Reproduction in selected New Zealand native ferns and their suitability for revegetation

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ABSTRACT

The potential to use New Zealand native ferns for revegetation was assessed in laboratory, nursery and field experiments. Laboratory experiments indicated that the three native fern species, *Blechnum novae-zelandiae*, *Cyathea medullaris* and *Dicksonia squarossa*, had different maximum levels of spore germination. These differences also varied in response to seasonal changes in the environment. The effect of three soil conditioners on the germination of the same three species was minimal. Gametophytes appeared to be tolerant of low levels of maceration, as they were able to continue to grow and develop normally. Additional laboratory experiments indicated that *B. novae-zelandiae* employs a mixed mating system, which utilizes an "antheridiogen" signal.

The development of fern spores, laboratory propagated gametophytes and segmented rhizomes, was assessed in the nursery. Each experiment was applied with a hydroseeding mix of paper fibre, tackifier, fertilizer and water. Spore of *B. novae-zelandiae*, *C. medullaris* and *D. squarossa* failed to produce any long-lived gametophytes. The survival of laboratory propagated gametophytes of *B. novae-zelandiae*, *B. discolor* and *B. colensoi* was low. However, a large proportion of surviving *B. novae-zelandiae* gametophytes produced sporophytes. *B. novae-zelandiae* rhizome segments produced healthy young ferns within 3 months of application.

Field experiments were conducted on a sandstone/loess bank, 5 km east of Palmerston North. Aspects of the substrate were analysed including, pH, N, P and organic matter. The results indicated that the bank had a high soil pH, was deficient in several macronutrients and had no organic matter. Hydroseeding was applied using spore of the species *B. novae-zelandiae*, *C. medullaris* and *D. squarossa*. Hydroseeded spore failed to produce any visible gametophytes. Rhizome experiments using *B. novae-zelandiae* and *Microsorum pustulatum* were also established. Low water availability resulted in poor rhizome establishment.

The results suggest that there is great potential for utilizing native ferns in revegetation. *Blechnum novae-zelandiae* is the best species for revegetation in accordance to the

results. Propagation via rhizome segmentation and gametophyte hydroseeding appear to be the most successful methods for establishing native ferns.

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"Nature will forever endeavour".

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1.1 New Zealand native ferns

New Zealand is home to over 194 native fern species along with 18 fern allies. Of these, 89 (46%) are endemic and 105 (54%) are widespread (Brownsey, 2001). Although the number of species endemic to New Zealand is few compared to tropical countries, the abundance of native ferns growing in the wild and domestically is very high (Metcalf, 2003). Ferns make up a large part of the vegetation growing on the forest floor and vary morphologically, from large tree ferns to small water ferns. The variation in morphology of New Zealand ferns is a result of their adaptation to the different environments New Zealand presents. As a result New Zealand's native ferns are distributed from cool alpine to warmer coastal regions.

1.1.2 DISTRIBUTION

The natural geographical regions of New Zealand consist of an array of environments from sub-tropical rainforest, to alpine regions, to desert. Each of these regions supports its own native flora (Dawson, 1993), and native ferns can be found growing in a wide range of these ecological niches.

Native subtropical rainforest and subantarctic forests are home to the majority of native fern species. The native subtropical rainforest (dominated by Podocarps and Araucarians) is consistent with a warm, humid, wet environment, which is generally considered to be optimal for the growth of the majority of native fern species. The subantarctic forest (dominated by *Nothofagus* sp.) provides slightly less optimal conditions than sub-tropical forest, and as a result a less diverse population of fern species is present. Within these forests it is common for different species of fern to inhabit areas such as stream banks, fallen trees, disturbed sites, and clearings (Stewart et al., 1991). These areas often provide the ferns with the resources required for growth including sufficient water, low light levels and a suitable substrate. It has been suggested that species distribution is mainly affected by environmental gradients such as soil wetness (Norton, 1994). New Zealand native ferns are also distributed in more

demanding locations, such as saline coastal environments (*Blechnum blechnoides* and *B. durum*) and alpine regions (*Blechnum penna-marina* and *Polystichum cystosgia*) (Chambers & Farrant, 1996; Brownsey & Smith-Dodsworth, 2000). The degree to which New Zealand's native ferns can cope with extremes of heat and frost has previously been researched (Bannister & Smith, 1983; Bannister, 1984). Several native fern species common to the Otago and Fiordland regions of New Zealand were included in the research. Comparisons were made with northern hemisphere ferns, and the results suggested that New Zealand ferns are less well adapted to cope with extreme conditions, which is unsurprising given New Zealand's generally maritime climate. Research has also shown that New Zealand native ferns form symbiotic relationships with mycorrhizal fungi. In *Histiopteris incisa* and *Pteridium esculentum* inoculation with mycorrhizal fungi caused substantial growth stimulation compared to controls (Cooper, 1975).

Several native fern species are widely distributed across farmland and forestry. This observation is evidence of the ability of native ferns to colonize land in competitive environments. *Pteridium esculentum* (bracken) and *Paesia scaberula* are examples of native ferns that infest pastureland. *Pteridium esculentum* is also a major contributor to the under-storey vegetation of pine plantations (Hollinger, 1987). The native tree ferns *Dicksonia squarrosa*, *Cyathea medullaris* and *C. smithii* are also significant members of the under-storey of older pine plantations (Ogden et al., 1997). In addition, *Cyathea dealbata, C. medullaris* and *Dicksonia squarossa* have been shown to predominate in bush margins (Young & Mitchell, 1994). Experimental evidence suggests that the gametophytes of several tree fern species preferentially grow at bush margins compared to the forest interior (Bernabe et al., 1999). This observation suggests that tree ferns behave as pioneer species and have the ability to colonize deforested areas such as road cuttings.

Statistical models have been designed to predict the distribution of native fern flora with respect to environmental conditions associated with geographic locations. Models are based on the concept that environments associated with preferential growing conditions would harbour common fern species in abundance. As a result, common species are generally modelled accurately, whereas rare species are not (Lehmann et al., 2002).

1.2 The homosporous fern life cycle

A fern is typically defined as a non-flowering vascular plant that has chlorophyll and reproduces sexually by means of spore rather than seed (Brownsey & Smith-Dodsworth, 2000). A fern's sexual life cycle consists of two separate free living generations called the gametophyte (gamete plant) and sporophyte (spore plant). The main difference between these two stages is that all gametophytes are haploid and all sporophytes are diploid.

A homosporous fern is characterized by the growth of a single spore into a gametophyte, followed by the initiation of sexual organs, fertilization and the development of the sporophyte (Fig. 1.1).

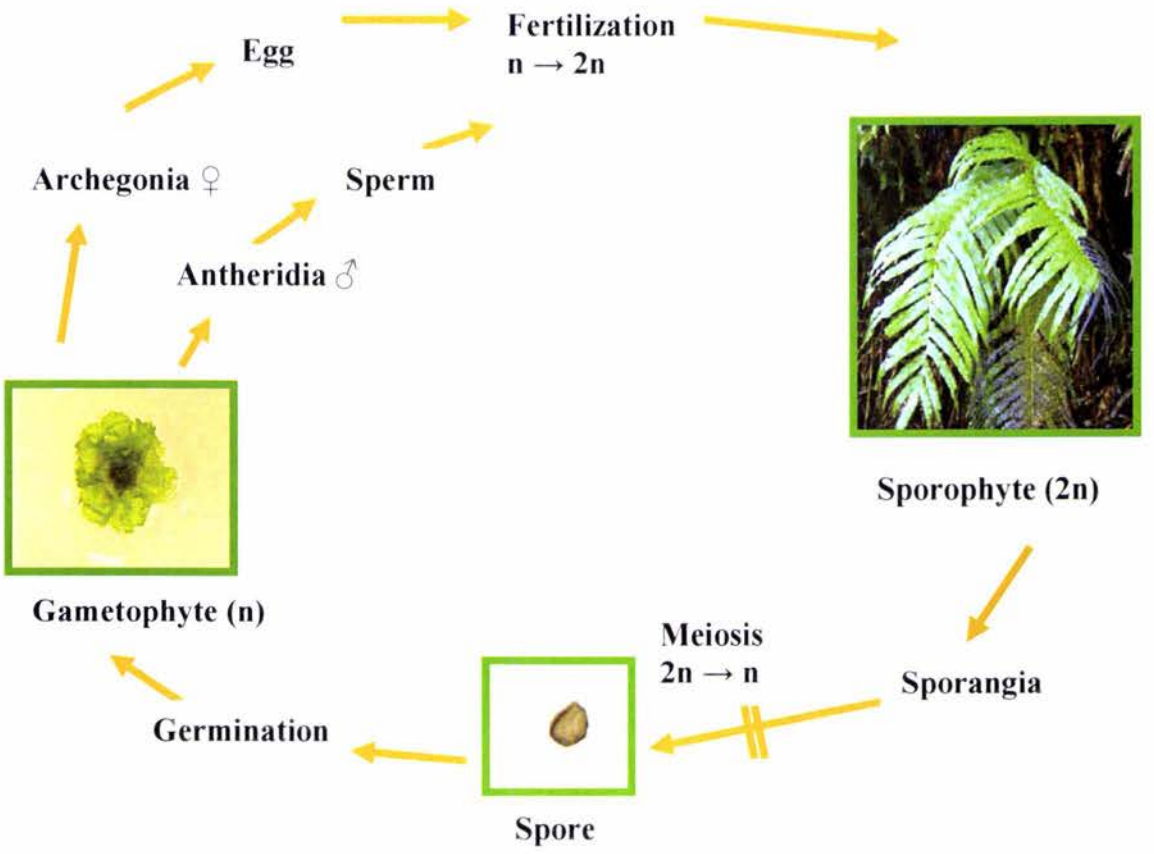


Figure 1.1 The homosporous fern life cycle of *Blechnum novae-zelandiae* (Kiokio), showing both the haploid gametophyte stage and the diploid sporophyte stage.

The sporophyte is the plant commonly observed growing in the bush or along roadsides. As it develops, spore is produced on the underside of fronds, in sporangia. The spore is shed and in the presence of water and red light, germinates and develops into the gametophyte. The gametophyte produces the sexual organs termed the antheridia (♂) and archegonia (♀). Sexual union occurs via fusion of the egg cell with a motile sperm cell. One important feature of this stage of the life cycle is that water is absolutely required as the sperm has to swim from the antheridia to the archegonia in order to initiate fertilization. The duration of the homosporous fern life cycle varies significantly between species of fern. The fastest growing ferns take only around 120 days to complete one cycle, whereas slower growing species can take several years (Hoshizaki & Moran, 2001). The age at which a sporophyte becomes competent to produce spore has also been investigated in several ferns native to Japan (Sato, 1985). Ferns growing on rocky banks, mossy rocks or tree trunks tended to reach a sexually competent stage earlier than those ferns growing on the forest floor. Therefore, the speed at which a fern produces spore is not only due to endogenous factors but also exogenous environmental factors.

The homosporous fern life cycle is unique in plant development as it offers the ability to study various phenomena at both the developmentally simple haploid gametophyte stage, and at the more complex vascular sporophyte stage. Its uniqueness has attracted interest in several scientific fields (Hickok et al., 1995). The water fern *Ceratopteris richardii* has been the preferred species in these studies because of its rapid life cycle and ease of culture (Hickok et al., 1987). *Ceratopteris richardii* produces large amounts of spore that can be mutagenized and screened. In addition, the ability to generate homozygote gametophytes from a single selfing event provides a powerful way of isolating mutations. The diploid sporophyte also provides a typical vascular plant system for developmental, genetic, biochemical or physiological research. The use of the fern *C. richardii* in early vascular plant research could perhaps be compared to the role of the angiosperm *Arabidopsis thaliana* in higher plant studies.

1.2.1 SEXUAL MATING SYSTEMS AND ANTHERIDIOGEN

The sexual stage of the fern life cycle occurs in the gametophyte. Gametophytes can be unisexual, having either male antheridia or female archegonia. In some species gametophytes can also be bisexual or hermaphroditic, having both male and female organs.

There are three basic sexual mating systems that ferns use, depending on the sexual nature of the gametophyte. Bisexual gametophytes may self-fertilise, which is often termed intra-gametophytic selfing (Verma, 2001). This phenomenon involves the union of sperm and egg cells from the same individual gametophyte. Most ferns are diploid therefore intra-gametophytic selfing often results in homozygous offspring. It is also possible that two gametophytes originating from the same sporophyte may initiate sexual union. This form of selfing is termed inter-gametophytic selfing. A degree of genetic variation is achieved with inter-gametophytic selfing because spore from the same sporophyte has undergone meiosis, and is therefore genetically diverse. The third possible mating system is termed inter-gametophytic crossing, where gametophytes from different plants establish close to each other and sexual union is achieved. Inter-gametophytic crossing results in a predominantly heterozygous population, which is advantageous in terms of increasing genetic variation within a population (Haufler & Welling, 1994).

Some species of fern have developed a pheromone system in order to promote inter-gametophytic selfing and crossing, and in turn, limit intra-gametophytic selfing. This pheromone is commonly known as "antheridiogen". Antheridiogens are produced by mature female fern gametophytes and can influence the sexual ontogeny of their neighbours (Haufler & Welling, 1994). The archegonia of female or bisexual gametophytes release antheridiogen into the surrounding substrate.

Antheridiogen stimulates the germination of surrounding spore and, furthermore, the development of antheridia bearing, male gametophytes (Wen et al., 1999). In the absence of antheridiogen, spore develops into female or hermaphroditic gametophytes (Banks et al., 1993). The concentration of the antheridiogen in the media determines its overall effectiveness to promote germination (Haufler & Welling 1994). When the

concentration is high, more spores are induced to germinate and develop as male. At lower concentrations the amount of spore affected by antheridiogen is decreased. A key property of antheridiogen is its ability to substitute for the light requirement of fern spore and induce dark germination. Like seeds, spore remains dormant until certain factors promote its germination (Dyer, 1994). The soil spore bank is a biotic component of native plant communities and is especially important for regeneration and conservation of fern species (Ranai, 2003). The ability of antheridiogen to induce dark spore germination has implications for sexual reproduction via the utilization of spore banks. Spore buried in the soil can be induced to germinate and produce functional, antheridia bearing, male gametophytes. These male gametophytes are non-chlorophyllous and produce sperm that is capable of swimming to the gametophyte from which the initial antheridiogen signal was produced (Haufler & Welling, 1994). Therefore, the antheridiogen signal promotes successful inter-gametophytic crossing between gametophytes.

Antheridiogen has been proven to exist in a diverse range of fern species, including *Blechnum spicant, Bommeria hispida, B. ehernbergiana, Ceratopteris richardii, Cryptogramma crispa, Lygodium microphyllum, L. japonicum, L. reticulatum* and *Pteridium aquilinum* (Banks et al., 1993; Haufler & Welling 1994; Fernandez et al., 1997; Pajarón et al., 1999; Wen et al., 1999; Kurumatani et al., 2001; Lott et al., 2003).

It has been shown that some antheridiogens have similar structures to gibberellins and in fact are derivatives of this group of plant hormones (Kurumatani et al., 2001). Initially, antheridiogens were thought to have a similar structure to gibberellins because their male induction response was able to be inhibited when gametophytes were treated with gibberellin biosynthetic inhibitors (Warne & Hickok, 1989). However, simple gibberellins (e.g. GA_3) were unable to stimulate the male induction response typical of all antheridiogens, suggesting that antheridiogens were chemically divergent from gibberellins. Interestingly, abscisic acid (ABA) acted antagonistically to the antheridiogen male induction response, which is similar to its antagonistic role with gibberellins (Banks et al., 1993; Wen et al., 1999). This research laid the foundation for the identification of the chemical structure of antheridiogen. Gas chromatography-mass spectrometry was used to successfully identify methyl esters of GA9 and GA73 in media supporting the growth of the species *Lygodium microphyllum* and *L. reticulatum*

(Kurumatani et al., 2001). These compounds acted as functional antheridiogens and were able to induce male development. These two species were chosen because it was previously noted that they produced large amounts of antheridiogen.

The ability to outcross is essential for maintaining genetic variation within a population. However, in some instances outcrossing is difficult. When the abundance of gametophytes is very low, or the soil spore bank is either non-existent or feeble, intra-gametophytic selfing becomes a viable mating option. The ability to reproduce through intra-gametophytic selfing promotes long-distance dispersal of a species (Lott et al., 2003). This form of mating system allows for a single gametophyte to establish a new population. A species that uses intra-gametophytic selfing can potentially radiate into areas devoid of a spore bank or resident fern population. Research indicates that only some species are able to produce viable sporophytes this way (Soltis & Soltis, 1992). Most species prefer to outcross using an antheridiogen male-inducing system. It may be possible that a species has a mixed mating system and can use both outcrossing and selfing mechanisms, depending on environmental conditions.

1.2.2 ASEXUAL REPRODUCTION

Apart from the well characterized homosporous sexual life cycle, ferns can also reproduce asexually by vegetative means or gametophytic apomixis. These characteristics are a variation on the basic fern life cycle and in some cases are results of adaptation to environmental stress.

Asexual reproduction leads to offspring that are genetically identical to the parent plant. Vegetative reproduction in ferns can be executed either by the production of new ramets (shoots) through rhizomatous growth, fragmentation and re-establishment, or via the formation of viviparous bulbils in the frond margins (Koptur & Lee, 1993). *Asplenium bulbiferum* (hen and chicken fern) is a well known New Zealand native that reproduces vegetatively via the production of bulbils (Perrie et al., 2005). Many native New Zealand ferns, including *Paesia scaberula* and *Pteridium esculentum,* spread vegetatively via underground rhizomes. Once these ferns establish new ramets (shoots),

rhizogenous connections to the parent can be terminated, thereby establishing an independent plant.

Apomictic reproduction involves the development of the sporophyte from the gametophyte, without the fusion of gametes. This phenomenon has been observed in several fern species and has been studied in the model fern *Ceratopteris richardii* (Hickok et al., 1995). Interestingly, some fern species have completely lost the sporophytic stage of their sexual life cycle. *Appalachian vittaria*, which is native to the eastern United States, is an example of one of these species. For this fern, vegetative reproduction of the gametophyte generation has evolved as an adaptive advantage, as it has permitted the growth of this species beyond its normal geographic range (Farrar, 1990). This phenomenon suggests that a ferns sexual reproductive mechanism is intolerant to an environment where water is unavailable.

Research on the homosporous life cycle of ferns allows for the further understanding of how ferns develop and how they utilize variations in sexual reproduction successfully. In addition, some species have evolved variations on the basic sexual homosporous life cycle to allow them to reproduce successfully in unsatisfactory environmental conditions.

1.3 Spore germination and light

A fern spore is an equivalent structure to a seed of a higher plant. Both structures give rise to a new generation and both require similar basic environmental parameters to stimulate germination. However, these two structures differ dramatically in size and ploidy level. Spore is dust-like, and individuals can only be observed clearly using a microscope, whereas seeds are generally much larger. In addition, all spores are haploid whereas the haploid generation in higher plants occurs prior to the formation of the diploid seed. Spores are periodically produced on mature sporophytes and released into the environment after a maturation period.

The ability of a spore to retain its capacity to germinate has previously been used as a measure of viability (Perez-Garcia et al., 1994). Spore is generally at its most viable soon after it has been released from the parent sporophyte, although it has been shown that *Platycerium bifurcatum* spore may require an after-ripening period (Camloh, 1999). Green spores have high water content and an average life span of 7 weeks (Perez-Garcia et al., 1994). This suggests that the environment in which these spores are deposited promotes conditions favourable for rapid germination. In comparison, non-green spores generally have low water content and can remain viable for a long period of time when subjected to the appropriate storage conditions.

The conditions that favour high spore viability vary between different species. The conventional approach is to store spore in dry, cool conditions, similar to seed storage conditions. However, research indicates that some non-green spores remain viable for at least two years when subjected to moist but dark conditions (Lindsay et al., 1992; Perez-Garcia et al., 1994). When taken in context, these findings seem plausible as spore stored in the soil is subjected to wet, dark conditions. Other research suggests that when spore is subjected to wet or dry conditions, there is little difference between the two treatments in terms of viability (Simabukuro et al., 1998). Therefore, the viability of fern spore varies enormously among fern species because of the physiology of the spore and the conditions to which it is adapted. These factors have implications for the dynamics of spore banks in terms of the representation of different species in different environments. As stated previously, spore banks are thought to be important for

promoting outcrossing using antheridiogen. Therefore, the viability of spore in a spore bank influences the success of inter-gametophytic crossing in antheridiogen producing species.

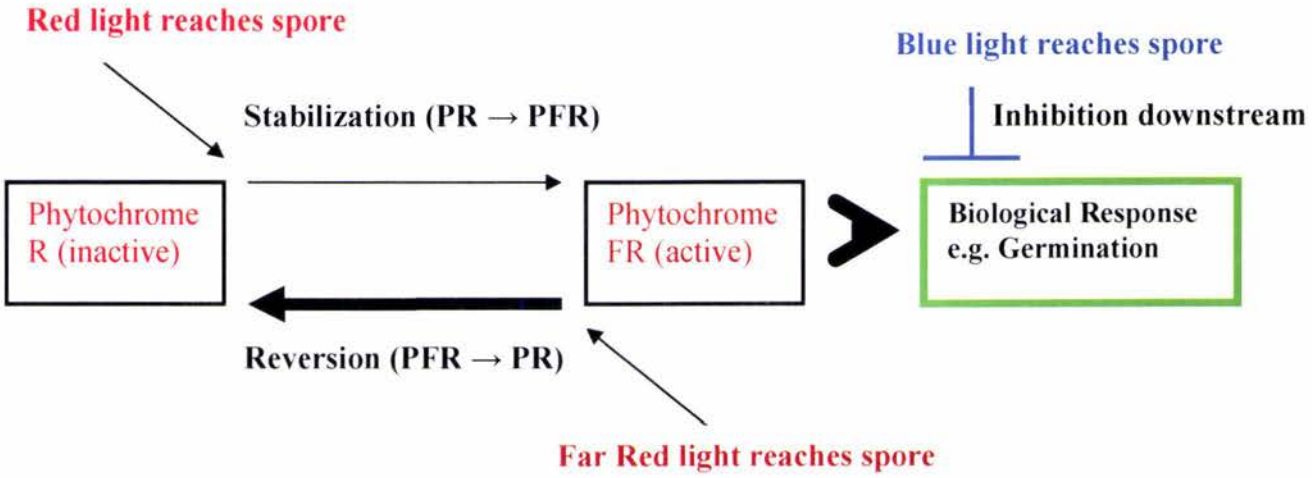
Fern spore viability has also previously been assessed using the fluorescein diacetate test (Large & Braggins, 1991). This test was previously developed for the evaluation of pollen viability (Heslop-Harrison & Heslop-Harrison, 1970). Fluorescein diacetate (FDA) is actively absorbed by viable spores and once it enters the cytoplasm the acetate group is cleaved by cytoplasmic esterases. This reaction forms a fluorescent compound. If the cytoplasm of the spore is dysfunctional, the esterases will also be dysfunctional and the fluorescent compound will not be formed. Therefore, viable and non-viable spore can theoretically be distinguished under a fluorescent microscope.

In the laboratory, sterilizing spore may detrimentally affect its viability. It is standard procedure to sterilize spore prior to sowing. Sterilization kills any microbes, fungi or bacteria that may cause detrimental effects to the developing gametophyte. In addition, spore being kept in wet storage should also be sterilized in order to prevent contamination. Relatively recent data suggests that the sterilization process directly affects the viability of spore depending on the chemicals or techniques used (Simabukuro et al., 1998; Camloh, 1999). Some chemicals are very effective at preventing contamination, but in turn can dramatically affect the viability of the spore. The concentration of each chemical used should be optimized for preventing contamination but not reducing spore viability.

After sterilization and sowing, spore is usually incubated at around 20 - 30°C, under low light conditions (20 μmol m^{-2} s^{-1} – 100 μmol m^{-2} s^{-1}) and at high humidity (Pangua et al., 1994; Camloh, 1999; Fernandez et al., 1999; Cox et al., 2003; Nondorf et al., 2003; Pangua et al., 2003). The photoperiod reputedly used for incubation varies from constant light to 12 h darkness. Once imbibed a single flash of red light is enough to initiate the germination response in some species (Haupt, 1985). Fern spores are predominantly photoblastic and use the well characterized phytochrome light-sensing system to sense and respond to light (Tomizawa et al., 1983; Cooke et al., 1987; Haupt & Psaras, 1989). This system involves the stabilization of phytochrome in the presence of red light. When red light reaches the spore it causes one form of phytochrome (PFR)

to stabilize. When enough PFR is stabilized, the germination response is initiated. However, far red light can cause reversion of this process and inhibit germination. The amount of red light required to initiate germination in spores varies between species. In addition, blue light can totally inhibit the phytochrome light-sensing system. This suggests that blue light influences factors downstream of the phytochrome mechanism in the spore germination induction pathway (Fig. 1.2).

Figure 1.2 A simplified, schematic diagram of the phytochrome light-sensing system in fern spore. Red light causes PFR to become active. If PFR remains active for a certain period of time a biological



response will be induced. If the spore perceives Far Red light PFR will be deactivated and reversion will occur. Reversion occurs instantly whereas the period of time it takes for PFR to stabilize and induce a biological response varies between species from a single pulse of red light to several hours.

1.4 Fern propagation

New Zealand native ferns are successfully propagated commercially, with the tree ferns *Cyathea dealbata, C. medullaris* and *Dicksonia squarrosa* forming the bulk of production. New methods of propagation are being sought in order to increase production, as it can take at least a year for species to develop from spore to plants fit for sale. *In vitro* propagation of fern material has been researched extensively. *In vitro* propagation provides a method for growing species that are difficult to propagate using conventional methods. In addition, *in vitro* propagation can potentially be used to conserve endangered fern species.

1.4.1 *IN VITRO* PROPAGATION

In vitro propagation involves growing ferns on specialised growth media, usually under laboratory conditions. Scientists tend to use this approach because the technique is sterile and it is possible to manipulate growth conditions easily. This method of propagation has the potential to produce a large amount of fern material in a short period of time compared to conventional propagation methods. The preferred medium for *in vitro* propagation of spore is half strength Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962), supplemented with agar.

In addition to spore, vegetative fern tissue can be grown on media containing appropriate concentrations of the plant hormones auxin and cytokinin (Fernandez et al., 1999). Rapid generation of adventitious shoots can be accomplished relatively quickly compared to *in vitro* propagation of spore (Camloh et al., 1994; Ambrozic-Dolinsek et al., 1999). Various concentrations of sucrose, jasmonic acid and charcoal have all been shown to affect the rate of shoot formation. The addition of sucrose to the medium seems to give variable results. High concentrations of sucrose have been shown to inhibit development of the sporophyte, whereas jasmonic acid and charcoal promote growth (Pasha & Chakraborty, 1982; Ambrozic-Dolinsek et al., 1999; Camloh et al., 1999). It has also been shown that addition of sucrose to the growth media can significantly enhance the growth rate of gametophytes (Goller & Rybczynski, 1995; Kuriyama et al., 2004).

Recent research has concentrated on the homogenization of *in vitro* established gametophytes and sporophytes, and their regeneration capacity in MS liquid medium as well as more conventional media (Janssens & Sepelie, 1989; Fernandez et al., 1993; Fernandez et al., 1999). It has been suggested that for species with rapid life cycles, homogenization of gametophytes can be considered an excellent method for high yield propagation in a short amount of time. Species that are apomictic are also excellent candidates for homogenization. Homogenization of rhizomes has also proved to be successful, with an estimated 500 sporophytes being produced from 0.5g of vegetative tissue (Fernandez et al., 1997). Another method of *in vitro* propagation of spore involves the use of an airlift fermenter. Spore of the ferns *Pteridium aquilinum* and *Anemia phyllitidis* were successfully cultured and subsequent growth and development was monitored (Sheffield et al., 1997). Using this technique, both species produced more biomass than any other solid or liquid based culture system tested. Gametophytes grown in the airlift fermenter appeared characteristically similar to soil-grown plants, indicating development of the gametophyte was not impaired by this method of propagation.

In vitro propagation has also been proposed as a useful method for propagating several endangered fern species. The two experiments reviewed implement different propagation techniques with varying success. *In vitro* propagation of spore was attempted for the conservation of *Shizea dichotoma*, which is native to Australia. However, poor germination rates hampered the ability to propagate the spore of this species (Cox et al., 2003). In comparison, the South African tree fern *Cyathea dregei* has been successfully propagated via a method similar to the homogenization method described above, suggesting that vegetative propagation may be a more successful technique (Finnie & van Staden, 1987). The ability to propagate a particular fern mainly depends on the specific growth characteristics of that species.

In vitro propagation techniques for the multiplication of fern tissue is an active field of research. The generation of large amounts of young ferns using this method has implications in commercial propagation, propagation of endangered fern species, and also in scientific experimentation.

1.5 Revegetation

The establishment of vegetative cover over an area of land is the most successful way of controlling the phenomenon of erosion (Grace, 2000). Vegetation is able to hold the surface layer of soil together promoting stability and cohesiveness. Revegetation is particularly important in areas of land that are disturbed by civil engineering works. Erosion along roadsides affects the quality of the road and leads to large annual maintenance costs. It has been estimated, that in North America, forest roads account for as much as 90% of all sediment production from forested land (Grace, 2002). Since European deforestation in New Zealand began, sediment production has largely been determined by landslide events (Smale & McLeod, 1997; Glade, 2003). This research implies that land devoid of vegetation is prone to the processes of erosion and establishing vegetative cover is the best solution for this problem. In addition, establishing appropriate vegetation cover on substrates such as mine tailings and old quarry sites is the fastest way to rehabilitate these areas, which are usually infertile and sometimes highly toxic to plant growth.

Disturbed land is naturally revegetated by a process known as succession. Succession is an ecological concept which states that there is a definable sequence of successive stages through which an ecosystem will pass. The speed at which succession occurs is influenced by certain factors associated with the specific area including temperature, rainfall, topography, slope, solar radiation and distance from intact forest (Leathwick & Rogers, 1996). Succession can be divided into two forms termed primary and secondary succession. Primary succession occurs in areas previously unoccupied by vegetation, whereas secondary succession occurs in areas where vegetation has been recently removed. In certain areas where environmental factors are unsuitable for rapid plant establishment and growth, succession occurs at a rate that is too slow to greatly reduce erosion. As a result, technology is continuously being developed that focuses on establishing vegetation in challenging environments as quickly and as cost effectively as possible.

1.5.1 HYDROSEEDING

Hydroseeding, also referred to as hydraulic seeding, is a common seeding practice used to sow areas of land. Hydroseeding involves the application of an aqueous slurry, commonly consisting of seed, fertilizer and tackifier, onto a target site via a high pressure hydraulic pump. The aqueous suspension is contained within a tank on the back of a slow moving tanker, and is progressively sprayed over the target area at an appropriate consistency. The slurry is designed to stick to the target site and promote germination and development of the incorporated seed. Hydroseeding has become particularly popular as a means of revegetating roadsides batters and verges. The main reason for this is that it is a cost effective way of establishing vegetation quickly across large areas of land that are sometimes inaccessible to traditional seeding equipment. It is prominently used for the reclamation of motorway embankments (Carr & Ballard, 1980; Andres & Jorba, 2000).

The ingredients included in the hydroseeding mix promote germination and development of the seed. Research has been carried out in order to identify which ingredients promote the best conditions for germination. However, results vary considerably as the specific environment into which the mix is introduced greatly influences the success of the seed.

Seed is usually chosen to suit the conditions of the site. Mainstream hydroseeding involves the sowing of mainly grasses and herbaceous legumes (Roberts & Bradshaw, 1985). These plants are chosen on the basis that they develop quickly, minimising the time it takes to successfully establish a suitable vegetative cover, and are able to grow in conditions that are less than optimal (i.e. low water availability, high light conditions). Legumes are particularly important inclusions in the seed mix because soils targeted for hydroseeding are usually devoid of organic material and are low in nitrogen (Roberts & Bradshaw, 1985). Legumes are known to form mutualistic relationships with soil borne microbes that fix N into forms that are accessible to the plant. Initially, young seedlings have plenty of N as the fertilizer included in the mix is still available in high concentrations. However, as the fertilizer is used by the plants and leached out of the soil, the N source becomes depleted. Legume species help to maintain the amount of

available nitrogen in the soil and, therefore, sustain the growth of the plants growing in the surrounding area.

Several types of mulch are used in hydroseeding mixes including wood fibre, peat mulch, straw mulch and paper mulch. Research indicates that the mulch increases the chance of successful establishment of seedlings (Sheldon & Bradshaw, 1977). The mulch has water retaining capabilities and can act as a water source for the germinating seed. The seeds and seedlings are, therefore, less prone to drying out. In addition the mulch, in conjunction with tackifier, creates a protective coat over the top-soil immediately after application. This coat has the ability to resist erosion to a degree, and provides a physical barrier that protects the seed from predation and simply being washed away. The degradation of the mulch over time also adds to the organic matter in the soil, which can be important when hydroseeding on land that contains no top-soil, e.g. subsoil, roadside verges and mine tailings.

In the past, debate has arisen over the requirement of fertilizers in the mix. Experiments have shown that in some cases fertilizer can be phytotoxic and can reduce the germination rate of the seeds, when compared to non-fertilized plots (Sheldon & Bradshaw, 1977). These results were affected by the type of soil that was being used in the experiments. When fertilizer is applied to a porous, fine soil type such as sand, germination rate was negatively affected. It was thought that if water was not readily available, the toxicity of the fertilizer became a factor. In some cases fertilizer is absolutely essential (e.g. on sites that are deficient in several of the macronutrients). Fertilizer can provide the site with elements essential for normal growth and as discussed earlier, is also important in providing the initial N source commonly lacking in areas that are chosen for hydroseeding. In some cases it has been suggested that fertilizer could be applied later, when the seedlings have established. This would reduce possible toxicity effects of fertilizer on germination. An alternative is to use slow release fertilizer that is not physically available in such high concentrations during germination and provides nutrition for longer periods of time.

The last component of the hydroseeding mix is often termed the tackifier or hydrocolloid. These chemicals are often polyacrylamides and act as soil conditioners and stabilizers. Properties of these chemicals include the ability to be dissolved in water,

to successfully enable germination and subsequent seedling growth, to be environmentally friendly, to limit erosion by creating a steadfast crust and to be cost effective. In the past it has been questioned whether these compounds were actually detrimental to seed development in some cases (Morrey et al., 1983). More recently research contradicted this evidence suggesting that modern hydrocolloids have no adverse affects on germinating seeds (Merlin et al., 1999). This paper conducted experiments to determine the effectiveness of these compounds on adhering seed to a sloped soil bank. It was shown that most of the compounds tested were able to induce high levels of adhesion of the seed to the soil.

Each site is unique in both abiotic and biotic factors. In order to obtain the best possible result, an appropriate mix has to be prepared depending on the nature of the site. Factors that influence the success of the hydroseeding application include the roughness of the substrate, the density of the substrate, available nutrients, soil acidity, slope and solar radiation, as well as seasonal implications (Cano et al., 2002).

The soil substrate can be modified prior to application of the hydroseeding mix in order to alleviate some of the abiotic constraints. Scarification of the soil may be required when the soil is so compact that roots are unable to penetrate the surface. Scarification also increases the surface area of the face and provides a better substrate to which the hydroseeding mix can adhere. In addition, scarification creates microsites which promote successful plant establishment.

Often the substrate chosen to be hydroseeded is deficient in several macronutrients. This deficiency can be compensated for by including a suitable fertilizer in the slurry. Acidic soils, such as those found at disused mine sites can be neutralised via the application of large amounts of lime ($CaCO_3$). If this method is chosen, tests on the substrate have to be carried out in order to determine the correct amounts of lime to be applied (Bradshaw, 1997). The alternative is to choose certain species to hydroseed that are naturally adapted to acidic or toxic soils. The fern *Pteris vittata* has been studied recently on its ability to grow on arsenic contaminated soils (Chen 2002; Visoottiviseth, 2002). This work has revealed that *Pteris vittata* is actually a hyperaccumulator of arsenic. As a result, a role for this fern has been proposed in the phytoremediation of arsenic containing soil, which can be prevalent at disused mine sites.

The gradient of the cut-slope is known to greatly affect hydroseeding success (Merlin et al., 1999). As discussed earlier, factors included in the mix, such as tackifier and mulch, can help to combat the effects of the slope on the establishment of the seed.

Water availability and solar radiation are correlated with the seasons. Therefore, when planning the logistics associated with hydroseeding it is important to identify the most appropriate time of year to hydroseed. In New Zealand the summer months are usually the worst time to hydroseed as water availability is low and solar radiation is high. Seedlings sown on steep batters are unable to source sufficient amounts of water to cope with the higher temperatures. Therefore, the best time of the year is autumn, when temperatures are mild and water is more frequently available.

Hydroseeding is a technology that requires varied applications for different sites. As more experiments are conducted and alterations to the basic method are implemented, the technology becomes more successful, as well as more flexible. An example of this flexibility is the development of hydroseeding applications for native species.

1.5.2 REVEGETATION WITH NATIVE PLANTS

The use of New Zealand native plants in revegetation offers a unique way to maintain areas of New Zealand's natural heritage. Instead of planting costly container plants, hydroseeding could potentially be used to establish native species in protected areas, reducing the immigration of exotic plants while maintaining the native plant flora. Road cuttings through native bush would be the main target for this treatment as legislation impedes the use of exotic species in revegetation projects within the boundaries of National Parks (National Parks Act, 1980).

Hydroseeding with native species in New Zealand has produced mixed results (Simcock & Ross, 1997; Smale et al., 2005). Efforts have focused on using seeds of native shrubs. The most successful species at two different sites were *Coprosma robusta* (karamu) and *Phormium cookianum* (mountain flax). Factors thought to influence the outcome of these experiments included hare and rabbit predation and the colonization of plots by weedy foreign species. Interestingly, scarifying the surface of the batter did not improve the ability of seedlings to establish compared to smooth surfaces. Native

species tend to be slow to establish, therefore it is important to include in the mix an appropriate soil stabilizer and in some cases, a nurse species. Nurse species have the ability to germinate and grow quickly without inhibiting the growth of the native species. The nurse species provides initial protection against erosion until the native seed has germinated.

In New Zealand hydroseeding has been used successfully to establish native mosses on mine tailings (Ross et al., 2003). Mosses are colonizers and have the ability to grow on infertile substrate. Over time moss collects soil particles, building the beginnings of a fertile substrate into which seeds and spore can germinate and grow.

Ferns are involved in the natural succession of plant growth on disturbed land (Kitayama et al., 1995; Rivera et al., 2000; Slocum, 2000). They generally establish after bryophytes, and are able to grow in relatively infertile conditions. These characteristics promote their use on infertile substrates, such as mine tailings and subsoil. Ferns are also naturally involved in secondary succession. In mature stands of *Pinus radiata*, native tree ferns are known to establish themselves naturally in the under-storey (Brockerhoff et al., 2003). New Zealand native ferns are found naturally on roadside slopes. Ferns such as *Blechnum novae-zelandiae* and *Microsorum pustulatum* are commonly found growing along roadsides in New Zealand (Isolde et al., 1995). These species of New Zealand native fern are especially adapted to growing on banks. They characteristically have a creeping rhizome with a mat of roots growing into the substrate. As a result, these characteristics suggest that there is potential for the use of native ferns in roadside revegetation.

1.6 Project aims

- To evaluate the potential use of several New Zealand native ferns for revegetation by conducting laboratory experiments focused on particular stages of their life cycles.

- To assess the ability of native ferns to establish in the nursery and field, using various revegetation methods, including hydroseeding.

SPORE COLLECTION AND LABORATORY ANALYSES

2.1 Overview

The experimental work in this thesis has been divided into three separate chapters in order to separate laboratory experiments, nursery experiments and field experiments. The work in all three chapters was conducted in accordance with appropriate experimental design, and the results were subjected to suitable statistical analysis.

The first chapter includes the description of where, when and how fern spore was collected along with the design, results and discussion of several experiments conducted in the laboratory. Laboratory experiments were carried out in order to acquire information on various aspects of the life cycle of several New Zealand native ferns. The germination of spore, collected mainly from the Manawatu district, was assessed under various conditions, using different procedures. Aspects of the mating system of the species *Blechnum novae-zelandiae* are described for the first time. The robustness of gametophytes of several native fern species is also considered. The information provided by these laboratory experiments was used to consider the potential of several native fern species as candidates for revegetation, as well as to increase the knowledge base on several common New Zealand native ferns.

2.2 Materials and Methods

2.2.1 SPORE COLLECTION

Spore was collected throughout the project for use in laboratory, nursery and field experiments. Most of the spore was collected in the Tararua Ranges, east of Palmerston North, New Zealand. Figure 2.1 shows the location of the main collection sites from which both spore and rhizomes were sourced.

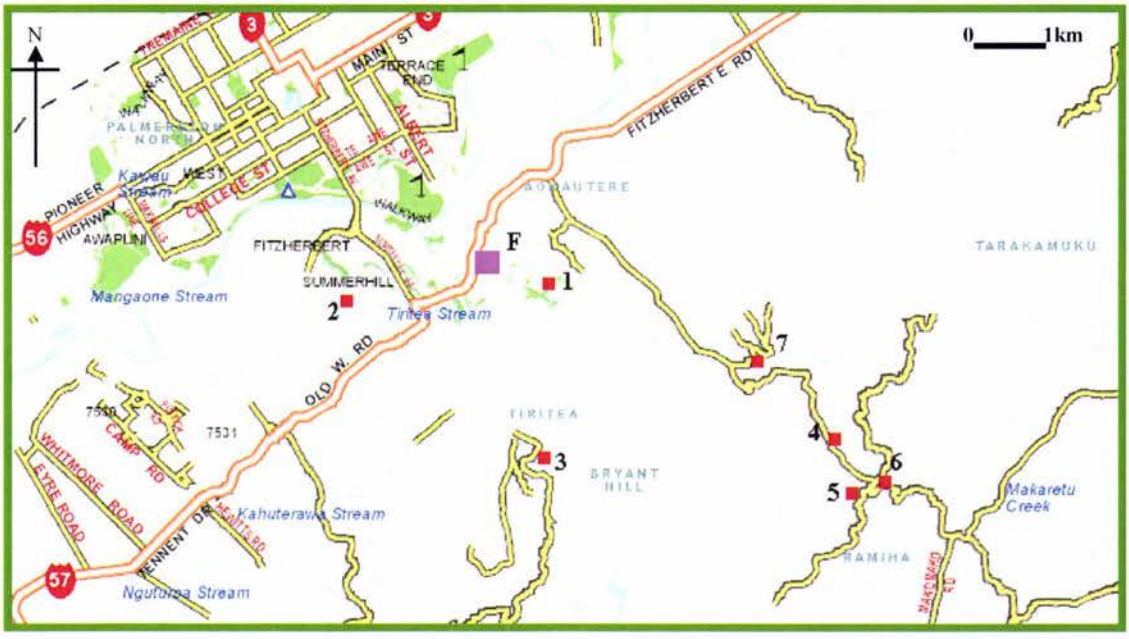


Figure 2.1 Collection sites are marked by a red square. **1.** Moonshine Valley Road. **2.** Massey Fernery. **3.** Greens Road. **4.** Pahiatua Track West. **5.** South Range Road. **6.** Pahiatua Track East. **7.** Harrison Hill Road. **F.** Location of Field site (purple square). Refer to herbarium for map references (via Appendix 1).

Species collected from the Manawatu region included *Blechnum novae-zelandiae* T. C. Chambers et P. A. Farrant (sites 2, 4, 5 & 7), *Cyathea medullaris* G. Forst (site 2 & 3), *Dicksonia squarossa* G. Forst (site 1), *Asplenium bulbiferum* G. Forst (site 6) and *Microsorum pustulatum* G. Forst (site not shown).

Microsorum pustulatum was only required for the rhizome field experiments and was collected from Hall Block Road, near the Manawatu Gorge. *Asplenium bulbiferum* spore was initially of poor quality, and therefore, this species was excluded from any of the experimental work. Only two collections took place outside of the Manawatu

district. Spore of *Blechnum colensoi* (Hook. f.) N. A. Wakef and *Blechnum discolor* G. Forst, was collected by Dr Craig Ross from the upper reaches of the Buller Gorge, in September 2004. A second collection of *Blechnum discolor* was made from the lower reaches of the Buller Gorge in February 2005.

The collection process involved harvesting fertile fronds of the required species and transporting them back to Massey University. Fertile fronds were laid out onto fresh newspaper with the sori facing downwards. The fronds were left in this state for about 7 days to allow them to dry. Once spore had been shed, it was separated from other debris that had collected on the newspaper, including sporangia, scales and hairs. The separation step involved gently shaking the newspaper at a downward angle. Sporangia, hairs and scales slid down the gradient faster than the spore, due to the greater mass of these structures. Once the separation process was complete the spore was tipped into envelopes and labelled with the species name, the date of collection, and the date of packaging. If spore was not required for use straight away it was kept in an unsealed paper envelope at 4°C.

The existence of self-incompatibility mechanisms in the species of interest was unknown. Therefore, to protect against this complicating factor, care was taken to make sure samples were taken from different ferns of the same species even when only a small sample was required.

Voucher specimens were collected from each site. These specimens provide direct references to the species and populations used in the project. The samples were dried, pressed and labelled prior to inclusion in the Ella Campbell Herbarium at Massey University. Each sample was given an MPN number for future reference (Appendix 1). Unfortunately samples from the first Buller Gorge collection were unable to be included as suitable specimens were not collected at the time.

The main species used in this project were *Blechnum novae-zelandiae, Cyathea medullaris* and *Dicksonia squarossa*. All of these species were available in the Manawatu district. In addition, these three species are predominant spore producers. Spore was required throughout the project for various experiments.

2.2.2 SPORE GERMINATION

2.2.2.1 Media preparation

The medium used in all experiments was Murashige and Skoog medium (MS) (Murashige & Skoog, 1962) supplemented with 1% agar (Life Technologies, Scotland). This medium was decided upon due to its continual mention in the literature as a propagative medium for fern spore e.g. (Fernandez et al., 1993; 1999; Cox et al., 2003). Its specific effect on the species of interest was unknown as no scientific literature mentions the propagation of native New Zealand ferns on an artificial medium of this kind. The standard concentration of MS is 4.4 g L^{-1} of water. Throughout the text Murashige and Skoog medium will be abbreviated to ½ MS, as this is the concentration used for spore germination and gametophyte development. Each batch of medium was prepared by adding appropriate amounts of MS, agar and water to a 1 L Schott bottle. The solution was then roughly mixed via swirling and placed in an autoclave at 121°C and15 psi, for approx.1 h. The solution was then kept warm in a 60°C water bath in order to keep the medium liquid prior to being poured into petri dishes. Pouring was carried out in a lamina flow cabinet in order to keep conditions sterile. Once cool, the Petri dishes were collected, labeled and stored in a refrigerator at 4°C until required.

2.2.2.2 Spore surface sterilization and sowing

It was important that spore was surface sterilized prior to sowing as contamination of plates with other microorganisms may have affected the germination process, as well as the growth and vigor of the gametophyte. Spore was initially removed from an envelope using a clean spatula and tapped into a sterile 1.5 ml micro-centrifuge tube. Around 0.05 ml – 0.1 ml of dry spore was a sufficient amount to sow ten plates. The next step required adding 1 ml of water to the spore to initiate imbibition and germination. The spore was then left to hydrate for at least 10 minutes. A solution of 4.5 g L^{-1} of sodium hypochlorite, Tween20 (Sigma chemical Co, USA) and water was used to surface sterilize the spore. The Tween20 acts as a wetting agent as the spore tends to be hydrophobic if it has only recently been exposed to water. Tubes were then centrifuged at 2000rpm for 3 min in order to pellet the spore and separate it from the water. The supernatant was pipetted off and discarded, and 1 ml of fresh

hypochlorite/Tween20 solution was added. Dispersal of the spores in the solution was facilitated by swirling or shaking the tube vigorously. The spore was then allowed to soak for 3 min before the tubes were centrifuged once again. After centrifugation the supernatant was pipetted off, and 1 ml of autoclaved water was added to the pellet. In total three sterile water washes were conducted in order to remove any trace of the hypochlorite/Tween20 solution. Spore was sown onto fresh ½ MS, 1% agar plates in a lamina flow cabinet. 0.1 ml of the sterile spore and water suspension was added to each plate. In addition, 2 ml of sterile distilled water was added to disperse the spore on the surface of the agar and facilitate germination. Plates were sealed with parafilm to reduce contamination by airborne micro-organisms. This process was successful at killing most microorganisms including paramecium, green algae and various fungi. In some cases contamination remained prevalent. However, due to the fact that each experiment used many replicates, contaminated plates were able to be discarded if necessary without compromising statistical analysis or the reliability of the results.

The effect of this method of spore sterilization on the spore used in this project was tested. Germination rates were compared between non-sterilized spore and sterilized spore, subjected otherwise to the same conditions.

2.2.2.3 Assessing germination

The germination of spore was used as a key parameter to test the performance and viability of spore collected throughout the project. Once spore was sterilized and sown it was usually transferred to a SANYO growth cabinet, model number MLR-350H. Plates were spread out on racks in order to maximise the amount of light available to them. The growth cabinet parameters were kept constant throughout the project. Constant white light was used at an intensity of ~ 40 – 60 $\mu mol\ m^{-2}\ s^{-1}$, relative humidity was kept at 75% and temperature at 23°C $\pm$ 0.5°C. In some cases spore was kept under different conditions to those in the growth cabinet.

Germination was recorded every two days after sowing. The number of replicates varied for each experiment. However, at least 3 replicates were used in order to validate the data. Germination was usually recorded for 20 days, or until germination was complete. Plates were positioned on a standard compound light microscope at 40x magnification

and 50 spores were scored for each replicate. The emergence of the primary rhizoid indicated positive germination. In addition, spores with a broken exine and visible chloroplasts were also recorded as positive germinates if rhizoids could not be seen. For each treatment at least three replicates were examined. In some cases gametophytes grown from germination experiments were subsequently used in other experiments.

One experiment analysed spore incubated in more "natural conditions" in the Massey University fernery and glass house. Light intensity, humidity and temperature in the Massey University fernery and glasshouse were measured using a Hobo™ data logger. Spore germination was assessed over a 20 day period and compared to a similar sample of spore grown in standard, growth cabinet conditions. In addition, dark sowing, using a green safe-light (0.01 μmol m^{-2} s^{-1}), was utilized in order to observe the effects of darkness on spore germination. Dark sown spore was left for 10 days in the dark, whereupon its germination rate was measured at day 10. It was then incubated for 10 more days in standard, growth cabinet conditions with germination being recorded every two days.

Throughout 2005, spore from the species *Blechnum novae-zelandiae, Cyathea medullaris* and *Dicksonia squarossa* was collected from the collection sites shown (Fig. 2.1) and sown as described above. Repeated collections were carried out at regular intervals in order to obtain information on the time of year that spore germination was at its highest in these species. It was perceived that this information would be valuable for planning future fern hydroseeding applications using these species.

In another experiment germination was used to compare the effect of different polyacrylamides on spore of *Blechnum novae-zelandiae, Cyathea medullaris* and *Dicksonia squarossa*. Polyacrylamides are often used as soil conditioners and stabilizers, and are incorporated into the hydroseeding mix. The three polyacrylamides used in this experiment were Soilfix™, FLOBOND and Aquasorb. Soilfix™ and FLOBOND are simple soil conditioners and act to bind soil particles together. Aquasorb has an added property that allows it to absorb and store water as well as act as a soil conditioner. Spore was surface sterilised and sown onto standard ½ MS, 1% agar plates. For each polyacrylamide two concentrations were tested. Plates were either subjected to a standard concentration of 1 g L^{-1}, or a very high concentration of 10 g L^{-1}.

For each treatment there were at least three replicates. Controls were included that only contained water without any polyacrylamide for each species.

2.2.2.4 Fluorescein diacetate spore viability test

The fluorescein diacetate (FDA) test was adapted from a pollen viability test for testing the viability of fern spore (Shivanna & Rangaswamy, 1992). It had previously been suggested that this method could be used to ascertain the viability of spore of New Zealand fern species (Large & Braggins, 1991). Each time this test was carried out a fresh stock solution of FDA (Sigma Chemical Co, USA) was prepared by adding 80 mg of FDA powder to 40 ml of acetone. Spore chosen for the test was freshly collected, and testing was carried out in conjunction with germination experiments. In addition "reduced viability" spore was also prepared for use as a negative control by baking it in an oven at ~ 90°C for 2-3 h. Spore was imbibed overnight prior to incubation with FDA. Seven tubes were prepared for each spore sample. Five of these tubes contained various sucrose solutions (0.1M, 0.2M, 0.3M, 0.4M and 0.5M) in order to establish osmolarity effects of water on the spore. Several drops of FDA were added to each tube containing sucrose and one tube containing water. The seventh tube was a negative control and only contained water. This spore was expected to show only background fluorescence. These solutions were added to small samples of spore and the mixture was left to incubate for 1–2 hours. Incubation was carried out on a slide or in a microcentrifuge tube. "Reduced viability" spore was also incubated with various solutions of FDA, sucrose and water. Samples were mounted on slides and viewed under an Olympus BX51 fluorescence microscope with a mounted digital camera. Viable spores were presumed to be able to change the fluorescein diacetate into a fluorescing compound, through the action of cytoplasmic esterases. If the cytoplasm of a spore was not intact, the spore would not have any esterase activity and therefore would not fluoresce, rendering the spore non-viable. Photos of treated spore were taken and spore was scored in terms of its ability to fluoresce.

2.2.3 SEXUAL MATING SYSTEMS

2.2.3.1 Sporophyte production and gametophyte sexuality

The New Zealand native fern *Blechnum novae-zelandiae* was used in this experiment to study the sexual development of its gametophyte. Initially gametophytes were grown from spore in the growth cabinet in order to see if they were capable of producing sporophytes, and hence carry out the fertilization process *in vivo,* under laboratory conditions. Photos were taken using a hand-held Sony DSC-P8 digital still camera (Fig. 2.8). Once it was discovered that this was possible an experiment was designed to test the ability of *B. novae-zelandiae* gametophytes to successfully carry out intra-gametophytic selfing, inter-gametophytic selfing or inter-gametophytic crossing. Initial experiments, using only 4 replicates, were conducted looking primarily at intra-gametophytic selfing. *Blechnum novae-zelandiae* spore was surface-sterilized, sown on standard growth media, sealed with parafilm and placed in a controlled environment as described previously. The spore germinated and produced small multicellular gametophytes. Four single, presexual gametophytes were isolated 40 days after sowing and transplanted onto four fresh ½MS, 1% agar plates. In addition, 1 ml of sterile water was added to each of the four plates to facilitate growth and development. Isolated gametophytes were then left to develop for an additional 32 days before they were flooded with 10 ml of sterile water in order to facilitate fertilization. Each plate was left unsealed to allow the water to evaporate. Gametophytes were then periodically checked to see if they had produced sporophytes over the following 4 weeks. Photographs of antheridia/archegonia and sporophytes were taken with a *Leica* MZ12 stereo microscope using dark-field microscopy.

A more extensive experiment was also designed looking at all three possible mating systems i.e. intra-gametophytic selfing, inter-gametophytic selfing and inter-gametophytic crossing. Spore from two separate populations of *B. novae-zelandiae* (Fig. 2.1, sites 4 and 7) were collected within two weeks of each other. These two populations were separated geographically, and therefore, it was anticipated that they would be slightly different genetically. The gametophytes of the Pahiatua Track West population were 50 days old when they were transplanted. Those of the Harrison Hill population were 37 days old. 18 replicates were set up for each treatment. The first

treatment used single, isolated gametophytes in order to test for intra-gametophytic selfing. The second treatment involved growing two gametophytes from the same plant in proximity to each other, to test for inter-gametophytic selfing. The third treatment involved growing two gametophytes from different plants in order to test for inter-gametophytic crossing. The Pahiatua Track west gametophytes were used in all treatments. The Harrison Hill gametophytes were required for the third treatment only. Gametophytes were watered with sterile water periodically and left to develop. All plates were grown in the same conditions and positioned randomly in the growth cabinet. 72 days after setting up the experiment gametophytes were flooded with 5 ml of sterile water in order to facilitate fertilization. Gametophytes were assessed every week after flooding for the production of sporophytes over a period of 11 weeks.

2.2.3.2 Antheridiogen

Antheridiogen experiments were conducted using the species *Blechnum novae-zelandiae, Cyathea medullaris* and *Dicksonia squarossa.* The first experiment was intended to test for the presence of antheridiogen. This was done by designing an experiment that utilized the knowledge that antheridiogen is able to compensate for light, and cause the initiation of spore germination in the dark. Initially gametophytes of each species were grown on plates in batches of 50 gametophytes per plate. It was proposed that if these species utilized antheridiogen it would be exuded into the medium. After 87 days these gametophytes were removed from the plates and the medium was used as potential antheridiogen containing medium. Spore was sown onto both antheridiogen containing medium and normal medium in the dark using a green safe light. The normal medium was used as a dark control and would presumably result in non-germinated spore. In addition, spore was also sown in the light and kept in the growth cabinet at standard conditions. Spore was unable to be sterilized due to the fact that the equipment required for sterilization was only available in lighted areas. Dark sown spore was placed in envelopes and then sealed in a black plastic bag. Spore was left for 10 days. After the ten days treatment, dark, and light control plates, were examined for germination by counting 100 spores on each replicate plate. All plates were then transferred to the growth cabinet. After 4 days they were examined again for any changes. After 22 days in the light the number of male gametophytes observed on 3 replicates plates for each species and each treatment were recorded and compared.

The second experiment was designed to test the effect of a mature gametophyte on the ontogeny and development of surrounding spore. Presumably if a mature gametophyte produced antheridiogen, surrounding spore in close proximity to the gametophyte would predominantly develop as male gametophytes. 87 day old gametophytes of each species were isolated and transplanted onto fresh ½ MS, 1% agar plates. Each plate contained a single gametophyte. Six replicates were set up for each species. Plates were watered with 2 ml of sterile water after transplantation. After 23 days of isolation, freshly collected spore was sterilized and sown around the gametophytes corresponding to that species. Control plates, missing mature gametophytes, were also sown. 46 days after sowing, the sexuality of gametophytes growing on both treatment and control plates was assessed. The sexuality of 50 gametophytes was determined at three different distances from the mature gametophyte, for each treatment plate. These distances were 0-1 cm, 1-2 cm and 2-3 cm from the mature gametophyte. Samples were removed using a clean spatula and were mounted on a microscope slide. At each distance the first 50 gametophytes, that were clearly distinguishable, were counted and sexed. Overall, the sexuality of 150 gametophytes was recorded for each plate, including controls. Photographs were taken for both antheridiogen experiments using an Olympus BX51 compound microscope (Figs.2.12, 2.13 & 2.14).

2.2.4 GAMETOPHYTE TRANSPLANTATION AND MACERATION

2.2.4.1 Transplantation of gametophytes

Gametophyte transplantation was required throughout the duration of this project in order to reduce competition between growing gametophytes and to provide fresh growing medium for gametophytes when old medium became exhausted. Transplantation was also carried out when setting up some experiments. The main challenge when transplanting gametophytes was to limit the amount of contamination from air and water borne microorganisms. Therefore, transplantation was carried out in a lamina flow cabinet. Gametophytes were uplifted using sterile toothpicks and were positioned on fresh plates. Lids were kept on plates at all times except when transporting gametophytes between them. Sterile water (2 ml) was added to each plate to facilitate growth and development of the gametophytes.

2.2.4.2 Maceration of gametophytes

Experiments to assess the ability of gametophytes to withstand a low level of maceration were conducted using *Blechnum novae-zelandiae, Cyathea medullaris* and *Dicksonia squarossa* gametophytes. The degree of maceration was based on what may be expected to happen to these gametophytes in practice (i.e. when pumped through a hydroseeder).

Spore samples from each species were sterilized, sown and incubated in the standard growth cabinet conditions for 46 days. After this period of time the gametophytes were large enough to be transplanted (i.e. 5 mm in diameter). 300 gametophytes of each species were transplanted onto fresh ½ MS, 1% agar plates (50 per plate). Two days after transplantation gametophytes were watered with 1 ml of sterile water per plate. Fourteen days after transplantation a further 2 ml of sterile water was added to each plate. 41 days after transplantation the gametophytes were sufficiently large to macerate.

Maceration was carried out in a lamina flow cabinet under sterile conditions. Gametophytes were macerated using a sterilized scalpel blade that had been soaked in 70% ethanol and passed through a Bunsen flame. 25 pieces of chopped gametophyte were transplanted onto fresh medium. Five replicates plates of 25 gametophytes were transplanted for each species. In addition, 25 whole gametophytes were also transplanted onto fresh plates and replicated 5 times for each species. These whole gametophytes were controls. Survival of the chopped and whole gametophytes was assessed every 7 days for 6 weeks. Photos of the plates were also taken one day after maceration as well as 4 weeks after maceration using a hand-held Sony DSC-P8 digital still camera.

2.2.5 ESTIMATING SPORE QUANTITY

A serial dilution was carried out in order to obtain an estimate of the amount of spore used in laboratory, nursery and field experiments. This estimate would also provide an indication of the amount of spore sown on each plate.

Spore (0.1 ml), each of *Blechnum novae-zelandiae, Cyathea medullaris* and *Dicksonia squarossa*, was used in this dilution. The spore was diluted 1000-fold in order to obtain counts within the appropriate range of 30 to 300 spores per 0.1 ml of solution. This was done by initially adding 1 ml of water to the 0.1 ml of dry spore and thoroughly mixing the solution. Following this, 0.1 ml of the solution was added to 0.9 ml of water creating a 10-fold dilution. This 10-fold dilution was repeated two more times in order to obtain a 1000-fold dilution. Throughout the serial dilution, solutions were thoroughly mixed in order to take uniform samples. Each dilution was carried out 3 times for each species and in addition 3 counts were made for each replicate. The amount of spore was counted for each replicate and an average was taken. The initial concentration of spore was then calculated for each species.

2.2.6 STATISTICAL ANALYSES

All germination curves were plotted using mean germination data at each time point, calculated from at least three replicates. In some figures the mean $\pm$ the standard error was also included to show variability between replicates. ANOVA was used to determine the significance of several data sets including the effect of sterilization on spore germination (Fig. 2.2), the effect of polyacrylamide concentration on spore germination (Fig. 2.6 A, B & C) and the difference between two inductive environments (Fig. 2.4). All treatments were tested for significant difference at $P < 0.05$ unless otherwise stated. Replicate plates were positioned randomly when placed in growing conditions in order to reduce sampling bias, and possible environmental effects within the plate environment e.g. light, humidity, temperature.

2.3 Results

2.3.1 SPORE GERMINATION

2.3.1.1 Surface sterilization

The germination of surface sterilized spore was compared with that of non-sterilized spore for the three species *Blechnum novae-zelandiae, Cyathea medullaris* and *Dicksonia squarossa* (Fig. 2.2). The mean germination for each two day interval has been plotted. All species germinated within 6 days of sowing and nearly all treatments reached maximum germination after 6-10 days. Sterilized *B. novae-zelandiae* spore had an overall lower germination rate than that of non-sterilized spore of the same species (50% and 70% respectively). However, analysis of the mean data, using ANOVA indicated there was no significant difference between the non-sterilized and sterilized spore germination data of *B. novae-zelandiae.*

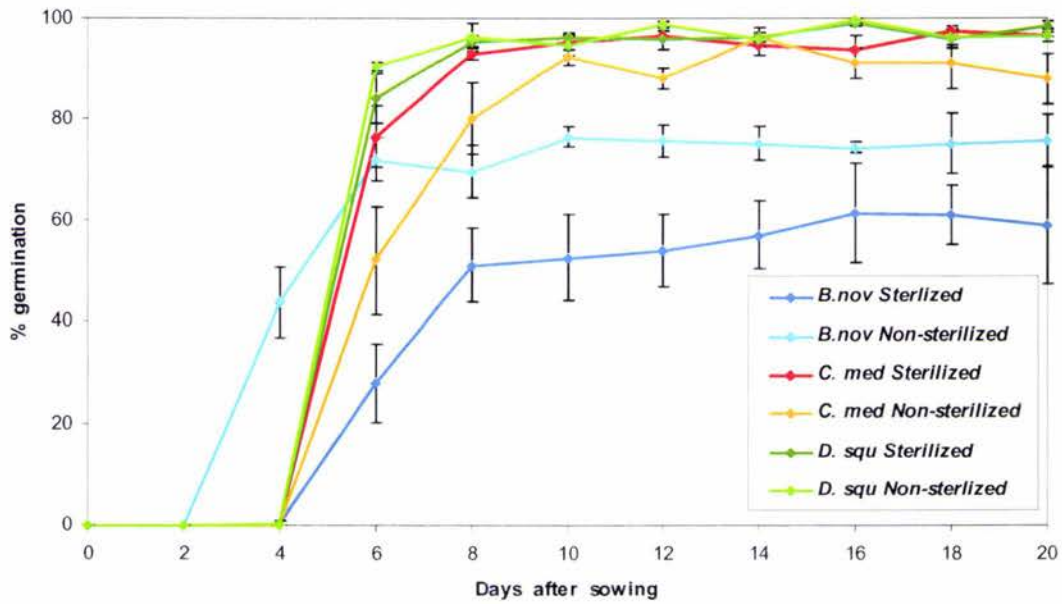


Figure 2.2 The effect of surface sterilization on spore germination of three native New Zealand fern species, *Blechnum novae-zelandiae (abbrev. B. nov), Cyathea medullaris (abbrev. C. med)* and *Dicksonia squarossa (abbrev. D. squ).*

Cyathea medullaris and *D. squarossa* spore germination curves showed very little difference between sterilized and non-sterilized spore. This was supported by one-way ANOVA, which showed that there was no significant difference between the data of sterilized and non-sterilized spore of *C. medullaris* and *D. squarossa*. *Cyathea medullaris* and *D. squarossa* spore showed around 85% - 99% germination when the maximum germination potential was reached, regardless of sterilization. Standard error bars indicate that sterilized *B. novae-zelandiae* spore exhibited the most variation between replicates. These data suggest that the sterilization process described in this project did not significantly affect spore germination in these three fern species.

2.3.1.2 Spore germination in controlled, dark and natural conditions

Blechnum novae-zelandiae, Cyathea medullaris and *Dicksonia squarossa* spore was germinated on standard media, in controlled, dark, and natural conditions (Fig. 2.3). Under controlled, growth cabinet conditions, spore germinated between 4–6 days after sowing for all three species. Germination reached its maximum potential for all three species 10 days after sowing. This is indicated by a plateau visible on each curve. *Blechnum novae-zelandiae* spore germination plateaued at about 60%. *Cyathea medullaris* and *D. squarossa* spore germination plateaued at around 95%. These results are similar to the corresponding curves seen in Fig. 2.2.

Spore sown in the dark did not germinate until it was transferred to the growth cabinet, 10 days after sowing, whereupon germination commenced 4 to 6 days later. This indicated that *B. novae-zelandiae, C. medullaris* and *D. squarossa* spore is photoblastic, i.e. requires light for germination. Germination plateaued at similar levels to the spore grown in the controlled environment, with one exception. *Blechnum novae-zelandiae* dark grown spore reached a higher maximum germination potential than spore grown in the controlled environment.

Spore grown in natural conditions did not germinate after 20 days, except for a very low percentage (< 1%) of *B. novae-zelandiae* spores, from 18 – 20 days.

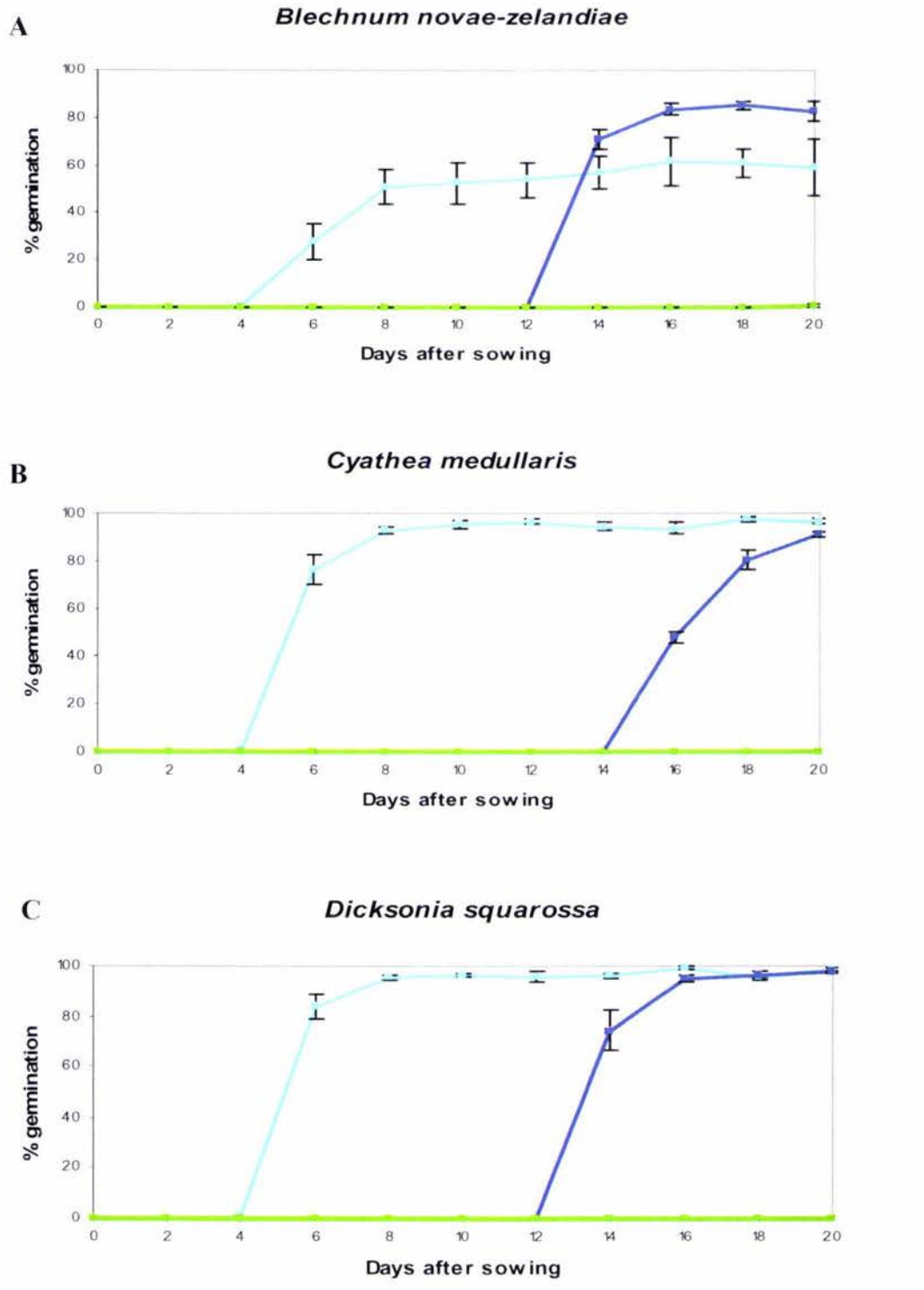


Figure 2.3 Germination of (A) *B. novae-zelandiae*, (B) *C. medullaris* and (C) *D. squarossa* in controlled, dark and natural conditions.

The spore used in each treatment was originally from the same collection, suggesting that even the non-germinated spore, grown in natural conditions, was potentially viable. Twenty-two days after sowing, this spore was transferred from natural to glasshouse conditions to test its ability to germinate. The changes in three environmental parameters (temperature, relative humidity and light intensity) compared with the germination of the three species, can be observed in Fig. 2.4. After four days in the glasshouse all three species germinated. Ten days after the change in conditions, all three curves plateaued, though germination reached a level substantially lower than that seen in controlled conditions (Fig. 2.3).

A change in temperature can be observed 22 days after germination, corresponding to the change in conditions (Fig. 2.4A). When the temperature data for the two different conditions is compared, ANOVA indicates there is significant difference between the temperature of the natural conditions and the temperature of the glasshouse conditions ($P < 0.0001$). The mean temperature increased by 7.5°C corresponding with the onset of spore germination for each of the three species.

A change in relative humidity was also observed (Fig. 2.4B). After plates had been transferred to the warmer glasshouse the relative humidity surrounding the plates decreased by 27.5%, an effect that was significantly different ($P < 0.0001$). These data suggest that a drop in humidity surrounding the incubation plates was associated with spore germination. However, the results shown here do not accurately measure the micro-environment to which the spore was exposed. The plates containing the spore were sealed with parafilm and, therefore, the humidity surrounding the plates (which is shown here) would only influence the humidity inside the plates indirectly, depending on how well sealed the plates were. It was observed that plates had dried out and lost most of their condensation by the conclusion of the experiment, indicating that water vapour was slowly escaping from the plates due to the lower humidity outside of the plates.

There was no consistent difference in light intensity between the natural conditions and the glasshouse conditions (Fig. 2.4C). The mean light intensities for the two environments were not significantly different.

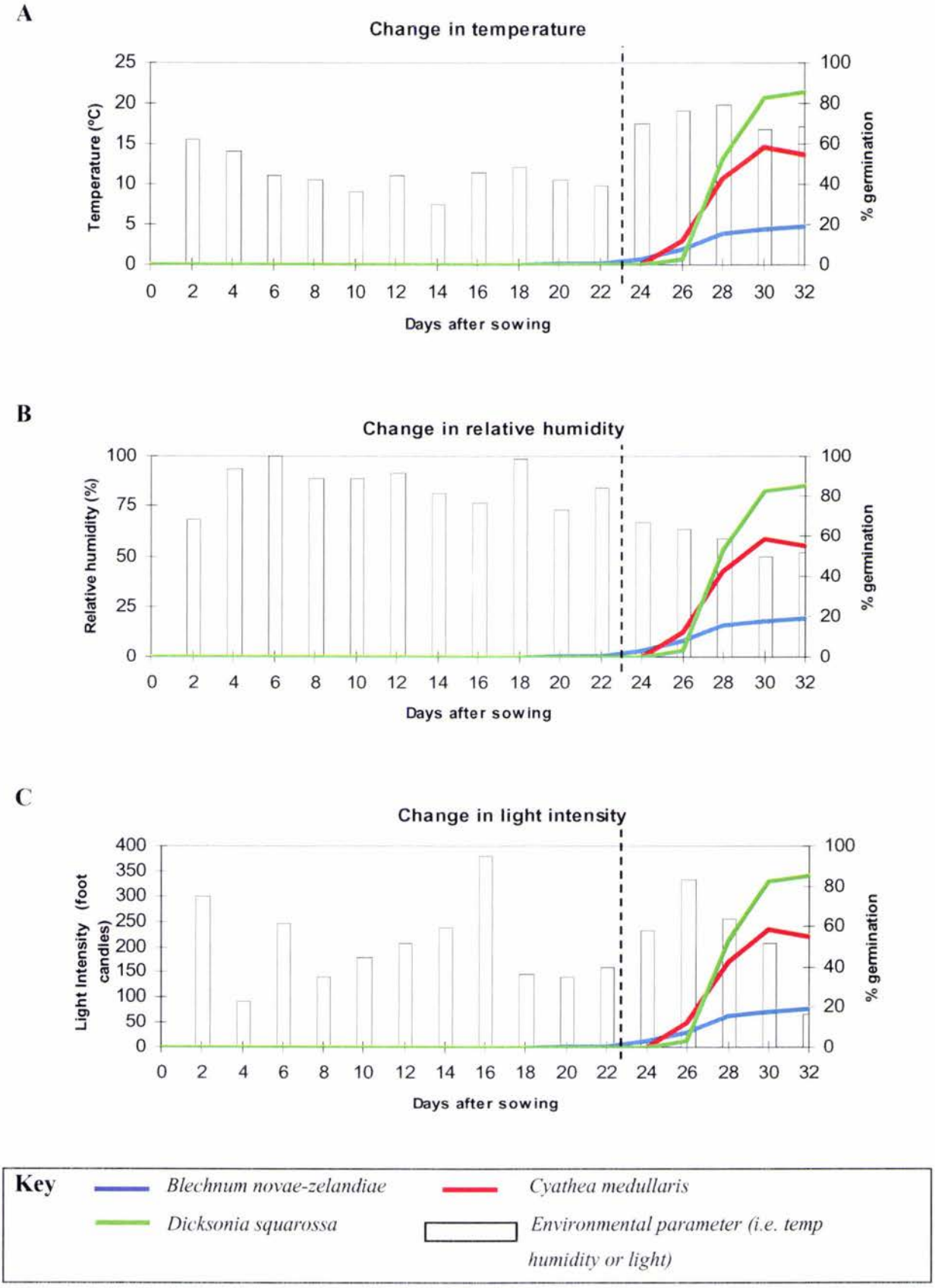


Figure 2.4 Spore germination of *B. novae-zelandiae, C. medullaris, D. squarossa* in natural conditions (0-22 days), and after transfer to glasshouse conditions (24 – 32 days). Changes in temperature (6A), relative humidity (6B) and light intensity (6C) are shown.

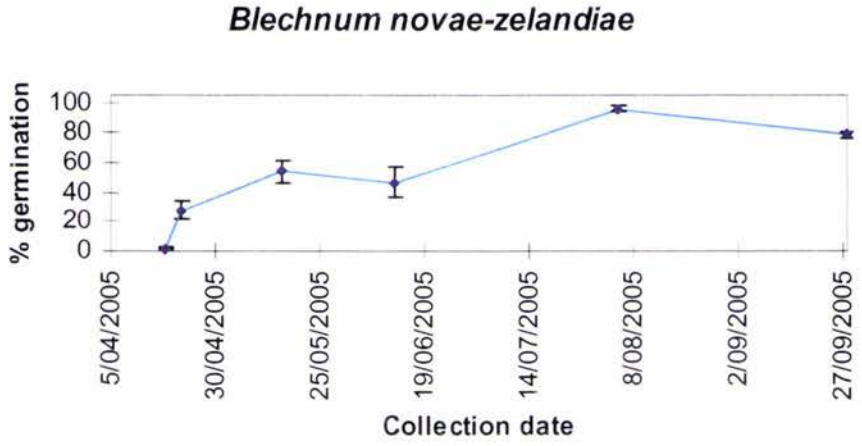
2.3.1.3 Seasonal variation in spore germination

The viability of spore of *Blechnum novae-zelandiae*, *Cyathea medullaris* and *Dicksonia squarossa* was assessed from April 2005 until September 2005, by measuring spore germination (Fig. 2.5). The early increase in spore germination seen in *B. novae-zelandiae* and *C. medullaris* corresponded with the maturation of the immature spore through autumn and into winter. The onset of spore maturation in *Dicksonia squarossa* is not shown. The period of spore maturation varied between the three species. *Blechnum novae-zelandiae* spore matured over a 5 month period (April – August) and reaches its highest germination rate in late winter. *Cyathea medullaris* spore matured in mid April and maximum germination was reached several weeks alter. The maturation of *D. squarossa* spore was not clearly expressed. However, its maximum germination peaked in the late autumn, similar to the other tree fern species *C. medullaris*. All three species had highly viable spore in the late winter/early spring.

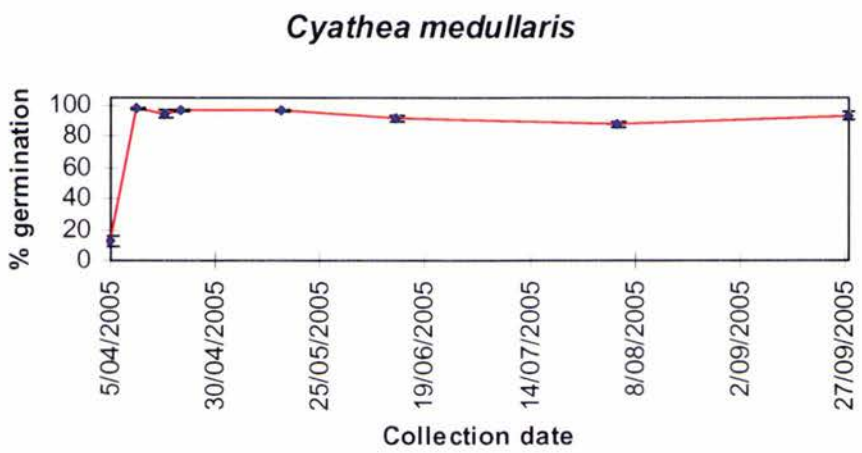
Through field observations it was clear that by September the quantities of spore produced by each species, was in dramatic decline. The season's spore production was decreasing as indicated by the spent fronds. However, the small quantities of spore retained their ability to germinate.

The variability within sample dates is shown by the standard error of the mean bars, located at each data point. Interestingly, for *B. novae-zelandiae* and *D. squarossa* the most variability within the samples was at the beginning of the season. *Cyathea medullaris* spore germination showed little variability at all stages.

A



B



C

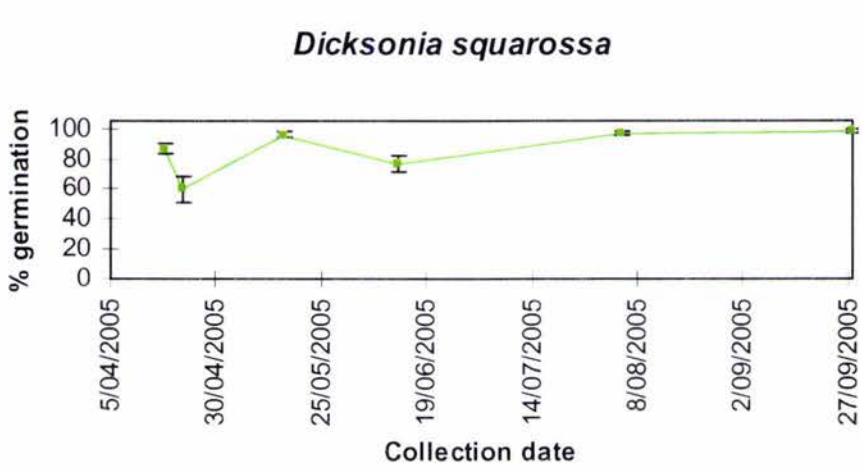


Figure 2.5 Mean germination rates of *B. novae-zelandiae* (A), *C. medullaris* (B), *D. squarossa* (C) showing germination rates from April 2005 to September 2005. Each time point shows the mean ± standard error after 12 days of germination.

2.3.1.4 The effect of three polyacrylamides on spore germination

The three chosen polyacrylamides; Soilfix™, FLOBOND and Aquasorb, did not influence the germination of *B. novae-zelandiae, C. medullaris* or *D. squarossa* spore (Fig. 2.6A, B & C). Using ANOVA it was determined that the increase in concentration from 1g L^{-1} of polyacrylamide to10g L^{-1} showed no significant change in germination compared to controls. The most variation between the three treatments was observed in *B. novae-zelandiae* spore. However, this variation is not statistically significant.

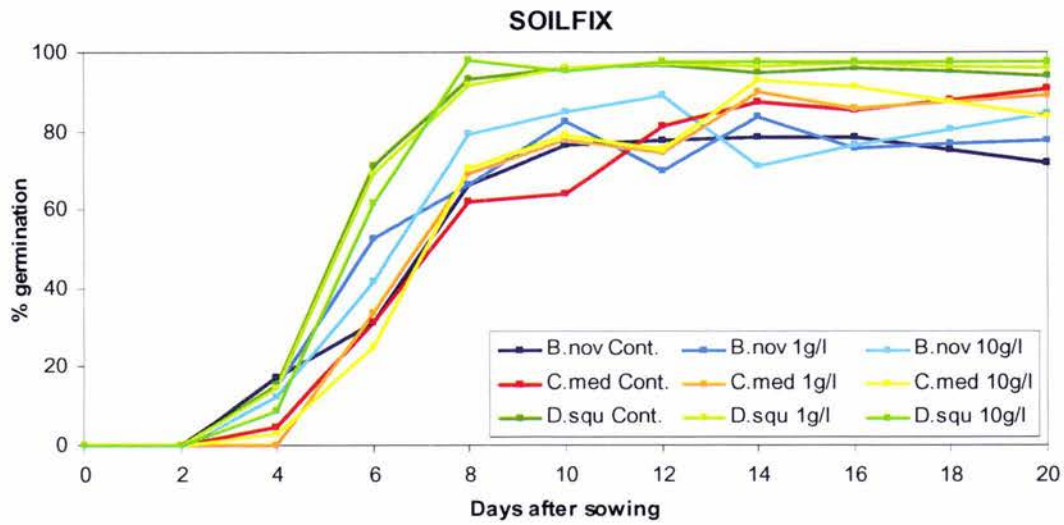


Figure 2.6A The effect of Soilfix™ on the germination of *B. novae-zelandiae, C .medullaris and D .squarossa* spore.

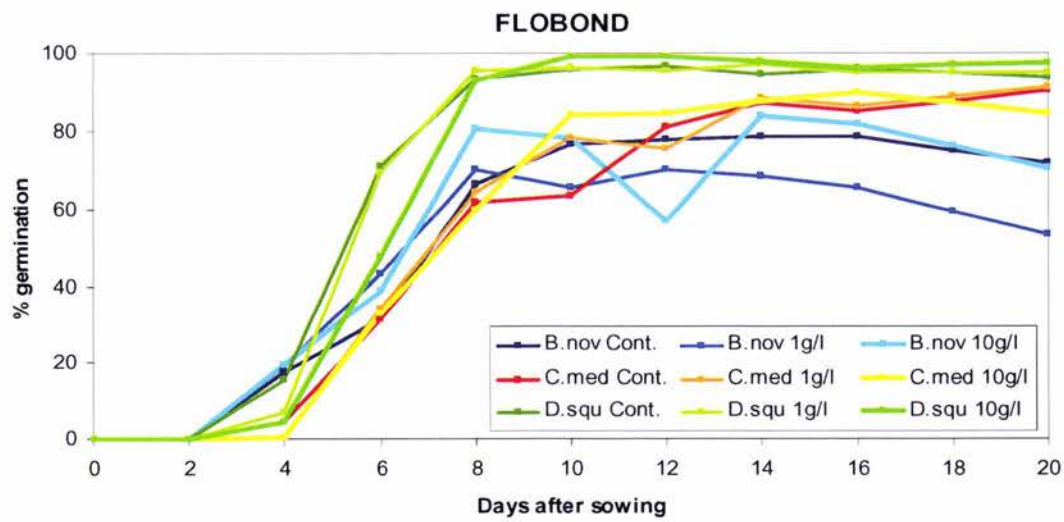


Figure 2.6B The effect of FLOBOND on the germination of *B. novae-zelandiae, C .medullaris and D .squarossa* spore.

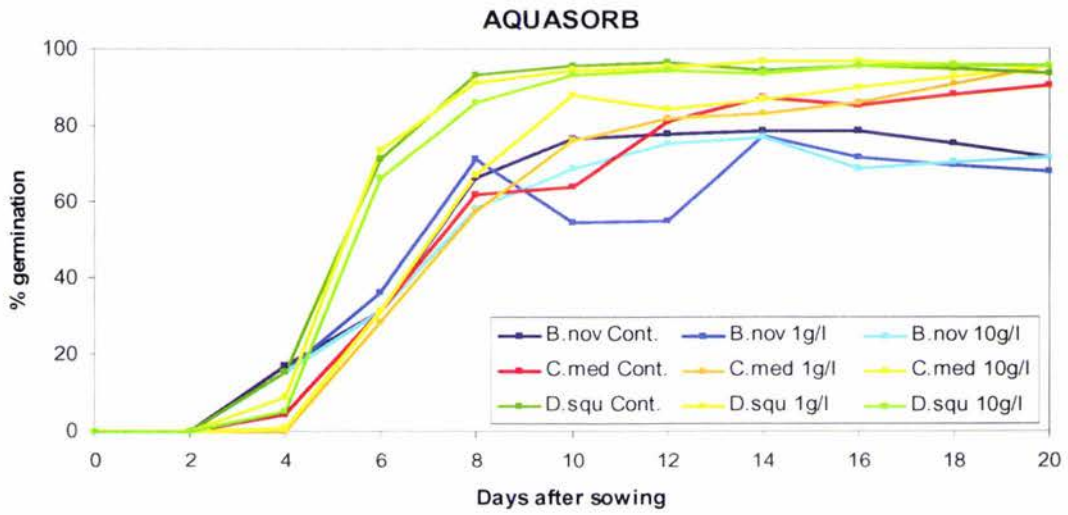


Fig 2.6c The effect of Aquasorb on the germination of *B. novae-zelandiae, C .medullaris and D .squarossa* spore.

2.3.1.5 Fluorescein diacetate spore viability test

The results for the fluorescein diacetate (FDA) test were inconclusive. In some cases the test appeared to work successfully. However, when repeated, varying results were obtained. Figure 2.7 shows a sample of several of the photos taken using the fluorescence microscope. When considering the three species together, viable and "reduced viability" spore treated with water only appeared to have no fluorescence. Therefore, it was possible to ascertain the background fluorescence, which in the case of these photos was minimal.

When FDA was added and left to incubate with the spore, several observations were made. Viable *B. novae-zelandiae* spore appeared to fluoresce, with some spores fluorescing more brightly than others. However, "reduced viability" spore also had a low and comparable level of fluorescence. Viable *C. medullaris* spore did not fluoresce at all when exposed to FDA. However, one spore seen in the *C. medullaris*, FDA treated, "reduced viability" section, fluoresced brightly (Fig. 2.7). FDA treated *D. squarossa* spore fluoresced regardless of whether it was viable or "reduced viability" spore. Several attempts of the FDA test failed to generate intelligible results, producing photos of FDA treated spore that had absolutely no fluorescence (data not shown).

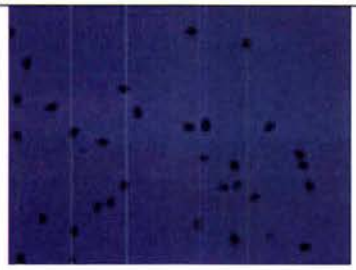
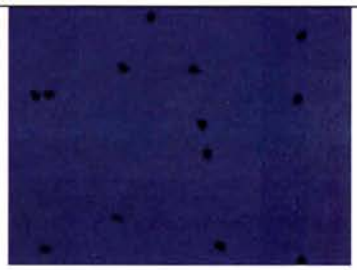
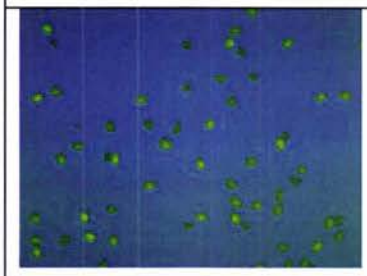
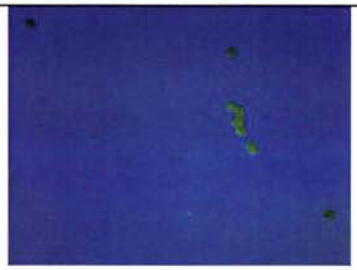
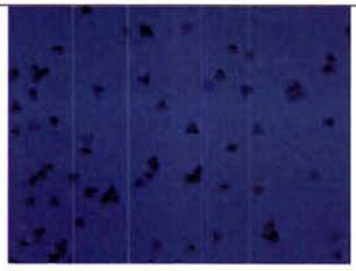
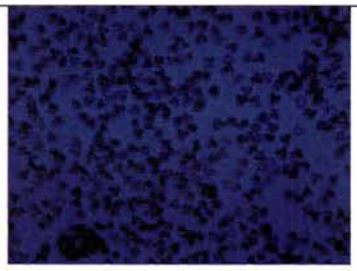
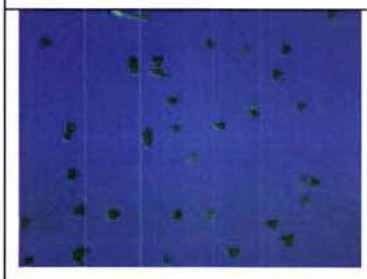
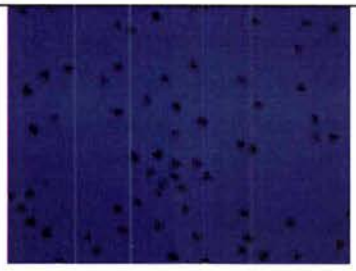
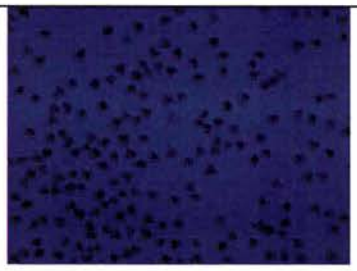
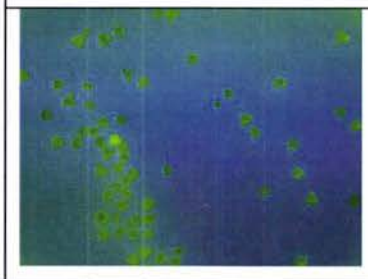
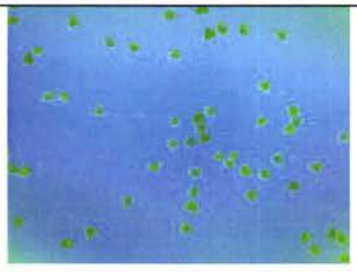
Species	Treatment	"Viable" spore	"Reduced viability" spore
B. nov.	Control (H_2O)		
	FDA		
C. med.	Control (H_2O)		
	FDA		
D. squ.	Control (H_2O)		
	FDA		

Figure 2.7 Photos of viable and "reduced viability" spore treated with water (controls) and fluorescein diacetate (FDA). *B. nov.* = *B. novae-zelandiae. C. med.* = *C. medullaris. D. squ.* = *D. squarossa.*

2.3.2 SEXUAL MATING SYSTEMS

2.3.2.1 Sporophyte production and gametophyte sexuality

Blechnum novae-zelandiae was successfully cultivated from spores, to sporophytes, under laboratory conditions (Fig. 2.8). The ability to produce sporophytes in the laboratory suggests that conditions were optimal for both antheridia and archegonia production. In addition, fertilization was able to be successfully induced by flooding gametophytes with sterile water. Antheridia (♂) and archegonia (♀) were identified using a compound microscope. In addition, the release of spermatozoids from a single antheridium was observed (Fig. 2.9). In one plate nearly the entire life cycle of *B. novae-zelandiae* was observed in 66 days (i.e. from spore to sporophyte).

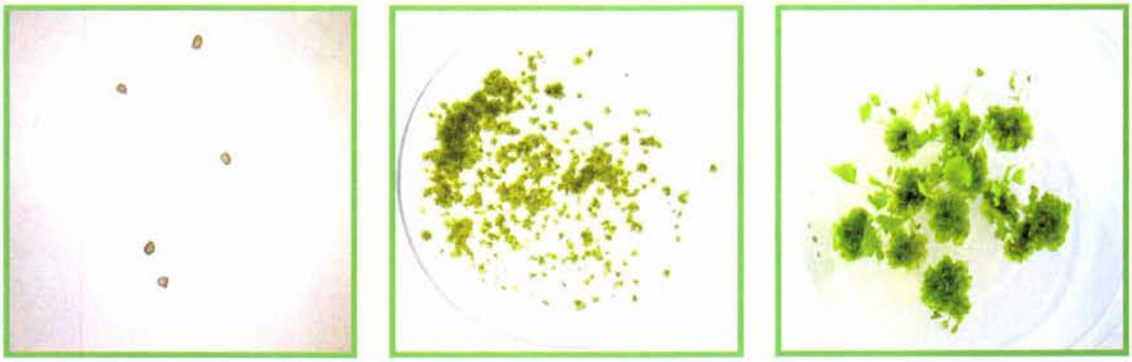


Figure 2.8 The progression of laboratory grown *Blechnum novae-zelandiae* spore (100x mag.), to gametophytes, and finally sporophytes.

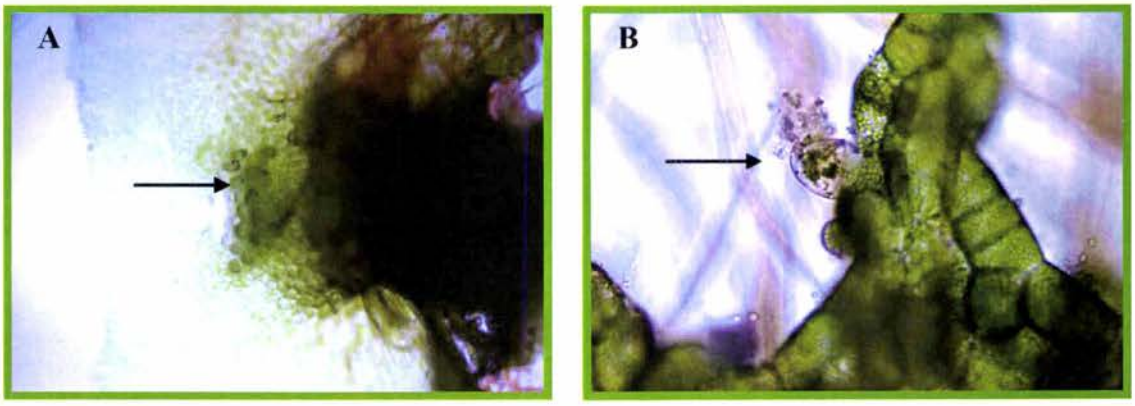


Figure 2.9 A. The arrow indicates the position of a cluster of *B. novae-zelandiae* archegonia next to the apical notch of the gametophyte (40x mag.). B. The arrow points to a single antheridium of the same species. Attached to the antheridium is a cluster of sperm (100x mag.).

Initial experiments suggested that *B. novae-zelandiae* was capable of intra-gametophytic selfing. One out of four isolated, presexual gametophytes successfully selfed and produced visible sporophytic structures (Fig. 2.10). It appears that several fertilization events occurred at the same time, as three sporophytic structures can be observed growing from the gametophyte.

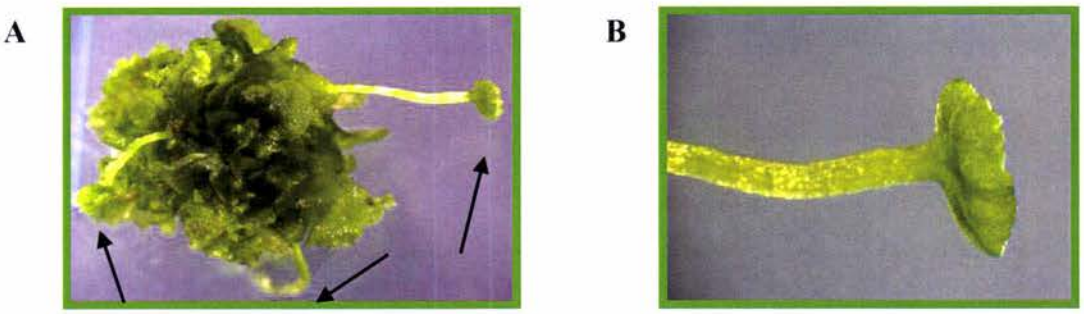


Figure 2.10 Intra-gametophytic selfing. A. The arrows indicate the position of three sporophytes (10x mag.). B. A close up view of one sporophyte (20x mag.).

In later experiments *B. novae-zelandiae* was found to be capable of both intra-gametophytic selfing and inter-gametophytic crossing, when grown in standard laboratory conditions (Fig. 2.11). A low proportion of gametophytes subjected to inter-gametophytic crossing, produced sporophytes. This type of mating was the most common. A lower proportion of gametophytes, subjected to intra-gametophytic selfing, produced sporophytes. Interestingly, gametophytes subjected to inter-gametophytic selfing did not produce any sporophytes. The data suggest a mixed mating system for *B. novae-zelandiae*.

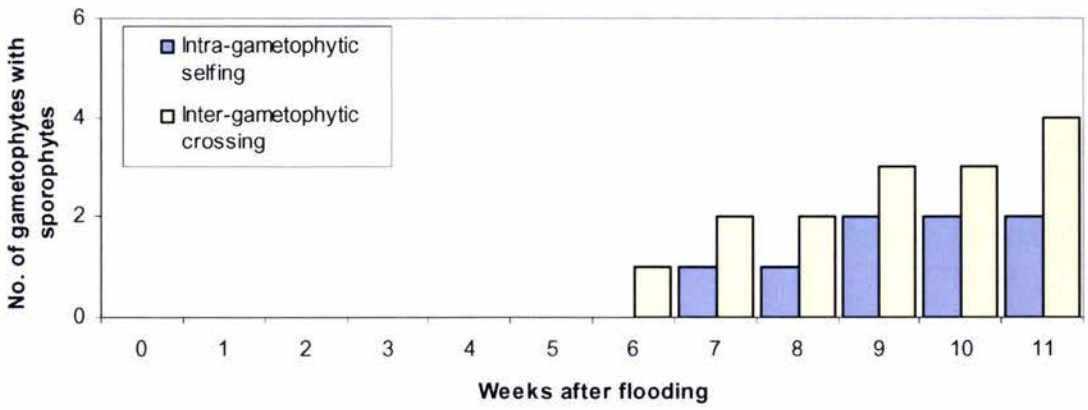


Figure 2.11 A comparison of potential mating systems in *B. novae-zelandiae*. Inter-gametophytic selfing was not observed over the 11 week experimental period.

2.3.2.2 Antheridiogen

The presence of antheridiogen was tested for in the species *Blechnum novae-zelandiae, Cyathea medullaris* and *Dicksonia squarossa*. It was expected that spore grown in the dark on media that originally supported mature gametophytes, would germinate if antheridiogen was present in the medium. After 10 days in the dark only *Blechnum novae-zelandiae* produced any germinants on the treatment plates (Fig. 2.12). No spore germinated on the dark grown control, suggesting that the germination observed on the treatment plates was due to the presence of a substance previously excreted by the original gametophytes. The number of *B. novae-zelandiae* spores that germinated on each treatment replicate plate varied from 0 to 7%. The emerging rhizoids observed on the treatment plates appeared dark and stunted compared to the rhizoids from the light grown gametophytes. Light grown spore indicated that the spore samples used in this experiment were viable.

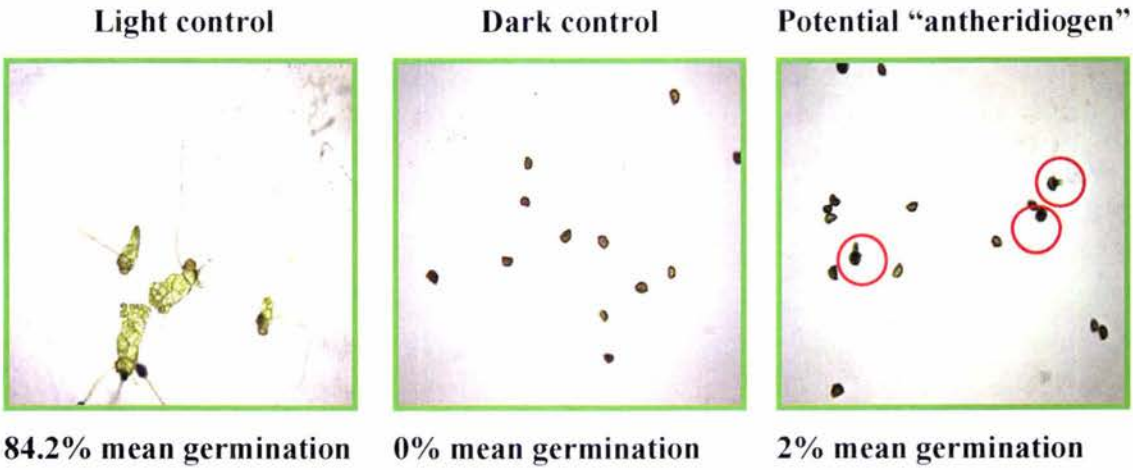


Figure 2.12 *B. novae-zelandiae* spore exhibiting the effects of 10 days exposure to light, dark and dark + "antheridiogen" (100x mag.). Red circles indicate spore with emerging rhizoids growing on plates that previously supported gametophyte growth. The mean amount of spore that germinated from 5 replicates is also shown.

Four days after the transfer of all plates to the light, plates were assessed again. Only *Blechnum novae-zelandiae* showed any sign of a possible antheridiogen effect. Large semi-chlorophyllus gametophytes were found on treatment plates (Fig. 2.13). It is possible that these gametophytes were overlooked during the initial assessment of the treatment plates due to the fact that there was only a small quantity. When compared to

light grown gametophytes of the same age, these treatment gametophytes were much larger. These gametophytes exhibited mature antheridia after only 14 days growth, and furthermore, produced active sperm when exposed to water. The germination of the initially dark grown controls confirmed the viability of the spore.

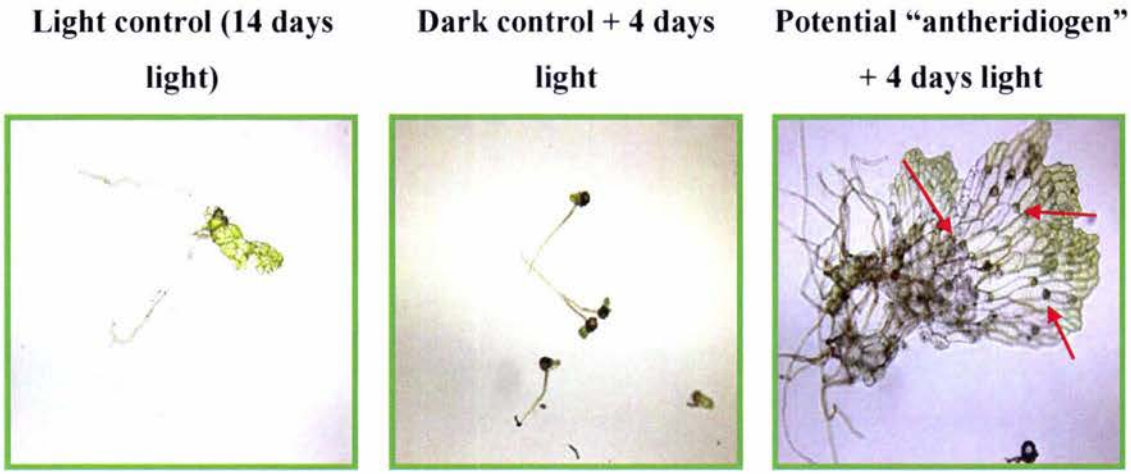


Figure 2.13 Observations of *B. novae-zelandiae* gametophytes 4 days after transfer to the light. The large semi-chlorophyllus gametophyte found on the potential "antheridiogen" plate bears multiple sperm producing antheridia (see arrows).

To further confirm the fact that a male-induction system was present in *B. novae-zelandiae*, the sexuality of gametophytes was recorded in light control, dark control and potential "antheridiogen" plates, 32 days after sowing (Table 2.1). The sex of gametophytes on the three potential "antheridiogen" replicates varied substantially from 2% - 76% male, indicating that the concentration of the substance causing male induction may have varied between the replicates.

Table 2.1 The mean % sexuality of 32 day old gametophytes, from light control, dark control and treatment plates.

	Gametophyte sex		
	% Male	% Female	% Presexual
Light control	0	0	100
Dark control	0	0	100
Potential "antheridiogen"	38.3	0	61.7

The ability of a mature gametophyte to influence the ontogeny of surrounding spore was assessed in *B. novae-zelandiae, C. medullaris* and *D. squarossa.* Similar to the results shown above, only *B. novae-zelandiae* showed any detectable response (Fig. 2.14). The morphology of the young 46 day old *B. novae-zelandiae* gametophytes, grown in the vicinity of the large mature gametophyte, appeared very different from that of the control. Control plates were grown without the influence of any mature gametophytes and exhibited a thalloid morphology, typical of young gametophytes. Interestingly, the morphology of *B. novae-zelandiae* gametophytes grown on the potential "antheridiogen" plates appeared filamentous. The filamentous morphology had also been observed previously for 32 day old male and presexual treatment gametophytes represented in Table 2.1. In addition, the total number of cells making up the treatment gametophytes was substantially smaller than those in control gametophytes of the same age. These results suggest that the ontogeny of the spore and developing treatment gametophytes was affected by the presence of the mature gametophytes.

When the sexuality of *B. novae-zelandiae, C. medullaris* and *D. squarossa* gametophytes was recorded, all *C. medullaris* and *D. squarossa* gametophytes were found to be presexual. Both control *B. novae-zelandiae* gametophytes and *B. novae-zelandiae* gametophytes grown on the potential "antheridiogen" plates exhibited a small mean percentage of male gametophytes (control = 2.2%, treatment = 1.4%). These levels were insignificant compared to the percentage of males recorded previously (Table 2.1).

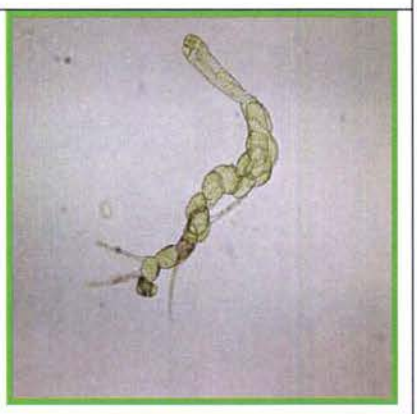
Species	Control	Treatment
B. novae-zelandiae		
C. medullaris		
D. squarossa		

Figure 2.14 Forty-six day old *B. novae-zelandiae, C. medullaris* and *D. squarossa* gametophytes, grown on fresh medium (control) or in the vicinity of single, mature gametophytes (treatment). All photos are taken at 100x magnification.

2.3.3 GAMETOPHYTE TRANSPLANTATION AND MACERATION

Gametophytes of *B. novae-zelandiae, C. medullaris* and *D. squarossa* were all able to survive a low level of maceration. Forty two days after maceration only one single *D. squarossa* whole gametophyte had died, due to contamination (data not shown). The survival of whole gametophytes indicated that transplantation did not affect the viability of gametophytes. After transplantation and maceration, gametophytes were able to continue normal growth (Fig. 2.15). A previous experiment also showed that macerated gametophytes were able to produce sporophytes (data not shown).

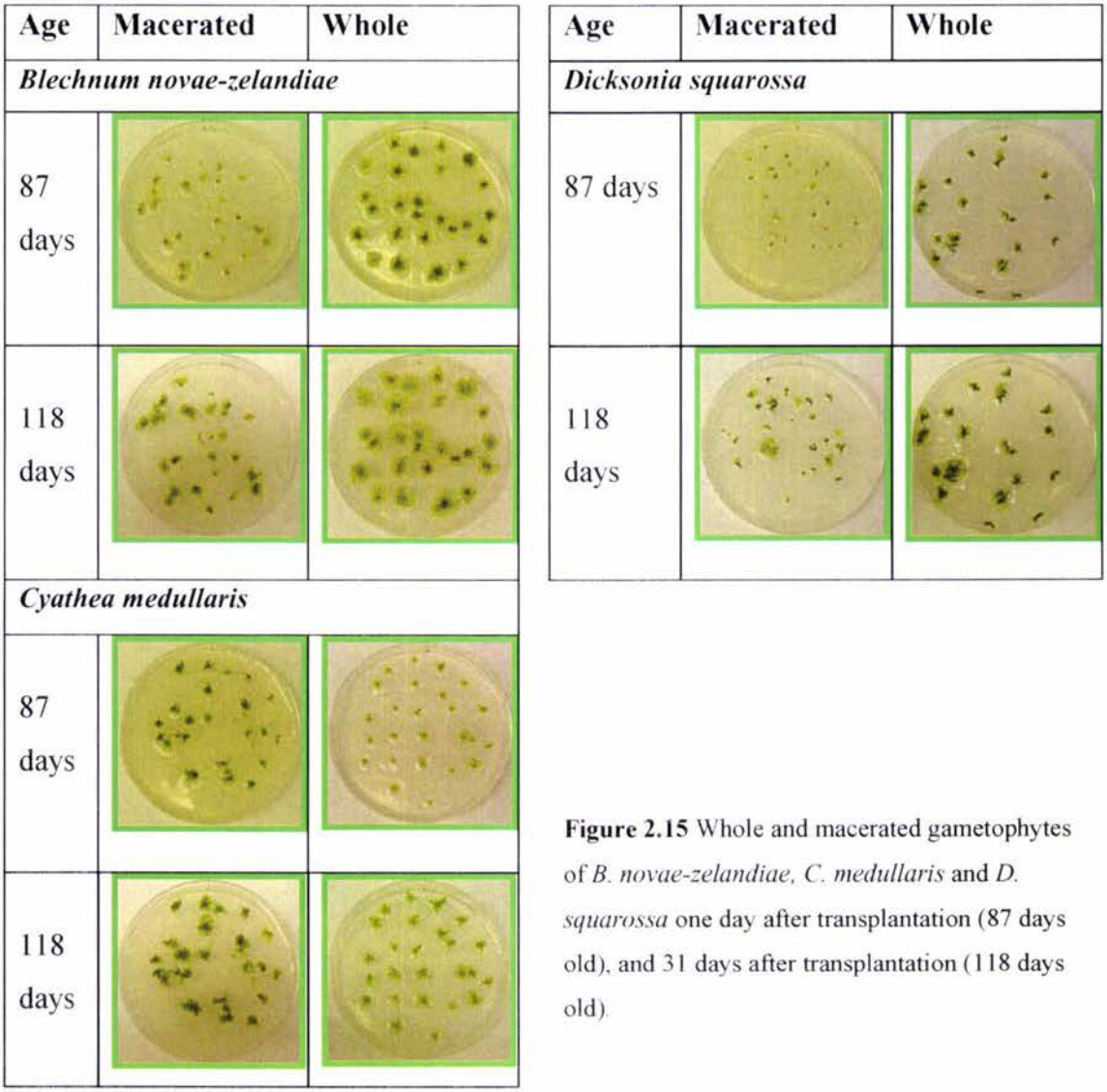


Figure 2.15 Whole and macerated gametophytes of *B. novae-zelandiae, C. medullaris* and *D. squarossa* one day after transplantation (87 days old), and 31 days after transplantation (118 days old).

2.3.4 ESTIMATING SPORE QUANTITY

The number of *B. novae-zelandiae, C. medullaris* and *D. squarossa* spores in a 1 ml volume was estimated by carrying out a serial dilution (Table 2.2). The estimated amount of dry *B. novae-zelandiae* spore in 1 ml was substantially less than the estimated amount of dry *C. medullaris* and *D. squarossa* spore. This can be explained by the difference in size of the spore, as a single *B. novae-zelandiae* appears slightly larger than a single spore of *C. medullaris* or *D. squarossa.*

Table 2.2 Estimated quantities of dry spore in a 1 ml volume.

B. novae-zelandiae	*C. medullaris*	*D. squarossa*
9.6×10^6	1.92×10^7	1.73×10^7

2.4 Discussion

Very little is known about the vegetative and sexual development of New Zealand native ferns, from the tiny spore, to the sexual gametophyte stage. Most of the current research relates to taxonomical issues, such as determining one species from another (Perrie et al., 2005). Previous research on New Zealand native ferns has also focused on the ability of several species to tolerate extreme environmental conditions, including frost and heat (Bannister & Smith, 1983; Bannister, 1984). The bulk of the literature that mentions New Zealand native ferns is associated with ecological studies (Chambers & Farrant, 1996; Heenan et al., 1999; Norton, 1999; Brockerhoff et al., 2003), and reveals little about the early development of individual species at a detailed level.

The flexible nature of this project has meant that several aspects of the life cycles of these native ferns could be analysed. The laboratory analysis of spore germination, gametophyte mating systems and gametophyte robustness in *Blechnum novae-zelandiae*, *Cyathea medullaris* and *Dicksonia squarossa* is original research, reported for the first time. The results of this research can be compared to research on species in other countries, where analysis of the early developmental of ferns has been researched.

2.4.1 SPORE GERMINATION

Spore germination is the initial step in the process of establishing a fern and is similar in many ways to seed germination. The emergence of a single rhizoid, or in some species the first chlorophyllus leaf, indicates that a spore has germinated (Pangua et al., 1994). Germination is commonly used to determine the relative viability of fern spore (Perez-Garcia et al., 1994). Accordingly, spore germination was used throughout this project to test the viability of freshly collected spore, as well as spore that had been treated in various ways. It is important to note that it is possible that some spore may also be dormant. This spore will not necessarily germinate, but is in fact viable. Germination curves were often used to show the results of a particular germination experiment. Each curve represented the first 20 days of spore incubation. This time period was chosen based on initial germination observations of the species of interest. All of the species

normally germinated within the first 6 days, suggesting 20 days was a sufficient amount of time. These results were consistent with the literature, which suggests that viable fern spores germinate within a week of being exposed to inductive conditions (Pangua et al., 1994; Simabukuro et al., 1998; Nondorf et al., 2003; Huang et al., 2004). Data were recorded for only 20 days because after this period non-germinated spore was often difficult to see, due to the increased gametophyte cover on the plates. Maximum germination was often achieved after 10 days in inductive conditions and could be visualized by a plateau in the germination curves (Fig. 2.3).

The spore surface sterilization process appeared to have no significant effect on the potential germination levels of the spore used in this project. However, Camloh (1999) reported that an increased rate of germination was obtained with non-sterilized *Platycerium bifurcatum* spore. These conflicting results could be explained by the variation in the methods used, as well as the fact that the two species of spore being sterilized are morphologically and perhaps physiologically different. Camloh (1999) also reported that the exine of immature spore was often damaged after treatment with hypochlorite. This was indeed observed when immature spores of *B. novae-zelandiae*, *C. medullaris* and *D. squarossa* were surface sterilized. It is also possible that sterilization may increase germination rate through the process of scarification, whereby the sterilization method increases the permeability of the spore wall. This was not observed, as there was no great difference between the germination of sterilized and non-sterilized spore. The effect of contamination on spore germination of non-sterilized replicates is unclear. It is possible that contamination may have affected the germination of the non-sterilized spore, resulting in germination levels lower than what could be expected. However, *Dicksonia squarossa* spore reached maximum germination levels close to 100%, regardless of sterilization (Fig. 2.2). This result suggests that non-sterilized spore of this species could conceivably reach its maximum germination potential, when growing in low levels of contamination.

In some cases the germination curves show a lot of variation between sampling dates. This can be observed for *B. novae-zelandiae* spore in Figures 2.6 A-C. This variation could have been a result of several factors, including sampling method and contamination. Sampling produced variation mainly due to the non-uniformity of spore on the replicate plates. The distribution of germinated and non-germinated spore varied

across a plate. Only sampling 50 spores per plate compounded this problem. In addition, there were also possibly small differences between the spore on replicate plates, including density and uniformity. Steps were taken to reduce the variation between plates including positioning plates in a non-biased fashion in the growth cabinet. When sowing spore, excess replicate plates were sown, as plates often became contaminated. Therefore, the problem of contamination could be managed.

Non-green spore germination initially requires water for imbibition, followed by a period of red light (Haupt, 1985). Green spores do not require imbibition as they already have high water content (Perez-Garcia et al., 1994). All spore used in this project was of the non-green type. In the laboratory freshly sown spore was incubated at 23°C, with humidity set at 75% and constant white light at an intensity of 40 – 60 $\mu mol\ m^{-2}\ s^{-1}$. These conditions were suitable for inducing spore germination. However, they differed greatly from realistic environmental conditions, and resulting gametophytes were extremely large and leafy. Spore was also incubated outside in the Massey fernery in order to test the effect of more natural, variable conditions on spore germination. The spore was subject to cool winter nights and failed to germinate. A similar result was observed for spore of *Cryptogramma crispa* when it was subjected to various low temperatures (Pangua et al., 1999). The delay in germination due to cold temperatures is thought to be a dormancy effect. Interestingly, when the spore in the Massey fernery was transferred to slightly warmer conditions a portion of the spore germinated. The degree of germination was lower than that of control spore grown in the laboratory, which suggests that some of the spore may have remained dormant (Fig. 2.3). These observations suggest the possibility that spore dispersed prior to winter, may enter into a dormancy period, until temperatures increase in the following spring. Therefore, temperature also appears to play a key role in fern spore germination.

Spore of *B. novae-zelandiae*, *C. medullaris* and *D. squarossa* all exhibited a photoblastic response (Fig. 2.3). This suggests that spore from these species absolutely requires light to germinate. Previous research suggests that fern spore is predominantly photoblastic and utilizes phytochrome to sense light quality (Tomizawa et al., 1983; Cooke et al., 1987; Haupt & Psaras, 1989). Figure 2.3A showed a higher maximum germination for *B. novae-zelandiae* spore initially given a 10 day dark period, in comparison to controls. Cooke et al. (1987) reported that an initial dark period of 7 to

10 days results in *Ceratopteris richardii* spore reaching maximum germination. Although a dark period was not employed for the majority of germination experiments in this project, it may be a useful technique to increase overall germination, especially in *B. novae-zelandiae* spore.

The germination potential of *B. novae-zelandiae*, *C. medullaris* and *D. squarossa* spore was assessed over a period of 6–7 months which included autumn, winter and spring. Over this period the fertile fronds of these three species changed dramatically. *B. novae-zelandiae* produces entire fronds dedicated to producing spore. In April they are green in colour. As they matured they become a dark brown colour. *C. medullaris* produces sori on the underside of its fronds. The immature *C. medullaris* spore was a light brown colour. As the spore matured it became darker until it was almost black. *D. squarossa* also produces sori on the underside of its fronds. Its spore was much lighter and initially appeared bright yellow. As it matured, the spore darkened slightly and became a light brown colour during the late winter months. As stated previously, spore germination is often used as a measurement of viability (Perez-Garcia et al. 1994) but fails to include dormant spores as being viable. It is unclear whether fern spore of these three species becomes dormant. In addition, the environmental stimulus that may alleviate spore dormancy in these species is also unknown. It is commonly recognized that some higher plant seeds of New Zealand natives require a period of cold weather, known as stratification, to alleviate dormancy (Fountain & Outred, 1991; Moore et al., 1994). *Blechnum novae-zelandiae* spore germination reached its maximum rate after the cold winter period (Fig. 2.5). It is possible that some of the spore sampled for this species prior to winter was dormant, explaining the low germination values in early samples.

Spore maturity also greatly influenced the early stages of the seasonal germination experiment. The variation in the early samples shown in Figure 2.5 can be explained by the sampling method. It is highly possible that the sampled sporophytes were at different stages of spore production and, therefore, a mix of immature and mature spore was obtained. In later samples the same plants had all developed further and were releasing only mature spore. The three species failed to show a marked decrease in spore viability near the end of sampling. However, field observations indicated that the amount of spore left on the sporophytes at the end of September was low. Sporophytes

of *B. novae-zelandiae*, *C. medullaris* and *D. squarossa* continued to shed viable spore over the season, and become spent prior to summer.

The effect of polyacrylamides and other soil stabilizing compounds on germination of various plant species had previously been researched (Morrey et al., 1983; Merlin et al., 1999). In some cases the phytotoxicity of the compounds detrimentally affected the germination and growth of the plants. However, most of the compounds tested had little to no effect. As soil science technology has become more advanced "phyto-friendly" soil stabilizers have been created. The effect of relatively modern soil stabilizers on the germination of fern spore had never been tested before. The three compounds Soilfix™, FLOBOND and Aquasorb were chosen due to availability. Soilfix™ is commonly used by the associated hydroseeding company (RST). Each of the three polyacrylamides had no effect on fern spore germination. However, it is unclear whether they could influence the growth of the resulting gametophytes, as this was not tested.

The Fluorescein diacetate test was adapted for use on fern spore from the FDA pollen viability test which is commonly used to elucidate the viability of pollen grains (Shivanna & Rangaswamy, 1992). It was suggested by Large & Braggins (1991) for use on native New Zealand fern spore. It is unclear exactly why the test gave inconsistent results. Controls were set up in order to establish that spore treated with only water (no FDA), and spore that had "reduced viability" (oven baked spore) would not fluoresce. These treatments acted as negative controls. As a positive control, portions of the spore that were tested were also sown on ½ MS, and the viability was measured by counting germination. It was noted that some spore may not germinate due to dormancy effects. As the results suggest the data were inconclusive (Fig. 2.7). The most surprising result is that a small portion of the oven baked control spore treated for 2 -3 hours at 90°C fluoresced. In addition, a low level of oven baked spore also retained the ability to germinate (data not shown). This in itself is an interesting observation in relation to the physiology of fern spore and its ability to withstand extreme conditions. The few photos that were analysed were difficult to interpret accurately as there was considerable variation between fluorescing spores. Some spores fluoresced brightly, whereas other spores had only small regions of fluorescence. A threshold level of fluorescence was chosen in order to obtain replicate counts from

different photos. However, the threshold level varied between different tests because all the photos were not taken at exactly the same exposure and light intensity. Determining the threshold level of fluorescence that constitutes a viable spore was also ambiguous. In some samples background fluorescence was very high and it was difficult to distinguish fluorescing spore from non-fluorescing spore. Often the test failed to produce any fluorescence, which suggested that the entire spore sample was non-viable. This was contradicted by the positive germination results. Germination curves were carried out for the spore shown in Figure 2.7. They failed to correlate with the results shown in the FDA test (data not shown). After numerous attempts it was decided that simple germination analysis was a suitable test for assessing spore viability, as the majority of the literature uses this method

2.4.2 SEXUAL MATING SYSTEMS

The success of much of the experimentation in this project relied on the ability to produce gametophytes, and sporophytes, in laboratory conditions. The native species *Blechnum novae-zelandiae* performed ideally and was easily manipulated. The tree ferns *Cyathea medullaris* and *Dicksonia squarossa* failed to produce any sporophytes in laboratory conditions. The speed at which the gametophytes of *B. novae-zelandiae* reached sexual maturity and produced sporophytes was comparable with other ferns with fast lifecycles. The species *Woodwardia virginica* has been shown to produce sporophytes in ~30 days (Fernandez et al., 1999). Ferns with slower life cycles (e.g. *Asplenium nidus*) can take at least 6 months to produce gametophytes with sporophytes from spore. As mentioned before it took only 66 days to produce *Blechnum novae-zelandiae* sporophytes from spore (Fig. 2.16).

Figure 2.16 Sporophytes of *Blechnum novae-zelandiae* (indicated by yellow arrow), produced only 66 days after sowing.

It was observed that transplantation affected the development of the gametophytes. Transplanted gametophytes grew a lot faster because there was less competition from other developing gametophytes. *B. novae-zelandiae* sexual organs were often difficult to identify on growing tissue. This was mainly due to the leafiness of the gametophytes. Antheridia were easier to identify than archegonia because they were more numerous, and active sperm could be visualised after adding water to the gametophytes.

Intra-gametophytic selfing was shown to exist in isolated *B. novae-zelandiae* gametophytes. Multiple fertilization events appeared to occur at the same time (Fig. 2.9). This is plausible because gametophytes contain a large number of antheridia and archegonia. Sperm is released after the gametophytes come in contact with water. A mass of sperm swim along a pheromonal gradient until they reach the archegonia. Haufler and Welling (1994) reported that sperm of the genus *Bommeria* are viable for only 4 – 8 minutes. *Blechnum novae-zelandiae* sperm was also observed to be active for a limited period of time, suggesting that multiple fertilization events are achieved quickly. The ability to carry out intra-gametophytic selfing promotes long-distance dispersal (Lott et al., 2003). Individual gametophytes of *B. novae-zelandiae* are able to establish at a great distance from the parent population, carry out intra-gametophytic selfing and establish a new population. The evidence of intra-gametophytic selfing in *B. novae-zelandiae* helps to explain its dominance in New Zealand and also promotes its use in hydroseeding and revegetation.

Blechnum novae-zelandiae also produced sporophytes via inter-gametophytic crossing. This system of reproduction is important for producing genetic variation within a population (Haufler & Welling, 1994). However, it is possible that what was interpreted as inter-gametophytic crossing could have in fact been intra-gametophytic selfing. The only way to positively determine which system was used would be to carry out genetic analysis on the sporophytes, as all sporophytes produced by intra-gametophytic selfing would be homozygous. This analysis was not an objective of this study.

No sporophytes were produced when gametophytes were subjected to conditions promoting inter-gametophytic selfing (Fig. 2.10). Verma (2001) suggested that some ferns may utilize a self-incompatibility mechanism. This mechanism would have to be specific to inter-gametophytic selfing for *B. novae-zelandiae*, as it was shown that this species is capable of selfing via intra-gametophytic selfing. The fact that no sporophytes were observed in inter-gametophytic selfing plates was probably due to other experimental factors, like the time of flooding. It is possible that the gametophytes had not produced active sexual organs at the time of flooding, and therefore, no sporophytes were produced. As stated earlier, the speed of gametophyte development varied between replicates, even if gametophytes were of the same age.

Blechnum novae-zelandiae was the only species to show characteristics of an antheridiogen male-inducing mating system. These characteristics included the ability of spore to germinate in the dark when exposed to antheridiogen, and the development of spore into male gametophytes when grown on medium that had previously supported the growth of mature gametophytes. The presence of antheridiogen is often determined by these very factors (Banks et al., 1993; Haufler & Welling, 1994; Wen et al., 1999). Within 14 days of sowing a large, semi-chlorophyllus, antheridia bearing gametophyte was observed on media that had previously supported mature gametophytes (Fig. 2.13). The spore had been initially kept in darkness for 10 days followed by a 4 day light period. The chlorophyllous cells appeared near the outer edge of the gametophyte. Therefore, it is plausible that the chlorophyll in these cells was manufactured in the four days of light. After a 3 week period the putative "antheridiogen" containing plates were shown to have a large proportion of male gametophytes when compared to controls. Taken together, these characteristics were indicative of antheridiogen influence.

Haufler & Welling (1994) concluded that the concentration of antheridiogen was related to the level of antheridiogen response observed. It is possible that the concentration of antheridiogen between *B. novae-zelandiae* replicates varied. This would explain the dramatic differences in dark spore-germination and male gametophyte development between replicates. The concentration levels in each plate were ultimately determined by the 50 mature gametophytes that had been previously grown on that particular medium. It is plausible to expect a difference, as it was commonly observed that gametophytes developed at different rates between replicates. The absence of any antheridiogen responses in *C. medullaris* and *D. squarossa* could perhaps be the result of a low concentration of antheridiogen, due to the immaturity of the gametophytes used.

The effect of a single mature gametophyte on the development of surrounding spore was also tested. The amount of antheridiogen produced from the single mature gametophytes was not enough to show any difference from control plots in terms of gametophyte sexuality. However, there was a marked effect on gametophyte morphology in *B. novae-zelandiae* replicates (Fig. 2.14). Gametophytes appeared filamentous compared to the controls. The mature gametophyte appeared to be influencing the morphology of the developing gametophytes, possibly via a diffusible signal. It is unclear if this response is related to antheridiogen, or to a completely different factor. Spiro et al. (2004) indicated that cytokinins were able to influence the morphology of developing *Ceratopteris richardii* gametophytes in dark conditions. The only way to test if antheridiogen is involved in this observation would be to isolate the *B. novae-zelandiae* antheridiogen compound and test its ability to influence the morphology of developing spore.

The discovery of a possible antheridiogen system in *B. novae-zelandiae* supports the earlier result that inter-gametophytic crossing is possibly an active mating system in this species. Using this system, a mature female gametophyte is able to stimulate surrounding spore in the spore bank, to develop as males (Haufler & Welling, 1994). This spore has the ability to germinate in the dark (under the soil) and produce active sperm rapidly. The results shown here suggest, for the first time, that a male-induction system is functional in *Blechnum novae-zelandiae*. It has been shown that a diffusible signal is able to induce dark germination, male development and influence gametophyte

morphology. The concentration of this signal seems to be a determining factor, in terms of the level of response seen in developing gametophytes.

Overall, the results from this section suggest that *B. novae-zelandiae* has a rapid life-cycle and utilises a mixed mating system, employing both intra-gametophytic selfing and an antheridiogen based outcrossing mechanism. This mixed mating system provides flexibility and partly explains why this species is so widespread and dominant in New Zealand. In addition, this information promotes *B. novae-zelandiae* as a key candidate for nursery and field experiments, as well as commercial revegetation projects.

2.4.3 GAMETOPHYTE TRANSPLANTATION AND MACERATION

The reason for carrying out these experiments was to ascertain whether gametophytes could be propagated *in vitro* and potentially be included in a hydroseeding mix. The hydroseeder could damage the gametophyte as a period of heavy mixing and high powered spraying is involved.

Fern gametophytes were found to be tolerant of low levels of maceration. After maceration, gametophytes were able to continue growing normally, indicated by sporophyte production in wounded tissue. Homogenization of gametophytes has previously been shown to be a useful way of increasing the amount of fern material *in vitro* (Janssens & Sepelie, 1989; Fernandez et al., 1993; 1999). The multiplication of gametophytic tissue, shown here via maceration and sub-culturing *in vitro*, provides a method for creating a large amount of hydroseedable fern material. These experiments positively promote the use of gametophytes for use in hydroseeding. The experiments described in this project used MS media as the substrate (Murashige & Skoog, 1962). In terms of commercial application, it would be beneficial to carry out more experiments using homogenized tissue on a natural substrate such as wood fibre or compost.

NURSERY EXPERIMENTS

3.1 Overview

The third chapter of this thesis will focus on various applications of fern material in a nursery environment. Nursery experiments were carried out to develop techniques for establishing native fern material in a more natural, but controlled environment. The success of applications of spore, laboratory propagated gametophytes, and rhizome segments were analyzed at three week intervals. All applications were mixed with a standard hydroseeding mix of paper fibre, tackifier, fertilizer and water. The nursery experiments specifically targeted practical applications of fern material.

3.2 Materials and Methods

3.2.1 NURSERY SITE PREPARATION AND EXPERIMENTAL DESIGN

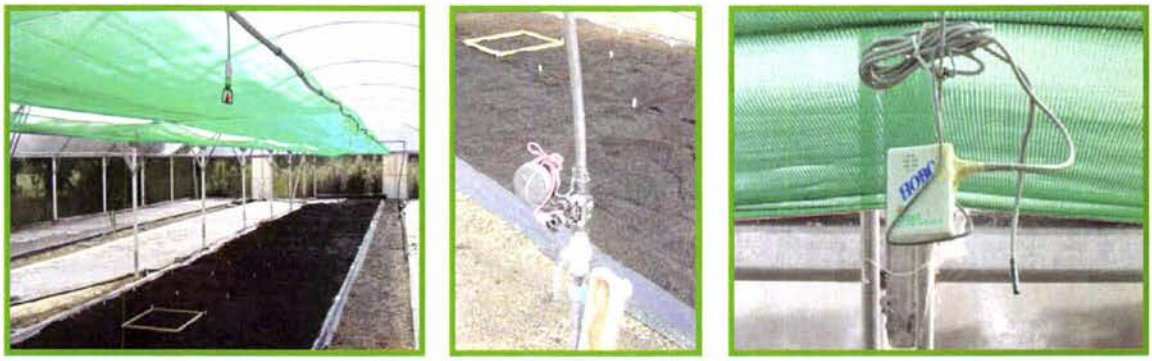
3.2.1.1 Nursery site preparation

Nursery experiments were established at Rural Supply Technologies' Newbury nursery, on the 23rd of March, the 30th of March and the 27th of April 2005. The Newbury nursery is situated about 10 km North of Palmerston North, NZ. Gametophyte experiments were established first followed by rhizome experiments, and finally spore experiments.

All experiments were conducted in shade house conditions utilizing sterile compost as the substrate. In order to keep weeds under control the shade house was initially sprayed with the herbicide glyphosphate 360, at the standard levels of 2 ml per litre of water. Fresh weed matting was then laid out prior to the spreading of sterile compost. Extra shade cloth was assembled over the plots to increase humidity and reduce the amount of light reaching the substrate to a rate equivalent to 5% full sunlight. Sterile compost was kept moist using a built in irrigation system supplemented with capillary matting. Irrigation was controlled by a Hunter ™ single station battery operated valve irrigation system. This system was programmed to irrigate for 10 min periods 9 times per day throughout the experimental period. The sprinklers produced a fine mist, which was advantageous as large droplets of water may have disturbed the contents of the plots and affected results. Two Hobo™ data loggers (Scott technical instruments, Hamilton) were positioned above the plots. Their function was to record temperature, light and humidity fluctuations over the entire nursery experiment period. Images have been included showing how the nursery was set up prior to application of the various treatments (Fig. 3.1).

Each nursery experiment consisted of several 0.5 m^2 plots, treated with different hydroseeding mixes. The ingredients of the standard hydroseeding mix included 5 g of Nitrophoska blue (12% N, 10% P, 10% K) fertilizer solubilized in water, 500 g of cellulose paper mulch, 1 ml of Soilfix™ polyacrylamide and 10 litres of water. Due to

the limited amount of propagative materials available (chiefly gametophytes) the complexity of the experiments was kept to a minimum, in order to incorporate a suitable number of replicates. All photographs in this chapter were taken using a hand-held Sanyo DSC-P8 digital still camera.



A B C

Figure 3.1 A. The layout of the nursery prior to application, including the position of the shade-cloth, irrigation, and the size of each plot (indicated by the 0.5 m^2 quadrat). **B.** The Hunter™ single station battery operated valve irrigation system. **C.** A Hobo™ data logger.

3.2.1.2 Gametophyte propagation and establishment

Gametophytes of *Blechnum novae-zelandiae, Blechnum discolour* and *Blechnum colensoi* were used in this experiment. These species were used because sufficient amounts of gametophytic material had been produced for each of these species in the laboratory, using sub-culturing methods. Information on the age of the gametophytes used and their collection dates and sites were recorded (Table 3.1).

Table 3.1 Information on the propagation of gametophytes of. *Blechnum novae-zelandiae* (*B. nov.*), *Blechnum discolour* (*B. dis.*) and *Blechnum colensoi* (*B. col.*) for nursery experiments.

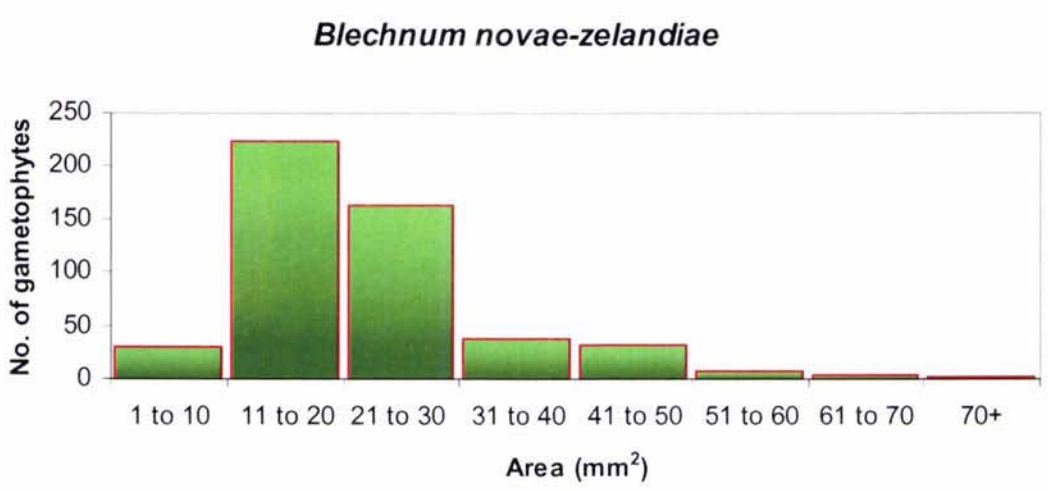
Species	Collection date	Collection site	Sowing date	No. of times sub-cultured	Age upon application
B. nov.	3rd Aug 04	South Range Rd	8th Aug 04	3	229 days
B. nov.	3rd Aug 04	South Range Rd	29th Sep 04	3	177 days
B. dis.	19th Sep 04	Buller Gorge	29 Sep 04	2	177 days
B. col.	19th Sep 04	Buller Gorge	29 Sep 04	2	177 days

This experiment required 12 plots, which included three replicates for each species and three control plots. The hydroseeding mix for each plot was made up in two buckets with half the contents being allocated to each bucket. This was done in order to facilitate the spreading of the mixture over the plot. Each half bucket consisted of 5 litres of water, 250 g mulch, 0.5 ml of Soilfix™ polyacrylamide, 2.5 g fertilizer (dissolved in water) and 83 gametophytes. Initially buckets were filled with water and the mulch was added. Large clumps of mulch were broken up by hand. The 2.5 g of fertilizer had been previously solubilized in water in order to obtain a more even distribution across the plot. The fertilizer was added, followed by the polyacrylamide, and finally the gametophytes.

Prior to the experiment, all of the gametophytes were measured and chosen for inclusion with respect to their health and size (Fig. 3.2). Gametophytes were excluded from the experiment if they exhibited necrotic regions. After adding the gametophytes the mixture was stirred with a stirring rod. The area where the plot was to be established was levelled using a rake. A 0.5 m^2 quadrat was laid on the sterile compost in the position randomly allocated for that specific plot. The hydroseeding mixture was poured out into the space confined in the quadrat. Care was taken in order to completely cover the area with the mix. After spreading had been completed the plot was labelled and recorded on a map (Fig. 3.5).

Every three weeks the gametophyte survival rate was recorded for each plot, as well as the presence or absence of sporophytes. Despite efforts to reduce the invasion of weed species (using sterile compost and pre-spraying of the weeds in the shade house) plots became infested with weeds quickly, presumably from wind-borne seed. A sheet of plastic was erected around the plots to impede wind blown seeds. Hand weeding was carried out frequently across the entire duration of the experiment. Toothpicks were inserted to mark each surviving gametophyte once it was realized that weeds were going to be a problem. They also aided sampling as the same gametophytes were more easily identified at each sampling date. If a gametophyte had died between sampling dates the toothpick was simply removed and the gametophyte was not counted. The presence or absence of sporophytes was also noted for each gametophyte observed. The experiment was terminated after 24 weeks on 7th September 2005.

A



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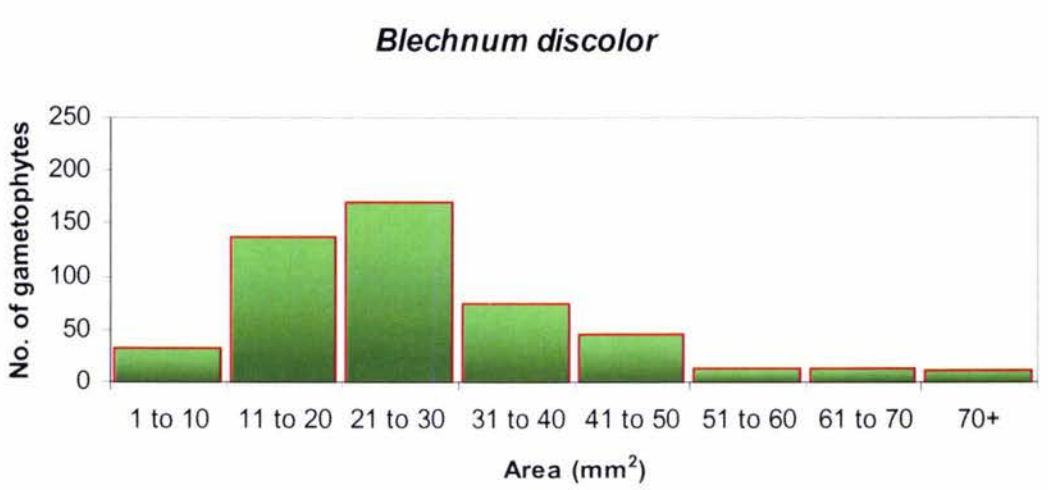

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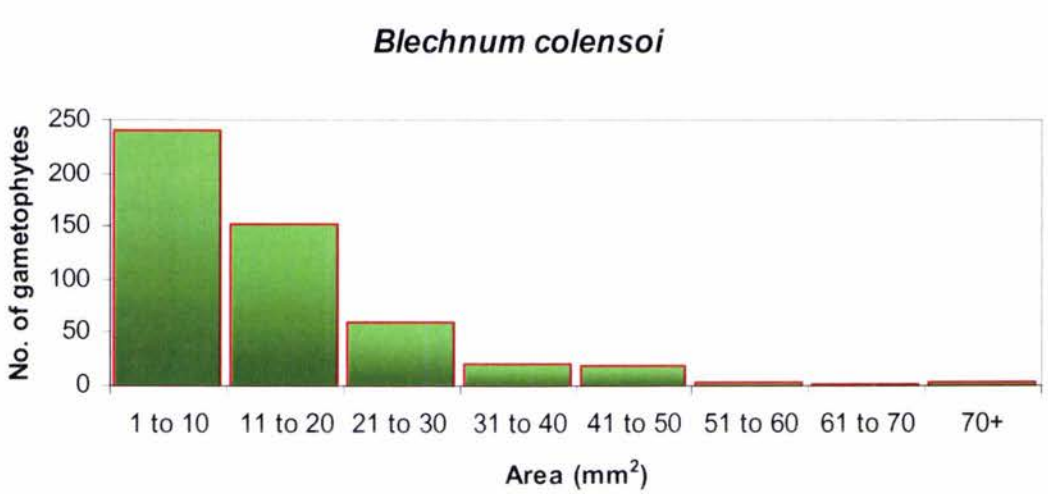


Figure 3.2 The distribution of size (length x width) of 500 laboratory propagated *B. novae-zelandiae*, *B. discolor* and *B. colensoi* gametophytes, prior to inclusion in the nursery gametophyte experiments. The size distribution is significantly different between species ($P < 0.05$).

3.2.1.3 Rhizome establishment

Six *Blechnum novae-zelandiae* plants were collected from banks on South Range Road (Fig. 2.1, site 5) and transported to the nursery. At the nursery the fronds were removed from the rhizomes along with the mass of roots. The rhizomes were then sliced into 2 cm segments in preparation for planting in the plots. Hydroseeding mixes were prepared in half bucket portions in order to facilitate spreading of the mixture from the bucket onto the sterile compost. Each half portion included 250 g of paper mulch, 0.5 ml of Soilfix™ polyacrylamide, 2.5 g of fertilizer and an average of 143 g of rhizome material. The mixes were spread within a 0.5 m^2 quadrat. Three replicate plots were poured along with a control plot (Fig. 3.3).

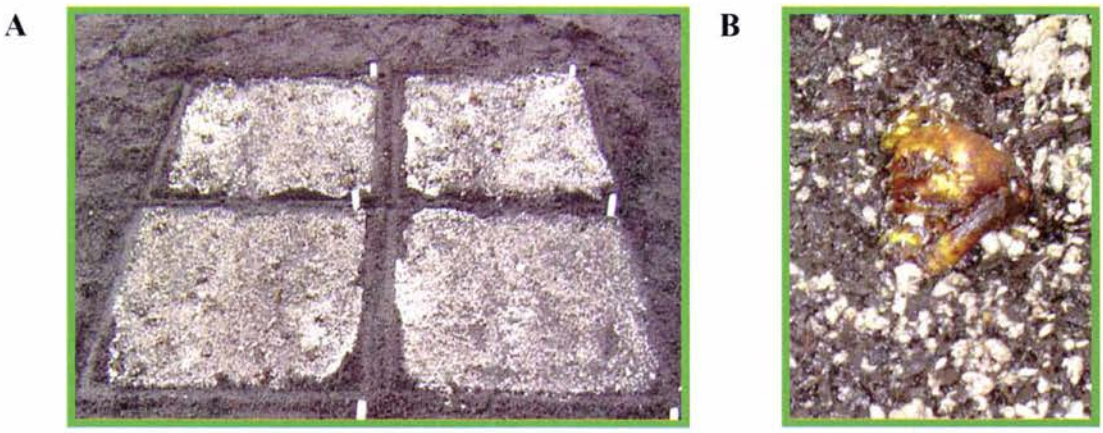


Figure 3.3 A. The three replicate rhizome plots with the control plot situated in the bottom right hand corner of the photograph. **B**. A close up photograph of one rhizome segment.

Rhizomes were assessed every three weeks for viability by assessing the number of rhizome segments with shoots. The length of each shoot was also measured and recorded at each sample date throughout the 27 week period. Plots were hand weeded periodically. Photos of the *B. novae-zelandiae* rhizome root system were taken on the 5th Oct 2005, at the conclusion of the experiment.

3.2.1.4 Spore establishment

The application of the spore experiment was subject to the availability of mature spore in the Manawatu district. Spore bearing fronds of *Blechnum novae-*zelandiae, *Cyathea medullaris* and *Dicksonia squarossa* were collected on the 23rd of April 2005. Care was

taken to include spore from several different plants in order to prevent any possible self-incompatibility problems. Spore selected for use in the nursery experiments was also taken to the laboratory in order to test its germination potential.

Twelve plot sites were chosen at random and prepared for application (Fig. 3.5). Each species was represented in three replicates. Three control plots were also included. Prior to application of the mix, the plots were weeded and raked. In keeping with the standard application procedure, half portions of each mix were made up for each plot, in order to facilitate spreading of the mixture. Initially buckets were filled with 5 L of water and 250 g of cellulose paper mulch. After physically breaking up the mulch by hand 0.5 ml of Soilfix™ polyacrylamide was added along with 2.5 g of water solubilized nitrophoska fertilizer. To each bucket 0.1ml of dry spore was added (see Table 2.2 for spore quantity estimate). The contents of each bucket were thoroughly mixed with a mixing rod in order to distribute the ingredients evenly. The contents of the two buckets were then poured over the ½ m^2 plot. Once the mix had settled on the sterile compost the quadrat was removed and rinsed clean. The mixing rod was rinsed between applications in order to minimize the chances of cross contamination between plots. The plots were organised into sub plots in order to facilitate sampling. The design and dimensions of a plot and its sub plots are shown in Figure 3.4.

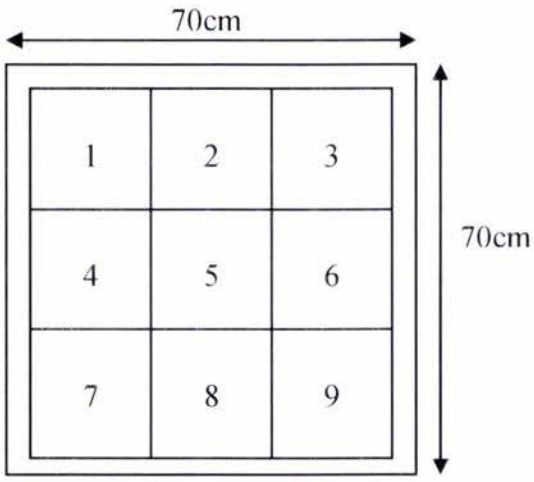


Figure 3.4 A diagrammatical representation of a single 0.5 m^2 plot. The nine regions represent 22 cm x 22 cm sub plots. The grey region represents a 2 cm border around the entire plot.

A border region of 2 cm was included around each plot in order to reduce edge effects. Every three weeks an equivalent sub plot was examined and excavated from each

replicate plot. Samples were scrutinized for the presence of gametophytes or other adventive species. Once individual sample specimens were isolated they were mounted on slides and photographed on a Leica MZ12 stereo microscope using dark-field microscopy and a mounted digital camera.

Photographs of adventive species were also taken with the Leica MZ12 stereo microscope using dark-field microscope (Fig. 3.18). The only exception was the photograph of the green filamentous algae in the same figure, which was taken with an Olympus BX51 compound microscope.

3.2.2 STATISTICAL DESIGN AND ANALYSES

A randomized block design was used to determine the sampling locations for each specific plot for the three nursery experiments (Fig. 3.5). The spore experiment plots were divided into sub plots (Fig. 3.4), with successive sub plots sampled at random, every three weeks.

Data are presented as graphs using either mean data or percentage data. Raw data have also been included where appropriate (e.g. Fig. 3.12 & Table 3.2). Standard errors have been included in some figures to indicate variation between replicates. In some experiments ANOVA has been used to determine whether data is significantly different between species and treatments. All treatments were tested for significant difference at $P < 0.05$.

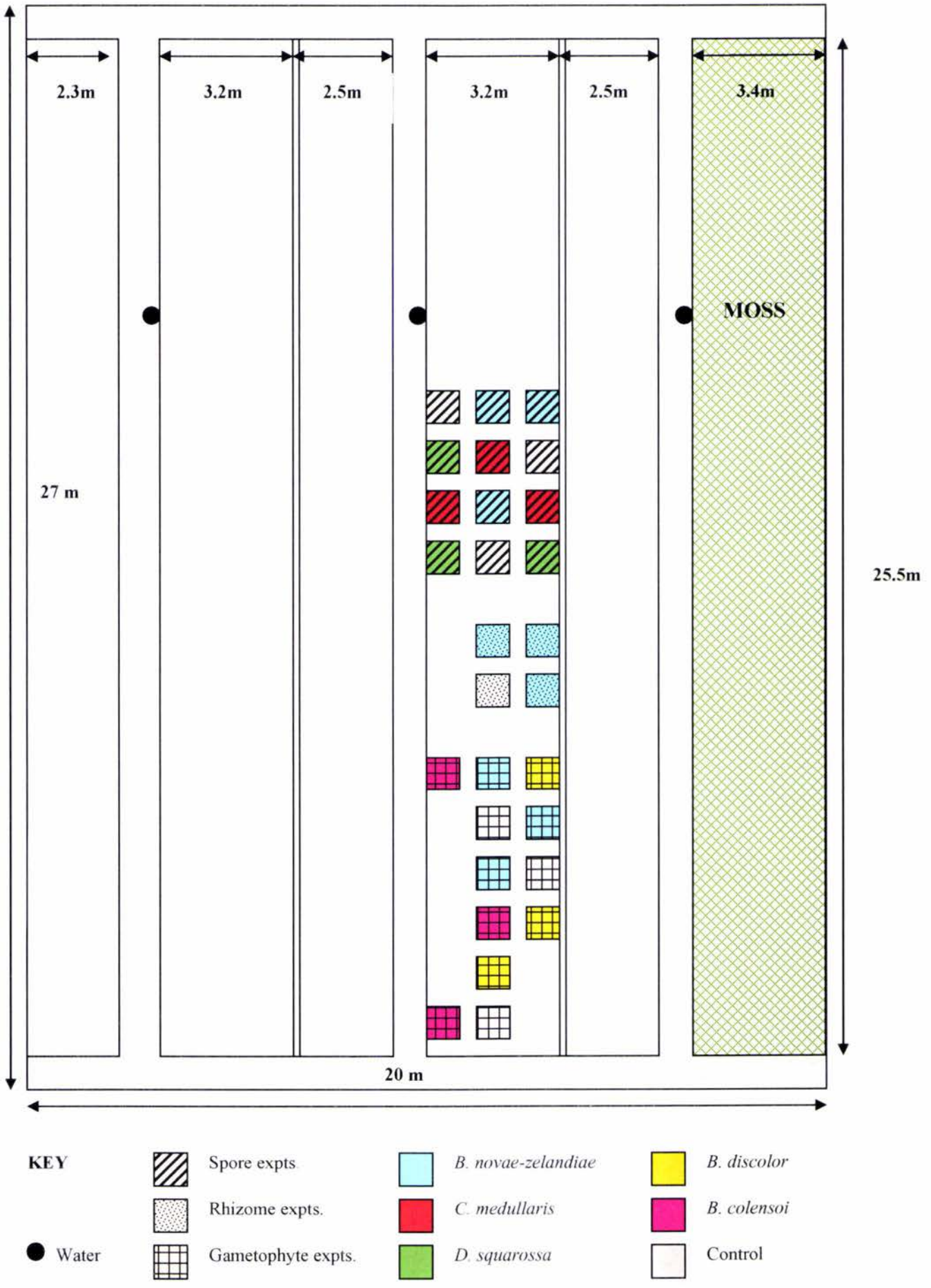


Figure 3.5 A diagrammatic map of the Newbury shade-house showing experiments and individual plots.

3.3 Results

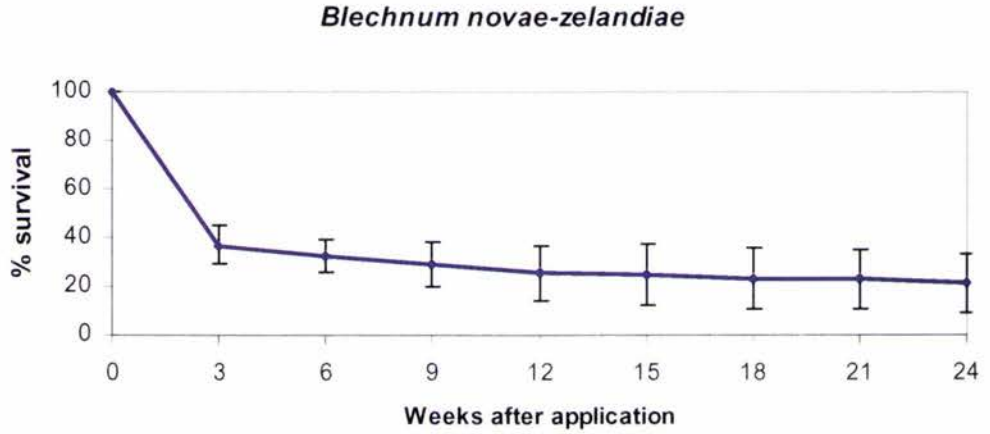
3.3.1 GAMETOPHYTE EXPERIMENTS

3.3.1.1 Gametophyte survival

Laboratory propagated gametophytes of *Blechnum novae-zelandiae, B. discolor* and *B. colensoi* were assessed for their ability to survive in nursery conditions. The propagated gametophytes were of varying age and health prior to application (Table 3.1). It was also apparent that gametophytes that had been sub-cultured many times had reduced vigour. *B. discolor* gametophytes appeared the most vigorous followed by *B. colensoi* gametophytes and *B. novae-zelandiae* gametophytes. However, *B. colensoi* gametophytes were very small compared to the other two species. All three species showed a dramatic decline in survival during the initial three weeks of the experiment (Fig. 3.6).

Blechnum novae-zelandiae gametophytes showed a continuous decrease in survival. After 24 weeks the mean survival rate of *B. novae-zelandiae* gametophytes was 21%. There was considerable variation between survival rates in replicate plots of *B. novae-zelandiae* gametophytes. *Blechnum discolor* showed a slight increase in gametophyte survival after the initial decrease. This was possible because some gametophytes had been missed in the initial counts due to being covered by sterile compost and paper fibre. After 24 weeks, *B. discolor* had a mean survival rate of 50%, which was the highest of the three species. Variation between plots of *B. discolor* gametophytes was evident throughout the experiment. *Blechnum colensoi* survival rate produced a curve that levelled off at a mean of 12% survival after 24 weeks. This was the lowest overall survival rate of the three species. This curve also showed the least variation between replicates. ANOVA revealed that the survival rate data was significantly different between the three species. Control plots failed to produce any gametophytes, suggesting that the experiment was not affected by any outside influence. Taken together, these data indicate that there was variation in gametophyte survival between replicates, and between the three different species. In all cases, a substantial proportion of gametophytes did not survive the application process.

A



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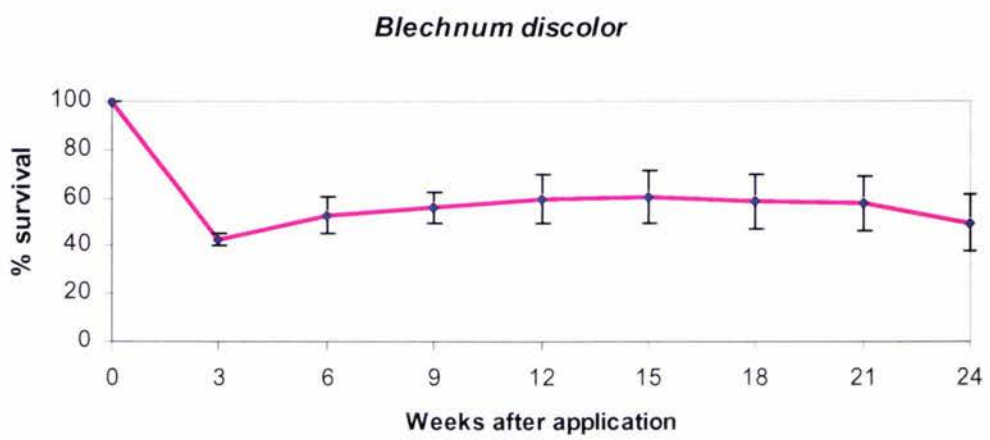

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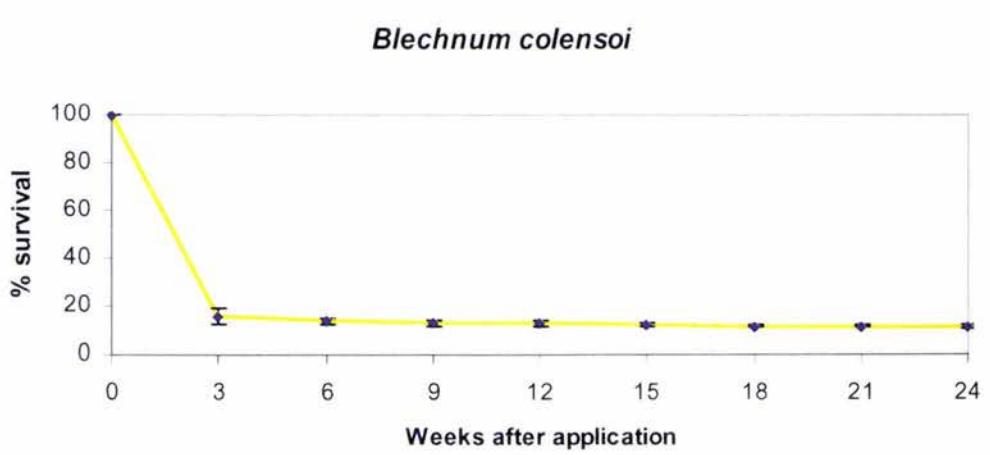


Figure 3.6 Survival of laboratory propagated *B. novae-zelandiae*, *B. discolor* and *B. colensoi* gametophytes, in replicated nursery plots, over a period of 24 weeks.

3.3.1.2 Sporophyte production on surviving gametophytes

Sporophyte production was also used to measure the success of this experiment. Figure 3.7 shows the sequential development of a *Blechnum novae-zelandiae* sporophyte, from the initial application, to a healthy 24 week old sporophyte.

Recently applied gametophytes (0 weeks after application) | **Very young sporophytes (18 weeks after application)** | **A young sporophyte (24 weeks after application)**

Figure 3.7 Photographs showing from left to right, the development of *Blechnum novae-zelandiae* sporophytes, on gametophytes that had been previously cultured in the laboratory.

All three species successfully produced sporophytes. However, there was a difference in the number of sporophytes produced between species (Fig. 3.8). All species began producing sporophytes between 6 – 9 weeks after the initial application. *Blechnum novae-zelandiae* had on average the highest number of sporophyte bearing gametophytes per replicate after 24 weeks. *Blechnum discolor* plots produced an average of 11 gametophytes with sporophytes per replicate plot. *Blechnum colensoi* only produced an average of 3 gametophytes with sporophytes per plot, after 24 weeks. There was a large amount of variation between replicate plots for each species. The sporophyte production data was significantly different between species as indicated by ANOVA.

It was also interesting to compare the proportion of surviving gametophytes that had produced sporophytes, for each species (Fig. 3.9). *Blechnum novae-zelandiae* produced the highest average proportion of sporophyte bearing gametophytes at 41% after 24 weeks. *Blechnum discolor* and *B. colensoi* had similar proportions of sporophyte bearing gametophytes, at 11% and 18% respectively after 24 weeks. ANOVA revealed

that the species data was significantly different. The standard error bars indicate that there was a large amount of variation between replicate *B. novae*-zelandiae and *B. colensoi* plots. When compared to the gametophyte survival rates of each species (Fig. 3.6), it is interesting to note that even though *B. discolor* had the highest number of viable gametophytes, these gametophytes failed to produce the most sporophytes. The trend of the graph (Fig. 3.9) suggests that the proportion of sporophyte bearing gametophytes was still increasing at the end of the experiment, for all species. This trend is supported by the observation that un-fertilized gametophytes often became necrotic, whereas fertilized gametophytes, with sporophytes, survived.

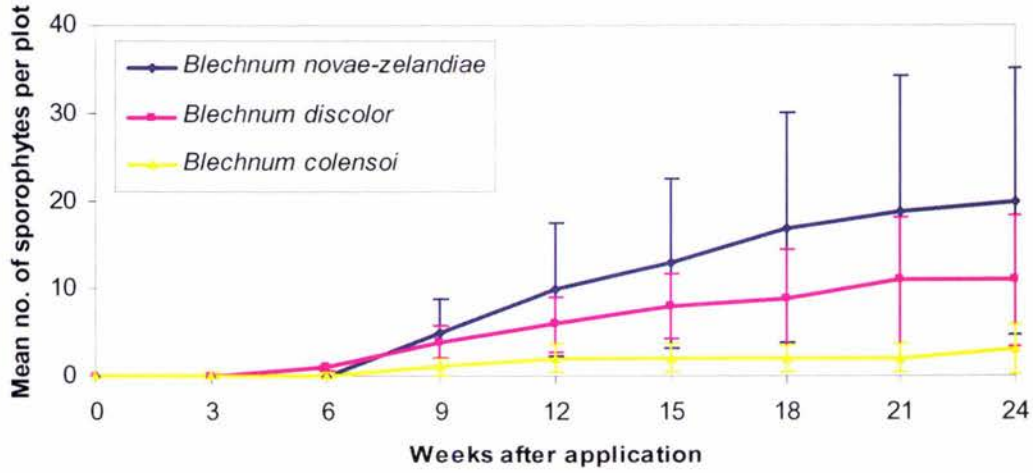


Figure 3.8 The mean number of gametophytes with sporophytes per replicate recorded for each species at each time point.

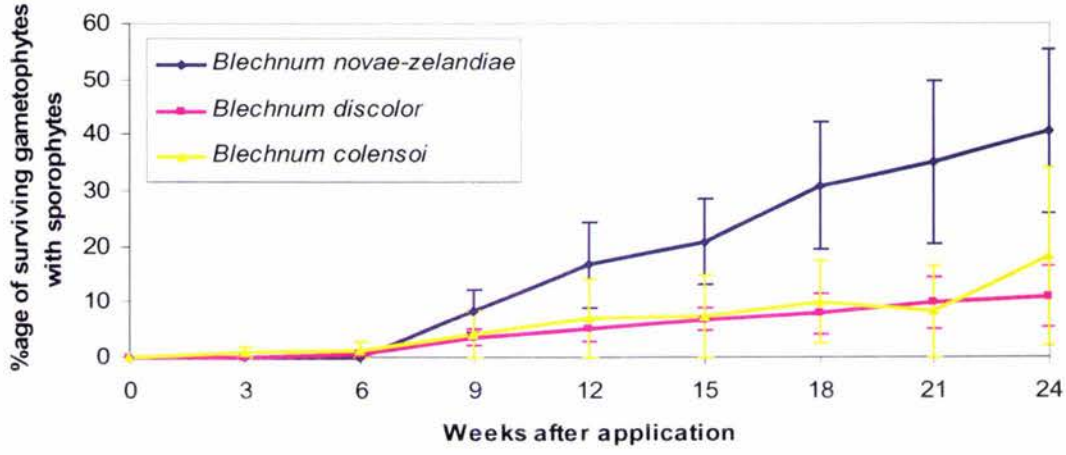


Figure 3.9 The number of gametophytes with sporophytes as a proportion of the total number of surviving gametophytes at each time point.

3.3.2 RHIZOME EXPERIMENTS

3.3.2.1 Shoot emergence and growth

Segmented rhizome pieces were assessed for their ability to produce shoots. From the initial 6 plants used in this experiment, 34 individual plants were successfully established. Figure 3.10 shows the sequential development of a rhizome segment, from submergence in the initial hydroseeding mix, through to establishment of shoots.

Figure 3.10 The sequential development of *B. novae-zelandiae* shoots on chopped rhizome segments.

Figure 3.11 shows the period of time that was required for the rhizome segments to produce their first shoots and indicate their viability. Overall, 45% of the 75 rhizome segments were viable. Two rhizome segments produced shoots within the first three weeks after the hydroseeding/rhizome mixture was applied to plots. Different rhizome segments continued to produce their first shoots up until week 24. The percentage of viable rhizome segments did not increase greatly after week 18, indicating that the majority of viable rhizome segments had produced their first shoots by this time.

The total shoot length for each replicate plot was recorded at each three week interval. Shoot length provided a way of measuring the growth rate of the rhizome shoots. Figure 3.12 shows the mean shoot length of the three replicates. Shoot length gradually increased throughout the duration of the experiment. The standard error bars shown indicate that there was some variation between replicate plots. However, this difference was not significant, as indicated by ANOVA. In the last 6 weeks of the project the length of the shoots doubled. It would be expected that the shoots would continue to

grow, until they reached their maximum size. This would presumably result in the curve reaching a plateau. Due to the time restraints on this project measurements were carried out for 27 weeks.

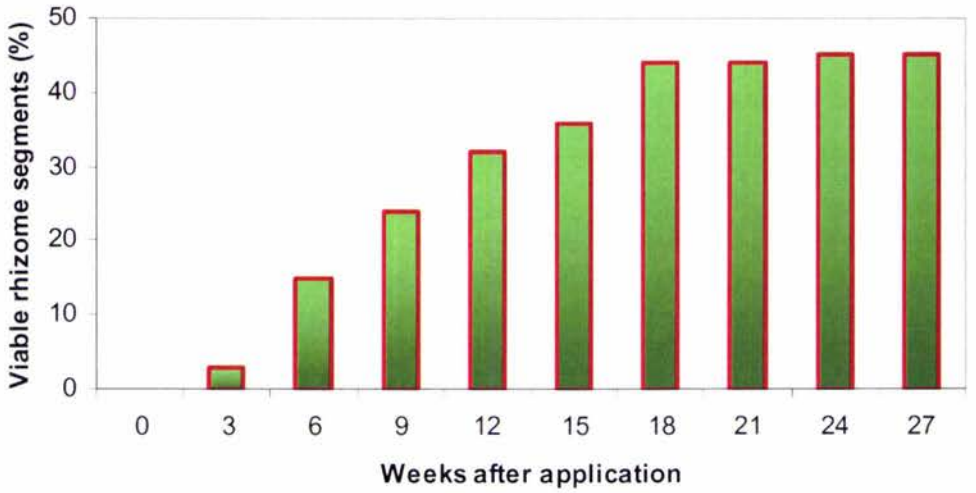


Figure 3.11 Percentage viable rhizome segments produced over time and assessed by production of the first shoots.

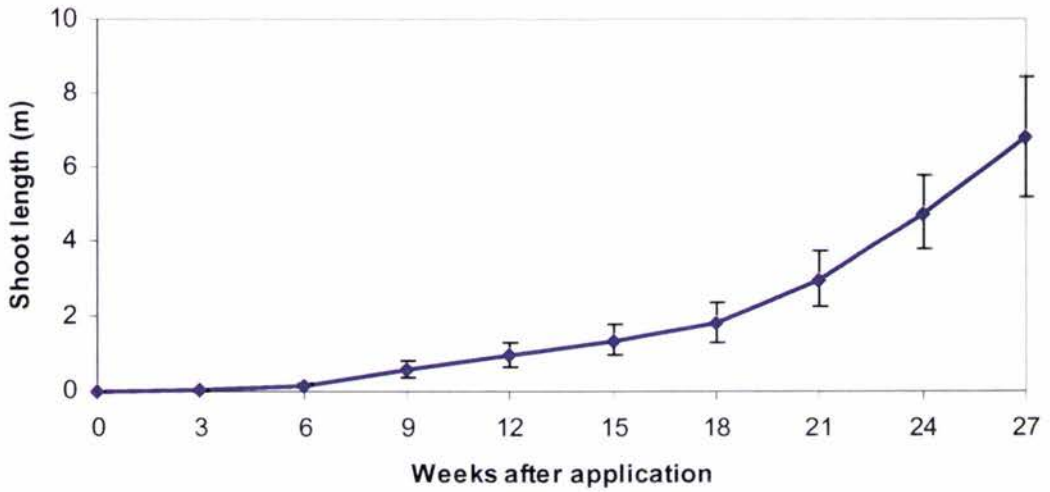


Figure 3.12 The total length of *Blechnum novae-zelandiae* rhizome shoots (m) recorded every 3 weeks for 27 weeks.

3.3.2.2 The rhizome root system

At the conclusion of the rhizome experiment the root system of the rhizome segments was uprooted and analyzed. Figure 3.13 shows the positions of roots, shoots and young fronds on differentially orientated rhizome segments. Rhizome A is positioned in the same orientation as a "normal" rhizome. Roots emerge form the basal surface of the rhizome. One end of the segment is committed to initiating the development of young fronds. Rhizome B is positioned in the opposite orientation to rhizome A. These rhizomes similarly produced roots on their basal surface. They often had more than one active meristem. Interestingly, neither shoots nor roots were initiated from the cut surfaces.

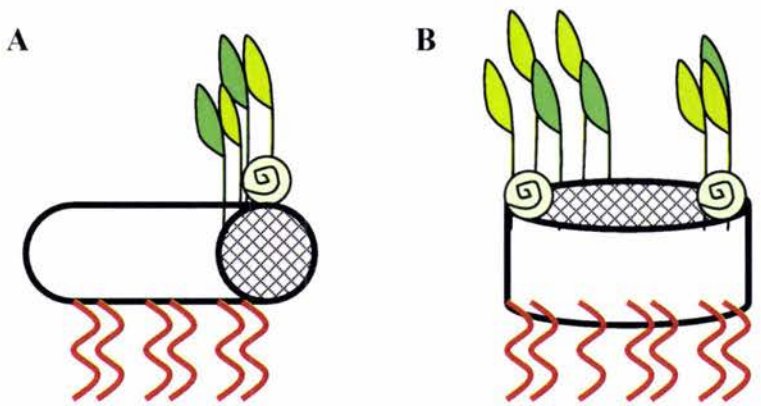


Figure 3.13 A & B show a diagrammatic representation of the position of roots, shoots and young fronds on chopped rhizome segments of different orientations. The cross-linked pattern represents the cut surface.

The extent of the root system for the rhizome segments was also photographed (Fig. 3.14). The photographs show that the rhizomes produced a thick mat of long roots, which provided anchorage for the plant and increased stability of the substrate.

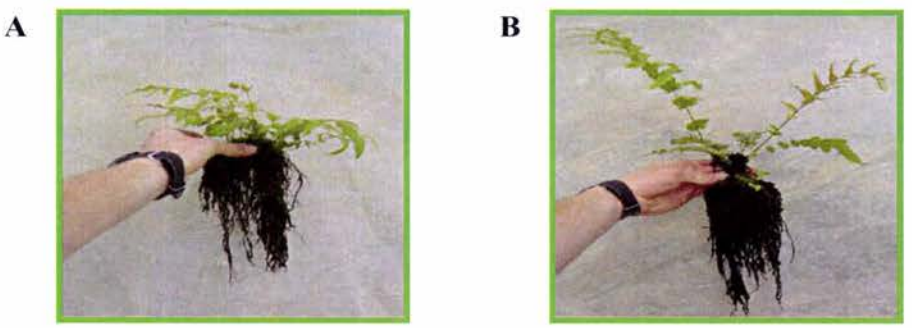


Figure 3.14 A & B The extent of the root system in *B. novae-zelandiae* rhizome segments.

3.3.3 SPORE EXPERIMENTS

3.3.3.1 Spore germination in the laboratory

The germination potential of spore used in the nursery experiments was initially assessed in the laboratory, using standard laboratory growth conditions (specified in section 2.2.2.3). Figure 3.15 shows the germination curves of laboratory sown *Blechnum novae-zelandiae*, *Cyathea medullaris* and *Dicksonia squarossa* spore. All spore germinated within 4 days. The *C. medullaris* spore sample reached its maximum germination of 99%, 14 days after sowing. This was the most viable sample for the three species. The *D. squarossa* sample reached its maximum germination of 81%, 18 days after sowing. The *D. squarossa* curve plateaus at around 80%. *B. novae-zelandiae* showed variation between replicates. This variability is common for *B. novae-zelandiae* germination curves (compare with Fig. 2.2 and Fig. 2.3). *Blechnum novae-zelandiae* spore reached its maximum germination after 10 days. The curve plateaus at around 40% germination. This was the lowest overall germination of the three species. ANOVA indicated that the germination data for the three species was significantly different. Taken together, these results indicate that the spore of the three native fern species used in this nursery experiment was potentially viable.

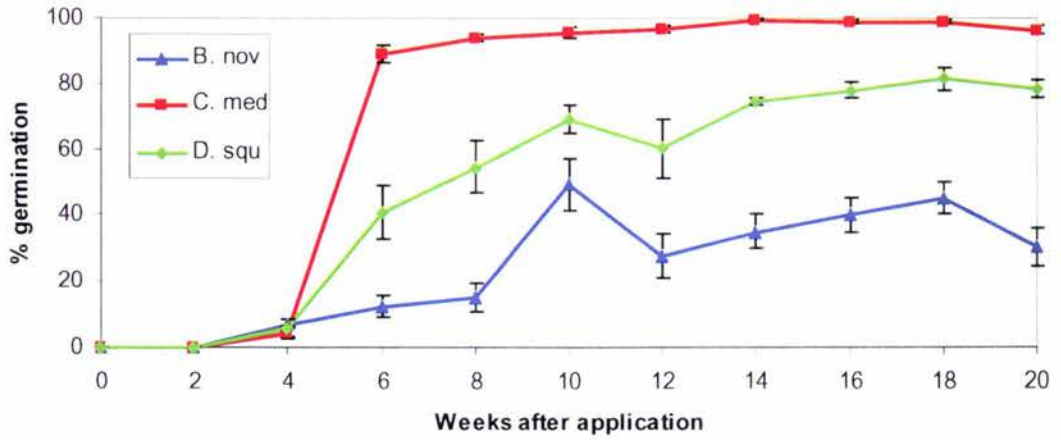


Figure 3.15 Mean germination of samples of *B. novae-zelandiae*, *C. medullaris* and *D. squarossa* spore in laboratory conditions.

3.3.3.2 Gametophyte establishment in spore experiments

Spore experiments successfully established a small number of gametophytes of the species *Blechnum novae-zelandiae*, *Cyathea medullaris* and *Dicksonia squarossa.* Gametophytes were first observed three weeks after the initial application (Table 3.2 and Fig. 3.16). The speed at which these gametophytes grew from spore was surprising as gametophytes of this size were never seen in the laboratory after 3 weeks growth. A very small number of gametophytes were found up to 12 weeks after the initial application whereupon successive sampling revealed no more. Interestingly, gametophytes were also found in the control plots (Table 3.2 and Fig. 3.16).

Table 3.2 Spore experiment raw data showing; the number of gametophytes found, in which plot and sub plot they were found, and at what week after application they were found.

Weeks after application	Sub Plot no.	*B. novae- zelandiae* replicates			*C. medullaris* replicates			*D. squarossa* replicates			Control replicates		
		1	2	3	1	2	3	1	2	3	1	2	3
3	# 8	**3**	0	**4**	0	0	**1**	**2**	0	0	0	0	0
6	# 4	**1**	0	**1**	0	**1**	0	0	0	0	**1**	0	0
9	# 9	0	0	0	0	0	0	**5**	0	0	0	**2**	0
12	# 3	**1**	0	0	0	0	0	0	0	0	0	0	0
15	# 1	0	0	0	0	0	0	0	0	0	0	0	0
18	# 7	0	0	0	0	0	0	0	0	0	0	0	0
21	# 6	0	0	0	0	0	0	0	0	0	0	0	0
24	# 5	0	0	0	0	0	0	0	0	0	0	0	0
27	# 2	0	0	0	0	0	0	0	0	0	0	0	0

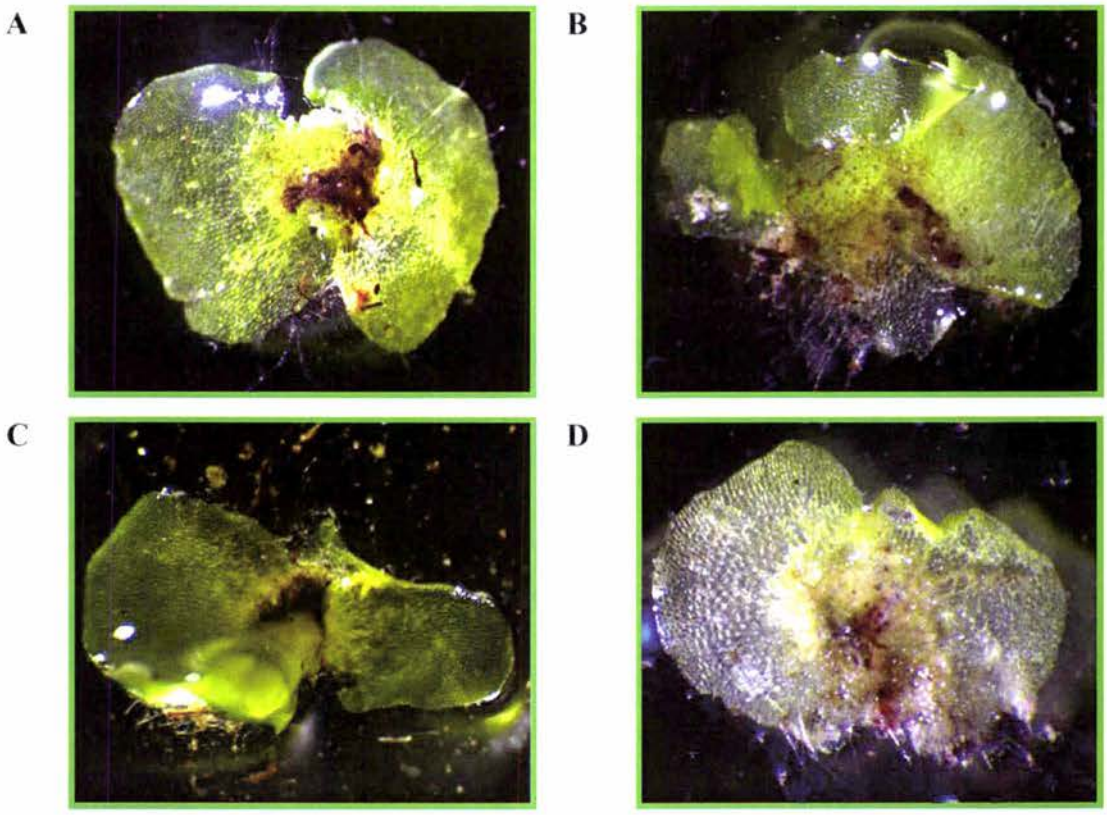
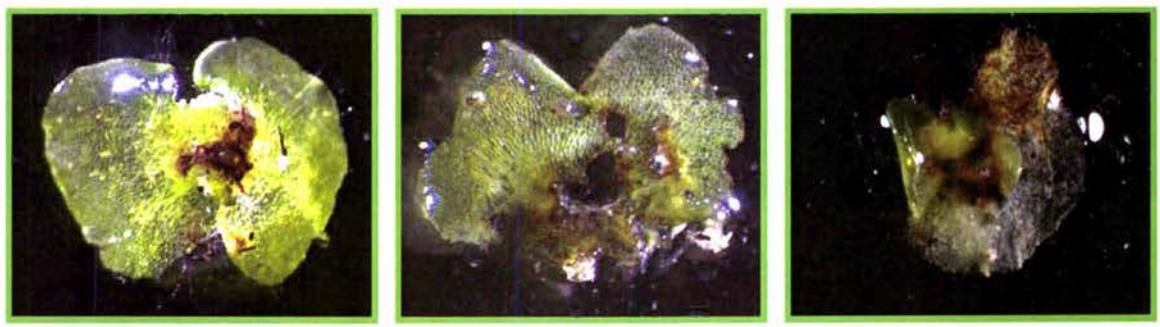


Figure 3.16 Gametophytes of (A) *B. novae-zelandiae*, (B) *C. medullaris*, (C) *D. squarossa* and (D) unknown gametophytes found on control plot. (A), (B) and (C) = 20x mag. (D) = 40 x mag.

The absence of gametophytes after 12 weeks could perhaps be explained by the photographs of *B. novae-zelandiae* gametophytes in Figure 3.17. Gametophytes sampled three weeks after application looked reasonably healthy. However, gametophytes sampled six and twelve weeks after application, exhibited increasing regions of necrosis.



3 weeks after application **6 weeks after application** **12 weeks after application**

Figure 3.17 *Blechnum novae-zelandiae* gametophytes sampled at different dates, showing increasing amounts of necrosis from left to right (20x mag.)

3.3.4 ADVENTIVE PLANT SPECIES IN THE NURSERY

Despite efforts to prevent invasion of weeds/adventive species, the nursery experiments were highly contaminated with a diverse range of plant life. In order to reduce possible competition effects weeding was routinely carried out to remove the more persistent broad leaf weeds. Figure 3.18 shows several of the more dominant adventive species.

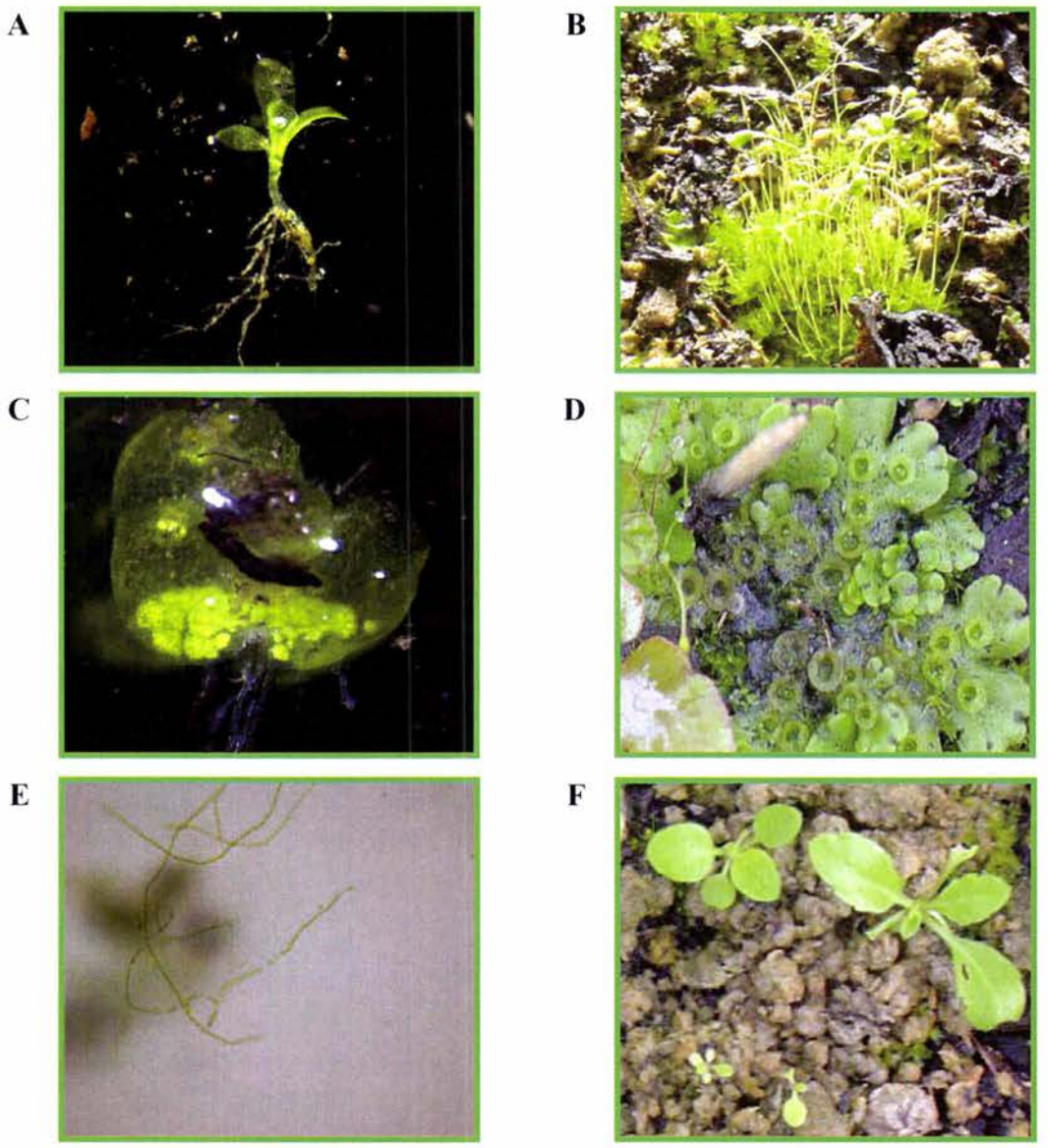


Figure 3.18 (A) A young specimen of *Funaria hygrometrica* (32x mag.). (B) *Funaria hygrometrica* in its mature form with sporophytes. (C) A young *Marchantia foliacea* liverwort with a fluorescent thallus (40x mag.). (D) Mature *Marchantia foliacea* showing cylindrical gemmae cups. (E) A filamentous green algae, thought to be Ulothrix (400x mag.). (F). Young broadleaf weeds, which were periodically removed from experimental plots.

Interestingly, rhizome plots produced a mixture of adventive species which included young fern gametophytes, often with sporophytes. It is possible that some of the soil attached to the rhizomes at the application stage contained fern spores. Figure 3.19 shows rhizome segments with ferns at various stages of development, 27 weeks after the initial application of rhizomes in hydroseeding mix.

A B C

Figure 3.19 (A) A "self-seeded" gametophyte with sporophytes. (B) A thalloid fern gametophyte attached to the base of a *B. novae-zelandiae* rhizome segment. (C) A young sporophyte of unknown species, possibly of the *Hypolepis* genus.

3.4 Discussion

Most of the scientific literature in relation to fern propagation describes *in vitro* propagation, whereby fern material is propagated in highly controlled conditions, usually on artificial media e.g. (Janssens & Sepelie, 1989; Fernandez et al., 1993; Camloh et al., 1994; Ambrozic-Dolinsek et al., 1999; Fernandez et al., 1999). This part of the project was more focused on achieving a method of propagation that would lead to a very high output of viable, hardy propagates, on a natural substrate, and which specifically utilized revegetation application techniques like hydroseeding. Literature directly relating to these experiments is scarce as hydroseeding fern material is an original concept.

3.4.1 GAMETOPHYTE EXPERIMENTS

Blechnum novae-zelandiae, *B. discolor* and *B. colensoi* gametophytes were successfully established in the nursery. A proportion of gametophytes of each species survived and produced sporophytes. *Blechnum novae-zelandiae* gametophytes performed the best as they had a medium survival rate and produced a substantial number of sporophytes (Figs. 3.6, 3.8). *Blechnum discolor* had the highest survival rate but only a low percentage of gametophytes produced sporophytes. Of the small number of *B. colensoi* gametophytes that survived around 15% produced sporophytes. These experiments demonstrated that this method of propagation was possible for these three different species. There are a number of key factors that may have influenced the success of these experiments.

The survival of gametophytes of each of the three species suffered a dramatic drop within the first three weeks of sampling. The size of the laboratory propagated gametophytes may have had a direct influence on their ability to establish in the nursery environment. Figure 3.2 clearly shows the distribution of gametophyte sizes for each of the three species used in the gametophyte experiment. When compared to the survival curves shown in Fig. 3.6 it seems that species with larger gametophytes had a better survival rate. *Blechnum discolor* had on average the largest gametophytes and also had

the highest survival rate, whereas *B. colensoi* had on average the smallest gametophytes and the lowest survival rate. Future propagation of this kind may benefit from using gametophytes of a certain size, e.g. at least 20 mm^2 in area.

The age of the gametophytes may have also been a factor contributing to the initial decrease in survival rate. Propagation of *Baloskion tetraphyllum* (Koala fern) via somatic embryogenesis was shown to be more successful if younger explant material was used (Panaia et al., 2004). Table 3.1 displays the range of ages of the gametophytes used in this experiment. Gametophytes of all three species were at least 177 days old. When compared with 66 day old sporophyte producing gametophytes grown in the same conditions (Fig. 2.16), these gametophytes are considerably older. In addition, these gametophytes were also sub-cultured several times in order to increase their numbers (Table 3.1). Periodic observations of sub-cultured gametophytes indicated that highly sub-cultured tissue did not appear as green and vigorous as tissue that had not been sub-cultured. It is possible that age and constant sub-culturing could have reduced the vigour and health of these gametophytes and, therefore, influenced the survival rates seen in Fig. 3.6. In future, it would be beneficial to use younger, less sub-cultured gametophytes when using this propagation method.

Plantlets produce via *in vitro* propagation often require a hardening off period prior to exposure to a more variable, natural environment (Sudha & Seeni, 1994). The laboratory propagated gametophytes were not given a hardening off period. The laboratory environment under which these gametophytes were grown was highly controlled with very little variation in temperature, light intensity and humidity. The sudden exposure to the nursery environment would have affected the physiology of these gametophytes dramatically. In future it would be beneficial to include a hardening off period, where gametophytes can acclimatize themselves to the environment prior to application. The initial laboratory propagation of gametophytes in a more natural environment, (i.e. with variations in light, temperature and humidity) and on a more natural substrate, may increase the survival rate of these gametophytes upon application. Pangua et al. (1994) and Herrero et al. (2002) use compost, sand and soil as the propagative substrate for their experiments with fern gametophytes. It would be even more advantageous if the gametophytes were propagated in a nursery environment under variable conditions.

In most cases the survival rate of gametophytes increased or decreased at a slow rate after the initial 3 week period. The increase in gametophyte numbers was initially unexpected but can be attributed to the emergence of initially buried gametophytes and also the increase in size of small, previously obscured gametophytes. In addition, it was also observed that several previously unrecorded gametophytes arose by the division or budding, of the large, leafy, laboratory propagated gametophytes. This asexual means of reproduction was prevalent in *B. discolor* plots.

A substantial proportion of the surviving gametophytes produced sporophytes (Fig. 3.9). This suggests that the environmental conditions in the nursery were adequate for successful fertilization. The most important environmental parameter in relation to fertilization is water availability, as fern spermatozoids require a film of water to swim from antheridia to archegonia (Brownsey & Smith-Dodsworth, 2000). The age of the gametophytes used in this experiment suggests that they were sexually competent upon application. In addition, 10 sporophytic structures were noted on the 1500 propagated gametophytes prior to application, confirming their competence. Sporophytes were more prone to death upon application and only one *B. colensoi* sporophyte survived application. The existence of this single pre-application sporophyte was excluded from Fig. 3.8 and Fig. 3.9 data, as the sporophyte was produced prior to the beginning of the gametophyte experiment.

It can be expected that if data collection had continued after 24 weeks the proportion of gametophytes with sporophytes would have increased. This assumption was made in respect to the trend lines seen in Fig. 3.9 and in conjunction with the knowledge that fern gametophytes are short-lived, having a defined life-span (Brownsey & Smith-Dodsworth, 2000). As more gametophytes produce sporophytes, the proportion of gametophytes with sporophytes will undoubtedly increase.

Overall the gametophyte experiments were relatively successful. All species were able to continue their normal life-cycle and produce sporophytes from gametophytes. Large scale *in vitro* propagation of fern gametophytes would have to be optimized before commercial hydroseeding of fern gametophytes could take place.

3.4.2 RHIZOME EXPERIMENTS

Rhizome fragmentation is a natural way for some native fern species to establish new plants (Koptur & Lee, 1993). *Pteridium esculentum* (bracken) is exceptionally good at using this method to produce new plants (Burge & Kirkwood, 1992). This species produces new ramets through rhizogenous growth and then terminates the underground connection to the parent plant, establishing a new, genetically identical plant. It is also possible that rhizomes may suffer damage via landslide events. The ability to fragment provides an interesting way of establishing new plants. This propagative system was analysed further using *Blechnum novae-zelandiae* rhizomes.

A rhizome is a horizontal creeping stem and, therefore, can be thought of as having a young meristematic region at one end and a non-meristematic region at the other end (Chambers & Farrant, 1998). The first shoots to arise on different rhizome segments did so gradually across a period of 24 weeks (Fig. 3.11) with two segments having shoots within the first three weeks. It is unclear why the first shoots were not initiated on each different rhizome segment at the same time. It is possible that the younger tissue near the growing end of the rhizome was able to de-differentiate faster than older tissue further away from the growing shoot. Therefore, when this rhizome is cut transversely, segments that arise closer to the growing end would produce shoots faster than segments arising from the older, woodier end (Fig 3.20).

The orientation of the rhizome on the substrate may have also influenced the speed of shoot formation. A segment orientated in the correct manner (Fig. 3.13A) would not have to re-orientate itself to environmental stimuli such as gravity, light, and water. However, a segment positioned in the opposite orientation (Fig. 3.13B) would have to re-orientate itself, which may take time and hence slow shoot initiation. To test these two hypotheses a simple experiment could be carried out whereby a rhizome is cut transversely several times and its segments are positioned from young meristematic segments through to older, woodier segments. In addition, a similar set of rhizome pieces could be positioned the same way but in the opposite orientation. Another separate experiment could be carried out to determine the minimum size one segment has to be to produce viable shoots.

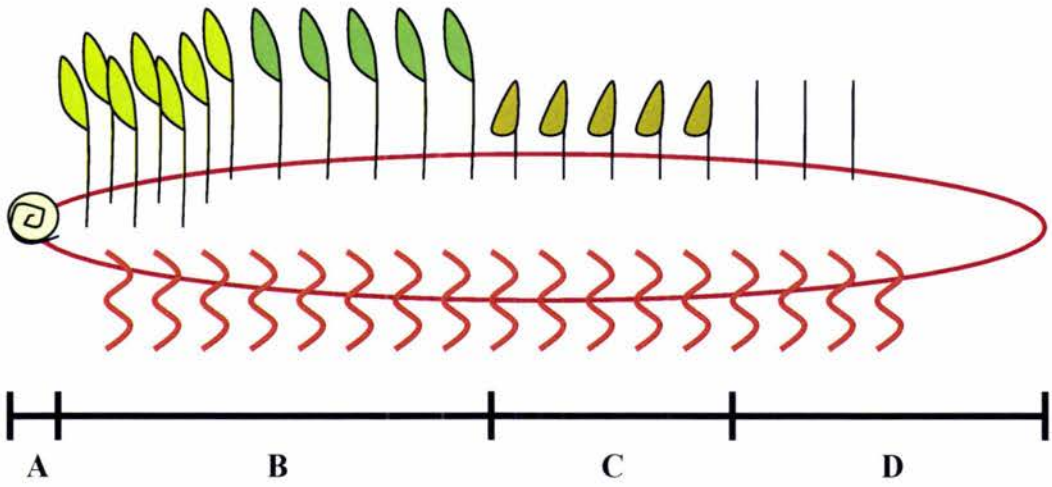


Figure 3.20 A schematic representation of a *Blechnum novae-zelandiae* rhizome indicating regions of maturity and growth. (A) The meristematic zone where new fronds are produced. (B) The growth zone containing young and mature fronds. (C) The frond senescence zone where older fronds are senescing. (D) The oldest, woodiest part of the rhizome containing no fronds.

Total shoot growth (m) was measured in order to express the growth of the rhizome segments over the experimental period. Shoot growth was chosen as the measurable parameter because it was easy to perform and could be measured at regular intervals throughout the experiment. Root dry weight could have also been a useful parameter to measure. However, it was decided that photographs of the rhizome segment root system, together with shoot length, would be sufficient to describe the extent of growth of these segments over a 27 week period. The photographs of the rhizome segment root system (Fig. 3.14A & B) show a large mass of roots. In the field *B. novae-zelandiae* is often found growing on steep batters with very little top-soil. It is the mass of roots that presumably allows these plants to grow on steep gradients. In terms of revegetation, the ability of the *B. novae-zelandiae* roots to impede soil erosion is of direct importance to this project. The limits of this project mean that research has not been carried out to establish if this is the case.

It is unclear if *B. novae*-zelandiae rhizomes can be propagated all year round. The rhizome used in the nursery experiments was collected in the autumn when *B. novae-zelandiae* plants were naturally producing new fronds and spore. The success of the nursery rhizome experiments may have been heavily influenced by the fact that the ferns were in their major growth period. It would be interesting to test the seasonal viability of rhizome segments throughout the year in a similar experiment to that carried

out in the laboratory with fern spore (Fig. 2.5). This would provide valuable information that could be directly utilized in the management of fern rhizomes for revegetation.

A number of adventive species, possibly *Blechnum novae-zelandiae* and *Hypolepis* species were found growing exclusively in the rhizome plots. Fern gametophytes and sporophytes were found growing directly upon rhizome segments (Fig. 3.19). It is assumed that these young ferns were transported with the rhizome material from where the rhizomes were initially removed, because control plots (without rhizome) contained no gametophytes whatsoever. Fern leaf litter is commonly used as a propagative substrate (Nhut, 2005). The frond litter is also potentially a source of spore and would be a useful inclusion in any fern revegetation method. In addition, it is also possible that beneficial microorganisms may have also been present in the soil around the rhizomes, if not in the roots of the rhizomes. Mycorrhizal fungi have been known to form mutualistic relationships with a number of native fern species including those of the *Blechnum* genus (Cooper, 1976). The niche environment around the rhizome segment may have also offered some sort of physical advantage. Fern leaf litter could potentially be used as an inoculant as well as a source of spore.

3.4.3 SPORE EXPERIMENTS

The nursery spore experiments showed signs of gametophyte development but failed to produce any mature gametophytes or gametophytes with sporophytes 27 weeks after application. In the related literature, fern spore is often propagated on natural substrates such as sand, soil and peat, but it is rarely grown outside of sterile laboratory conditions (Pangua et al., 1994; Bernabe et al., 1999; Herrero et al., 2002). This section of the discussion will suggest reasons why spore germination in the nursery was low and why gametophytes failed to survive and produce sporophytes.

The germination of a sample of nursery spore in the laboratory revealed that the spore used in the nursery experiment was viable, but the viability ranged from 30% - 95% depending on the species (Fig. 3.15). A total of 0.2 ml of dry spore was added to each plot which equates to an estimate of 1.9 million *B. novae-zelandiae* spores, 3.84 million

C. medullaris spores and 3.46 million *D. squarossa* spores per plot. If only 30% of the spore from each species was viable this would equate to a large number of gametophytes. However, the results of this experiment only produced 22 gametophytes for all three species. There are several factors that may have contributed to the low number of gametophytes produced including spore dormancy, spore predation by micro-organisms, lack of or excess of environmental parameters (light, water, temperature) and competition from other plants. A more controlled environment with sterile soil and no foreign species may result in the production of more gametophytes. Future work could be carried out to optimize the production of gametophytes from spore on a large scale.

The first gametophytes in the nursery spore experiment were discovered only three weeks after application. This was a surprising result as gametophytes produced under laboratory conditions had taken around 2 months to mature to the same stage. Ross et al. (2003) reported that the addition of fertilizer to a substrate improved the establishment and growth of New Zealand mosses and lichens. It is possible that the addition of fertilizer to the hydroseeding mix may have caused the rapid growth of these gametophytes.

It is interesting to make a comparison with the rhizome experiments (section 3.3.4), which unexpectedly produced healthy gametophytes, often with sporophytes. The two experiments were only several metres apart in the nursery but gave different results. It can be speculated why spore brought in with the rhizomes germinated and produced viable, sporophyte-producing gametophytes, whereas hydroseeded spore did not (refer to section 3.4.2).

Interestingly, gametophytes were also found in the control plots. This was possibly a direct result of contamination from adjacent plots. Continuous irrigation may have washed or splashed spore from one plot to another. Also during the application process even the slightest breath of wind could have caused spore to contaminate adjacent plots. It is also possible, although highly unlikely, that spore could have drifted in from an outside source. However, control plots in the other two experiments showed no gametophyte growth whatsoever.

The destructive method of sampling was perhaps not the best way to assess this experiment. Initially it was thought that it would be possible to see germinating spore on the medium surface at a microscopic level. However, it was soon realised that there was very little chance of finding any germinating spore as it was too small and there was too much sterile compost to sift through (22 cm x 22 cm per sub plot). Hence, the sub plots were subsequently assessed *in situ* for the production of gametophytes. It would have been more useful to use a non-destructive method whereby newly identified gametophytes were marked with a toothpick and assessed for survival every three weeks, similar to the gametophyte experiments.

FIELD EXPERIMENTS

4.1 Overview

The fourth chapter of this thesis describes the application, assessment and results of spore and rhizome field experiments, over a period of six months. The first experiment was designed to test the ability of spore to establish in the field using hydroseeding as the method of application. In addition, rhizome segments were also applied to the bank and assessed as a separate experiment. Both experiments were assessed at monthly intervals. Characteristics of the field site, including the substrate, were also analysed in order to provide information on the micro-environment of the field site.

4.2 Materials and Methods

4.2.1 FIELD EXPERIMENT SITE PREPARATION AND ANAYLSIS

4.2.1.1 Site specifications

A cut-slope situated near the settlement of Aokautere on State Highway 57, 5 km from Palmerston North city, was chosen as a suitable field site (Fig. 2.1). The batter faced northwest and ran 80m along an inclination. The average slope of the cut-slope was measured at 45° using an inclinometer. The batter was sheltered from most directions except the north-west. In the winter it received direct sunlight in the morning and was shaded in the afternoon. Gradually the bank became more exposed to sunlight as the days lengthened, leading up to summer. The cut-slope was relatively fresh and therefore mainly bare of vegetation. However, the initial establishment of moss and several isolated stands of exotic weed species were evident. The vegetation above the cut-slope consisted of several pine trees surrounded by agricultural grasslands. Pine trees were also situated on the opposite side of the road, along with several native shrubs including *Coprosma robusta* and *Leptospermum scoparium*. The substrate consisted of both sandstone and loess, with the sandstone situated along the base of the cut-slope and the loess situated on the upper reaches (Fig. 4.1).

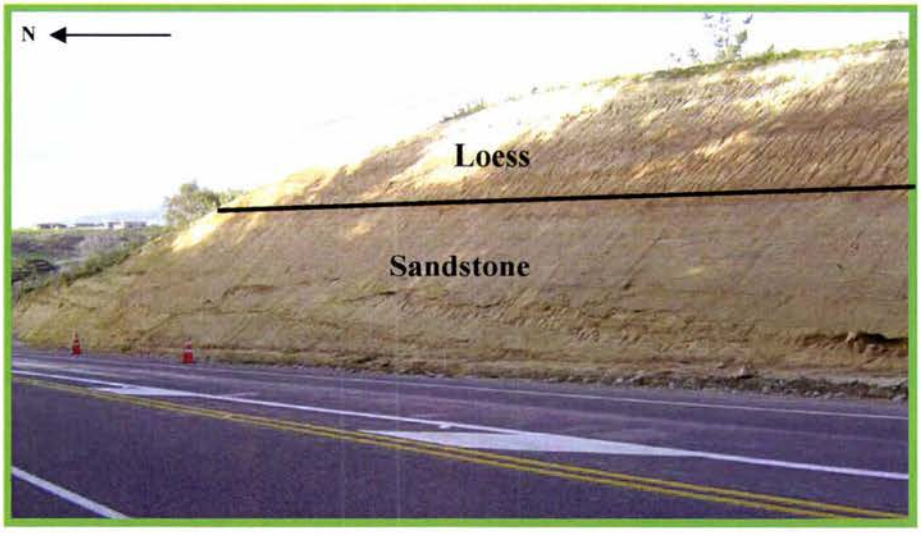


Figure 4.1 The field site prior to application of the experiments, indicating the two different substrates, with loess situated above the black reference line and sandstone below.

4.2.1.2 Plot preparation

Prior to application it was decided that each plot would be 3m wide and 3m in height. The field site was divided into 15 plots spanning an overall length of 45m along the base of the batter. Plots were allocated to a site at random using a split plot design. Due to the location of the experiment, at the bottom of the batter, all plots were subsequently established on the sandstone substrate (Fig. 4.1).

Plots were pegged out to provide a reference for application and also for assessment purposes. Five plots at the northern end of the batter were chosen for the rhizome experiments. Dimples were made in this area using a small trowel in order to imitate a dimpling process, usually carried out by an excavator. Dimpling the bank enabled the rhizomes to be easily positioned on the steep slope and also aided in their adherence to the batter. These five plots, including one control plot, were treated as a separate experiment. *Blechnum novae-zelandiae* and *Microsorum pustulatum* rhizomes were used in this experiment.

The ten southern plots were designated two different spore treatments, which were each replicated four times. These two treatments consisted of a shotgun spore mix only, and a shotgun spore mix containing two nurse species. Two control plots of hydroseed mix only (no spore or seed), were also included. The shotgun spore mix contained a mixture of *Blechnum novae-zelandiae*, *Cyathea medullaris* and *Dicksonia squarossa* spore. *Agrostis capillaris* (brown-top) and *Trifolium subterraneum* (subterranean clover) were used as the two nurse species.

4.2.1.3 Application

On the 27th of May, 2005 the first hydroseeding application took place at the field site. Prior to the application the hydroseeder was rinsed twice with clean water in order to avoid contamination from previous use. The hydroseeder's hose was also washed out to remove any seed remnants. The standard mix contained 90 kg of cellulose fibre mulch, 5 kg of nitrophoska fertilizer, 750 ml of Soilfix tackifier and ~ 500 litres of water. These ingredients were thoroughly mixed and pumped through the hose in order to create a homogenous solution. Initially the three control plots were sprayed. 78.9 ml of

fern spore was then added to the tank and the resulting solution was mixed thoroughly (Fig. 4.2A & B). In order to obtain a homogenous mixture the slurry was sprayed through the hose and back into the tank (Fig. 4.2C). The spore mix was applied to the four designated plots (Fig. 4.2D). 72 g of *Agrostis capillaris* (brown-top) and 360 g of *Trifolium subterraneum* (subterranean clover) seed was added next. These exotic species are able to rapidly establish and therefore provide initial erosion control. *Trifolium subterraneum* is also able to fix nitrogen and therefore provide a source of available N for the growing ferns. Care was taken to use a low rate of these species in order to prevent them from becoming too dominant, allowing for ferns to establish simultaneously. The new mixture was mixed and pumped through the hose back into the tank prior to spraying the remaining four plots.

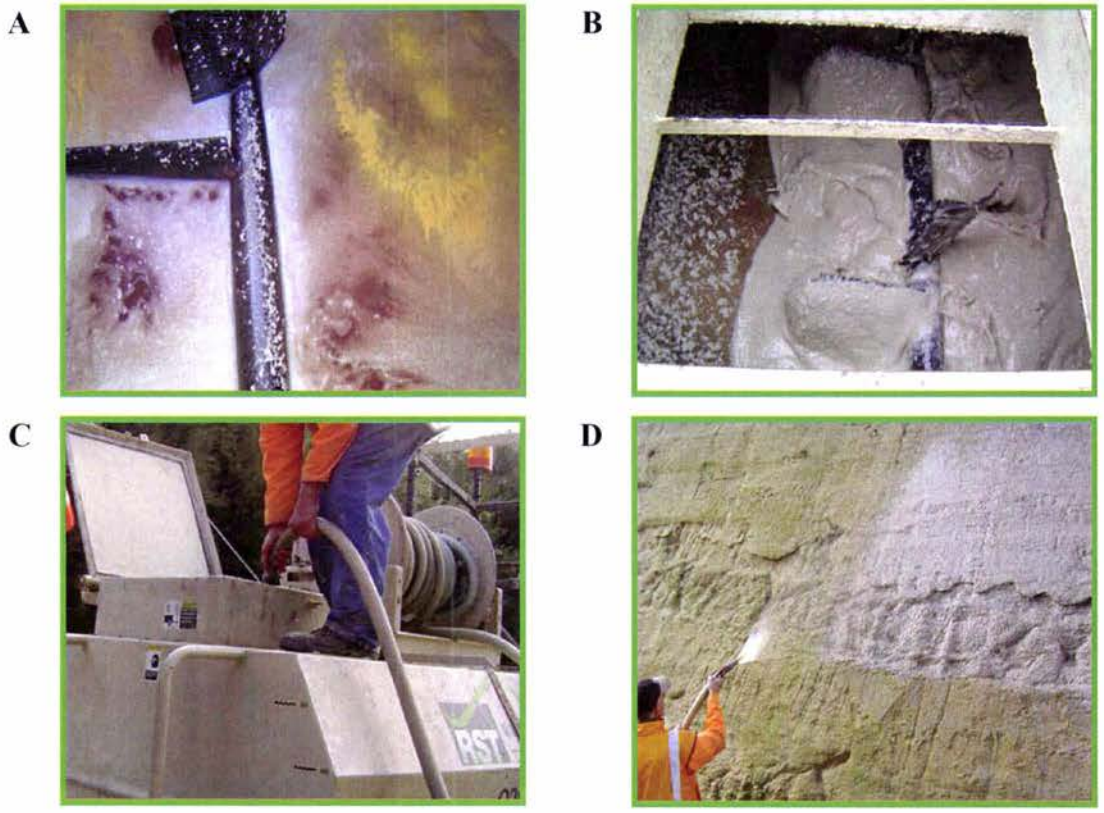


Figure 4.2 (A) Spore of *Blechnum novae-zelandiae, Cyathea medullaris* and *Dicksonia squarossa* in the hydroseeder's tank. (B) Mixing of the spore, paper fibre, fertilizer, tackifier and water. (C) Back pumping the mix in the tank in order to get a homogenous mixture in the tank, as well as in the hose. (D) Spraying of the field experiment using a hose and a fan spray nozzle.

One rhizome of each species of *Blechnum novae-zelandiae* and *Microsorum pustulatum* was placed in each dimple created in the rhizome plot area (Fig. 4.3B). Rhizomes were not added to the control plot. Once each dimple was filled the mix left in the tank (spore + seed) was sprayed over the rhizomes in order to help cement them in place. In total 150 pieces of rhizome of each species were used.

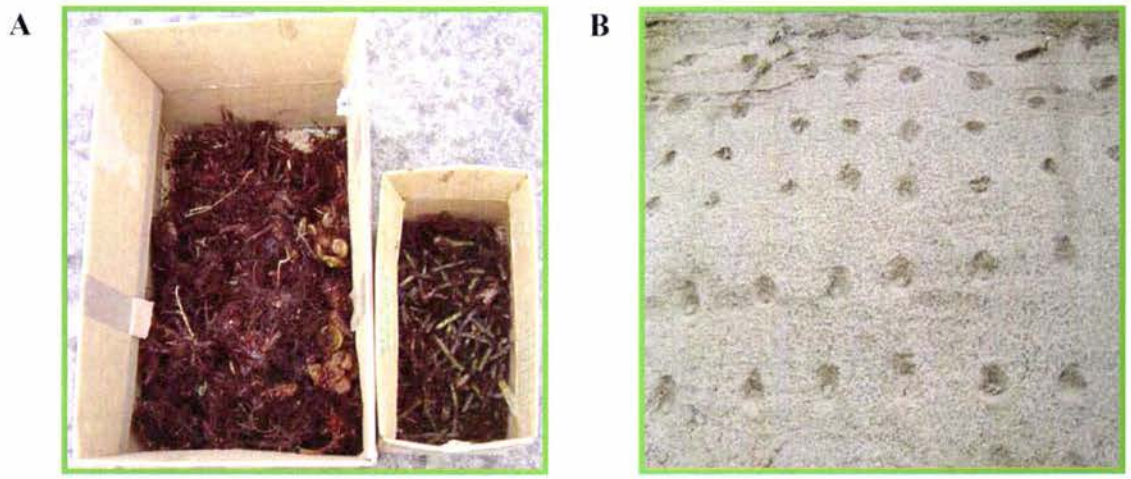


Figure 4.3 (A) *B. novae-zelandiae* rhizome segments (left) and *Microsorum pustulatum* rhizome segments (right). (B) Rhizome segments positioned in dimples and sprayed with hydroseed.

Due to light showers immediately after the first hydroseeding application, it was estimated using a vegetation ground cover chart (Payton et al., 1999) that about 55 - 65% of the original application had washed off (Fig. 4.4).

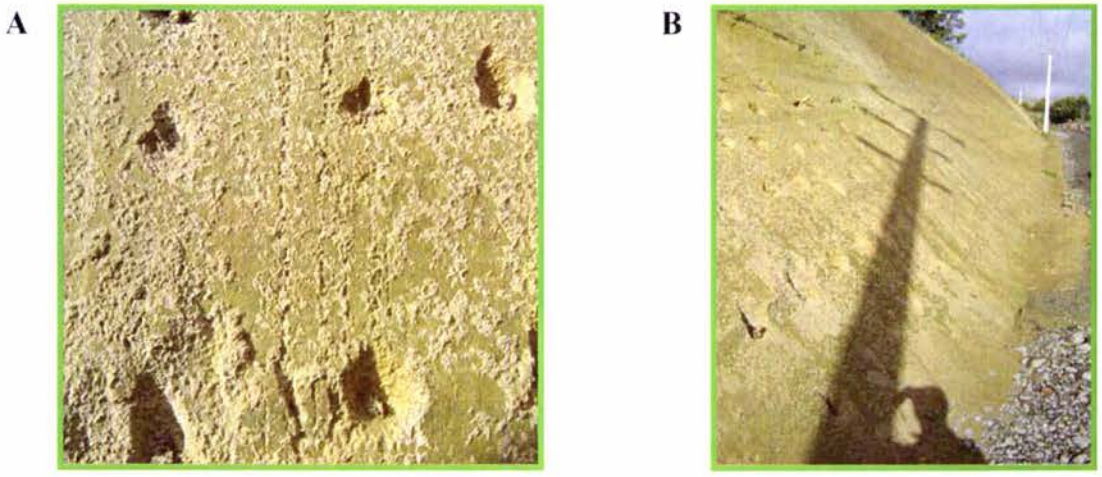


Figure 4.4 (A) A front on view of the field experiment after the first application which was affected by rain. (B) A side on view of the field experiment showing the affect of rain on the first application.

A second hydroseeding application was carried out on 23rd of June, 2005, in a similar manner to the original application. The only difference to the method was that 61.8ml of spore was used instead of 78.9 ml. The applied hydroseed had time to adhere to the bank before showers arrived in the evening. The second application resulted in sufficient cover of the bank.

4.2.1.4 Assessment

Three sub-plots were chosen at random as sample sub plots for each 3 x 3 m plot. Pegs were erected in the top two corners of each sub plot in order to support a 0.5 m^2 quadrat. The location of the pegs was never changed and therefore sub plots were able to be routinely identified and assessed every month. Plot assessments were carried out monthly from the 23rd of June until the 21st of December 2005. A ladder was used to scale the face of the bank when sampling and recording data, as the steepness of the slope impeded accessibility. The vegetation cover was measured for each sub plot using a vegetation ground cover chart (Payton et al., 1999). A copy of this chart has been included (Appendix 2). Counts of gametophytes/sporelings and rhizome shoots were made at each assessment date. The establishment of adventive species to the plots was also noted. Three soil samples were collected from the bank and sent to Hill Laboratories Ltd. for analysis of various parameters including pH, macronutrient concentration, available N and organic matter composition. The samples were collected as specified by the instructions provided by Hill Laboratories Ltd. A Sanyo DSC-P8 digital still camera was used to take all of the field experiment photos.

4.2.2 STATISTICAL DESIGN AND ANALYSES

A randomised block design was used for the field experiment site. Each replicate plot had 9 representative sub plots. Three sub plots were randomly chosen from each replicate plot for assessment. Mean data was used for both the spore germination graph (Fig. 4.5) as well as the vegetation cover graph (Fig. 4.7). Standard error bars were included in both or these graphs to indicate variation between replicates. In order to display the rhizome segment viability data, raw data were plotted. ANOVA was used to compare vegetation cover on replicate spore experiment plots at the level of $P < 0.05$.

4.3 Results

4.3.1 PRE-EXPERIMENTAL ANALYSES

4.3.1.1 Soil/Substrate analysis

The three soil samples taken from the sandstone bank were tested and the results were compared with standard pasture soil figures (Table 4.1). The pH of the sandstone bank was consistently high compared with pasture soil. The macronutrients P, K and Ca were only available at extremely low levels. However, sample two produced an average rate of P that was just below the pasture soil figures. The macronutrients Mg and Na were present in adequate and high amounts. Available N and organic matter levels were very low and possibly non-existent. Overall these measurements suggest that the pure sandstone substrate is very different to pasture soil and is not ideally suitable for plant growth.

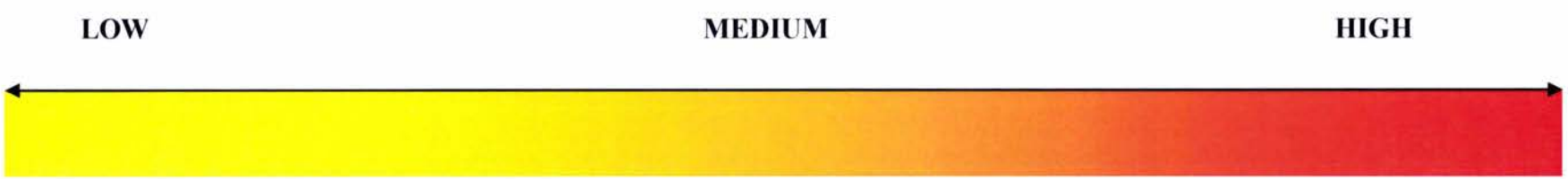
4.3.1.1 Spore germination in the laboratory

Spore used in both the initial field trial application and the re-spray was also sown in the laboratory, in order to assess germination potential. Figure 4.5 shows the germination rates of *Blechnum novae-zelandiae*, *Cyathea medullaris* and *Dicksonia squarossa* spore, used in both the initial hydroseeding application and the respray.

Spore collected for the initial trial appears to have a higher germination potential in all species, than spore collected for the respray. The individual results are consistent with previous germination curves showing *C. medullaris* with the highest overall germination potential, followed by *D. squarossa* and *B. novae-zelandiae.* As indicated by the germination data the spore applied to the field trial did in fact have a degree of viability, and if conditions were correct, it could be expected that young gametophytes would be produced.

Table 4.1 Analysis of various parameters of the field experiment substrate. * The medium range of values are those commonly found in pasture soils. The coloured reference bar indicates the relative levels of each parameter compared with those found in pasture soils. The actual values are also included.

	pH	P (mg/L)	K (me/100g)	Ca (me/100g)	Mg (me/100g)	Na (me/100g)	Available N (kg/ha)	Organic matter
Medium range*	**5.8 – 6.5**	**25 – 50**	**0.50 – 1.00**	**6.0 – 12.0**	**1.00 – 3.00**	**0.00 – 0.50**	**100 - 150**	**7.0 – 17.00**
Sample One	**7.2**	**1**	**0.11**	**2.7**	**3.18**	**0.78**	**< 10**	**< 0.2**
Sample Two	**7.2**	**23**	**0.14**	**2.7**	**2.93**	**0.97**	**< 10**	**< 0.2**
Sample Three	**7.1**	**2**	**0.11**	**2.9**	**2.37**	**0.31**	**< 10**	**< 0.2**



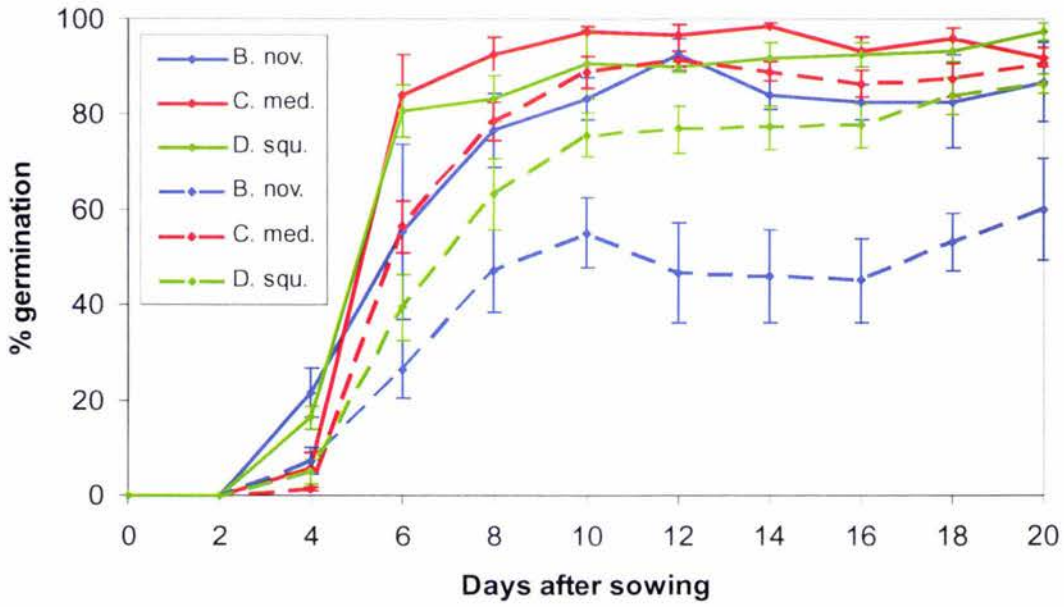


Figure 4.5 Laboratory germination of *B. novae-zelandiae, C. medullaris* and *D. squarossa* spore, used in both the initial field trial hydroseeding application (unbroken lines) and the respray (broken lines).

4.3.2 HYDROSEEDING FERN SPORE IN THE FIELD

4.3.2.1 Spore germination and gametophyte establishment

The field experiments failed to produce any observable gametophytes after 6 months of analysis. This was despite the fact that the spore used in this experiment was proven to be viable (Fig. 4.5). The establishment of ferns via hydroseeding spore was unsuccessful at this particular site.

4.3.2.2 Vegetation cover

The amount of vegetation growing on the field plots increased considerably over the 6 month period. Vegetation cover was classed into three types; nurse species, moss and adventive species. Areas that were not hydroseeded were void of any class of vegetation (Fig. 4.6). The extent of vegetation cover varied between different treatments (Fig. 4.7).

The "spore + nurse species" treatment plots (S+) produced the highest average vegetation cover at each assessment date. The "spore only" treatment plots (S-) produced vegetation cover rates equivalent to control plots (C). ANOVA indicated that there was no significant difference between control replicate plots. S- plots only showed a significant difference between replicate plots assessed 2 months after application. S+ replicates showed significant differences in the 1st, 5th and 6th month after application. These variations between replicate plots are also indicated by the standard error bars included in Figure 4.7.

A decline in vegetation cover is evident in the 3rd and 5th months after application. In the 3rd month of assessment it was observed that vegetation cover in the S+ plots had declined. The 5th month of assessment showed a decline in vegetation cover for all treatments, including the control plots. Interestingly, according to rainfall data collected in the area, these periods had long dry spells without rain (Appendix 3). A common observation at these dates was that moss cover had decreased considerably.

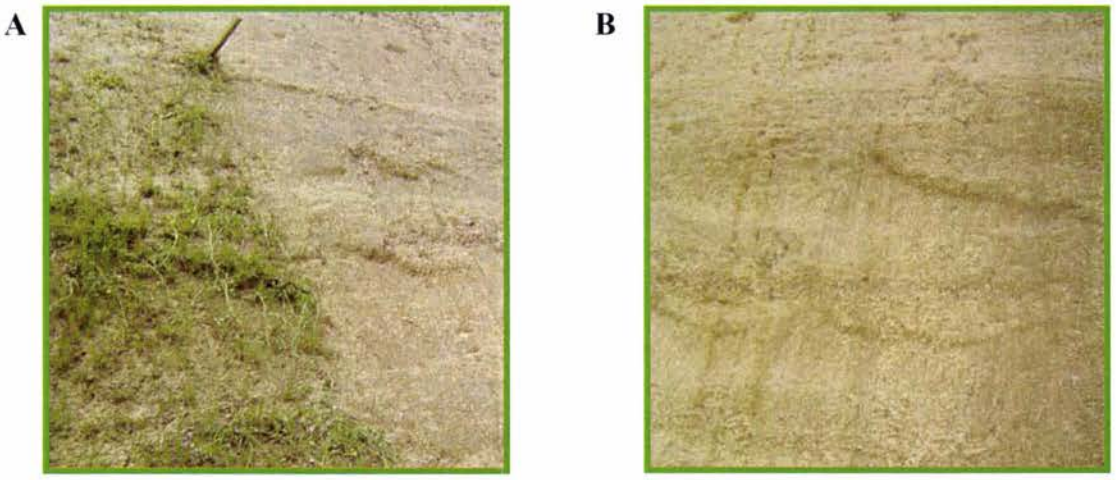


Figure 4.6 (A) A comparison between a hydroseeded area of the field site (left) and a non-hydroseeded area (right). (B) A front on view of the non-hydroseeded region showing that there is no visible vegetation 6 months after the field site was hydroseeded.

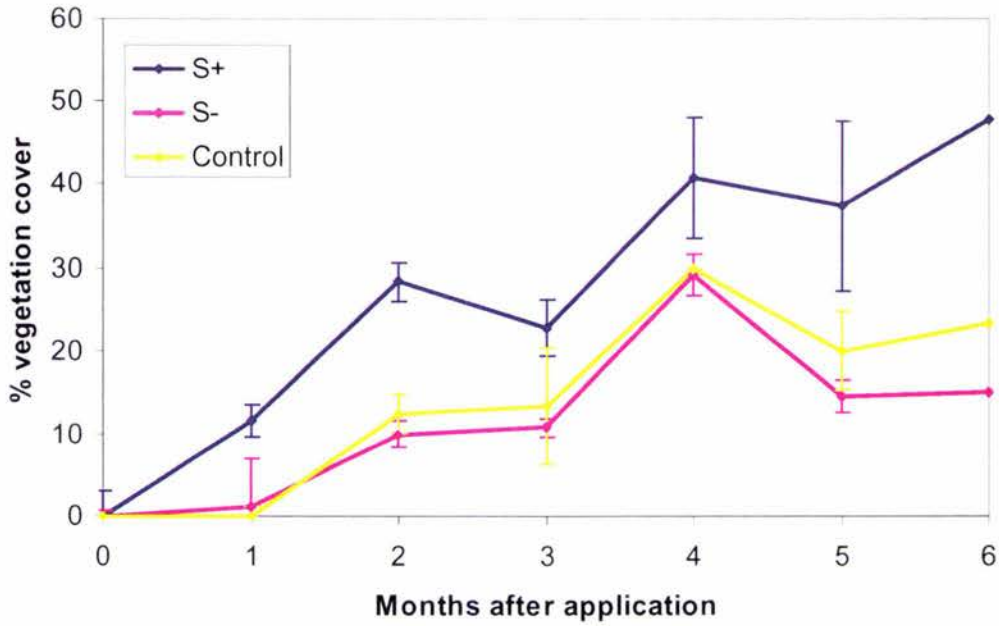


Figure 4.7 Vegetation cover for the two different treatments and control plots included in the field experiment. S+ = spore + nurse species. S- = spore only. C = control.

4.3.3 FERN RHIZOME EXPERIMENT

4.3.3.1 Rhizome establishment

The creation of dimples on the sandstone face made rhizome establishment possible. Dimples combined with the overlaid hydroseeding mix supported the rhizome segments sufficiently. As a result not one of the rhizome segments was misplaced from the bank.

4.3.3.2 Rhizome viability

The two species used in this experiment both produced a small number of viable rhizome segments, of which most died within the 6 month period. *Blechnum novae-zelandiae* produced 17 viable rhizome segments in total, however, only three survived. *Microsorum pustulatum* produced 3 viable segments of which only 1 survived. The viability of rhizome segments of both species was extremely low over the course of 6 months (Fig. 4.8). The first two months of assessment show the number of viable

rhizome segments increasing. Two months after application the number of viable rhizome segments remained constant for a short period followed by a marked decrease, until just over 1% of the rhizome segments exhibited viability.

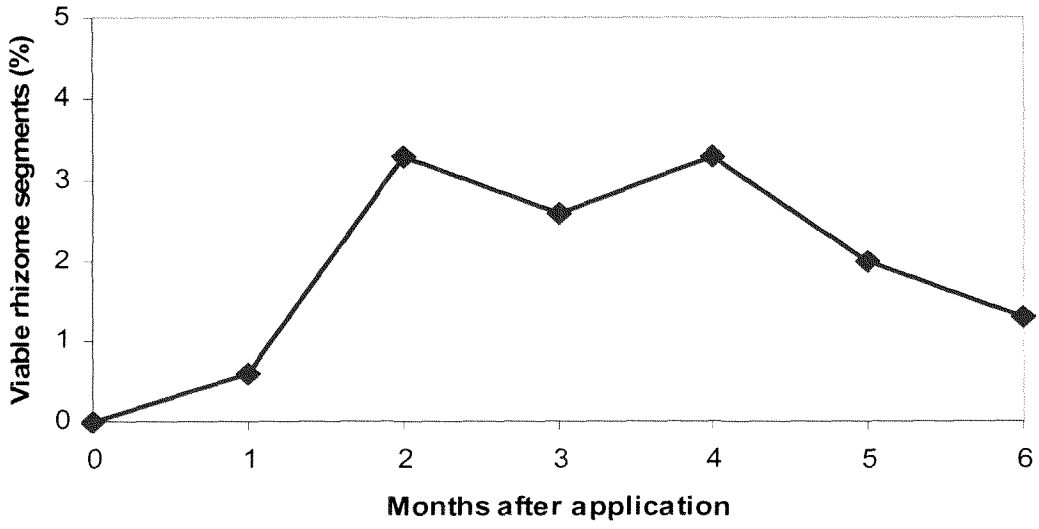


Figure 4.8 Rhizome segment viability in the field experiment, over a 6 month period.

4.4 Discussion

The concepts of hydroseeding fern spore and using rhizome segments in revegetation are both original. The field experiments described in this chapter aimed to assess the success of fern establishment and vegetation cover on a roadside bank. Previous research has often concentrated on using relatively standard hydroseeding techniques in situations where the areas to be hydroseeding offer different challenges. This research includes studies on hydroseeding mined areas (Brofas & Varelides, 2000), sand slopes (Roberts & Bradshaw, 1985) and roadsides (Carr & Ballard, 1980; Cano et al., 2002). Other examples of hydroseeding research aim to optimize the technique itself, usually via the addition of various ingredients (Morrey et al., 1983; Merlin et al., 1999) or via the preparation of the area to be hydroseeded (Montalvo et al., 2002). All the literature agrees that the local microclimate has a substantial role in the success of the various revegetation methods described.

Native hydroseeding is a relatively new concept in New Zealand. Literature exists on the success of hydroseeding New Zealand native mosses (Ross et al., 2003) and New Zealand native shrubs (Smale et al., 2005). Utilizing native ferns in revegetation is a plausible continuation of this recent work.

4.4.1 SITE CHARACTERISTICS

The field site used for this project was far from ideal for revegetation. However, a large number of sites that are required to be revegetated are often not favourable (Roberts & Bradshaw, 1985; Brofas & Varelides, 2000). They are often very steep sites and are almost always lacking fertile top-soil. In addition, other aspects such as water availability, irradiance and soil substrate characteristics dramatically influence the success of revegetation practices (Cano et al., 2002).

The Aokautere field site had a steep gradient and top-soil was absent. In addition, the site was exposed to direct sunlight for a substantial part of the day. The field experiment was set up in late autumn and assessed throughout winter, spring and into

early summer. It is common practise in the industry to hydroseed in autumn as water availability is high and will generally remain high until late spring time. These conditions provide an opportunity for the initial establishment of hydroseeded material.

The soil assessment of the sandstone substrate provided extra information about the site. The availability of several macronutrients was extremely low and the pH of the site was relatively high (Table 4.1). The lack of several macronutrients was not unexpected as sub-soil is often devoid of macronutrients. The ingredients of the hydroseeding mix were purposefully included to combat some of these deficiencies. Nitrogen and phosphorus deficiencies were combated with the addition of fertilizer to the hydroseeding mix. In addition, the inclusion of subterranean clover in the mix would potentially help to alleviate the N deficiency over time. The inclusion of paper fibre as a mulching material aimed to boost the amount of organic matter on the site.

The soil substrate analysis data were consistent across the three replicates, except for one phosphorus reading (Table 4.1). Sample two exhibited a very high P level, compared to the other two samples. This variation can be explained by the seams of rock running through the site, which possibly partly consisted of phosphorus rich apatite (Fig.4.9). Sample two was taken in the vicinity of one of these rock seams. To my knowledge there is no literature that defines the acceptable levels of macronutrients that are required for the growth of New Zealand native ferns. It is possible that ferns have very different requirements to pasture species. For this particular site both K and Ca were measured at low levels. The hydroseeding mix did nothing to combat this deficiency. It may be beneficial to assess the macronutrient requirements of ferns in future experimental work, in order to ascertain which macronutrients are the most important for the growth of native ferns.

Figure 4.9 A possibly phosphorus- rich seam of rock running through the field experiment site.

The pre-assessment of the field experiment site suggested that is was not ideal for plant establishment. Its steepness, high irradiance, high pH and lack of key macronutrients meant that it would be a challenging site to revegetate. However, the ability of native ferns to establish and grow on a site such as this was unknown.

4.4.2 HYDROSEEDING NATIVE FERN SPORE

Hydroseeding native fern spore of *Blechnum novae-zelandiae*, *Cyathea medullaris* and *Dicksonia squarossa* failed to produce any visible gametophytes or sporophytes after 6 months. The nurse species, *Agrostis capillaris* (brown-top) and *Trifolium subterraneum* (subterranean clover) germinated within a month after application and became a dominant part of the vegetation on the site after 6 months. The growth of these species indicated that the site was fertile enough to support plant growth. Moss cover was dominant in the early stages of the field experiment. The growth of moss is thought to be a direct result of the fertilizer in the hydroseeding mix. Previous research suggests that native New Zealand mosses respond positively to fertilizer (Ross et al., 2003). This response was also evident in adventive species that had colonized the plots prior to the hydroseeding application. Moss cover appeared to fluctuate within the 6 month assessment period (Fig. 4.10), possibly due to fluctuations in water availability.

2 months **4 months** **5 months** **6 months**

Figure 4.10 Moss cover in a "spore only" treatment sub plot at successive intervals within the 6 month assessment period. Moss cover appeared healthy and dense after 2 months. Its dominance within the same sub plot varied over the 6 month period. The growth of adventive species is also apparent.

Despite the limitations discussed earlier in terms of the ability of this site to support vegetation, there are other biotic factors that may have influenced the success of spore establishment and development. The field site was totally absent of top-soil and,

therefore, was also missing a soil spore bank. Spore banks are important biotic components of fern communities and play an important role in the regeneration of fern species (Ranai, 2003). Earlier laboratory work suggested that *B. novae-zelandiae* may use an antheridiogen mating system, which is thought to utilize spore already present in the soil. Therefore, the application of spore via hydroseeding may be more successful if a soil spore bank is already present. Alternatively, the inclusion of fern leaf litter into the mix as an additional source of mature spore may be beneficial. It is also possible that the seemingly viable spore that was applied to the site may have become dormant in the less than ideal growing conditions. The lack of water on the steep site may have caused spore to enter dormancy. Little is known about fern spore dormancy. A longer assessment period may have revealed different results in terms of fern establishment.

The vegetation cover results suggested that there was significant variation between some of the replicate plots (Fig.4.7 and accompanying text). As with any site, slight variations in characteristics such as slope angle, substrate composition (e.g. P levels in sample two, Table 4.1), irradiance and water availability exist. These small variations would have influenced the growth of the vegetation in each replicate plot and therefore help to explain the differences observed.

The data collected for this field experiment concentrated specifically on the success of establishing vegetation to stop erosion. Several plots exhibited small areas of erosion (Fig. 4.11). It would have been useful to measure erosion as well as vegetation cover, as this would have provided more information on the success of these experiments. However, this was not the specific aim of this experiment.

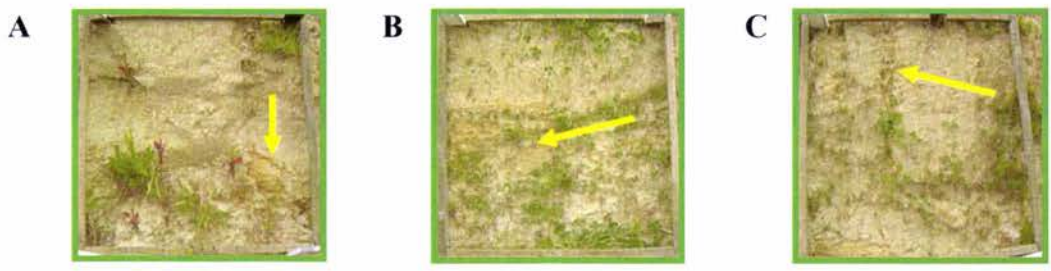


Figure 4.11 (A) A "spore only" plot showing a small area of erosion. (B) Erosion in a "spore + nurse" plot. (C) A "spore + nurse" plot showing rilling i.e. vertical channels made by water flow.

4.4.3 REVEGETATION WITH RHIZOME SEGMENTS

The rhizome experiment was also greatly affected by the environmental conditions at the field site. As the field experiment entered its 3rd month, previously viable rhizome segments began to die. This decrease in viability was attributed mainly to the lack of available water on the steep sandstone site. In addition, exposure to high irradiance and also low humidity may have also caused a loss in viability. Fronds under these types of environmental stresses characteristically show browning of the pinnae (Fig. 4.12).

4 months (Oct) **5 months (Nov)** **6 months (Dec)**

Figure 4.12 A *Blechnum novae-zelandiae* frond established from a rhizome segment, exhibiting frond browning and cell death over a period of three months.

Dimpling proved an effective method for establishing rhizome segments on a steep site. The nurse species hydroseeded over the rhizome segments tended to prefer to grow in the dimples. This suggests that the dimples were successful at allowing for the establishment of vegetation on a steep site. They created a more supportive and shaded microenvironment.

The collection of the rhizome segments for the field experiment occurred during winter. It is possible that rhizome segments may have improved viability at other times of the year. It would be beneficial to ascertain if there is a seasonal difference in rhizome viability so collections could be carried out at optimal times.

GENERAL DISCUSSION

5.1 Overview

The final chapter of this thesis aims to discuss the significance of the findings of the first three chapters and how these results have collectively contributed to our understanding of using native New Zealand ferns for revegetation. In addition, future research pathways, relating specifically to the work contained in this thesis, will also be suggested. The last section will list the overall conclusions of this project.

5.2 General discussion and future work

5.2.1 THE SUCCESS OF *BLECHNUM NOVAE-ZELANDIAE*

The New Zealand native fern species *Blechnum novae-zelandiae* was used in experiments throughout this project. This fern was initially highlighted as a key species in these studies, as it is commonly distributed throughout New Zealand and is a major inhabitant of roadside embankments (Simcock et al., 2005). Other aspects of this particular species that lead to its success in this project were its spore producing capabilities, and the regeneration capabilities of its rhizome. Specifically for this project, *B. novae-zelandiae* was successful in *in vitro* laboratory experiments as well as *in situ* nursery and field experiments. The successes in these areas lead to an increase in our knowledge about this species as well as the development of several commercial revegetation technologies.

Experimentation in the laboratory was carried out in order to understand more about the life cycle of several native ferns. *Blechnum novae-zelandiae* performed well in all experiments and was the only species to successfully produce sporophytes from spore *in vitro* (Fig. 2.8). *Blechnum novae-zelandiae* was able to produce *in vitro* sporophytes from spore at a fast rate (Fig. 2.16). This characteristic suggests *B. novae-zelandiae* would be useful in revegetation because plants that establish quickly are beneficial as they reduce the amount of time an area is devoid of vegetation and exposed to erosion. The ability of *B. novae-zelandiae* to produce sporophytes *in vitro* opened up several more avenues to research, specifically mating systems and antheridiogen.

Several experiments indicated that *B. novae-zelandiae* had a mixed mating system. Isolated gametophytes were able to produce sporophytes, indicating that self fertilization or intra-gametophytic selfing was possible in this species. Fern species with this characteristic are thought to be able to radiate and establish new populations easily, as they are not required to outcross with other gametophytes (Lott et al., 2003). In addition to intra-gametophytic selfing, results indicated that *B. novae-zelandiae* responded to a male-inductive signal commonly referred to as antheridiogen (Figs. 2.12, 2.13 and Table 2.1). This signal promotes outcrossing within a species by inducing

spore in close proximity to a female gametophyte, to develop as male. Spore can be induced to germinate by antheridiogen even in the absence of light. Therefore, spore in the soil spore bank can be induced to germinate when a female gametophyte is growing in close proximity, resulting in sexual reproduction via outcrossing. *Blechnum novae-zelandiae* appears to utilize both selfing and outcrossing mechanisms, depending on the situation of the gametophyte. This mixed mating system may be one reason why this species is so dominant in the New Zealand flora.

The discovery that *B. novae-zelandiae* utilizes an antheridiogen mating system has implications for how it should be used in revegetation. When hydroseeding gametophytes it would be beneficial to include the substrate the gametophytes were cultivated on, in the hydroseeding mix. The substrate would contain antheridiogen and could potentially induce spore in the spore bank of the revegetation site, to germinate and fertilize the hydroseeded gametophytes. It would also stimulate spore on the soil surface to germinate, resulting in a foreseeable increase in fertilization and sporophyte initiation. Through the use of a mass spectrophotometer it would be possible to identify the antheridiogen signal of *B. novae-zelandiae*. Once positively identified in structure and function, the antheridiogen may even be able to be synthesized on a large scale and included in a hydroseeding mix. However, synthesizing large amounts of antheridiogen would almost certainly be too costly.

The spore of *Blechnum novae-zelandiae* was often the most abundant, but also always showed the most variability in germination curves, e.g. (Fig. 4.5). It is possible that this observation may be the result of spore dormancy. Figure 2.4A indicated that after a marked increase in temperature, spore was induced to germinate. However, the *B. novae-zelandiae* seasonal spore germination curve reached its highest rate after winter (Fig. 2.5), suggesting that a process such as stratification may also alleviate dormancy in spore. To date, little is known about fern spore dormancy. Future work could concentrate on determining that dormancy exists in fern spore, followed by the elucidation of environmental stimuli that result in and alleviate spore dormancy. This research would have implications for hydroseeding, as pre-treating spore to alleviate dormancy would result in a higher germination rate. In addition, inducing dormancy may have beneficial effects in terms of storing spore for long periods of time.

From the knowledge gained in the laboratory it was clear that *Blechnum novae-zelandiae* would be an interesting species to propagate. Nursery experiments resulted in the successful production of *B. novae-zelandiae* sporophytes on hydroseeded gametophytes, and the establishment of a high proportion of viable rhizome segments. Further analysis showed that the rhizome segments grew into densely rooted young ferns within 27 weeks. Spore experiments were not so successful, possibly due to a number of factors such as, spore dormancy, spore predation by micro-organisms, lack of or excess of environmental parameters (light, water, temperature) and competition from other plants. Replication of both the spore and rhizome experiments in the field resulted in a better understanding of conditions required for fern establishment suing these methods. The site characteristics were far from ideal and as a result, spore hydroseeding failed to produce any gametophytes and only a very low percentage (1%) of rhizome segments survived. It could be expected that these applications would be more successful at a site with lower irradiance, higher water availability and a more fertile substrate.

5.2.2 THE DEVELOPMENT OF COMMERCIAL TECHNOLOGY FOR NATIVE REVEGETATION

One of the main aims of this project was to develop revegetation technology for the commercial benefit of Rural Supply Technologies Ltd, specifically using native ferns. Three propagation methods were studied, focusing on different stages of the homosporous fern life cycle. These methods included propagation of spore, gametophytes and rhizome segments. The three methods showed varying degrees of success. The potential of each method, including foreseeable improvements is discussed in this section.

Propagation with spore using hydroseeding as the method of application produced relatively poor results. Spore propagation had the most challenges associated with it, as its success is influenced by complicating factors such as viability, environmental conditions (light, water, and temperature), predation and possibly dormancy. In a controlled environment such as the laboratory, spore germination was routine. However, spore germination was less successful in the nursery and totally absent in the

6 month field experiment. Steps were taken to optimize the spore propagation method for future experiments, including the collection of information on seasonal spore viability for the species *Blechnum novae-zelandiae*, *Cyathea medullaris* and *Dicksonia squarossa*, and the discovery that several common polyacrylamides have no significant effect on spore germination. As mentioned previously, more research could be carried out on fern spore dormancy. In addition, finding out the best conditions for long term storage of fern spore would also be useful. Another alternative would be to conduct more field trials at sites that offer less challenging conditions, i.e. lower solar irradiation, smaller slope angle, higher water availability and also higher humidity.

The application and subsequent establishment of laboratory propagated gametophytes in the nursery, proved to be relatively successful. Gametophytes of *B. novae-zelandiae*, *C. medullaris* and *D. squarossa* all produced sporophytes, indicating that development of the gametophytes continued normally in the nursery. *Blechnum novae-zelandiae* produced the most sporophytes. Due to limitations in the number of gametophytes that could be propagated at one time, a similar experiment was not conducted in the field. It is proposed that gametophytes could be hydroseeded, offering a more developed level of fern revegetation compared to spore. Gametophytes were tested in the laboratory for their ability to withstand low levels of maceration, which could be expected in a hydroseeder. They performed remarkably well in maceration experiments, with nearly a 100% survival rate. Future work could focus on further gametophyte homogenisation techniques. In order to make this method of revegetation possible an up-scaling in gametophyte production is required. Propagation of gametophytes from spore, in a temperature controlled nursery, on a hydroseedable substrate is a plausible option. Trials would initially be carried out to determine the best conditions and most suitable substrate. As stated previously the inclusion of the substrate and possible antheridiogen signal in the hydroseeding mix may aid establishment and development of the gametophytes.

Revegetation using segmented *Blechnum novae-zelandiae* rhizomes proved to be the most efficient way of producing ferns. This method was very successful in the nursery and it also worked in the field. However, characteristics of the field site resulted in very low rhizome segment establishment. It is unclear what the best time to harvest rhizome is, and whether there is a seasonal effect on rhizome viability. It would be beneficial to

carry out a similar experiment to the seasonal spore germination experiment, using rhizome segments instead. The main limitation with this method is sourcing rhizome material. Often, sourcing of material is allowed to take place within a designated area around the site that requires revegetation. This is termed eco-sourcing. Eco-sourcing is possibly the easiest method, but often the amount of material required for the job may be more than what is available in the surrounding area. Another possibility is to strip a site of valuable vegetation such as fern rhizome before the land is disturbed by machinery. The health and vitality of the vegetation could be maintained in a nursery until it is required for revegetation. The same material could also be propagated, increasing the number of plants. The rhizome segment revegetation method has already been used commercially by RST in conjunction with their Filtrex™ system. The results from their commercial work have been very positive.

5.3 Conclusions

The results of this study suggest that New Zealand native ferns can be successfully used in commercial revegetation. The species *Blechnum novae-zelandiae* has exhibited characteristics in its life cycle that make it the preferred species for native fern revegetation in New Zealand. These characteristics include its abundance in the New Zealand flora, its mixed mating system, and its ease of propagation via spore, gametophytes and rhizome segments. Of the three methods of propagation used in this project rhizome segments were the most successful, followed by gametophytes and spore. In all cases, the conditions in which various fern material was propagated had a large influence on its viability and growth. Water availability, temperature and irradiation are possibly the most influential factors associated with growing fern material when using this approach.

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APPENDIX 1

Table 1.1 Information on the herbarium voucher specimens that were taken from various collection sites and deposited in the Ella Campbell Herbarium at Massey University.

Species	**Location**	**Collection Date**	**MPN no.**
Dicksonia squarossa	Moonshine Valley	16/05/2005	MPN 31072
Dicksonia squarossa	Ecology Building	4/08/2005	MPN 31073
Cyathea medullaris	Ecology Building	4/08/2005	MPN 31074
Blechnum novae-zelandiae	Massey University Fernery	4/08/2005	MPN 31075
Cyathea medullaris	Greens Road	15/03/2005	MPN 31076
Blechnum novae-zelandiae	South Range Road	15/03/2005	MPN 31077
Blechnum novae-zelandiae	Pahiatua Track	15/03/2005	MPN 31078
Blechnum novae-zelandiae	Harrison Hill Road	16/05/2005	MPN 31079
Blechnum discolor	Lower Buller Gorge	16/02/2005	MPN 31080
Asplenium bulbiferum	Pahiatua Track	15/03/2005	MPN 31081