role, if any, that oxidation of these compounds has in the regulation of dormancy. Moreover, determination of the extent of lignification, if any, in the seed coat of *M. hortensia* may provide further evidence for a role of the seed coat in constraining embryo growth in this species.

The rate of germination in control seed in this study is slow compared to those reported

in nurseries (A Butler, *pers. comm.*). The rate observed in this study does, however, fit with Metcalf's observation (Metcalf, 1995) that germination of seed of *M. hortensia* is slow and erratic and seed shed in summer may not emerge until the spring. The more "rapid" germination reported in the nursery may be partly because germination in the nursery is reported when first observed rather than at the median germination time reported here. Nonetheless the germination conditions used in this study eliminate any wetting and drying cycles that may promote germination in the soil or potting mix. Interactions between the seed and soil microbial activity are also removed. These may help weaken the coat and/or allow improved gas exchange, increasing the germination rate in the nursery situation.

5.4.3.2 Effect of Dormancy-breaking treatments on seedling development

The peeling, cutting and chipping treatments all involved major surgical manipulation of the seed. The peeling and cutting treatments appeared to have a damaging effect causing an increase in the number of seedlings that developed roots with a glassy appearance and also, in the first experiment, infected seedlings. Although once again the LSD indicated significant differences in glassy root development whereas the Tukey did not. As in the storage experiment (Chapter 4) this is most likely a function of the relatively small number of seeds used for each treatment (25 per replicate) contributing to the variability of the data. However, both the LSD and Tukey identified a significantly higher percentage of abnormal seedlings in peeled seed compared to control seed in seed harvested in 1997 in both the main dormancy-breaking experiments. The 1997 harvest seed used in the dormancy-breaking experiments had been in store for approximately twelve months. The development of glassy roots is often an indication of seed deterioration and was observed in this study in seed that deteriorated in open storage (Chapter 4). However, glassy root development in 1997 control seed was not significantly different to that in 1998 control seed suggesting that ageing is not the cause of the damage observed here. It is possible that ageing may have made the seed more susceptible to damage. The significantly lower percentage of seedlings with glassy roots in peeled seed from the 1998 harvest compared to seed harvested in 1997 gives some support to this idea. However, glassy root development was significantly higher in cut seed harvested in 1998 compared to chipped seed harvested in the same year. This, and the fact that glassy root development was not significantly different between 1997 and 1998 harvest seed in cut seed suggests that some damage, albeit to a lesser extent, may be

occurring in freshly harvested (1998) seed.

The significantly higher number of seeds with infected radicles or cotyledons observed in peeled seed in the first main dormancy-breaking experiment may simply reflect the fact that surgical manipulation of the seed was performed under conditions with a higher level of micro-floral contamination than in the second main dormancy-breaking experiment. Accepting that conditions in the first experiment allowed more contamination, the higher level of infection in peeled seed compared to control seed may also suggest that the seed coat plays a role in protecting the embryo from infection.

In peeled seed germinated on seed leachate a higher percentage of emerging radicles and cotyledons became infected and rotted. This suggests that while seed leachate does not prevent germination (radicle growth) *per se* it may nonetheless contain compounds toxic to the seed and developing seedling. Alternatively, the seed leachate of *M. hortensia,* which is likely to be nutrient-rich, may provide an enhanced environment for the growth of pathogens which are then able to invade the *M. hortensia* seedlings. This latter suggestion is supported by the presence of slime, characteristic of bacterial growth surrounding some seed (retaining its seed coat) on the germination blotters in both the dessication/storage and dormancy experiments. Moreover, Loria and Lacy (1979) found that *Pisum sativum* seedlings incubated in exudate from bleached *P. sativum* seeds had a higher disease rating (more necrosis) than those germinated on exudate from non-bleached seeds. A higher concentration of the three carbohydrates, sucrose, glucose and fructose was found in bleached seeds suggesting that the higher concentration of carbohydrates leached from bleached seeds provided a better environment for growth of fungi. Loria and Lacy (1979) found that bleached seed exudate caused 40% of chlamydospores of *Fusarium solani* (f. sp. *pisi*) to germinate compared to only 10% in unbleached seed exudate.

5.4.4 Conclusions

This study has demonstrated that seed of *M. hortensia* is dormant and that this dormancy is a function of the seed coat rather than the embryo. The dormancy mechanism involved is less clear but is likely to be a result of either restriction of physical growth of the embryo or restriction of gas exchange or a combination of both. Further study on the effect of seed coat removal on oxygen uptake and carbon dioxide evolution, the presence of

phenolic compounds in the embryo and coat, the effect of pricking the seed coat with a finer (< 0.2mm) needle on the point of coat rupture and the extent of lignification of the seed coat will be needed to clarify how dormancy is regulated in *M. hortensia*.

Despite uncertainty regarding the dormancy mechanism or mechanisms involved it is clear an effective technique for alleviating dormancy in this species is to prick the coat in the middle of the cotyledons.

CHAPTER 6 CONCLUSIONS

The broad aims of this research were to determine the germination and storage requirements of Chatham Island forget-me-not (*Myosotidium hortensia*).There was speculation in the scientific literature that seed of *M. hortensia* could be recalcitrant (Fountain and Outred, 1991). In contrast Metcalf (1995) reported that seed would store for 12 months or more. Metcalf (1995) also suggested that the germination of seed of *M. hortensia* was slow and erratic but other authors (Maloy, 1992) that the seed would germinate readily if fresh.

This study has determined that seed of *M. hortensia* can be desiccated to a moisture content of 7.5% without loss of viability and stored at that seed moisture and low temperature (5°C) for at least 21 months with no decline in germination. The seed is therefore not recalcitrant. While the seed appears to be orthodox in its storage behaviour, data from this study suggests that control of seed moisture content is critical to longevity in store. At 9.5% seed moisture germination was lost within 21 months despite a low storage temperature of 5°C. The seed storage reserves in *M. hortensia* are predominately lipid (24%). The loss of germination at 9.5% seed moisture is consistent with that observed in seed of other species containing similar levels of lipid in their storage reserves, but the almost complete loss of germination within 21 months at a the low storage temperature of 5°C is more rapid than that reported in other species. The 77% normal germination of the *M. hortensia* seed when placed in store suggests that the seed may have been of low vigour and that this may have contributed to the relatively rapid decline in germination. Timing of harvest may influence seed quality.

There was no difference in storage longevity between seed stored as nutlets or seed under any storage conditions evaluated. The recommendations for storage of *M. hortensia* are therefore to store the seed as either seed or nutlets at a moisture content of 7.5% and low temperature of 5°C. This would generally require use of water impermeable seed packaging for storage to maintain seed moisture at 7.5%.

M. hortensia, like some other members of the Boraginaceae, stores a high percentage of its food reserves as oil. *M. hortensia* can therefore be considered to be an oilseed. The seed oils of *M. hortensia* also contain a significant percentage (9%) of the commercially-

important fatty acid, γ-linolenic acid. Again this is also characteristic of some members of the Boraginaceae, most notably *Borago officinalis*, a commercial source of γ-linolenic acid. The use of seed of *M. hortensia* as a source of γ-linolenic acid can not be totally discounted but considerable research will be required, not only to determine the biological efficacy and safety of γ-linolenic acid extracted, but also to develop seed production systems.

This study has also confirmed the observations of Metcalf (1995) that germination of seed of *M. hortensia* can be slow and erratic, and determined that the reason for this germination behaviour is dormancy imposed by the seed coat. The precise dormancy mechanism remains unclear but is likely to be either a function of the seed coat physically constraining embryo growth or restricting oxygen uptake or a combination of both. This dormancy can be alleviated without reducing the normal germination percentage most effectively in the laboratory by pricking the seed coat with, for example, a dissecting needle.

At the end of this research our knowledge of the germination and storage behaviour of the seed of *Myosotidium hortensia* is substantially increased, however, as with any research, but particularly on a poorly studied species, there are still many question unanswered and new ones raised. There is, therefore, considerable scope for further research. This includes:

1. an evaluation of the longevity in store of different seed from different production environments and under a wider range of storage conditions, in particular, at lower (2-6%) seed moisture content, and higher temperature and for longer storage times

2. an assessment of the effect of seed vigour on the rate of germination loss of seed in store at low temperature

3. an assessment of the timing of harvest on seed quality

4. an evaluation of the effect of seed coat removal on oxygen uptake and carbon dioxide evolution and pricking the seed coat with a finer (< 0.2mm) needle on the point of coat rupture

5. an assessment of the level of phenolic compounds in the embryo and coat and lignification of the seed coat

6. an assessment of the total lipid content and fatty acid percentages of seed from different production environments.

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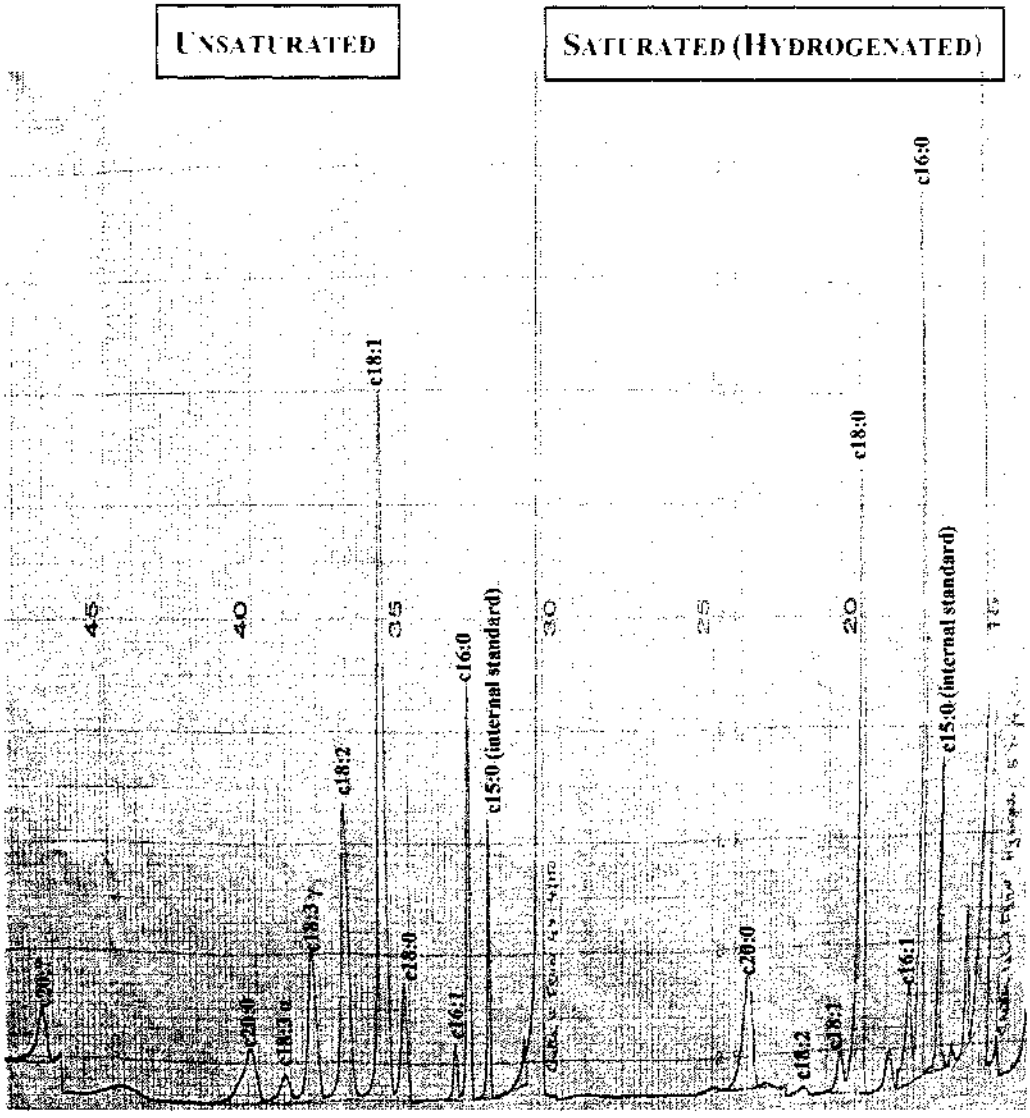
Appendix 1: Chromatograms of fatty acids extracted from *M. hortensia* seed separated on a packed column.

Fatty acids were extracted and methylated as described in ***3.2.5.1.1*** and hydrogenated as described in ***3.2.5.1.2***. Unsaturated and saturated(hydrogenated) fatty acids were separated on a 2.6 metre (15% ethylene glycol succinate (EGSS-X) on Chromosorb W A/W) packed column using a Shimadzu GC-8A Gas Chromatograph.



Retention Time (minutes)

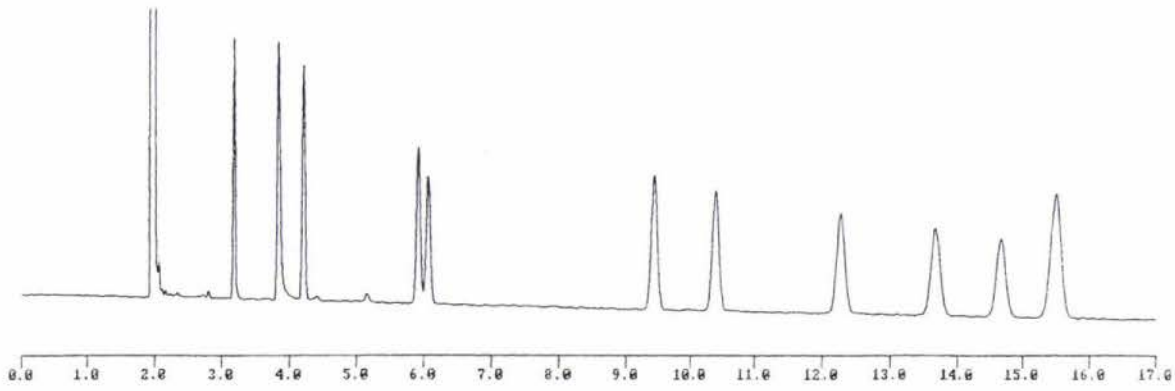
Plate A.1: Chromatograms of fatty acids before and after hydrogenation separated on a packed column. The attenuation was set to 4 except for the hydrogenated C_{18} fatty acids where it was set to 16.

Appendix 2: Specifications of the BPX-70 capillary column

SERIAL NO: 272602

CODE: 30QC5/BPX70 0.5
PART NO: 054620

COLUMN PERFORMANCE REPORT



PERFORMANCE DATA

PEAK	COMPOUND	RETENTION TIME (MIN)	CAPACITY RATIO (K)	KOVATS INDEX
1	SOLVENT			
	C14:0			
	TETRADECANOL			
	C16:0			
	ACENAPHTHYLENE			
	C18:0			
2	C20:0	9.43	3.96	2000.00
3	C20:1	10.39	4.47	2040.55
4	C20:2	12.28	5.46	2108.29
5	C20:3n6	13.66	6.19	2150.82
6	C20:4n6	14.67	6.72	2178.67
7	C22:0	15.50	7.15	2200.00

AVERAGE GAS VELOCITY (cm/sec)	26.3
(ml/min)	3.48
EFFECTIVE PLATES (Peak 3)	31137
THEORETICAL PLATES (Peak 3)	46639
EFFECTIVE PLATES/METRE (Peak 3)	1038
SEPARATION NUMBER (Peaks 2-3)	3.42
SKEW (C20:1)	0.91

COLUMN SPECIFICATIONS

LENGTH	30 METRE	FILM THICKNESS	0.5 micron
TYPE	BONDED PHASE	I.D.	0.53 mm
MATERIAL	FUSED SILICA	O.D.	0.68 mm
PHASE	BPX70 (very polar)		

OPERATING TEMPERATURES

MINIMUM TEMPERATURE	50 deg C
MAXIMUM CONTINUOUS TEMPERATURE	260 deg C
CONDITIONING TEMPERATURE	260 deg C for 1 hour
MAXIMUM CYCLING TEMPERATURE	290 deg C

TESTING CONDITIONS

COLUMN TEMPERATURE	175 deg C	TEST SAMPLE	TRF P
DETECTOR TEMPERATURE	280 deg C	SAMPLE SIZE	0.1ul
INJECTOR TEMPERATURE	240 deg C	SPLIT RATIO	60:1
CARRIER GAS	HYDROGEN	SENSITIVITY	32*10E-12 AFS
INLET PRESSURE	2.5 PSI	DETECTOR	FID

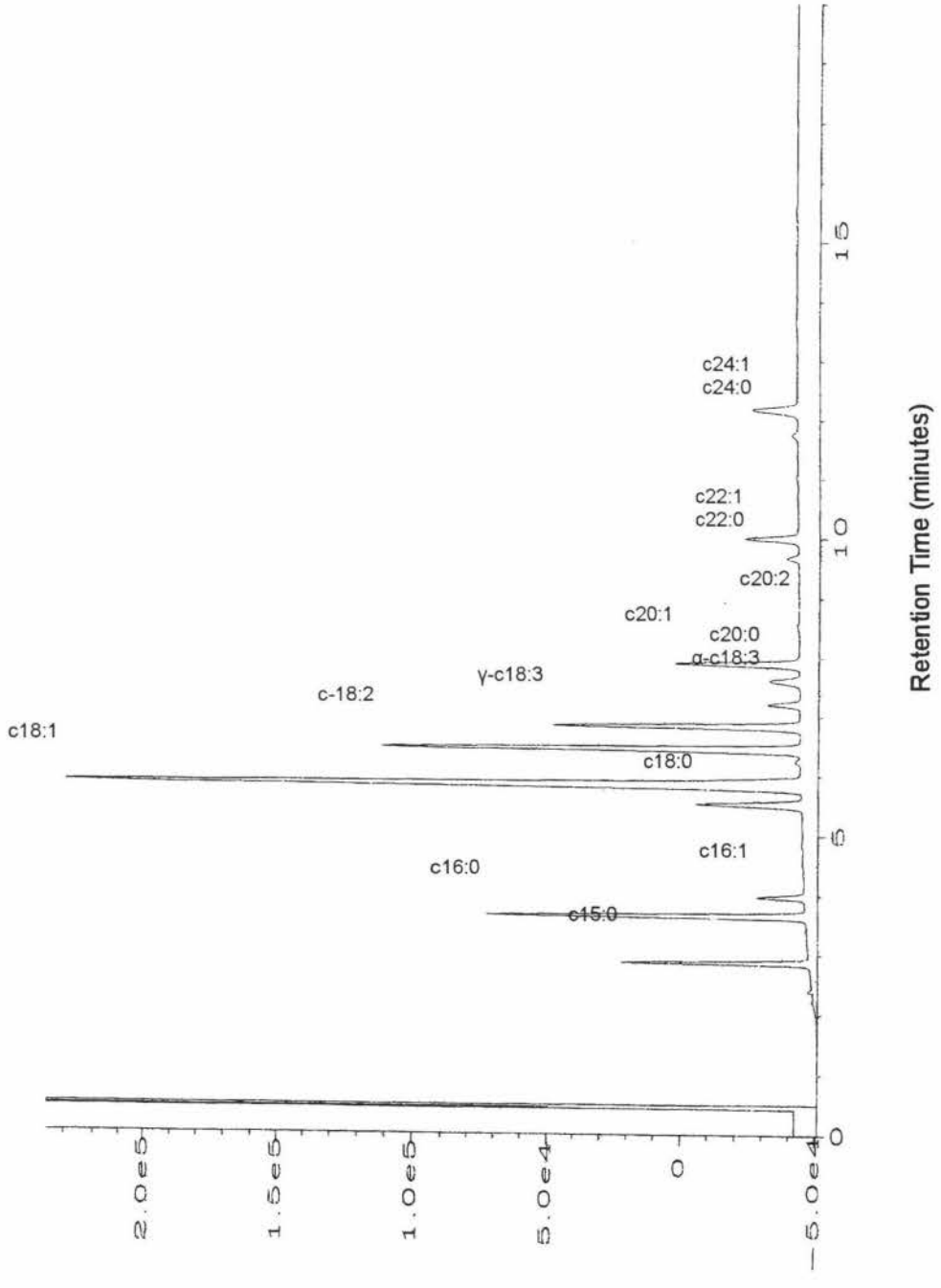
The enclosed capillary column has been individually tested to guarantee it meets required performance standards. The above chromatogram and data are from the enclosed column, which has been tested by CORINA-22

CHROMATOGRAPHIC PERFORMANCE DATA BY DELTA CHROMATOGRAPHY SYSTEMS

ISO 9002 CERTIFIED

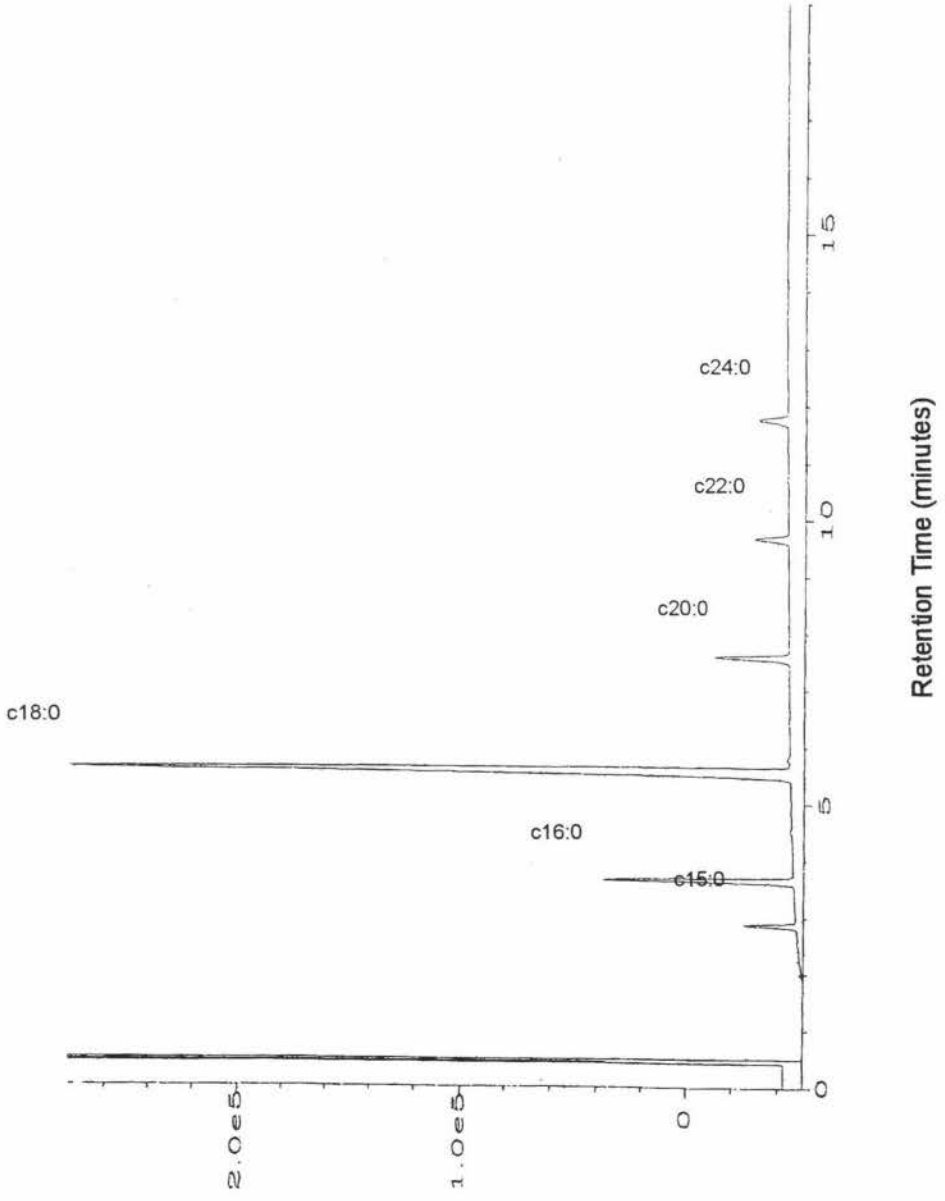
Appendix 3: Chromatogram of fatty acids extracted from *M. hortensia* seed separated on a BPX-70 capillary column.

Fatty acids were extracted and methylated as described in ***3.2.5.1.1*** and separated on a BPX-70 capillary column using a using a Hewlett-Packard 5890 Series II Plus Gas Chromatograph with a Hewlett-Packard 7673 GC/SFC injector attached. Hewlett-Packard Series II ChemStation software was used to generate the chromatogram.



Appendix 4: Chromatogram of fatty acids extracted from *M. hortensia* seed, hydrogenated and separated on a BPX-70 capillary column.

Fatty acids were extracted and methylated as described in ***3.2.5.1.1*** and hydrogenated as described in ***3.2.5.1.2***. Hydrogenated FAMES were then separated on a BPX-70 capillary column using a using a Hewlett-Packard 5890 Series II Plus Gas Chromatograph with a Hewlett-Packard 7673 GC/SFC injector attached. Hewlett-Packard Series II ChemStation software was used to generate the chromatogram.



Appendix 5: Germination of seed of *M. hortensia* with and without Thiram 80W

Four replicates of 10 seeds of *M. hortensia* with (dusted as described in **4.2.1.3**) and without Thiram 80W were set to germinate as described in **4.2.1.3**. Germination was monitored and the normal and abnormal germination percentages and percentage of dead determined as described in **4.2.1.3**, except if fungal growth was visible on the seed they were classified as infected. The student t-test was used to compare the means.

Table A.1 The effect of Thiram 80W on seed germination of *M. hortensia*

Treatment	Normal Germination (%)	Abnormal Germination (%)	Dead (%)	Infected (%)
No Thiram 80W	60 (±5.2)	4 (±2.3)	15 (±2.3)	21 (±5.9)
Thiram 80W	76 (±8.7)	5 (±3.0)	16 (±7.2)	3 (±3.0)

The normal germination percentage did not differ between seed dusted with Thiram 80W and seed not dusted but, the percentage of infected seed was significantly lower ($P<0.05$) in dusted seed compared to seed not dusted.

A comparison of the infection levels in dusted and non-dusted seed suggests dusting the seed with Thiram 80W provides some protection against infection. Although this is not reflected in a significantly higher normal germination percentage in dusted seed this is more likely a reflection of the high standard errors associated with the sample means rather than Thiram 80W not having a protective effect. The high standard errors are most likely a result of the small number of seeds assessed (10 per replicate for each treatment). Certainly dusting the seed with Thiram 80Wis not detrimental to normal seed germination.

Appendix 6: Normal germination percentage of *M. hortensia* seed after removal from the temperature gradient plate

Normal germination percentage of *M. hortensia* seed removed from the temperature gradient plate and set to germinate at 10°C/15°C. Seed is classified by washing treatment and germination temperature on the plate and peeling treatment when removed from the plate. Note the number of seed in each treatment ranged from 7 to 14 hence a difference in the germination percentage of 7% may only represent one or two seeds.

Table A.2 Germination of peeled and unpeeled seed of *M. hortensia*, with and without washing, after removal from different temperatures on a temperature gradient plate

Germination Temperature (°C)	Normal Germination Percentage (%)			
	Peeled		Unpeeled	
	Washed	Unwashed	Washed	Unwashed
5	90	90	83	93
6	91	79	73	91
7	83	83	92	85
8	91	82	91	82
9	90	92	83	92
10	91	90	92	83
11	91	80	92	100
12	91	100	100	100
13	89	100	100	90
14	89	100	100	100
15	100	100	100	90
16	91	100	100	100
17	100	89	91	100
18	100	100	100	100
19	100	100	100	100
20	100	100	100	100
21	100	90	100	100
22	90	86	80	89
23	100	100	80	80
24	100	100	80	90

Appendix 7: Seed moisture content data for the first dormancy-breaking experiment

Table A.3 Moisture content of 1997-harvest *M. hortensia* seed after pre-conditioning

Treatment	Seed Moisture Content (%)
control	7.4 (2.19)
1.5 hours imbibition	20.1 (3.03)
preconditioned only	40.9 (3.71)
24 hour washing	40.9 (3.70)
seed soaked for 24 hours	42.1 (3.74)
seed coat chipped at the distal end	40.8 (3.70)
seed coat cut above the embryo axis	44.1 (3.79)
peeled seed germinated on water	43.7 (3.77)
seed soaked for 24 hours, peeled and germinated on water	42.1 (3.74)
peeled seed germinated on seed coats	44.5 (3.79)
peeled seed germinated on seed leachate	44.4 (3.78)
LSD, $P<0.05$	0.107

transformed means are in brackets, LSD is for comparing transformed means.