THE BIOGEOGRAPHY, ECOLOGY AND ENDOPHYTE MYCORRHIZA OF THE NEW ZEALAND CORYBAS ALLIANCE (Orchidaceae) Specifically: *Nematoceras iridescens* (Irwin et Molloy) Molloy, D.L.Jones & M.A.Clem. (Species).

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Abstract

No research on the fungal endophytes in the green New Zealand terrestrial orchids has been published. Identification of the endophyte resident in *Nematoceras iridescens* roots was accomplished by comparing hyphal septal ultra-structure and using TEM imaging of the hyphal septa, all of which indicated that the genus *Tulasnella* was involved.

The *Tulasnella* species was identified using molecular techniques focused on sequencing of the ribosomal RNA locus on the ITS1–5.8S–ITS2 nuclear ribosomal gene. The endophytic fungi, resident in the host plant *N. iridescens* and the germinating seed of this species, were identified for the first time as strains of *Tulasnella calospora.*

Scanning electron microscopy (SEM), light microscopy (LM) and Confocal Laser Scanning Microscopy (CLSM) was used to investigate spatial distribution of the endophyte hyphae within the plant. The SEM results identified four morphological types of hyphae: initiating, divaricating, intercellular and necrotised, or undergoing lysis. Peloton formation only occurred in specific areas of the root; mainly within the sub-epidermal and mid cortex cells. No hyphal involvement within the stele or the immediately adjacent cortex cells occurred.

This thesis, based on meteorological information, proposes that the centre of origin of *Nematoceras* is likely to be Papua New Guinea, with on-going dispersal being direct or from Australia to New Zealand. Wind vectors and *Nematoceras* adaptations to seed dispersal, both local and long distance, were investigated and tend to support this hypothesis.

Germination of all known orchid seed requires an obligate mycoheterotroph, generally a member of the Basidiomyceteae. For the first time, CLSM has imaged the fungal endophyte within the seed embryo and this was identified by molecular techniques and found to be a unique strain of *T. calospora*.

Three methods of orchid seed germination were trialled: symbiotic, asymbiotic and field envelopes. After a 12-month period, only field envelopes produced germinating seeds of *N. iridescens*. In all other methods the seed failed to germinate.

All *Nematoceras* spp. are solitary leafed and classified as moist mesophytes. Leaves were found to be hypostomatous. Being single leaved, protection is essential and a number of adaptations to counter herbivoury were found: raphide crystals, wax cuticle, winter maturity with summer–autumn aestivation all provide an antiherbivoury component.

The relationship between the genera of *Nematoceras* plus *Singularybas* and the associated species, of the far more ancient Hepatophyte order of Metzgeriales, were investigated.   The genus *Aneura* contains peloton like vesicles of various strains of *T. calospora*. The majority of the Hepatophytes have a parallel geographic-ecological requirement. The *T. calospora* mycorrhiza of the Metzgeriale genus *Aneura* was found to have a close association with the Corybas alliance observed.

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Glossary and Abbreviations used

Δ?	Provisionally identified / diagnosed .
Abaxial	Ventral aspect of a leaf, the underside
Acicular	A needle like form, especially in crystals
Acotyledonous	Without seed cotyledons
Adaxial	Dorsal aspect of a leaf, top side
Aeolian	Borne on the wind, (Aeolus, the Greek wind god)
Aestivate	A period of dormancy / quiescence
AFLPs	Amplified Fragment Length Polymorphism
Agenous	Ontogenetic pathway creating stomata without subsidiary stomatal development. An unequal mitosis occurring in protodermal cells, leading to the development of meristemoids.
Amphistomatous	Stomata found on both sides of the leaf (Generally a higher proportion are found on the Abaxial side of the leaf)
Anamorph	Describes the fungus when reproducing asexually
Anemochory	The role of wind in seed dispersal
Anomycytic	No subsidiary cells surround the guard cells.
Anomycytic stomatal complex.	Mature guard cells surrounded by epidermal cells that do not deviate morphologically from other epidermal cells in the same plant.
Anthesis	A period in which a flower is fully open and functional.
Apiculus	A long sharp pointed tip
Apomorphic	A derived type, a more contemporary state character
ASL	Above sea level
AWCGS	Allan Wilson Centre Genome Services
Biseriate	Two cell layers in depth.
CA	Corybas alliance
CLSM,	Confocal laser scanning microscopy.
Connate	Joined together, a union between two similar parts
cpDNA	Chloroplast DNA
Crustose.	Crust like, having an epidermal surface characteristic.
Cyclocytic pattern	More than four subsidiary cells surrounding a central pair of guard cells.
Cyclocytic stomatal complex.	
	A pattern of subsidiary cells that occur when a greater number of similar subsidiary cells radiate out from the mature guard cells outer circumference.
Defensin	Small, basic peptides that can inhibit the growth of a broad range of pathogenic fungi but seem nontoxic to either mammalian or plant cells.
Dematiaceous	Having a dark colour, usually olive, grey, or black.
Dermatogen	External cuticle of plants in a forming condition.
Diacytic	Only two subsidiary cells surrounding the stomata.
Diacytic stomatal complex	Mature guard cells surrounded by a pair of subsidiary cells with their shared wall at right angles to the long axis of the guard cells
Disjunct	A plant species that is found in two or more widely separated areas with no evidence of that species being represented between the two sites
Distichous	Two leaves growing oppositely and alternately
dNTPs	Dinucleotide triphosphates

Dolipore septum	A cross wall found in Basidiomycetes and characterized by special swellings and membranes in association with the septal pore
Druse crystals	Small open topped clusters of calcium oxalate crystals
DSE's	Dark septate endophyte
Edaphic	When produced or altered by the soil
EDAX	Energy-dispersive X-ray spectroscopy
Endemic	Taxa which occur only in a specific area
Endophyte	In the context of this dissertation the term represents a fungal endophyte that has a significant part of its lifecycle residing internally and asymptomatically in various plant structures and distinguishable from mycorrhiza in not possessing external hyphae or mantels.
Endovelamen	Inner tangential wall thickening of the external velamen
Ensiform	Shaped in the form of a double edged sword.
Epivelamen	External tangential wall thickening of the velamen
e-SEM	Environmental scanning electron microscope
EtOH	Ethanol
Exo III	Exonuclease III
FIM	Fungal initiating media
GA	Gibberellic acid
Gynostemium	A fused or partially fused column of stamens and pistils.
Hemimesogenous	The meristemoid divides twice to produce a second order meristemoid.
Holoepiphytes	Epiphytes having a complete life cycle on the host tree.
Holomorph	Describes the whole shape.
Horizontal transmission	Transmission of the fungus by sexual or asexual spores.
Hyaline	Transparent, clear.
Hydrophytic	Living in /on or by water.
Hypostomatous	Leaf stomata found on the abaxial surface.
Isodiametric	Length width and height are of roughly the same dimensions.
LM	Light microscopy.
Ls	Life span.
Lotus effect	Leaf surface cleaning by water droplets running off a hydrophobic waxy cuticle.
Lysigenous development	A duct in tissue, formed following lysis of cells.
Malesia	The botanical area of Indo-malaysia and Australia
Mesogene cell	A second order meristemoid.
Mesophytic	Neither too wet nor too dry an environment.
MGS	Massey Genome Service
MMN	Melin-Norkrans agar
MMNL	MMN without agar, liquid media
Mya	Million years ago
NCBI	National Centre for Biotechnology Information
Neoendemic	Representing an evolutionarily young taxon that has not had an opportunity to disperse.
NGS	Next generation sequencing
nrDNA	Nuclear ribosomal DNA
OM	Orchid mycorrhiza
Paracytic stomatal complex.	Mature guard cells surrounded by two flanking subsidiary cells.

Term	Definition
Palaeoendemic	Representing relicts of a once broadly dispersed taxon.
Parenthosomes	Shaped like a parenthesis symbol "(". They are bow to dome-shaped double membranes thought to be a modified part of the endoplasmic reticulum (ER), that cover the dolipore and pore channel forming the septal pore cap.
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar, Difco
Pedicel	The stalk, internode, that arises from the peduncle and terminates at the distal end of the individual flower. Fahn (1997)
Peduncle	An inflorescence stalk bearing a solitary flower in a one-flowered inflorescence
Peloton	Intracellular coils of mycorrhizal fungal hyphae found within the root cortex cells in an endosymbiotic association, Burgeff (1936).
Perigene cells	Cells that arise during stomatal development by the division of protodermal cells around the stomatal meristemoid.
Petiole	The stalk of a leaf, attaching the blade to the stem
Phorophytes	Host trees
Plasmoptysis	The physiological state of mycorrhizal fungi after the cytoplasm has been ejected. See ptyophagy.
Plesiomorphic	An original type or primitive ancestral state character
Pseudovivipary	Production of offspring by apomictic or asexual propagules such as plantlets and bulbils.
Ptyophagy	Fungal cytoplasm is inserted into the transfer cells of roots by the specialized hyphae of the mycorrhizal mutualist.
PUA	Polyunsaturated aldehyde
rDNA	Ribosomal DNA.
RAPD	Random amplified polymorphic DNA
Raphides	Needle like crystals composed of calcium oxalate
Reniform	Kidney shaped (renal)
RFLP	Restriction fragment length polymorphism
Rosanoffian crystals	Crystals are found within a sheath, bundled within a membrane or specialist cell.
Schizogenous development.	Development of a duct by division of a common middle lamella that expands to form a lacuna.
SAP	Shrimp Alkaline Phosphatase
SEM	Scanning electron microscope
SPA	Septal pore apparatus
Teleomorph	Describes the fungus when reproducing sexually.
Tetracytic pattern	Four, roughly equal sized, subsidiary cells surround the stomatal guard cells.
Tolypophagy	Intracellular aggregates of coiled fungal hyphae that have been isolated by root or rhizoid cells prior to absorption or excretion.
TS	Transverse section
Uniseriate	One cell layer depth.
Wewelite	Calcium oxalate monohydrate crystal
Xeromorphic	Morphologically adapted for dry conditions

Chapter 1   Introduction and aims of the thesis

New Zealand has over 140 species of native orchids in over 40 genera (Dawson *et al.*, 2007). Five of these genera include endangered plants (Townsend, 2008). One species, *Anzybas carsei*, is listed as critical with only 30 individual plants counted at the last survey (de Lange *et al.,* 2004). Other members of the Corybas alliance (CA): *Nematoceras rivularis* and its varietal types ("Kaimai", "Kaitarakihi", "Rest area", "Rimutaka", "Whiskers"), and *Nematoceras* aff. *trilobum* varietal types ("Pygmy" and "Trotters") have not as yet been fully surveyed (Dopson *et al.,* 1999). Many of the other NZ orchid species have not been classified but anecdotal and personal observation suggests that numbers, as well as those mentioned above, of CA species, are in decline. A conservation strategy used in Australia (Clements, 1981; Chung *et al.,* 1981), in Europe (Rasmussen, 1995) and in the USA (Zettler *et al.,* 1995*)* was to initiate breeding programmes by collecting the symbiotically germinating seed and then planting the resultant seedlings in sterile media (Quay *et al.,* 1995; Batty *et al.,* 2001). *In vitro* seed germination is being utilized at Kew in an endeavour to conserve threatened species, including orchids, (Sarasan *et al.*, 2006). Seed propagation is used, in preference to tissue culture, in order to maintain a genetic diversity (Rasmussen,1995).

All orchids depend on mycorrhizal fungi for germination, an obligate mycoheterotrophism, and this appears to be the only universally common characteristic of orchids which have the greatest morphological diversity of any plant family (Williamson and Hadley, 1970; Warcup, 1981b; Arditti, 1992; Zelmer *et al.,* 1996; McKendrick *et al.,* 2002; Rasmussen, 2002; Cameron, 2004).

As early as 1824, certain fungi were known to associate with orchid roots (Link, 1824) and the observed fungal mycelia were thought to be pathogenic, utilizing the orchid as a host (Schleiden, 1849). In 1892, Frank, recognized the association with orchids and plant roots as being neither parasitic nor saprophytic and termed this fungal association with host roots a "mycorrhizal" association, describing it as a symbiotic association between particular soil fungi and orchid plants. Frank also proposed two broad divisions of root-associated mycorrhiza; an externally located fungus he termed ectomycorrhiza and an internally inhabiting fungus he termed endomycorrhiza. Unfortunately, none of these early observers realized the implication of the mycorrhizal fungi and the obligate mycoheterotrophism of all orchid seed.

This observation was later elaborated by Bernard, who described this characteristic in 1899 and published his seminal work in 1904 (Bernard, 1904), which was further elaborated on (Bernard, 1909). Prior to World War 2 (Burgeff,1936), reported that all orchid seed, he was successful in growing, required a fungal endophyte inoculum, otherwise the seed invariably failed to germinate. This dependence on a fungal endophyte, obligate mycoheterotrophism, applies to all known orchid seed. Narrow specificity of the obligate fungus does not occur and a broad spectrum of Auricales fungi appeared able to initiate germination, (Rasmussen 1992,1995; Rasmussen and Whigham,1998; Otero *et al*., 2002, 2004; Chapela and Garbelotto, 2004; McCormick *et al.*, 2004; Chase, 2005).

As the host orchid matures many orchids discard the primary fungal endophyte and form a mycorrhizal association with more specific fungal genera (Read *et al*., 2000;

McCormick *et al*., 2004). Investigations into orchid mycorrhiza, found that the Basidiomycete complex of Rhizoctonia, appeared to be an ubiquitous mycobiont to a large range of orchids (Harvais and Hadley,1967; Warcup and Talbot, 1967; Benzing and Friedman,1981; Henrich *et al*.,1981; Warcup,1981; Arditti,1992; Goh *et al*.,1992; Zelmer *et al*.,1996; Shan *et al*., 2002; Tupac *et al*., 2002; Rasmussen and Whigham , 2002; McCormick *et a*l., 2004; Hemborg and Bond, 2005) .

The specificity of certain fungi, with particular taxonomic orchid groups and the associated ecology of the environment, were reviewed by both, Burgeff (1936) and Curtis (1939). The hypothesis that the mycorrhizal fungi provided nutrients to the host plant was initially described by, Magnus (1900), who proposed that cellular digestion of the fungal hyphae occurs within the root cortex, by a process later termed "tolypophagy" (Burgeff,1936), where intracellular aggregates of coiled fungal hyphae are immobilized prior to being isolated by root or rhizoid cortex cells and then digested by the "host".

While significant research on ectomycorrhizal fungi has been accomplished in New Zealand, (Orlovich and Cairney, 2004), very little research has been conducted, in New Zealand, on identifying specific fungal endophytes that form mycorrhizal associations with native orchids. The author of this thesis can find no formal research that has investigated the identity of the fungal endophyte, the symbiotic germination of terrestrial NZ native orchid plants or their seed. The ability of plant seed to be dispersed and to then germinate is a critical area in the conservation of any endangered species.

To identify a plant species positively, in the field, it is necessary for the plant to be flowering. As the optimal time for removing pelotons from the plant tissue for mycorrhizal endophyte culture is at the early pre-digestive phase, some method of identifying the genera and species at leaf emergence rather than waiting for flowering would be advantageous.

Many botanists, both professional and amateur, have taken the *Corybas* flower as the principal character for species identification, (Oliver,1930; Hatch,1951; Molloy and Irwin, 1996; St George *et al*.,1996; Jane, 2001). Flower related taxonomy is not discussed in this thesis since there are many references, regarding NZ orchid flowers in the literature, from both professional botanists and amateur authors,(Hatch,1951; Allan,1962; Cooper,1981; St George and McCrae,1990; St George, 1991; Molloy and St George,1994; Jane, 2001; Kores *et al*., 2001; Bateman and Rudall, 2006).

The genera of *Anzybas, Corybas, Nematoceras*, and *Singularybas*, part of the CA, are reliant on only a single leaf that, combined with mycorrhizal fungi, provide the nutritional requirements of each species of plant, (van Royen, 1983; Arditti, 1992; Rasmussen, 1995; Williams and Eamus, 1997; Tung *et al*., 2000; Cameron *et al*., 2008).

Such reliance would suggest that plants within the taxa have developed effective antiherbivory defenses. In this thesis, observed predators on *N. iridescens* are discussed and their selectivity of leaf tissue could be related to calcium oxalate idioblast location within the leaf.

In angiosperms, stomatal complexes can be taxonomically important and these characters can be used in specific species diagnosis, (Stace, 1989). The patterns of guard and subsidiary cells surrounding the stomata have been utilized as key taxonomic characters (Rasmussen,1987).

The major aims of this thesis are to:

1. Establish a molecular protocol that can be used in future NZ orchid endophyte identification,

2. Investigate the initial root entry of the endophyte hypha an to observe the subsequent path and morphology of fungus through to peloton lysis and cortex cell re-establishment,

3. Investigate the seed germination of *Nematoceras iridescens* and seed dispersal mechanisms available to the New Zealand CA species,

4. Assist the conservation of at-risk NZ orchid taxa, specifically the NZ CA , *Nematoceras iridescens*, by identifying the mycorrhizal endophyte/s of both the host plant and in the developing seed embryo,

5. Examine the leaf epidermis, stomatal system and the seed testa anatomy of selected New Zealand CA species in an endeavour to identify the target species and other CA species prior to flowering or when flowers are absent, and

6. Record the predators of leaf tissue on *N. iridescens*, examine eating patterns and relate these to raphide developed within the crystal idioblast cells of the leaf.

This thesis, because of the diversity of research contained therein, has been split into three sections as well as individual chapters (Chapter 1 (Introduction), Chapter 2 (Methods and materials used), Chapter 3 (Description and distribution of the CA) and Chapter 7 (Conclusions).   Section A comprises the ecology of related results of the *N. iridescens* experiments, section B elaborates on the results of the investigation as to the identity of the fungal endophyte, mycorrhiza, inhabiting the pelotons of *N. iridescens* and related species, and section C comprises a summary of the thesis.

Chapter 2   Methods, materials and sampling sites used in the thesis

Since many of the methods and materials used in the various sections of this thesis are repetitive and all samples were obtained from the same areas, this chapter (2) has been established to present the methods and materials used in the respective experimental procedures.

2.1 Sampling

Plants, of the Corybas alliance (CA) studied in this work, were found growing on or associated with steep banks that had been formed from roadside cuttings, slips, or other soil disturbances. All, except *Anzybas* spp., *Corybas cheesemanii* and *Molloybas* sp., were located in moist shaded areas either on open south facing slopes or shaded northern aspects.

Most CA species are found growing on the west coast areas of both North and South Islands, in the higher North Island inland areas. The taxa is also found growing in coastal areas in Northland, the south of the South Island and in the Southern Islands,   (St George, 1992,1996; Laursen *et al.,* 1997).

The New Zealand endemic terrestrial orchid, *Nematoceras iridescens*, was selected as the experimental subject because of its local vigour. Largely undisturbed colonies were found growing on almost sheer banks where they remained free of large herbivore foraging (i.e. feral goats, sheep, deer and cattle). Population numbers are still reasonably high, thus the conservation status is currently classed as, not at risk.

All plant samples of *N. iridescens* were obtained from six independent sites within the Taranaki area over the period July – January 2004-2009 (see Fig. 2.1). Entire plants of *Nematoceras* spp. were obtained from moss and fern litter on either a mudstone (Moki, Uruti, Matemateaonga; or volcanic ash (Pukeiti, Tarata) base (Kamp, 1988).

At collection the samples were placed in rigid plastic 2L ice-cream containers together with 250-400 mL of loose litter that surrounded the plants. Approximately 100 mL of run off water was added to the specimen container to retain the humidity. The containers were placed in polystyrene "chilly bins" with ice-pads to maintain a cool temperature.

The rapid degradation of orchid endomycorrhiza, within 24 hrs of collection, has often been reported, (Rasmussen, 1995; Brundrett, 2004; Suárez *et a*l., 2006). This creates, from field collected specimens, a problem in obtaining fresh endophyte root material, for culture or microscopy. Samples were therefore collected as early as possible in the morning and immediately chilled in the "chilly bin" as described above. The samples were held in a refrigerator at $4^{o}C$, prior to transfer to the laboratory.

Herbarium collection specimens, immediately after collection, were placed in 250 x 155 mm plastic zip-lock bags together with 5g fine silica gel (Merck, Kieselgel 60 #35-70) for initial dehydration, (Chase and Hills, 1991). After seven days the dried specimens were placed in appropriately sized culture dishes together with a further 5-10g of silica gel (Merck, Kieselgel 60 #35-70), sealed then labeled, (Ecroyd, 2004), and deposited in the Dame Ella Campbell Herbarium, Massey University, Palmerston North. (See Appendix 1c for specimen voucher index).

2.1.1 The sample sites:

All of the sampling sites (Fig. 2.1) were located on shaded, moist road embankments in areas often overhung by Blechnum ferns. The steep sloping ground above the site embankments consisted of large areas of *Dracophyllum filifolium.*

Trees of black beech (*Nothofagus solandri*) and rewarewa (*Knightia excelsa*) were common within the North Eastern Taranaki sites of Moki and Uruti. Large mats of moss and hepatic *Aneura* spp. were inevitably associated with all of the *Nematoceras* spp. observed over the course of this research project.

Figure 2.1   Sampling site locations: Insert map (upper left) shows the location (green) of Taranaki within the NZ map.

1 Moki,   (39° 0' 31.97" S 174° 48' 55.00" E)

2 Uruti tunnel, northern approach,
     (39°.01S.174°.34'

3 Tarata tunnel, south approach
     (39°.07 S.174.21°E)

4 Pukeiti (39° 12' S.173° 59'E)

5 Matemateonga (39° 23' S. 174° 34'E)

6 Nursery plants, New Plymouth.
     (39° 03S.174°05'E)
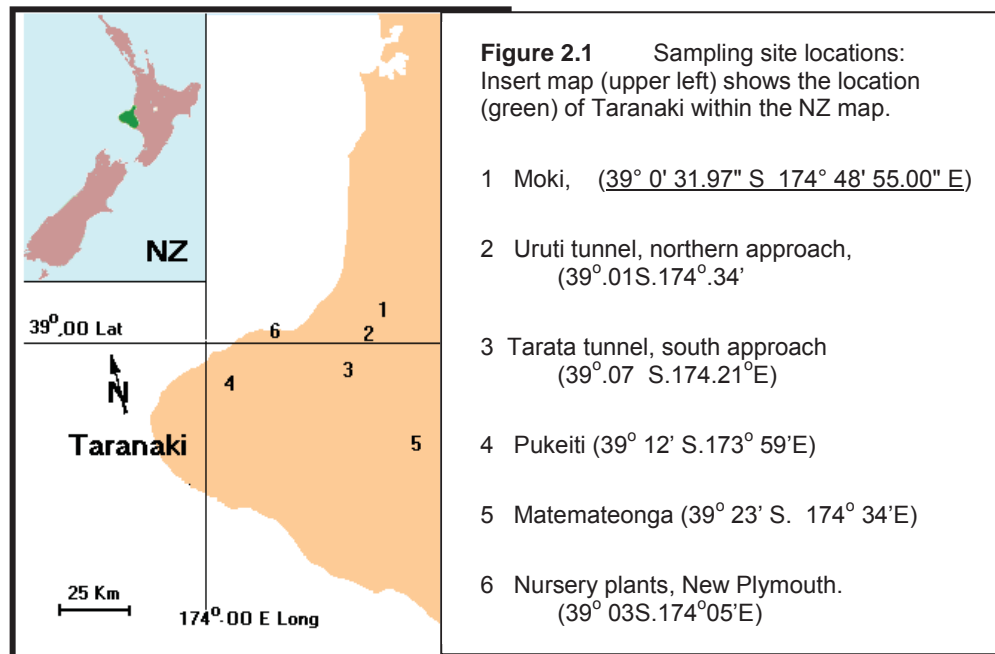
Photographs of each collection area can be seen in Fig. 2.2. The Tarata and Pukeiti sites were generally composed of a mixed conifer/ broadleaf, warm temperate rain forest.

At Matemateonga, large areas of secondary growth of kanuka (*Kunzea ericoides*), mahoe (*Melicytus ramiflorus*), tree ferns (various spp.) and high country pasture grasses have replaced the original forest remnants of rimu and totara forest that previously had been heavily milled. Apart from the "nursery" sites, at 10m above sea level (ASL), all were at altitudes of between 300-600 m. These altitudes provided the moisture and variable temperature range, via local air convection, prevailing humid westerly and northerly on-shore winds.

The cooler temperatures create an atmospheric condenser to the warm humid air and consequently ensure high rainfall areas. The endemic forests in the Taranaki area are classified as temperate rain forest, (Wardle, 1991). Dominant trees in these forests are kohekohe (*Dysoxylum spectabile)*, tawa (*Beilschmiedia tawa*), kamahi (*Weinmannia racemosa*), rimu (*Dacrydium cuppressinum*), northern rata (*Metrosideros robusta*) and a large diversity of ferns and bryophytes.
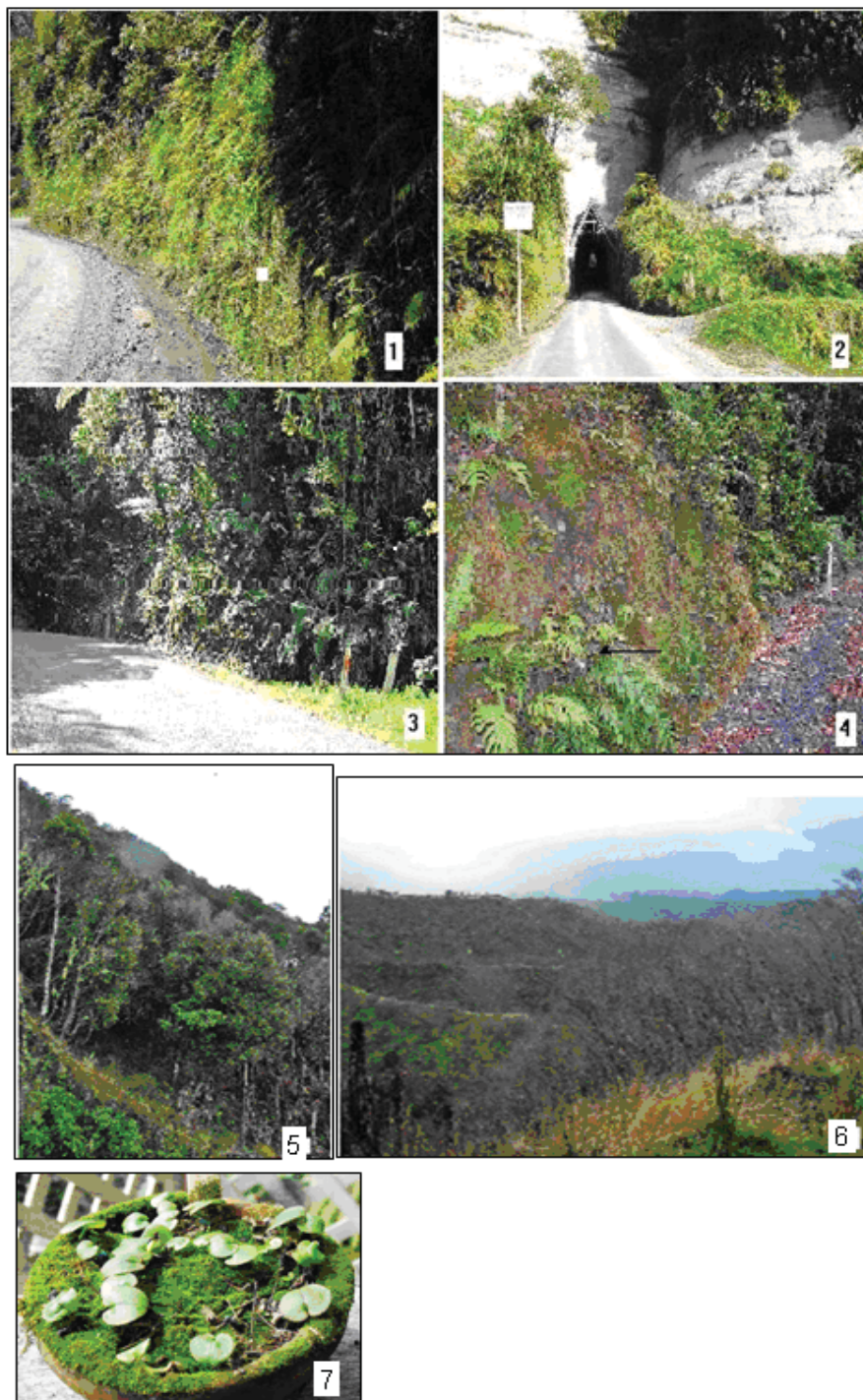
Figure 2.2 The site areas from which the samples of *Nematoceras* plants were found. 1= Moki, 2= Uruti tunnel, 3= Tarata tunnel (South), 4 = Pukeiti, 5, and 6 = Matemateonga sites 7 Nursery pot of *N.iridescens*

Light levels had previously been measured in areas inhabited by a number of CA species (*Nematoceras iridescens*, *N. macranthus* and *Singularybas oblongus*). These plants were growing in association with colonies of the Anthocerophyte, *Megaceros pellucidus*, which had been earlier investigated for chloroplast light response, (Watkins, 2002). The light strengths ranged from the *S. oblongus* site with 18 ± 2 μmoles photons $m^{-2}s^{-1}$ plus short period, "sunfleck", light levels, on *N. iridescens* and *N. macranthus*, of 556 ± 4 μmoles photons $m^{-2}s^{-1}$. This represents an optimal light range for the majority of Bryophytes, Hepatophytes, Anthocerophytes and fern colonizers, (Watkins, 2002). Ecological niche site preference by the CA plants suggests that the conditions of the moss colonized, previously disturbed soil, present favourable conditions for these specialist taxa to colonize this area successfully.

Unfortunately, the sites at Moki and Pukeiti were compromised by road earthworks (Figs.2.3 and 2.4) midway 2007, through the research phase of this thesis. Cutback of banks and road edges plus natural earth slips appears to be the greatest threat to colonies of *Nematoceras* spp.(*pers obs*). This phenomenon is however a blessing in disguise, since fresh sites are rapidly colonized by bryophytes and after a number of years provide suitable sites for fresh *Nematoceras* spp. Older sites tend to become overgrown with terrestrial ferns, other shade tolerant plants and leaf litter, thus restricting the light available for low lying terrestrial plants, (Watkins , 2002).
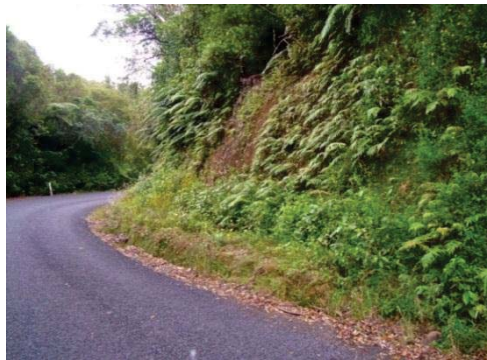
Figure 2.3   The original Pukeiti collection site, 2005, prior to roadworks.

Figure 2.4   Roadworks (2006-2007) have destroyed all plants on the right bank. Photograph, 2007, of the Pukeiti collection site.

2.2   Wind measurements

In this section of the thesis, a measure of the immediate wind velocity and direction was taken at the time of pod dehiscence.
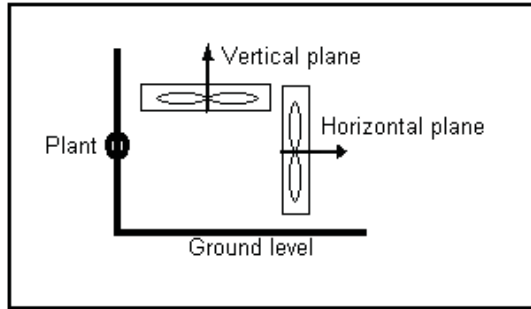
Figure 2.5   Illustrating Q1301 anemometer reading positions both vertical (A) and horizontal. (B) Each reading taken was the direction of strongest wind reading.

Plant loci were selected at 1m from ground level (all plants were growing in roadside banks). The wind-speed recordings were measured on a Q1301 anemometer which was used in two readings at each site; A = vertical wind current and B = horizontal wind current (Fig.2.5). A tape was stretched horizontally, at ground level, below the plant, at $90°$ from the plane of the bank surface (Fig.2.6). Measuring sites were established with a metre rule to give regular heights.   At both positions, automatic readings at 2-second intervals over a period of 5 minutes with the final readout giving the average wind velocity in ms^{-1} with an error of ± 5%. The reading range of wind velocities was 0.2 ms^{-1} to 14.3ms^{-1}.

Temperatures ranged from 20-21$°C$ at altitudes, Moki 380 m, Pukeiti 480m above sea level. Wind currents measured at the pod level of *N iridescens* gave a zero wind current velocity, the anemometer unable to record flows of less than 0.2 ms^{-1}.
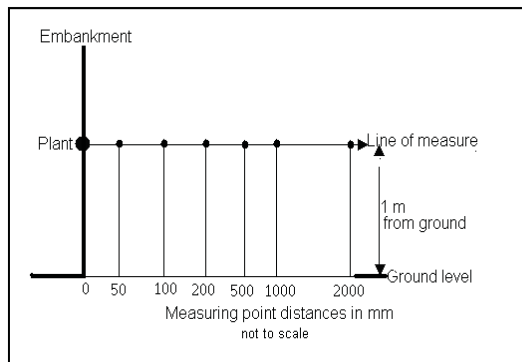
Figure 2.6   The measuring points used in collection of the wind data at Pukeiti and Moki.

While many orchid references mention peduncle post-anthesis extension, only, Ohno (1991), who investigated the effects of floral organ removal and phytohormone application on the peduncle elongation of a miniature *Cymbidium* hybrid, has investigated this characteristic in any detail. Ohno concluded that peduncle elongation in *Cymbidium* orchids is stimulated by auxins, possibly

supplied from the flower bud development, specifically from the anthers and that flower bud development is enhanced by a gibberellin (GA).

Peduncle elongation, they concluded, is a secondary effect of developing floral parts brought about by a gibberellic acid (GA). There are no specific reports, in any of the literature examined, of terrestrial orchid peduncle extension or any correlation to wind strength, temperature and humidity associated with their growth.

2.3 Pod harvest

Pods were harvested from six *N. iridescens* plant colonies in the site areas. Only a few seedpods, per colony, are produced annually, which necessitated a minimal harvesting protocol, this being dependant on the colony size and presence of further pods. To contrast *N. iridescens*, a pod from each of *N. papa, N. longipetalum* and *Singularybas oblongus* were similarly measured and recorded.

Each pod sample was tagged with red coloured wool, loosely tied around the petiole together with a plastic label, with a date and number, inserted into the ground alongside the plant. Care was taken to ensure the roots were not severed. The peduncle, from the adaxial leaf surface to the pod base, was measured with a CK3450 slide calliper (CK Toolshop), every ten days. When finally severed the peduncle length was measured then mounted as a display. The time / growth sequence data obtained was recorded and the growth rates graphed. Seed from the pods was collected, stored in 1.5 ml sealed centrifuge tubes and used for the establishment of physical parameters such as: weight, dimensions and seed germination; in later experiments.

Severing of all pods occurred as close to dehiscence as possible. Pods that had dehisced prior to harvesting were carefully collected and seed remaining within the pod extracted, using a small paintbrush. The extracted seeds were brushed off the paintbrush fibres into a 1.5 ml centrifuge tube.

2.4 Pod oscillation

After observing, the movement of mature pods in the wind currents and their "pepper pot" effect in liberating seed into the wind stream a search was made of the literature, to no avail. A small electrical fan (Goldair) was set up on a table, in an area of minimal air movement. At a slow fan speed selection, the resultant air current was measured with a Q1301 wind speed meter. Once the distance giving the required wind speed was calibrated the base of a full *N. iridescens* peduncle, of 125 mm in total length, and pod, approaching dehiscence, was clamped by a pair of mole grips and placed on the calibrated area with peduncle and pod at right angles to the table top. Measurements of the oscillations were read from a metal ruler clamped to a retort stand and orientated to lie parallel to the tabletop and to the airflow stream (switched on after the display had been set up and calibrated with the pod set at a height of 120 mm (Fig. 2.7).
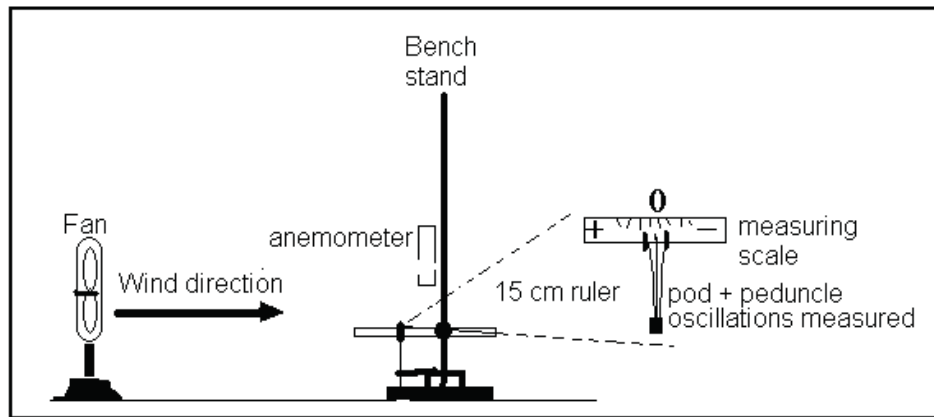
Figure 2.7 The experimental set up used to measure the pod oscillations in a lignified peduncle of *N. iridescens*.

2.5 Seed collection

Mature pre-dehiscent pods of *N. iridescens* were collected at the sampling sites over the period December - February through 2004-2008. Some pods, in the area, had already dehisced and no seed remained. The collected pods were placed in plastic specimen jars, numbered and dated. To conserve the natural seed number only a minimal number of pods were taken. The pods were surface sterilized, in a laminar flow hood, by dipping in 75% EtOH then washing three times successively in sterile distilled water.

The seed in pods that had just opened and prior to total seed liberation were considered unsterile and were collected in 1.75ml Eppendorf tubes with the lid of the Eppendorf holding the peduncle of the pod. These mature pods of *N. iridescens* appeared mid to late November, late pods were found as late as December. The ambient weather appears to "fine tune" dehiscence, if the weather was warm and had low humidity the mature pods dehisced early. However if the weather had a high humidity and temperatures were low the mature pods remained closed for a longer period (*Pers.obs*).

In the laboratory, the seedpods were again surface sterilized by immersion for two minutes in 3.2% w/v NaOCl, Janola®. After the two-minute period, the pods were washed three times in sterile water and left to dry before placing them in pre-sterilized Eppendorf tubes with lids opened. All operations were undertaken in a laminar flow cabinet. The Eppendorf tubes, containing pods, were then placed into a pre-sterilized, silica gel, desiccator jar until pod dehiscence occurred. The desiccator was then opened and the Eppendorf tube lids closed. The seeds were harvested by vigorously agitating the closed Eppendorf tubes by hand until the ripe seeds became dislodged from the pod interior. The closed tube was then immersed in 75% EtOH for 1 minute. The pod was removed from the container and in a laminar flow cabinet, the remaining seed within the seedpod were scraped out with a sterile dissecting needle and placed back into the Eppendorf® centrifuge tube that was then sealed and held in a silica bead desiccator, at 4°C, until required.

2.6 Seed morphology

A forceps "pinch" from each sample, 12, derived from three cohorts, two from Tarata and one from the Moki sites, was taken with a pair of blunt end forceps and

placed onto a microscope slide. The sample was covered with a cover slip. Each sample was examined under a Leica MZ12 binocular stereo microscope for the total number of seed (n), the various morphologies and the number comprising each morphological group.

The groups were labelled:

1	normal seed with embryo,
2	small seed (micro) with small embryo,
3	testa only, no apparent embryo,
4	damaged testa or diseased embryo.

The percentage, in each cohort sample of the various groupings, was calculated for totals and average number per group, along with standard deviation and sample error.

After sorting and counting the seed samples from each group the seed was placed into a 1.75 ml centrifuge tube and surface sterilized with 400 µl Janola® 3.5% NaOCl for a period of 1 minute and then irrigated x3 with 400 µl of autoclaved RO water, each for a period of 1 minute. Twelve pre-sterilized Petri dishes containing PDA and oatmeal media were inoculated with a seed sample then covered and sealed with Parafilm®.

Seed of *N. iridescens* was measured to obtain embryo and total volumes using CLSM. The maximum section out of 46 was taken as the basic measurement and details were calibrated by the CLSM software programme.

Total Seed and embryo areas were calculated from a series of point-measured micrographs (Fig.A1.8) and depth from z-axis component (Fig.A1.9).

2.7 Seed germination

All three seed germinating techniques as outlined in the introduction as:

1	symbiotic germination,
2	asymbiotic germination,
3	field germination,

were trialled and the remainder of this chapter has been partitioned accordingly.

2.7.1 Symbiotic germination of N. iridescens seed (series 1)

During the period 2005 - 2007 seed capsules from ten previously tagged *Nematoceras iridescens* plants were collected from four collection sites; Moki, Uruti, Tarata and nursery. Seed capsules, at maximum capsule peduncle extension immediately prior to dehiscence, were harvested from each of the four sites (November – early February).

To ensure that the seed and media remained sterile a vigorous surface sterilization of the external surface seed capsule with 70% EtOH and followed with 3.5% NaOCl, prior to opening the seed capsule, was undertaken. Each capsule was incised in a laminar flow cabinet using normal sterile techniques. The seed was then scraped into a sterile 90mm petri dish. Aliquots of this seed were transferred into sterilized 1.5ml Eppendorf tubes, (Rasmussen, 1993, 1995). All seed was stored at 4°C until required. Each batch of seed was then distributed and the new factions placed into duplicate 1.5ml Eppendorf tubes. To ensure the trial seed represented a generalised representation of the site the individual pod seed from *N. iridescens* was mixed. (Table 2.1)

Location	Pod seed	Seed mix
Moki	P1, P2, P3, P4.	Px
Uruti	U1 and U2	Ux
Tarata	T1 and T2	Tx
Nursery	N1 and N2	Nx

Table 2. 2 The coding of seed used in the symbiotic germination experiment.

All seed was surface sterilized in a 1% sodium hypochlorite solution (Janola © 3.5%) for a period of 3 minutes followed by three separate washes in sterile de-ionized water, (Rasmussen, 1993; Wilkinson *et al.*, 1994; Zettler, 1997; Rasmussen and Whigham, 1998). Seeds were pipetted, using a cut pipette tip proportional to the seed size, a forceps pinch of seed mix / 100μl water onto sections of quarter round autoclaved 70 mm Whatman #1 filter paper. The quarter papers were placed around the external area of an oatmeal plus Difco potato dextrose agar medium (OPDA) (Fig. 2.8) contained in a 90mm sterile Petri dish, (Rasmussen, 1995; Kauth *et al.*, 2008).

Figure 2.8 Outline of 90mm Petri-dish with 4 x ¼ segments of 70 mm Whatman #1 filter paper resting on PDA and H4 oatmeal agar medium. Each segment had 100μl of treated orchid seed suspended in sterile RO water placed in its centre. The centre of the media (o) indicates the placement position of the fungal plug.
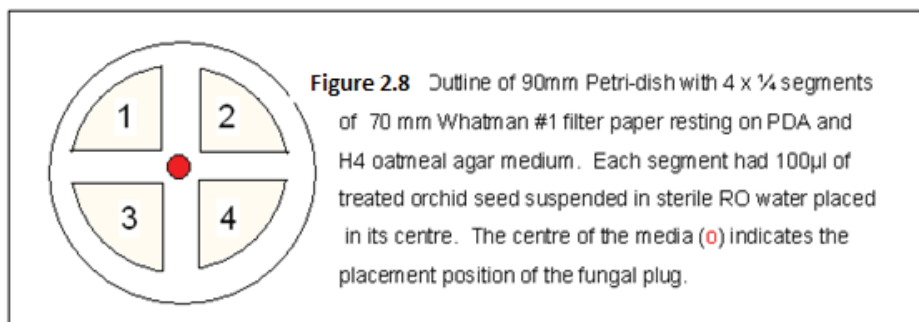
Plugs from 33 library fungal isolates, Table B3.1 and 2 (Series 1) were each plated onto OPDA media and incubated at 25°C for a period of 7 days. Single 6mm plugs, obtained from the edge of the incubated cultures, and were placed in the centre of the Petri dish medium, surrounded by the prepared seed filter paper sections. The plated dishes were covered and sealed with Parafilm® before being placed in a dark 24/24 incubator maintained at 15°C for a period of 45 days. Selection of seed, for seed germination experiments, was based on sample trials in which a forceps pinch of collected seeds were plated, onto PDA media for a period of 60 days. The plates were checked for contamination and seed for the ability to imbibe water (Table A1.5). Seed with clean embryos, no appearance of fungal growth on culturing and having no obvious damage to the testa over the test period, indicated which bulk seed mix to use in future experiments.

2.7.2   The symbiotic germination of *N. iridescens* seed (2)

A second series of symbiotic germination trials was undertaken after the poor results of the initial experiment. Three major modifications from the first series methods were undertaken.

The incubation temperature was altered to $12^{\circ}C$ to bring this parameter into line with a natural environmental range. A comparison of light regimes was also undertaken. The experimental conditions for this series were:

     1   a 24/24 dark series in a Gallenkamp-cooled incubator IH-270

     2   a 24/24 light regime in an Astell incubator,

     3   a 12/24 light regime also in an Astell incubator.

Plug samples were taken from fungal library specimens that had been DNA sequenced and identified to the nearest Genebank accession number (Table 2.2).

Cultures were grown from 5mm inoculant sample plugs taken from the culture perimeters. The plates were inspected for fungal growth and evidence of germination with a Leica MZ12 binocular stereo microscope. ...

Seeds were collected from nine surface sterilized pods (NaOCl 3.5% followed by a swab of 70% EtOH). The pods were cut from plants at Tarata on the 23/11/2006 and consisted of three green pods plus six mature pre-dehiscence pods. A,B and C one green pod seed each and seeds from six mature pods into each of six plates labelled D,E and F.

code	Library #	Closest Genebank accession #	Identity
N3	N3a1	AY634132	*Sebacinaceae* uncult.
A7	Mollyred	AY295325	*Neonectria* isolate
T3	T3a1	AY568066	*Ascomycete* spp.
A1	A1	AM260847	*Sebacinaceae* uncult.

Table 2.2   Selected library inoculants, which produced close match to Genebank accession numbers.

2.7.3   The asymbiotic germination of *N. iridescens* seed.

Seed was initially collected from mature but unripened pods after a surface sterilization of the external pod with 95% EtOH followed 5 minutes later by a wash in sterile RO water.

In a laminar flow cabinet, the pod was incised from the calyx to the petiole and the seed liberated from the opened pod into a sterile 90 mm petri dish using a sterilized probe. Seed from ripe pods, that had reached a point similar to pods held in the Eppendorf collection tubes were sterilized by immersing in a Janola™   (3.5% NaOCl solution), plus 5 µl of Tween 80™, for a period of 3 minutes and then washed 3x in sterile RO water, (Rasmussen, 1995). The procedure proved difficult, the minute seed and its cellular structure inhibited a thorough wetting therefore disinfection was inadequate and created high contamination rates in the plated out seed.   A new method of wetting and sterilizing stored dry seed was developed. The collected seed was placed into the top filter section of a QIAshredder™ Mini Spin Column, with a collection tube attached   (Fig 2.9).
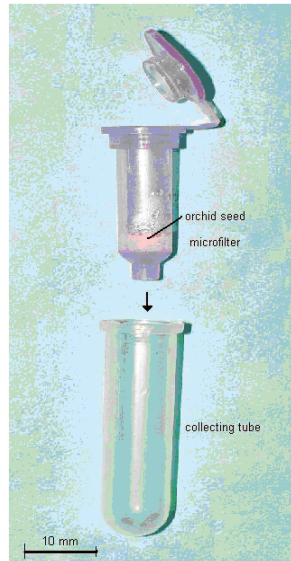
Figure 2.9 A QIAshredder™ Mini Spin Column (lilac coloured) and its 2 ml collection tube (clear).

At pod dehiscence, when gaps appeared in the seedpod pericarp, the seed contained were placed into a Petri dish and transferred into an Eppendorf tube. A disinfectant comprising 400 µl of 1% NaOCl w/v plus 5 µl of Tween 20 was introduced onto the seed in the QIAshredder™ Mini Spin Column.

The top was then closed and the column vortexed for three minutes.   The disinfecting solution was removed by centrifuging the combined column and collection tube at 6000 rpm for 1 minute on a Heraeus Biofuge (pico) centrifuge followed by three 400µl washings of sterile MilliQ water, each wash was placed onto the top of the seed in the filter section and then centrifuged at 6000 rpm for 1 minute.

A final immersion of the seed, in 200µl MilliQ water, was completed in a laminar flow cabinet and was not removed by centrifuge but left to create a seed-carrying phase for plate distribution. The QIAshredder™ Mini Spin Column was washed in 70% EtOH prior to adding the final water phase, after which a transfer of the surface sterilized seed from the QIAshredder™ Mini Spin Column onto the relevant media using a flame sterilized bacterial loop, was then carried out.   Seed was sown directly, with a drop (10µl) of sterile RO water, onto the media held in sterile Petri dishes. The seeded plates were labelled and sealed with Parafilm. A small hole was burnt, with a heated probe, in the top dish plate. The hole was then sealed with a strip of Parafilm. This hole allowed for watering, with autoclaved MilliQ water, using a sterile syringe and needle. This method allowed irrigation of the plated seed without having to remove the Petri dish lid thus reducing possible contamination of the seed and media.   The Parafilm tape sealed aperture was

swabbed, both before and after any irrigation procedure, with 70% EtOH and the syringe needle was flame sterilized between plate samples.

A number of media (see formulae Appendix 3) were trialled; initially the well tested orchid seed germinating media of: Knudson C, (Knudson, 1946) amended, (Anderson, 1990), N3f ammended, (Burgeff, 1936), Vacin and Went amended, (Withner, 1953; Clements, 1982).

For the second group of orchid seed the culture media was purchased from Phyto Technology Laboratories LLC (PTL). The selection of BM1 (Terrestrial Orchid Medium), M551 (Malmgren Modified Terrestrial Orchid Medium) and T842 (Terrestrial (Cypripedium) Orchid Medium) as media was based on the research of the experimental germination of selected North American terrestrial orchids by Kauth et al. (2008). (See the relevant media formulae in Appendix 2). Seed germination was monitored for 24 weeks in line with the six developmental stages (Table 2.3).

Stage	Orchid seed phase description
1	Imbibed seed, swollen greening still with attached testa
2	Seed enlarged, testa removed
3	Protocorm with pointed shoot apex and rhizoids
4	Protocorm shows leaf development and developing rhizoids
5	Seedling developing one or more leaves plus root development
6	Seedling with obvious roots and leaves

Table 2. 3 Kauth's six orchid seed germination stages of development, (Kauth *et al.*,2008).

2.7.4 Field germination of *N. iridescens* seed

Mature pre-dehiscent pods of *N. iridescens* were collected at the Tarata tunnel site over the period December- February through 2004-2008. Some pods in the area had already dehisced and no seed remained. The collected pods were carefully placed in paper specimen envelopes, numbered and dated.

To conserve the natural seed number present only two pods were collected from each of three separate colonies. Once in the laboratory, the pods were carefully surface sterilized, in a laminar flow hood, by dipping in a 75% sol. of EtOH then washing three times successively in sterile distilled water. Finally, the intact pods were immersed in a 3.5% Janola™ sol. for a period of 3 minutes before washing in three successive baths of sterile distilled water. The pods were dissected with a pre-sterilized scalpel and the seeds removed onto an autoclaved watch glass. Clumps of the evacuated seed were teased apart with the scalpel and a sterile needle probe. Forceps pinches of seed samples were inserted into sterile 1.75ml centrifuge tubes, without closing the lids. The tubes of seed samples were held in a desiccator jar to which sterile silica gel had been added. After a period of three weeks, the desiccator was opened, in a laminar flow cabinet, and the centrifuge tube lids closed. All of the tubes were placed in an aluminium foil closed 250ml beaker and retained in a 4°C fridge until required for sowing.

Germination of terrestrial orchid seed is enhanced if the seed is pre-treated with a 3.5% NaOCl solution prior to sowing, (Rasmussen, 1992, 1993, and 1995). This pre-germination treatment also provides an effective fungicide / bactericide to the seed, (Clements, 1981; Rasmussen, 1995; Vujanovic et al., 2000; Batty, *et al.,*

2001). Sample seed was therefore immersed in 3.5% NaOCl solution for three minutes prior to being irrigated x3 with sterile RO water.
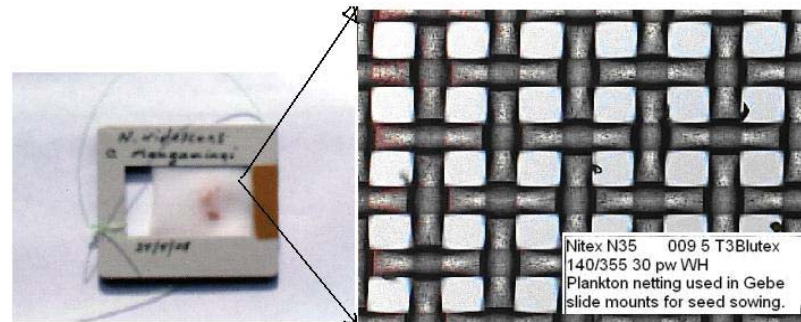
Figure 2.10 Left the completed orchid seed envelope. The brown centre stain is the orchid seed mass enclosed in the grid, as seen on the right; The grids mesh ≈ 30 μm^2.

The surface sterilized seed was used in field envelopes that were made up from rectangles of 30μm Nitex™ Plankton netting, (Fig.2.10). Each envelope strip was cut into 80mm x 40mm and laid out on a sterile surface (95% EtOH cleaned). A bacterial transfer loop was used to transfer approximately 50 *N. iridescens* seeds onto the centre of each open strip. The opposing ends of the plankton netting rectangle were drawn together to form an envelope which enclosed the seed in the fine plankton mesh. The envelope was then inserted into a plastic 35mm Grobe™ photographic slide frame and sealed with the Grobe clip device and masking tape.

Each slide was tied with a 60cm length of fishing nylon to a marker of soft #10 wire and then individually inserted, at a height of 1 – 1.5 m from the track edge, into the soil of the bank, with the length and breadth of the slide axis vertical to the track soil level, (Fig. 2.11).

The completed slides were placed into 3 sites at Mangamingi (Matemateonga) on the 5 May 2008. Each site possessed an active colony of, #1 site = *N. iridescens*, #2 site = *N. trilobum* and #3 site = *N. rivulare*. The three sites were located on a precipitous ridge in the damp exposed clay bank next to an old logging track. See Site illustrations Fig. 2.2 (5-6).

Three envelopes, labelled a, b and c were placed in each site and in a nursery tray of *N. papa*, which was held in the nursery collection. Each labelled slide remained in situ for at least 6 months, the first of each series (a,) was removed (15/12/08) for observation and analysis. The second series of slides (b) were removed for examination on (20/05/2009). The third set (c) was removed and examined in mid-December 2009 (Table A1.8).

Figure 2.11 A field marker at Mangamingi,

To ensure an initial degree of wetting, each envelope was moistened with distilled water prior to placing it into its respective site. Soil, from the immediate area was placed alongside the slide to ensure firmness and stability over the period of the experiment.

After the designated time lapse, the envelopes were opened in the laboratory and the seed examined with a Leica MZ12 binocular stereo microscope for signs of imbibition and germination. Any seed batch showing signs of germination was examined by Confocal Laser Scanning Microscopy (CLSM), DNA extraction, PCR amplification and sequencing to verify the identity of the fungal germinating mycorrhiza.

2.8 Leaf surface and crystal inclusions

A simple experiment, using two groups of nursery specimen plants, (*Nematoceras papa, N. triloba, N. iridescens* and *N. macranthum*) x2, were held at an ambient temperature and light situation over the period June 2004 to February 2005. One group was thoroughly watered every second day and held in wet drip trays with the other group kept in dry trays with only weekly watering. All water was collected from rain butts or the adjacent river (City water with constant chlorine levels could compromise soil fungi and orchid mycorrhiza, (Alexander, 1984).

To investigate the adaxial, abaxial leaf surfaces, stomata density and size, a series of SEM micrographs (60) were taken of seven CA spp. fresh leaf samples: *Nematoceras rivulare, N. macranthum, N. triloba, N. iridescens, N. papa, N. orbiculatum* and *Singularybas oblongus*.

The stoma of the Diurideae is characteristically anomycytic, (Pridgeon,1994), and this state was investigated in selected *Nematoceras*

Microscopy involved CLSM, scanning electron microscopy (SEM), environmental scanning electron microscope (E-SEM), (Danilatos, 1981), and energy-dispersive X-ray spectroscopy microanalysis (EDAX), to investigate the various leaf forms of selected species within the Corybas alliance; *Anzybas, Nematoceras, Singularybas* and *Corybas*. Details, of methods and techniques of LM, CSLM, SEM, and E-SEM used, are outlined later in this section.

A comparison of species, within the taxa, was investigated for any morphological divergence. Raphide composition was examined by X-ray diffraction (XRD) analysis using a FEI Quanta 200 ESEM with an EDAX (energy-dispersive X-ray spectrometer) facility. Sample crystals were located by ESEM prior to targeting with EDAX. Emission graph readings were compared with other graph results of calcium oxalate, which had also been chemically analysed for purity, (Franceschi, 2005; Perera, 2006; Prychid, 1999). EDAX readings of the empty mounting stub were taken as a control contrast.

All photographs, of external gross morphology, leaf diversity and entomology were taken with a Fuji "FinePix" S7000 macro lens digital camera. A Leica MZ12 binocular stereo dissecting microscope was used to observe and micrograph low magnification images of Lepidoptera and CA samples.

A *N. iridescens* leaf, showing obvious predation, harboured a solitary Lepidoptera larva. This larva was held at ambient temperature, in a 250ml glass beaker, covered with "Clingfilm", which was removed and replaced each day. The larva was fed daily with a fresh leaf of *N. iridescens*.

Micrography, with the Leica dissecting microscope, irritated the specimen and made it impossible to focus on the rapidly moving larva. Finally, the specimen was

placed in the freezing compartment of a fridge for 5-10 minutes until movement ceased, which then enabled microscopy to continue until the ambient temperature reactivated the sample.

No identification of the larva was possible and this initiated an effort to obtain a mature adult. The larvae, together with *N. iridescens* leaves, was held at room temperatures of between 19-25°C until late December 2007, when the larvae pupated. An adult moth emerged from its cocoon in July 2008 and was identified using the DSIR reference "Lepidoptera - annotated catalogue and keys to family-group taxa, Fauna of New Zealand (14)", (Dugdale, 1988).

2.9   Examination of the fungal endophyte

An initial screening of 3 standard media preparations was prepared to identify an optimal fungal growth medium:

1	Water Agar (Difco Bacterial Agar) 6g /400ml RO water,
2	Cornmeal Agar, CMA (Difco) 6.8g /400ml RO water,
3	Potato Dextrose Agar, PDA (Difco) 15.6g /400ml RO, water.

The three stock solutions were autoclave sterilized at 15lbs/in^{2} for 15 minutes, cooled and held in a water bath until the media temperature had reached 50°C. Twenty 90mm pre-sterilized Petri dish plates for each prepared solution were poured within an Oliphant HLF 3/L Laminar Flow Cabinet.

Samples were taken from the various sites, as previously outlined. Two other genera of the CA *(Singularybas* and two phenotypes of *Molloybas* (the Alba and red flower forms) were also root cultured for comparison.

A thick fleshy root of 80mm length and complete with a tuber was removed from plants, and washed under running tap water to dislodge any soil particles. The root was then dried between paper towels before being placed in a watch glass and surface sterilized by immersion in 3.5% w/v NaOCl   (as the commercial bleach Janola™), for one minute. The section was then washed x3 with sterile RO water to remove any remaining bleach. A final rinse of autoclaved RO water was used, within a laminar flow cabinet, as a final clean.

After preparation, the root and tuber were each transversely sectioned (t/s) with a sterile scalpel blade to create root and tuber sections of approximately 1-2 mm in length. Each section was removed with forceps and placed onto the prepared media plates, a cut side to the agar, (2x CMA plates, one with 4 tuber sections the other with 4 root sections) similarly with the PDA and Water Agar plates.

The six loaded plates were placed in a Gallenkamp IH-270 -cooled incubator, set at 15°C and examined weekly for a period of 8 weeks for signs of fungal growth. The 15°C incubation temperature was selected as being similar to the Taranaki Regional Council figures of the seasonal ambient temperature, over the period 1970 to 2002 proximal to the areas being sampled.

Plugs of 5mm samples of similar root tissue, that did not respond to culturing in PDA, CMA, or H$_2$O agar, were introduced to the more specialized media of: Melin-Norkrans agar,   (Marx, 1969; Taylor and Bruns, 1997), Bonnardeaux   media, (Bonnardeaux *et al.*, 2007) or FIM (Fungal initiating media), (formulae in Appendix 2a).

A total of 7 x 600ml flasks each of MMN and PDA plus 2 x 600ml flasks of MMNL were prepared and autoclaved at 121°C for a 30 min period. When the stock media was sufficiently cooled 30ml aliquots of each of MMN and PDA were plated

out into 160 plastic pre sterilized 90 mm Petri dishes A 10 ml aliquot of the liquid MMNL preparation was placed into each of 100 x 25ml sterilized Bijou bottles.

All media plate preparation, sampling, dissection and plating were accomplished in a lamina flow hood, observing recognized sterile protocols. Many of the early tissue cultures became contaminated with bacterial colonies necessitating a replating and the addition of antibiotics to the culture media. The choice of erythromycin or Novobiocin (Sigma) as a counter contaminate was made because of their thermo-stability, specificity and ease of use, (Stewart and Kane, 2006). Novobiocin was irrigated onto the pre-set media, at $50^{o}C$, in a Laminar flow cabinet at a dilution of 0.025g / 500ml distilled MilliQ water and adjusted to pH 5.8.

A total of 430 pre-sterilized Petrie dishes, sectioned into quarters, a, b, c and d were investigated for fungal development. One TS section of 1mm depth was placed onto each quarter of the media to create 4 samples in each plate, 1720 samples.

Each dish was labelled with an index number:

1 The site location letter   M = Moki, Mate=Matemateonga, U=Uruti, T= Tarata,
                                                  P= Pukeiti, N = Nursery.
2   The plant number   1 = first plant, 2 = second plant etc.
3   Sample status;   a = young root; b =, old root; tub = tuber.
4   Culture plate position number, 1-4 representing each quadrant.

Initial culture sample plugs, 5-7 days of age, were checked using a stain of Trypan Blue 0.05% w/v in lactoglycerol, (Brundrett *et al.*, 1996). A drop of the stain was placed onto the top of the sample plug and after 3 min had elapsed washed in a gentle stream of water prior to the cover slip being placed on top of the sample, (Sneh *et al.*, 1991; Rasmussen and Whigham, 1998).

Cultures, which showed a Rhizoctonia form, were sample plugged and the plugs transferred to sterilized 25ml Bijou bottles, each containing 10ml of sterile MilliQ water. Samples were indexed as library samples and were kept in a laboratory refrigerator at $4^{o}C$. The library cultures were re-cultured every 6 months, inspected, plugged and rebottled as before. Other plugs of similar size were placed in 1.75 ml micro-centrifuge tubes and used for later SEM, TEM and molecular analysis. These samples were held at $-18^{o}C$ in a freezer at the Allan Wilson Centre (AWC) Laboratory until required.

Sample identification by molecular sequencing was undertaken using the Basidiomycete primers ITS1 / ITS4 and ITS5 / ITS4, (White *et al.*,1990).

2.10   Microscopy used in this thesis

2.10.1 Light microscopy

Specimen samples of *N. iridescens* roots of 20 mm length were gently washed in running water trimmed into 5 mm pieces and fixed in FAA (Formalin 5% v/v, Glacial acetic acid 6% v/v, ethanol (EtOH) 89% v/v). These samples were then held for a period of 48 hours, at $4^{o}C$ prior to being dehydrated in an EtOH series (70%, 85%, 95%), each for a period of 60 minutes, then finally immersed in 100% EtOH overnight and completing the series process with a fresh immersion in 100% EtOH for 60 minutes.

After dehydration, the samples were transferred from the 100% EtOH immersion to100 percentage EtOH / 1% eosin stain for a period of 2 hours.

A Leica RM 2145 manual microtome, with S35 blades, was used to cut 8µm and 12µm sections from the prepared paraffin blocks. The general cellulose wall dye, Alcian blue, followed by SafraninO, (Battersby, 2004; Ruzin, 1999), was used to differentially stain the histological specimens after microtoming and clearing. Immediately following the staining procedures, the sections were dehydrated and mounted in DPX (dibutyl phthalate in xylene) synthetic resin mountant (Electron Microscopy Sciences).

The rehydrated slides were stained in a 1% w/v Alcian blue stain A secondary staining, of a 1% w/v SafraninO stain (1g Safranin O in 100 ml of 50% isopropyl alcohol), for a period of 10 minutes, followed the 1% w/v Alcian blue staining. After each stain was completed the slides were washed with four changes of RO (reverse osmosis filtration) water. A final dehydration, x 2, in 100 % EtOH was followed by x 2 Roticlear© after which the slides were mounted for microscopic examination in Practamount©, covered with cover slips and sealed with an acetate fingernail polish. The prepared slides were examined with a Zeiss Axiophot Compound Light Microscope with data imaging; the captured images were burnt onto Verbatim CD-R disks using the Nero programme.

2.10.2   Environmental scanning electron microscopy   (eSEM)

The advantage of this system lies in the specimen preparation, which is negligible and the low vacuum (10 Torr) that surrounds the specimen, (Danilatos, 1981). The sample was dissected out from the original root specimen and mounted on a stage. Drops of MilliQ water were added to the sample to create a "wet" sample. The sample was then imaged on the monitor of a FEI Quanta 200 eSEM.

2.10.3   Scanning electron microscopy   (SEM)

Samples measuring 2mm x 2mm (approx.) were immersed in a primary SEM fixative solution (3% v/v gluteraldehyde, 2% v/v formaldehyde in a pH 7.2 phosphate buffer).   The fixative containing the samples was held at room temperature for 24 to 48 hours after which they were gently washed for 10 -15 minutes each x3 with a 0.1M phosphate buffer pH 7.2. A serially graded dehydration immersion in EtOH (25%, 50%, 75%, 95% to 100%), with each stage taking 10-15 minutes and the final 100% stage for 60 minutes, was undertaken prior to critical point drying in liquid CO_2 . The dried samples were mounted onto aluminium specimen support stubs using double-sided tape prior to being coated with gold using a Balzers SCD 50 Sputter Coater. The mounted specimens were examined using a FEI Quanta 200 eSEM. Collected images were selected and burnt onto a Verbatim CD-R disk using the Nero programme (NERO AG).

2.10.4   Confocal laser scanning microscopy (CLSM)

The successful staining of fungal endophyte tissue within the host plant tissue was fraught with complexities. The use of Calcofluor White M2R, a fluorophore dye, commonly used in epifluorescence microscopy to enhance the imaging of fungal hyphae, is severely limited in CLSMs. The optimal excitation wavelength for Calcofluor White M2R is 347nm, has a peak emission wavelength of 450nm and has a low photobleach period making it unsuitable for CLSMs without UV excitation facilities.

An investigation for an alternative fluorescence stain for fungal wall chitin resulted in this author identifying two fluorophores, Solophenyl Flavine 7GFE 500 and Pontamine Fast Scarlet 4B, both had been used in identifying fungi in a human mycopathology laboratory, (Hoch, *et al.,* 2005).

Hoch reported that these dyes provided excellent alternatives to the more commonly used fluorophores, especially Calcofluor White M2R. Of the two fluorophores, only Solophenyl Flavine 7GFE500.(2-[4-[1-[(2-methoxyphenyl) carbamoyl]-2-oxopropyl],diazenyl-3-sulfo-phenyl]-6-methyl-benzothiazole-7-sulfonic acid from Ciba Specialty Chemicals NZ Ltd.) was available and was supplied, as a powder sample, courtesy of Mr. Chris McGregor of Huntsman Textile Effects, NZ Branch.

Solophenyl Flavine 7GFE 500 is a stilbene-based fluorophore dye, which was prepared for use as a 0.01% (w/v) stock solution in pH 9.2 carbonate / bicarbonate buffer (one BDH, pH 9.2 Buffer tablet 100mL-1 Milli-Q). The stock solution was further diluted to a working strength of 0.001% Solophenyl Flavine 7GFE 500.

Solophenyl Flavine 7GFE 500, depending on the filter sets used, portrays a fluorescently stained material in the blue to green wavelengths, (Fig.2.12) Hoch, *et al*. (2005), also reported a minimal incidence of photobleaching over an extended period. The fluorophore Solophenyl flavine 7GFE 500 in addition to being used at various spectral wavelengths from mercury arc sources in epifluorescent microscopy, can be used with laser sources providing the 488nm and 543nm line wavelengths that are common to most scanning confocal microscopes, (Pawley, 2006).
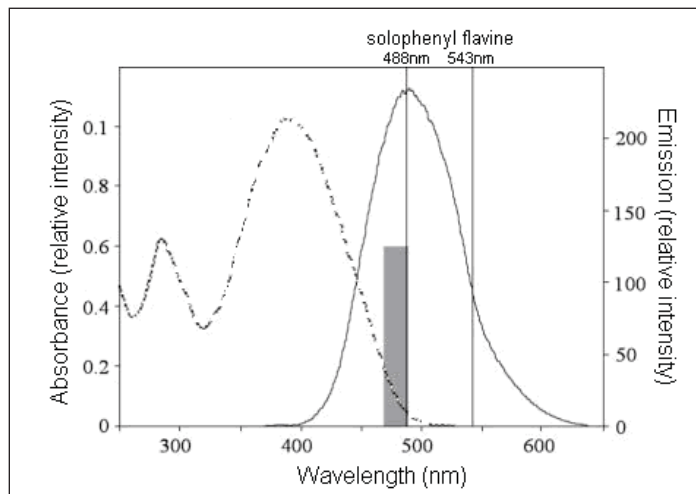
Figure 2.12 Absorbance (dashed line) and emission (solid line) spectra of Solophenyl Flavine (7GFE 500**).**

The prepared dye was added to the medium surrounding the specimen section for a period of 3 min. The dye was rinsed away with 50mM carbonate-bicarbonate buffer (pH 9.2) RO water to clear the stain. Each stained section was mounted on an Esco Polysine© slide in Shears mounting media and covered with a #1 Esco cover slip and the edge sealed with a clear acetate finger nail polish.

Fungal endophytes that were resident in the root cortex of *N. iridescence* were examined using epifluorescence using an Olympus BX51 microscope. The fluorescence values, 405nm and an emission of 450-500nm, were used for the calibration of the CLSM, Leica SP5 DM6000B. The fungal hyphal walls and peloton formations were observed using (n=50) x 5µm thick root section images, to form final images.

2.10.5   Transmission electron microscopy (TEM)

Dehydration of the samples was undertaken through a graded acetone series, 25%,50%, 75%, 95% and finally twice in 100% for a period of between 10-15 minutes each, with the final 100% acetone step taking 60 minutes. Anterior, peloton rich areas, of the root tip were selected using a Wild M3Z binocular dissecting microscope and sections of 5 mm length and between 20mm and 50mm were cut with a fresh razor blade to provide strips with maximum peloton presence. These were cut and placed into a primary fixative of 3% gluteraldehyde, 2% formalin in a 1M phosphate buffer of pH 7.2. Samples were vacuum infiltrated in the primary fixative for 3 cycles using a water pump after which the initial fixative was replaced with fresh fixative for a period of 2-3 hours at ambient temperature. A series of 3x 1M phosphate buffer washes of 10-15 minutes each was then applied.

The samples were placed into a 1% solution of osmium tetroxide, OsO4, in a phosphate buffer for 30 minutes at ambient temperature followed by 3x 1M phosphate buffer washes of 10-15 minutes each. Initially samples were infiltrated with 1:1 Procure 812 resin/acetone mixture, placed on a stirrer overnight and then followed by a 100% resin immersion for a final 8 hours on a continuous stirrer.

After removal from this phase, the samples were embedded in fresh resin using silicone rubber moulds and cured at 60°C for 48 hours. The specimen block (Fig 2.13) was removed from the mould and trimmed for the Reichert-Jung Ultracut E microtome ult. Sections of 100 nm (pale gold) for TEM were then cut on a using a diamond knife. The retrieved sections were "stretched" using a chloroform vapour technique (a drop of chloroform on an old grid wafted over the sample sections). Sections were attached to #200 copper grids using sellotape-chloroform cement and stained with a tincture of saturated uranyl acetate in 50% EtOH / water for a 4 minute period followed by a   lead citrate stain (0.25 g/100 ml), (Venable and Coggeshall, 1965), for a further 4 minutes.

All TEM observations were performed on a Philips CM10 transmission electron microscope and resultant images were collected using an SIS Morada 11 megapixel digital camera system. Selected images were recorded on a Verbatim CD-R disk using the Nero programme (Nero© 7 Premium).
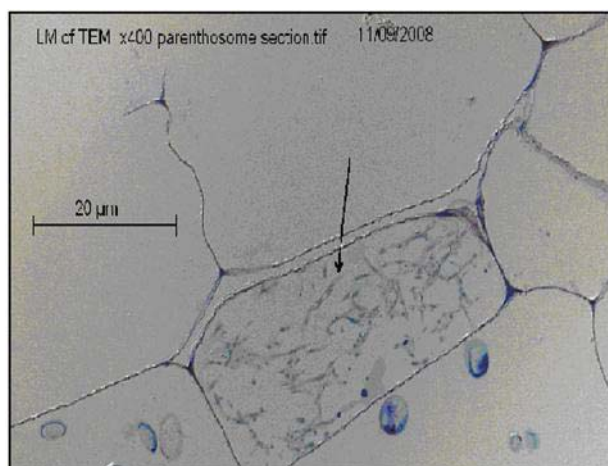
Figure 2.13. The resin block prior to ultra-microtoming (arrow indicates the hyphal section that was cut and examined by TEM).

2.11   Molecular analysis, DNA preparation

Prior to microscopy, a fine transverse section was cut from the sample root and placed onto a microscope slide. A stain of Trypan Blue 0.05% w/v in lactoglycerol, (Sneh, 1991; Burpee, 1978), was applied and left for 3 minutes prior to washing with water. The sample was then examined under an Olympus BH-2 microscope to ensure that root areas had evidence of active peloton activity within at least 3 layers of large mid cortex cells exhibiting, pre, active or early lysis.

Tissue samples, each of ≈ 5mm in length, were placed into a 1.7ml microcentrifuge tube with 1ml of sterile water. These samples were held at -20°C ready for DNA extraction. Fungal cultures for DNA extraction were prepared by scraping the culture surface and placing the scrapings into a 1.7 ml Eppendorf tube with 1ml of sterile water and holding at -20°C.

A number of glass pestles, equivalent to the number of samples to be analysed, were hand made by melting the tip of a glass Pasteur pipette in a Bunsen burner, drawing the molten tip out and forming a round head bead that fitted snugly into the bottom of a microcentrifuge tube. Individual samples of excised, NaOCl (3.5% w/v) sterilized and washed, *N. iridescens* root sections of 5mm length, or surface scrapings of fungal root isolate cultures were placed separately in 1.7 ml microcentrifuge tubes. After recording and indexing the tubes, the contents were flash frozen with liquid nitrogen for one minute. The frozen contents in each microcentrifuge tube were ground to a fine powder with its unique glass pestle. The DNA from the crushed samples was extracted using a Qiagen DNeasy® Plant Mini kit and the recommended protocols.

The DNA eluate sample was kept in a -20°Cfreezer ready for amplifying or, if time permitted, amplified immediately. An aliquot of extracted DNA (typically 5μl) was run on an agarose gel to check the molecular weight and intactness. The DNA quantities of the eluted samples were also checked by ultraviolet-visible (UV/Vis) spectrophotometry absorbance on a Nanodrop ND-1000, using Nanodrop Technologies software version 2.5.0.

The quantity of DNA from the extraction protocol was on average 8.57 ± 1.4154 (SE) ng / μl and the 260/280 reading was typically between 1.8 and 2 and suitable for polymerase chain reaction (PCR) amplification.

2.11.1   Selection of primers (Series 1)

Ecto-, Ericoid and arbuscular mycorrhizas have been identified to species level using fungal specific PCR amplification of the nuclear ribosomal internal transcribed spacer (ITS), (White *et al*., 1990; Gardes *et al*., 1991; Gardes and Bruns, 1993; Horton and Bruns, 2001; Redecker, 2002).

Fungal ITS sequences can be amplified with the primer pair ITS1-F / ITS4 or ITS1-F / ITS5 and annealed at 53°C, (Taylor and Bruns, 1994), the ITS1-F primer being Basidiomycete specific.

Primer	Sequence	Identifier
ITS1	TCC GTA GGT GAA CCT GCG G	(18S) (White, 1990)
ITS1-F	CTT GGT CAT TTA GAG GAA GTA A	(18S)(Gardes, 1993)
ITS4	TCC TCC GCT TAT TGA TAT GC	(25S) (White, 1990)
ITS5	GGA AGT AAA AGT CGT AAC AAG G	(18S) (White, 1990)

Table 2.4   Primers used in the first series of PCR amplifications

The relevant primers (Table 2.4) were obtained from Invitrogen. These were re-suspended at one nmol.µl^{-1} and stored at -80°C. A working stock of 10 pmol.µl^{-1} was diluted and stored at -20°C. A locus map of the initial primers used can be seen at Fig. 2.14.
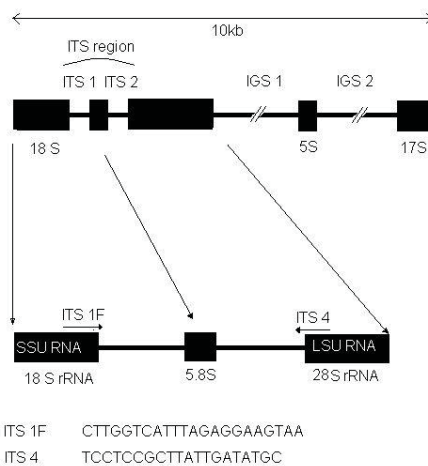
Figure 2.14   A locus map of specific primers, designed for Basidiomyceteae PCR.

ITS 1F   CTTGGTCATTTAGAGGAAGTAA
ITS 4   TCCTCCGCTTATTGATATGC

All extracted DNA samples from Series 1, plus a positive, (DNA that has previously yielded a product) and a negative (no DNA at all) control, were amplified by PCR using the primer pairs ITS1 / ITS4 and ITS5 / ITS4, (White *et al.*, 1990).

Amplification reactions were performed in a PCR buffer containing 1.5 mM MgCl$_2$ (Roche), 250 µM dNTPs, 1 M Betaine, 10 pmol each primer and one U Taq polymerase (Roche).

Samples were amplified in a Biometra Thermocycler programmed for a primary denaturation A = 94°C for 3 minutes; short denaturation B = 94°C for 30 sec; annealing C = 50°C for 30 sec ; extension D = 72°C for 45 sec; recycling D → B x 35 cycles; final extension E = 72°C for 5 minutes and held at 10°C.

The PCR products were visualised using gel electrophoresis, in a gel comprised of 1% (w/v) agarose in TAE buffer (40mM TrisAcetate, 1mM EDTA) containing 1X SYBRSafe DNA gel stain® (Invitrogen).

A 3µl aliquot of a PCR sample was mixed with 1µl of loading dye (0.16% (w/v) bromophenol blue, 0.16% (w/v) xylene cyanol, 25% (w/v) Ficoll) and 6 µl Milli-Q water prior to loading into a gel well. A size standard (1kb+ Ladder, Invitrogen) was loaded on each gel. Gels were run at 100V for a 45-minute period and then photographed using a Gel Doc 2000TM (BIORAD)

2.11.2 Sequencing

The PCR products were prepared for sequencing using 0.5U of shrimp alkaline phosphatase (SAP, USB) and 1.25U of exonuclease III (Exo III USB) which were added directly to each PCR reaction and then incubated in a PCR machine at 37°C for 30 minutes, followed by 80°C for 15 minutes.

Around 40ng of PCR product (2-5µl depending on the concentration of the PCR product as determined by gel electrophoresis) was mixed with 3.2 pmol of primer and water added to a total of 15µl.

The samples were submitted to MGS (Massey University Genome Sequencing Service) for sequencing. Two sequencing reactions, one from the forward primer and one from the reverse, were done for each PCR product. The resulting sequence data was imported into Sequencher (GeneCodesTM version 4.9). Forward and reverse sequences were edited against each other where possible and a consensus sequence was made. Sequences were used in a nucleotide BLAST search of Genebank for other similar sequences.

2.11.2.1. Series 2

The second series sampling was of *N. iridescens* plant roots from the Tarata, Pukeiti and Matemateaonga sites. Both the Moki and Uruti sites had been compromised by roadwork and bank excavations, (see Figs. 2.3 and 2.4).

Fresh samples were collected from September 2008 until March 2009. The DNA extraction protocol, apart from the tissue disruption and primers, was identical to the protocol as outlined in series 1. Some of the extracted samples from series one were also amplified with the new, more specific primers. Three (fine forceps pinch) samples of *N. iridescens* seed DNA from the germination field trial were extracted using the bashing beads and PCR amplification.

2.11.3 Tissue disruption

The tissue disruption of the samples, using individual pestles and liquid nitrogen prior to DNA extraction, may have failed to achieve a sufficiently fine and homogenous starting material. A trial was undertaken in which root samples were placed in a Zymo Research Corp, 2ml BashingBead Lysis tube with 0.5mm ceramic ZR BashingBeads™ and disrupted in a Roche MagNA Lyser for a period of 99 secs at 5000 rpm.

This method was compared with pestle ground samples from the same sources and run concurrently. Initially, lysis buffer was added prior to "bashing" but this produced very thick, gelatinous foam which inhibited the bead movement. Subsequent "bashing" was dry and the lysis buffer was added after the disrupter cycle was completed. Tissue disruption created a fine powdery starting material for all the tissue samples, except one section of

old root which required 3 cycles, each at a max speed of 7000 rpm for 99 seconds.

2.11.4 PCR for the 2nd Series

A number of specific primers, designed for *Tulasnella* PCR, (LeeTaylor and McCormick, 2008), were used in the second series for PCR amplification (Fig.2.15).
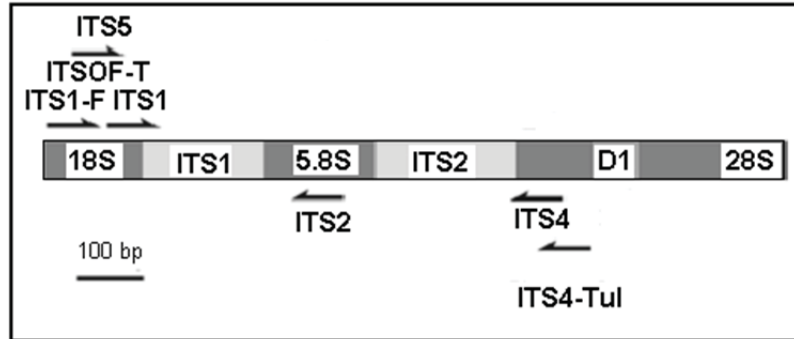
Figure 2.15   A locus map of specific primers, designed for *Tulasnella* spp.

The primers for the PCR protocol were changed to, ITS1-OF C + T mix (ITSx) / ITS4-OF and ITS4-Tul / ITS5. The annealing temperature was also changed from 50°C for 30 seconds to 54°C for 30 seconds. Otherwise the PCR protocol remained the same as for Series 1.

Chapter 3   Description and distribution of the CA

The taxa is mycoheterotrophic, usually saprophytic, with leaf reduction in those species inhabiting litter layers. Colonies comprise small rhizomatous herbs, together with Bryophytes and Hepatophytes, that form on banks (Fig.3.1) and mats on the forest floor. Plants aestivate during dry or cold climatic periods forming tubers from the enlarged nodes of the rhizome branches.

Plants of the CA produce a single leaf, occasionally two; the leaf base is double lobed and varies from broad-cordate, through oblong orbicular to acuminate, and can change shape from juvenile to adult. Leaves can often display various constrictions and lobes.

Figure.3.1 A *Nematoceras iridescens* colony on the bank of the northern entrance of the Tarata Tunnel (11/07/2008)

The general structure (Fig.3.2), of the taxa comprising the CA, is of a branching rhizome with alternating nodes and internodes. All growth originates from the nodes which contain primordia for the root and/or leaf, depending on whether they are above, or below the ground surface.   The roots are elongated internodes and the tubers are swollen nodes.
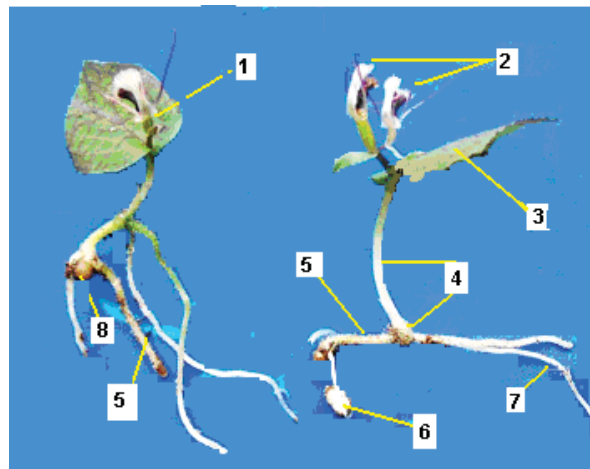
Figure 3.2 Plants of CA *Singularybas oblongus* from the Pukeiti site   4/04/2003.

Key:=1 = single flower   2 = rare double flower   3 = single leaf   4 = petiole   5 = rhizome   6 = tuber (dropper) 7 = new root   8 = old aestivate tuber

The CA taxa produce one, rarely two, flowers composed of a resupinate flower, labellum and dorsal sepal; the other segments are filiform and often reduced. The pod peduncle elongates after fertilisation of the flower.

Flower colours show various shades of green plus shades of red (Fig. 3.3). Occasionally the red pigment is absent or modified and the flowers then display light green to white colouration.

A full botanical description of *Nematoceras iridescens* (Fig. 3.3) appears in Appendix 1a. A Kew re-registration of the genera comprising the CA appears in Appendix 1e

Figure 3.3. Frontal view of *N. iridescens* (12/09/2005) at the Moki site.

3.1 The geographic footprint of the Corybas alliance (CA)

Genera that comprise the CA are located within the Acianthinae, a sub-tribe of the Diurideae, (Arditti, 1992; Dressler, 1981,1993) and consists of terrestrial orchids comprising approximately 100 species, (Dransfield, 1986; van Royen,1983). They range from India with 2 spp, China 2 spp, Taiwan 1 sp, Philippines 2 spp, Malayan Peninsula 5 spp, Sumatra 2 spp, Java 7 spp, Borneo 6 spp, Lesser Sunda Islands 1 sp., New Guinea 51 spp, Micronesia 1 sp., Solomon Islands 2 spp, New Hebrides 1 sp., New Caledonia 1 sp., Australia 11 spp, Samoa 1 sp., Society Is 1 sp., New Zealand 8 spp, Macquarie Island 2 spp., (Clements, 2007; Dransfield, 1986; van Royen, 1983), (Fig.3.4). Currently the centre of origin for the CA appears to favour New Guinea, based on the diversity of species (51), greatest dominance, density, (Dransfield,1986) and long distance concentric radiation patterns, (Stace, 1989). The area New Guinea, Australia and New Zealand share 66% of the total taxon with New Guinea having 49 % and the greatest diversity

Figure 3.4 The "footprint" of CA distribution (shaded area)

A molecular phylogeny of the taxon has to date been limited to the Australasian species, (Clements, 2002), in which three clades represent NZ CA endemics and 2 NZ representatives appear in two other largely Australian clades (Tables 3.1 and 3.2). Few, of the large number of species within New Guinea – Papua region, have been sequenced.

Table 3.1. The Acianthinae group classification showing the New Zealand genera

The bulk of the New Guinea "Corybas" will be included into the Nematoceras genus if the Type species is applied to the current New Guinea taxon, (van Royen, 1983; Dransfield, *et al*., 1986)

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3.2   Distribution within New Zealand

In New Zealand, the greatest plant concentrations of the CA occur inland to the West Coast (Fig.3.5 and Appendix 1d) at altitudes of between, less than 5m in the southern regions to greater than 2000m in parts of the North, (Hatch,1951; Moore, 1970; St George, 1990, 1996). A limited spread of the CA taxa, up to 5 species, occur on the east coast of New Zealand, Chatham Islands and Macquarie Island   This is consistent with the initial long distance wind trajectory carriage of orchid "dust" seeds.

Macquarie Island, contains the most southern of orchid species, represented by *Nematoceras dienemum* and *Nematoceras sulcatum,* (Clements and Jones, 2002).

Nematoceras is a reinstated genus, (Jones, *et al.,* 2002). Recognized New Zealand species include: *N.acuminata,. N. hypogaea, N. iridescens, N. longipetala,.N orbiculatus, N. pandurata, N. papa, N. papillosa, N. rivularis, N. triloba and N.macrantha.*

The NZ endemic genus, *Singularybas,* previously classified as belonging to the genus Corybas, has only a single named species, *Singularybas oblongus.*

Regional spread of *Nematoceras* spp., (Fig.3.5 and Appendix 1d) is concentrated on the western slopes of mountains and high ground with limited distribution in eco-provinces on the eastern sea board and eastern central areas. A literature search of the N.Z. Native Orchid Group journal (NZNOG) articles was made of CA distribution within the NZ ecological regions. The CA distribution within the New Zealand botanical area does include varietal forms of botanically recognized species however, the distribution pattern gives a good representation of the CA with the high varietal numbers included within the recognized species.
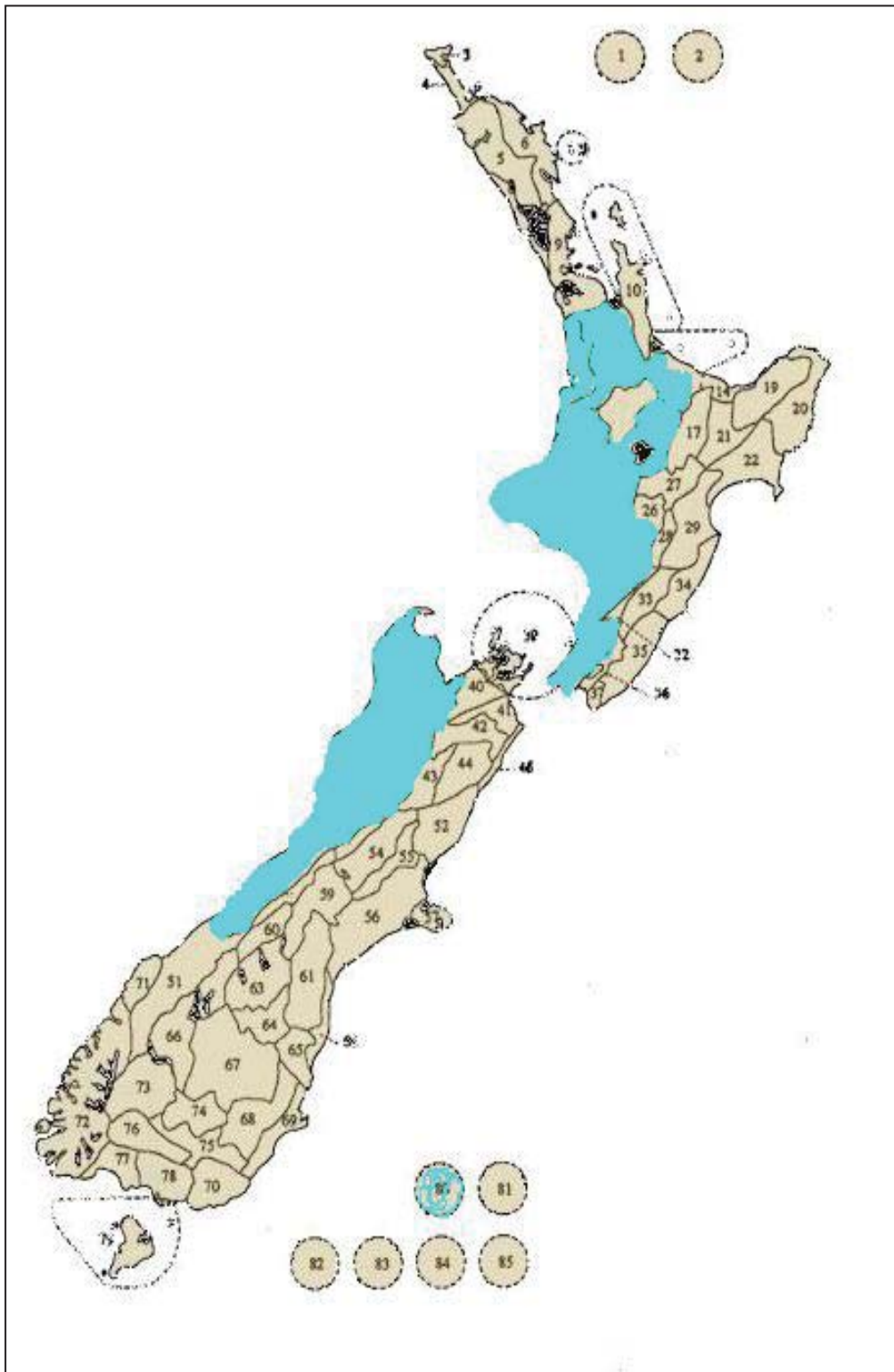
Figure 3.5. NZ ecological regions after MacEwen (1987). Shaded areas indicate where the genus *Nematoceras* has 6<9 species. The numbers explanation appears in appendix 1d.

The southern NZ Islands possesses a relative abundance of *Nematoceras* represented on the Chatham Islands and Macquarie Island on which the southern most plants of the CA occur, *Nematoceras sulcatum* and *N. dienemum*, (Clements, 2007). This pattern, has a likelihood of establishment from seed born by Föhn winds, leaving the Southern Alp barrier and carried above the dry East coast areas to moist areas around the Chatham Islands.

Flower morphology and anatomy provides 54% of the taxonomic characters used in orchid species identification, (Arditti, 1992; Bateman, 2006; Clements, 1995; Clifford, 1974; Dressler, 1993; Hatch, 1951; Rasmussen, 1982; Schweinfurth, 1959; Stace, 1989; van Royen, 1983). A count of characters used by, Clifford (1974) in his orchid taxonomy reveals: Leaf = 20, Root = 10, Stem = 4, Flower = 40.

Figure 3.6 The morphological difference between "helmet" (left) and "spider" (right) CA orchids.

One characteristic, that is apparent but has gathered very little attention in the literature, is the division of the morphological flower forms (Fig 3.6). In the CA (Table 3.2), the taxa are divided into two broad divisions, "Helmet types" and "Spider types". The Australian taxon are roughly divided into 82.35% helmet type and 17.65% spider type while the New Zealand taxa are divided into 21.43% helmet type and 78.57% spider type. Helmet types seem to be more xeric in location and adapted to the drier Australian environment than the "spider" morphology types which are mesophytic and adapted to mossy, high rainfall areas.

Location	"Helmet" form.	"Spider" form
New Zealand	*A. rotundifolius* *Anzybas carsei* *Corybas¨ cheesemanii*	*Molloybas cryptanthus* *Nematoceras acuminatum* *N. hypogaeum* *N. iridescens* *N. longipetalum* *N. macranthum* *N. orbiculatum* *N. papa* *N. rivulare* *N. trilobum* *Singularybas oblongus*
Australian	*Corybas abditus* *Corybas abellianus* *Corybas acontiflorus* *Corybas barbarae* *Corybas despectans* *Corybas fimbriatus* *Corybas fordhamii* *Corybas hispidus* *Corybas incurvus* *Corybas limpidus* *Corybas neocaledonicus* *Corybas recurvus* *Corybas undulatus* *Corybas unguiculatus*	*Corybas unguiculatus* *Corybas macranthus* *Corybas pruinous*
New Caledonia	*Corybas neocaledonicus*	
Macquarie Is		*Nematoceras dienemum* *Nematoceras sulcatum*

Table 3.2 Species distribution of "helmet and "spider" flowering forms of Australian and N.Z. Corybas species.

A disadvantage of using flower characters in the identification of plant species, is the delay between leaf emergence and mature flower production. The harvesting, of early peloton endophyte fungi, is optimal at initial leaf formation, prior to lysis and digestion of the hyphae. This necessitates either prior identification of plants for peloton endophyte analysis or use of characters other than the flower, that appear as the plant emerges. Colony flowers, at a point after root harvest, can verify the initial identification of the plant.

Apart from the "nursery" sites, at 10m above sea level (ASL), all were at altitudes of between 300-600 m ASL. These altitudes provided the moisture and variable temperature range, *via* local air convection, prevailing humid westerly and northerly on-shore winds. The cooler temperatures create an atmospheric condenser to the warm humid air and consequently ensure high rainfall areas. The endemic forests in the Taranaki area are classified as temperate rain forest, (Wardle, 1991). Dominant trees in these forests are kohekohe (*Dysoxylum spectabile*) , tawa (*Beilschmiedia tawa*), kamahi (*Weinmannia racemosa*), rimu (*Dacrydium cuppressinum*), northern rata (*Metrosideros robusta*) and a large diversity of ferns and bryophytes.

Light levels had previously been measured in areas inhabited by a number of CA species (*Nematoceras iridescens*, *N. macranthus* and *Singularybas oblongus*). These plants were growing in association with colonies of the Anthocerophyte, *Megaceros pellucidus,* which had been earlier investigated for chloroplast light response, (Watkins, 2002). The light strengths ranged from the *S. oblongus* site with 18 ± 2 µmoles photons $m^{-2}s^{-1}$ plus short period, "sunfleck", light levels, on *N. iridescens* and *N. macranthus*, of 556 ± 4 µmoles photons $m^{-2}s^{-1}$. This represents an optimal light range for the majority of Bryophytes, Hepatophytes, Anthocerophytes and fern colonizers, (Watkins

(2002). Ecological niche site preference by the CA plants suggests that the conditions of the moss colonized, previously disturbed soil, present favourable conditions for these specialist taxa to colonize this area. successfully

3.3 Climatic factors in seed dispersal

The main transport vehicle for the majority of very long distance seed dispersal is wind, generated by temperature fluctuations and air pressure relationships, (Burrows, 1975; Cain, 2000; Nathan, 2002; Tomlinson, 1973).

Plugs of 5mm samples of similar root tissue, that did not respond to culturing in PDA, CMA, or H_2O agar, were introduced to the more specialized media of: Melin-Norkrans agar, (Marx, 1969; Taylor and Bruns, 1997), Bonnardeaux media, (Bonnardeaux *et al.*, 2007) or FIM (Fungal initiating media), (formulae in Appendix 2a).

Germination of the seed requires a number of conditions:

1.	high moisture and regular rainfall,
2.	a suitable range of symbiotic fungi
3.	adequate shade,
4.	an adequate nutrient supply,
5.	suitable temperatures.

The above requirements are further defined by, (Arditti, 2000; Rasmussen, 1992, 1993, 1995). A study of the germination requirements, the long distance dispersal forces and the environment available in the high CA species number in the western areas, as outlined by the shaded areas in Figure 3.5, can be coordinated with weather patterns and the local geography.

3.3.1 General site climate.

As seen from the tables below (Tables 3.3 and 3.4) the annual equitable range of humidity, temperature, rain and sunlight for the high terrestrial orchid density areas (green highlight areas), within the NZ distribution map (Figure 3.5), can be readily ascertained

location	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	YEAR
NEW PLYMOUTH	82.3	83.8	80.4	80.3	83.4	84.6	84.1	82.9	80.4	80.4	77.3	80.7	83.5

Table 3. 3 Mean monthly values, of the relative humidity for the New Plymouth 1971-2000 period.

	Rainfall mm	Wet-days >= 1.0	Sun: hours	Temperature Mean °C	High °C	Low °C	Ground frost days	Wind mean speed km/h
KAITAIA	1334	134	2070	15.7	30.2	0.9	1	15
WHANGAREI	1490	132	1973	15.5	30.8	-0.1	11	16
AUCKLAND	1240	137	2060	15.1	30.5	-2.5	10	17
TAURANGA	1198	111	2260	14.5	33.7	-5.3	42	16
HAMILTON	1190	129	2009	13.7	34.7	-9.9	63	12
ROTORUA	1401	117	2117	12.8	31.5	-5.2	57	13
GISBORNE	1051	110	2180	14.3	38.1	-5.3	33	15
TAUPO	1102	116	1965	11.9	33.0	-6.3	69	13
NEW PLYMOUTH	1432	138	2182	13.7	30.3	-2.4	15	20
NAPIER	803	91	2188	14.5	35.8	-3.9	29	14
WANGANUI	882	115	2043	14.0	32.3	-2.3	7	18
PALMERSTON NO	967	121	1733	13.3	33.0	-6.0	38	17
MASTERTON,	979	130	1915	12.7	35.2	-6.9	60	11
WELLINGTON	1249	123	2065	12.8	31.1	-1.9	10	22
NELSON	970	94	2405	12.8	36.3	-6.6	88	12
BLENHEIM	655	76	2409	12.9	36.0	-8.8	60	13
WESTPORT	2274	169	1838	12.6	28.6	-3.5	26	11
KAIKOURA	844	86	2090	12.4	33.3	-0.6	27	15
HOKITIKA	2875	171	1860	11.7	30.0	-3.4	54	11
CHRISTCHURCH	648	85	2100	12.1	41.6	-7.1	70	15
MT COOK	4293	161	1532	8.8	32.4	-12.8	140	10
LAKE TEKAPO	600	78	2180	8.8	33.3	-15.6	149	7
TIMARU	573	81	1826	11.2	37.2	-6.8	84	12
MILFORD SOUND	6749	186	1800*	10.3	28.3	-5.0	56	9
QUEENSTOWN	913	100	1921	10.7	34.1	-8.4	107	12
ALEXANDRA	360	66	2025	10.8	37.2	-11.7	148	6
MANAPOURI	1164	129	1700*	9.3	32.0	-8.1	not measured	10
DUNEDIN	812	124	1585	11.0	35.7	-8.0	58	15
INVERCARGILL	1112	158	1614	9.9	32.2	-9.0	94	18
CHATHAM ISLAND	855	133	1418	11.4	28.5	-2.3	4	25

Table 3.4   A summary of climate information for selected NZ locations.
Data are mean annual values covering the period 1971-2000.

The Taranaki area, used for research in this thesis, is a high diversity *Nematoceras* spp. site. The normal rainfall is high over the winter and spring period, with drier weather not commencing until mid January to April-May. Even over the dry period humidity tends to generally remain over 80% (see Tables 3.3 and 3.4).

Rainfall appears as a critical factor in the growth and colony continuity for *Nematoceras* spp. Dry conditions encourage dormancy and moist conditions enhance the evergreen period of *Nematoceras* species. Data for comparison of a "very dry year" with a "normal" year can be seen in Fig. 3.7.
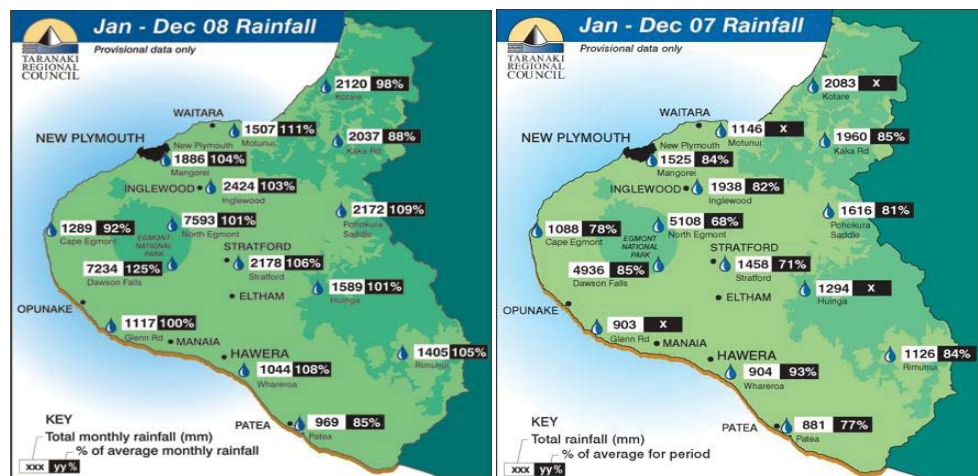
Figure 3.7   Rainfall maps of the Taranaki area. The rainfall figure is followed by the percentage of total average rainfall

3.2.2   Long distance seed dispersal

Aerobiology of orchid seed, is presented in this section of the thesis as having three basic phases; local wind carriage, long distance wind transport and new establishment. An investigation into the aerobiology of *N. iridescens* seed, in relation to long distance wind dispersal, local climatic observations and the putative origins of the NZ taxa was undertaken. Contact was established with National Institute of Water and Atmospheric Research (NIWA), (Nichol, 2004) regarding the New Zealand metrological wind trajectories and wind envelope transport hypothesis, (Sturman, 1997).

Species with dust-like seeds are dispersed by the wind, without the evolution of special aids, such as hairs and wings, (Baskin, 1998; Benzing, 1987; Ridley, 1993). The "dust seeds" of orchids have an innate aerodynamic ability. Species at the other end of the spectrum, are excluded from wind dispersal because of the heavier weight of their seeds, (Ran, 2002).

The dehiscence, of *Nematoceras* seed pods takes place in New Zealand at optimal local wind lift conditions. In the case of *N.iridescens* this occurs in late December – January to early February and were measured, as part of this thesis, at the time of pod dehiscence (Jan. 2005).

A comparison of other bio-organism long distance dispersal; Lepidoptera, (Ramsay, 1966; Fox, 1970; Tomlinson, 1973; Gibbs, 1969, 1980), birds, (Falla, 1958), fungal spores, (Spiers, 1989; Viljanen-Rollinson, 2002), fruit bats, (Daniel, 1975). Volcanic dust, (Burrows, 1975; Collyer, 1984; Dixon, 1903; Glasby, 1971; Healy, 1970; McGowan, 2000). All of these are considered either as deposits, natural vagrants or migrants to New Zealand. The literature, cited above and in various reports dating from 1855, hypothesised wind as the long distance transporting vector from Australia to New Zealand.

Little appears to be known, or recorded, regarding the various stages of seed dispersal in terrestrial orchids, following dehiscence of the mature pod. In the CA, the peduncle grows in two stages after fertilization. The peduncle remains static for a period, presumably while the seed matures, and eventually the seedpod extends to a more superior position than the initiating ovary. Rates and measurements, of *Nematoceras* peduncle extensions, were recorded from the field and personal nursery specimens.

Nowhere in the available literature, or in personal communications, does there appear any explanation of how seed, when released at pod dehiscence, moves from the point of release to enter a long distance wind flow. This pattern could account for the distribution of orchid species to and within New Zealand, the greatest diversity being found on the western rain forest areas, (Hatch, 1951; Moore, 1970; St George, 1990, 1996) explaining the species density along the west coast as outlined in Figure 3.5.

When the humid air from a cold front arrives in NZ it cools down as it impacts with the higher ridges and mountains, which lift the air into colder areas, thus producing condensation rain which precipitates out any material being carried by the winds that comprise the front squalls, (Tomlinson,1973).

While no empirical evidence exists of orchid seed being transported over the Tasman Sea, the shortest distance being 1500 km, there exists ample evidence

of both mineral and biological material being blown across the Tasman Sea in trajectory envelopes within the prevailing wind flows, (Close, 1978).
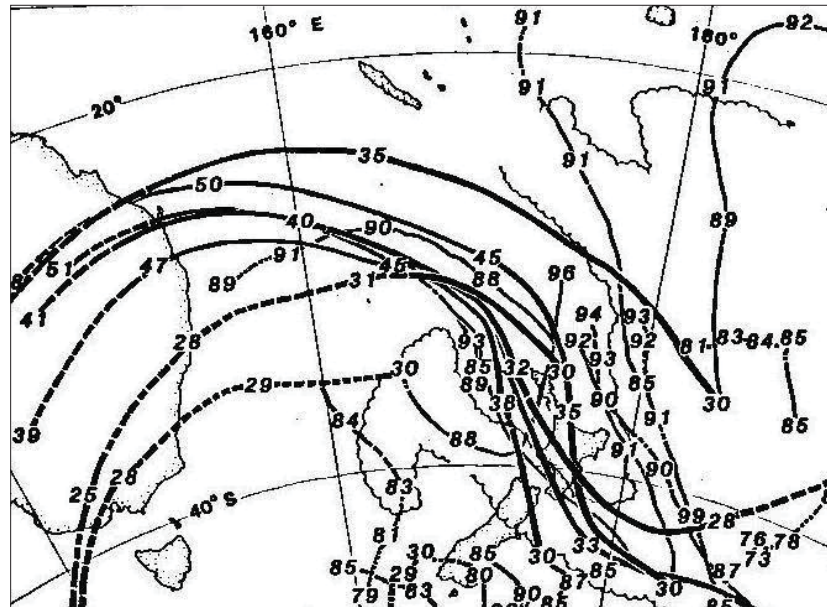
Figure. 3.8 Backtracked trajectory paths of air envelopes showing average measured trajectories spanning the Tasman Sea. Numbers on the trajectory paths indicate the number of trajectories measured at a point, (Tomlinson, 1973).

Trajectory air envelopes (Figs 3.8 and 3.9), can take between one to seven days to travel across the inter-Tasman sea distance, between Australia – New Zealand, (Nichol, 2004). If the wind speed is on average 5 ms^{-1} and the wind direction is steady for 10 hours, an air parcel is able to travel 180 km, (Nathan, 2002). Routine air sampling of generated four-day wind fields have shown that 10% of air trajectory parcels crossing at Baring Head (on the south coast of the North Island, near Wellington), originate from eastern Australia. The transport patterns are similar from season to season with the winter months tending to have more southerlies and westerlys than the summer months, (Nichol, 2004).
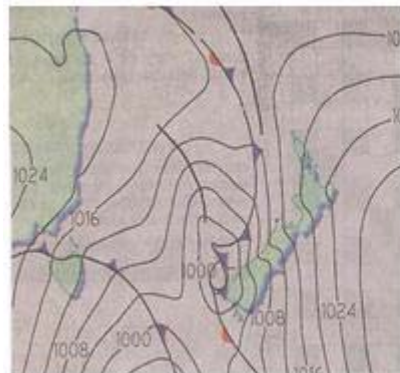
Figure 3.9 An isobaric weather map of the Tasman Sea and South West Pacific areas.

The transport cycle of particles and biological organisms is illustrated in Figs 3.10 and 3.11. Initially, uplift of particles occurs in areas of heightened temperatures. These areas produce air escalation, often with associated

cumulus and cumulonimbus cloud formations with severe air vortices. These climate conditions occur frequently in tropical areas such as the Papua New Guinea highlands and North Australia. The air current strength is sufficient to carry light objects into horizontal air currents via trajectory envelopes which are incorporated into long distance wind currents.

A major limitation, on long distance seed transport, is UV radiation at high levels, above 10,000m which has been found to affect seed viability, (Gradstein, 1999). Low-level minimum heights, below 2500m, allows the air envelopes to be perturbed by localized sea / land surface wind eddies, (Murren, 1998; Rantio-Lehtimäki, 1994). An optimal height, of between 3-4 thousand metres, for long distance seed transport has been suggested, (Bullock, 2000).

Solar energy
<400 W m⁻²

Temperatures increase on exposed sites. Hot air rises

Higher altitude and under heavy shade much lower air temperatures are produced

Movement of cloud

Mature Cumulus cloud

Rain area

Warm air

Cool air

Squall foot

Km
10
8
6
4
2
G.L.

Cumulus and cumulonimbus cloud forms posses very large vertical air currents within the vicinity. Optimal transference of low level air to high cooler sites. Particles can be conveyed from this area by trajectory wind flows.

Air envelope trajectory 17 days

Tasman Sea

Cold air is denser than warm air and so falls

(2) adapted from Tomlinson, A.I. "Meterological aspects of Tran-Tasman Insect Dispersal".

Figure 3.10 Symniotic meterological wind flows depicting the most likely vector for aerodynamic seed long distance dispersal from Australia and Papua-NewGuinea to New Zealand. Illustrated is a holistic air flow diagram. (1) cool air from the valley floor (purple arrow) is exposed to the sun. At the suns zenith they become warm with resultant air uplift (red arrow) (2) major air uplift diagram typical of tropical storms. It is this situation, when occurring during pod dehiscence that could provide the required escalation of orchid seed enabling them entry to long distance trajectory envelope flows (3) via an air envelope, optimally up to 3000m and then into a warm humid (5-6) west to east windflow (top of diag). When this air flow reaches high ground (B) the air is compressed (7) forcing the current up into cold regions (9) and condensing the vapour into rain (8). The air then regains heat because of compression and becomes (10) a strong dry Föhn wind, (Hill,1980; Palmer, 1942; Sturman,1996; Tomlinson, 1973).

39

N. iridescens pods dehisce as temperatures increase in the late morning to early afternoon (*pers.obs*.). This time frame of seed dispersal coincides with deep valley wind patterns (see Fig 3.11.)
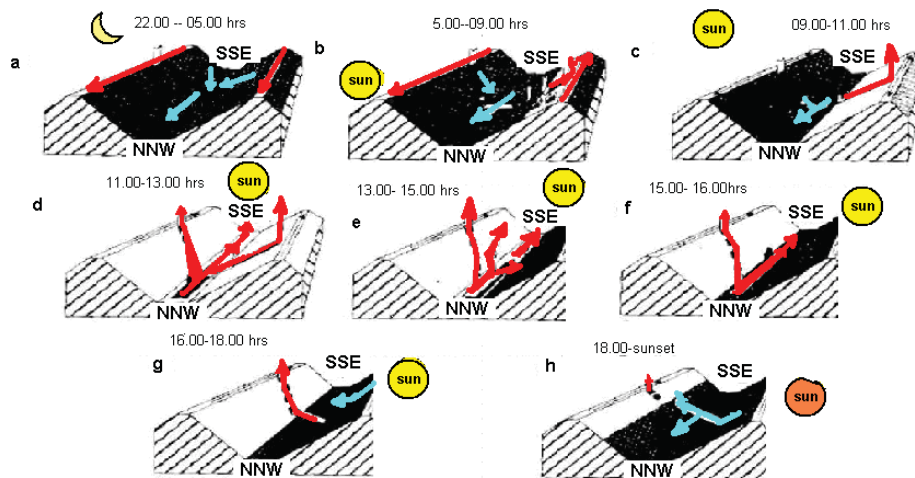
Figure 3.11. Diagramatic adaptation of wind flow directions in a deep alpine valley, Sturman (1983). **a** = night; cold air falls into the valley and flows down the valley floor, blue arrows, warmer ridge tops red arrows. **b** = early morning sunrise the top of the east facing ridge receives first light and commences to warm. **c** = mid-morning east facing slope fully exposed to sun light warming and convection currents are instituted. **d** = mid day sun at zenith, valley fully open to sun light, maximum heating occurs maximum warm upward convection currents, warm air current flows up the valley floor. **e** = mid afternoon western facing slope receiving full sunlight east facing slope shaded but retaining residual warmth. Warm upward air convection currents arise from western facing slopes, and the valley floor but convection currents on east facing slope loose updraft facility. **f and g** = late afternoon; greater shade cover of eastern slope , valley floor and lower west facing slopes, temperatures drop, humid cold air flows drop to valley floor creating mists, **h** = sunset, last warm air upward convection from western ridge top. Adapted from, Sturman (1983). Blue tracks = cold air Red tracks = warm air.

The mid-day valley wind currents have sufficient air uplift to elevate seed into local wind streams which can then distribute seed both locally and at a distance. All pods observed dehisce at higher day temperatures (from 11.00 hours to 14.00 hours *pers obs.* Air temperatures at high altitude drop dramatically from that of the ambient terrestrial temperature, dropping about 0.7°C for every 100 m of altitude (NIWA). Orchid seed germplasm has survived 10-day storage at -80°C and germination was initiated 19 days after sowing, (Mweetwa, 2006).

3.3.3 Wind velocity and direction at the plant site

Once orchid seed is released from the pod a group of factors affect the dispersal: the mass of the seed, the volume of the seed, the morphology of the seed, position of dehiscence, wind currents and their velocity, time of day, temperature and humidity, all influence the degree of seed transport to a greater or lesser extent, (Arditti, 2000).

The importance of seed flotation time is attributed to, Burgeff (1936), who rated the seed mass to volume ratio as being the critical factor. Burgeff's experiments, in laboratory conditions, used vertical glass tubes of 1.5 m length x 40 mm internal diameter, the seed fall being timed over this distance. The longer the fall time the greater the flotation attributes. No consideration was made of any external perturbations, such as wind strength and eddies. The Burgeff experiment was criticized by Arditti, (2000), as being non practical and not representing natural conditions. A more practical and well designed experiment by, Murren, (1998), used a ballistic model in which external forces contributed to the flight path and aerodynamic efficiency of a small particle, such as an orchid seed.

3.3.4   Results of wind site measurements

Measuring of wind strengths, at pod dehiscence, took place between 2nd and 4th of January 2005. The coastal wind at Port Taranaki, over this period, was WNW at a velocity of 9.8msec^{-1}, this being typical of summer prevailing winds for Western NZ regions). This wind was blowing directly into the sample sites and was locally moderated by the forest vegetation.
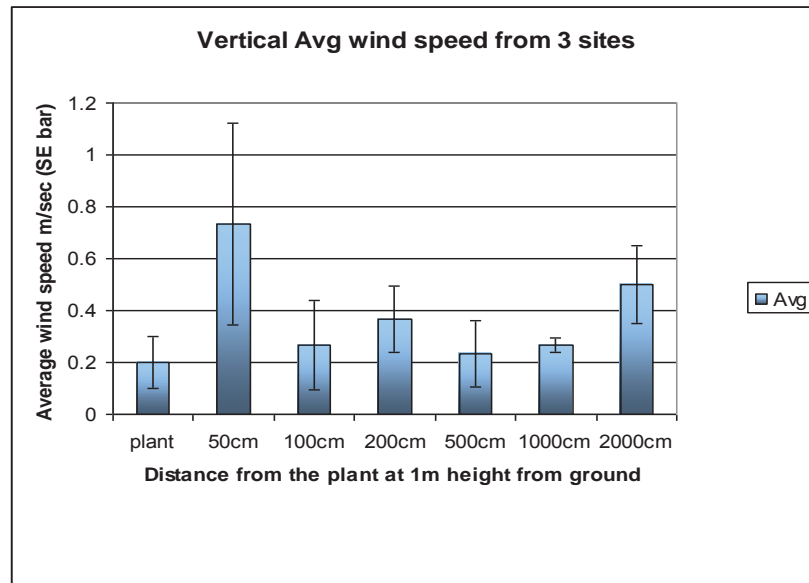
Figure 3 12 Vertical (parallel to the plant bank) average wind speeds The readings represent an average of readings taken at three sites.
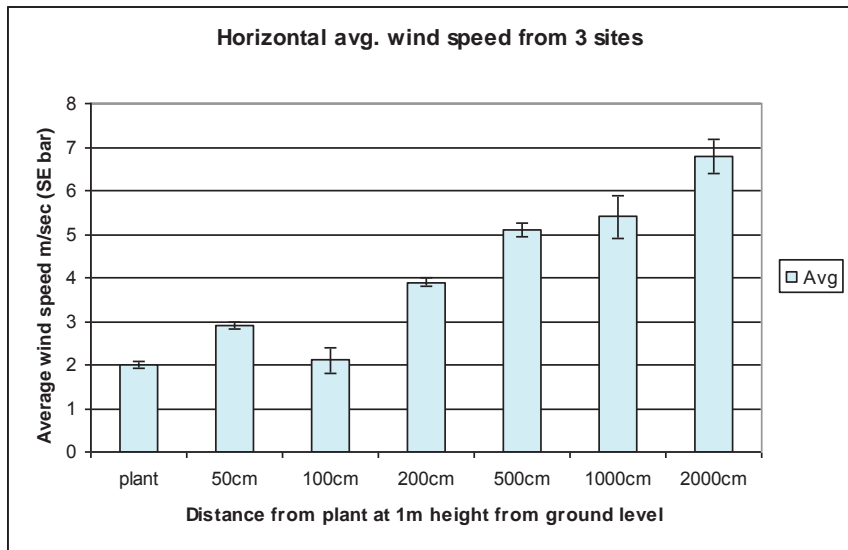
Figure 3 13   Horizontal average wind strength on a horizontal line from the plant. The readings represent an average of readings taken at three sites.
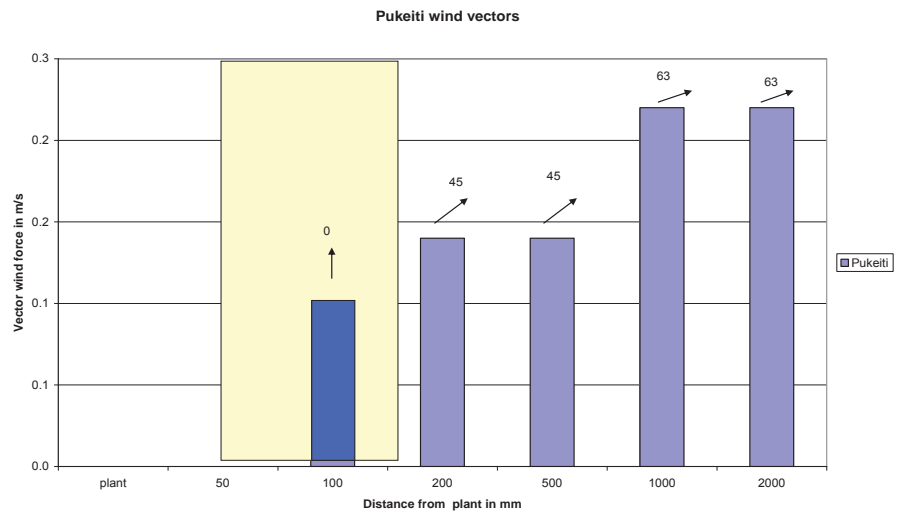
Figure 3 14. Wind strengths, in m sec^{-1} at Moki.   Blue bars indicate wind strength, arrows above the bars show vectors and direction of the vertical and horizontal components. The yellow highlight area represents extension lengths of peduncles.

Moki Wind vectors and direction
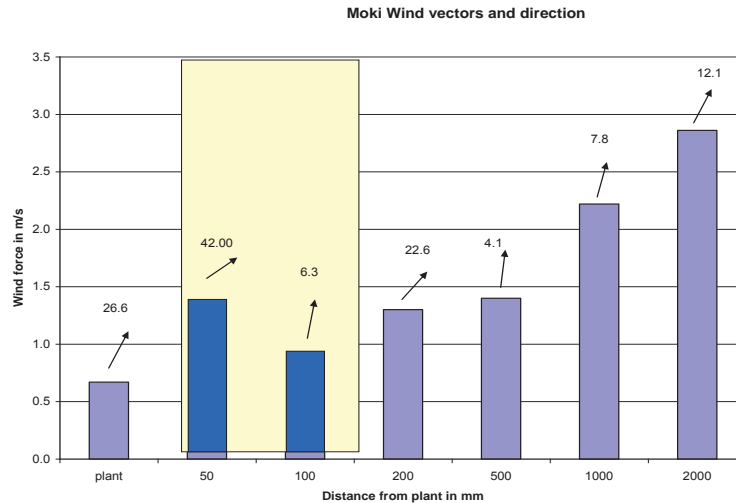
Figure 3 15 Wind strengths in m sec^{-1} at Pukeiti. Blue bars indicate wind
strength, arrows above the bars show vectors and direction of the vertical
and horizontal components. The yellow highlight area represents extension
lengths of peduncles.

An average wind velocity at 50 mm = 1.4 m sec^{-1}, 100 = 3.5 m sec^{-1}, 200 = 1.3
m sec^{-1}, indicates that an amplified airflow passed the dehiscing pods at this
time and day. The pod extension placed the mature pod into a suitable wind
flow. Wind currents measured in July at flowering and at leaf surface level
gave zero wind current velocity, the anemometer unable to record any wind flow
below the minimum reading of 0.2 ms^{-1}.

3.4   Discussion

This section, of the thesis has outlined a possible transport mechanism and
direction of orchid "dust seed" to New Zealand from dispersal from a North –
West arc. With the paucity of molecular evidence available, relating to CA
species, the centre of origin (CEO) is unable to be established. Based on
generalised rules, (Ricklefs,1993), involving; genera and species density, site,
and climate, it would appear that the CEO of the CA could be Papua New
Guinea with Australian or South-West Pacific Island steps to New Zealand.
Alternatively, or in combination with a far earlier introduction, orchids may have
been present prior to the Gondwana-New Zealand separation, (McGlone, 1985)
and adapted to their present morphology through time.

With the advent of molecular analysis, orchids have been accorded a far earlier
history than at first thought, (Chase, 2005). The most recent common ancestor
of extant orchids, occurred in the Late Cretaceous (76–84 Mya ago), (Ramirez,
2007). Based on the separation of New Zealand from Gondwana some
80mya, (McDowall, 2008; McGlone, 1985; Pole, 2001), it is possible that
ancestral fungi and orchid species have been in residence earlier than New
Zealand's separation. However, more recent thought, based on authenticated
orchid fossils and molecular evolutionary evidence, places the diversification of
Orchidaceae and Epidendroideae into the Early Eocene Climatic Optimum
rather than the earlier Late Cretaceous, (Gustafsson, 2010).

The early Miocene era 21-16.5mya, (Rait, 1992), currently provides evidence of orchids in the New Zealand landscape with the first epiphytic orchid fossils dated from this time being found in NZ, (Conran, 2009). Epiphytic orchids such as the fossils found (*Dendrobium* and *Earina*) which belong to the Australian basal clades of the Vandeae / Cymbideae, Epidendreae (*Earina*) and the Australasian clade of *Dendrobium* (*sensu lato)* suggest that the NZ orchid genera have arisen from long distance wind dispersal from Australia and/or New Guinea.

The universal character of the Orchidaceae is the obligate mycoheterotrophic requirement of orchid seed prior to germination, (Burgeff, 1936; Curtis, 1939; Rasmussen, 1995).   (see Section B Chapter 1).   The Dikarya : Glomeromycotina, Ascomycotina and Basidiomycotina are ancestral to the various mycorrhizal taxons, (Selosse, 1998), and have been recorded as being present in the Australian section of Gondwana, (Lepp, 2005).

Diversification events, estimated to occur from 30mya, were obtained from nucleotide substitution rates in the fungal 18S rDNA gene, Moncalvo (2008). This phylogenetic construct from a worldwide sampling of ITS rDNA sequences revealed eight clades: that correlate with the geographic origin of the strains.

> A.   One Southern Hemisphere clade.
>
> B.   One Southern Hemisphere, Eastern Asia clade.
>
> C.   Two temperate, Northern Hemisphere clades.
>
> D.   Three Asian clades.
>
> E.   One neo-tropical clade,

Paleoclimatology provides evidence, based on phylogenetic reconstruction of a worldwide sampling of ITS rDNA sequences of Basidiomycota, that fluctuations of climate occurred regularly throughout the South Pacific-Asian-Australasian area, (Parrish, 1982).   Shifts in the long-distance overall dispersion of the basidiomycete *Ganoderma applanatum* through the Asian- Australasian region by episodic events of dispersal within the Southern Hemisphere has been recorded, (Moncalvo, 2008).

Analysis of the Southern Hemisphere clade, indicated restricted gene flow with vicariance by distance and then island type strain development and speciation. This indicates that dispersal bias plays an important role in explaining the Southern Hemisphere distribution of many fungal taxa, (Moncalvo, 2008).

Fungal diversification and existence in New Zealand anteceded the orchid evidence. Indicating that potential mycorrhizal fungi could have been present to initiate germination of orchid seed embryos and implement the naturalization of vagrant long distance orchid species carried to New Zealand by long distance wind dispersal.   The mycorrhizal requirement of seeds of the Orchidaceae have a broad spectrum of orchid mycorrhizal diversity compared to the adult plant , (Hadley, 1970; McCormick, 2004; Rasmussen, 1995). Fungal endophytes of *N. iridescens* are identified and discussed in Section B

Winds over the southwestern Pacific, from the mid to late Campanian (Mid / Late Cretaceous) period 80-70 mya to the present time, shifted South and retracted Northwards in conjunction with the North hemisphere interstadial

periods, (Denton, 2010), thus,.giving a greater latitudinal range of origins for wind carried long distance seed dispersal over a period of time. .

Dehiscence in *N. iridescens* occurs between the December to January-February period and appears to be related to climatic conditions. Vegetative propagation occurs through the formation of root tubers "droppers" (Fig. 3.2) on the plant over the wet cool winter / spring season. As the summer season becomes dryer the droppers commence aestivation and thus provide protection to the plant over the summer autumn drought periods. When the dry period ends the winter wet period, June July, initiates the droppers to activate and produce the current seasons shoots and roots.   Floral production of *N. iridescens* then occurs over the period July—September and pod production starts , after fertilization, between September- November period.

Adaptation to local wind conditions and the escalator effect of these to high-level long distance trajectories can be observed and partially calibrated at the immediate plant site.   Only a small sample set was measured since the windows of recording opportunities were limited (timing of pod dehiscence, very few pods and lack of suitable weather to initiate the pod dehiscence).   The observations do however show that *Nematoceras* spp. pedicel elongation and dehiscence place the seed pod in a position of optimal wind strength able to support local distribution of *Nematoceras* "dust seeds" .

On the current evidence it would appear that the New Zealand endemic Orchidaceae have been derived from founder events arising from "dust seed" wind distribution from Australia and / or Papua New Guinea since 30 mya. The range of suitable fungal endophyte taxa was already established in NZ and able to initiate germination of the mycoheterotrophic orchid seed.

Section A. Ecology

Chapter A1 Aspects of seed morphology, anatomy and germination in *Nematoceras iridescens* seed

A1.1 Introduction

The generally accepted and descriptive term for orchid seed is "Dust Seed", a term that has been derived from a translation of the German term, 'StaubSamen', (Barthlott, 1976; Rauh, 1975), which aptly describes the size, lightness and number of seeds within orchid pods, for example: < 4×10^6 seeds per pod for *Cycnoches chlorochilo*, (Arditti, 2000).

Orchids produce the smallest of seeds. They range in weight from $2 \mu g$ to $14 \mu g$ and size from 0.4mm to 1.25 mm in length, with a breadth of between 0.08mm to 0.27 mm, (Withner, 1959; Dressler, 1981; Arditti, 1992, 2000). This dust-like character of orchid seed is typical of long distance wind dispersed plants, (Arditti, 2000; Eriksson, 2000; Levin, 2003; McMahon, 1973; Nathan, 2002, 2006).

Orchid seeds encompass a range of shapes: flattened, globular, lenticular, kernel shaped, ovoid, balloon, winged and thread-like, (Dressler, 1981; Baskin, 1998; Arditti, 2000). The structure and morphology of orchid seed is an important characteristic in orchid taxonomy, (Healey, 1980; Arditti, 2000; Tournay, 1960; Burgeff, 1936; Withner, 1959).

Orchid seed development, without a viable endosperm, initiates when a megasporangium is produced with the outer integument later forming the seed testa. The megaspore mother cell forms an embryo sac containing eight haploid cells. Double fertilization, typical of angiosperms, occurs when a pollen tube enters the ovule micropyle. One of the subsequent developments is the triple fusion (n + 2n) that occurs when one of the pollen tube sperm nuclei (n) fuses with the polar nuclei (2n) to produce a 3n primary endosperm nucleus. It is at this stage the monocotyledonous orchid seed differs from other angiosperms in that the primary endosperm nucleus degenerates and endosperm is not formed, (Sharma, 1987; Sood, 1986; Baskin, 1998). This absence of endosperm and an embryo of minimal cells provide the basis for the orchid seeds characteristic lack of weight and sustenance, (Arditti, 2000; Withner, 1959).

Apart from seven genera: *Arundina, Bletilla, Dendrochilum, Encyclia, Polystachya*, *Sobralia* and *Thunia*, (Arditti, 1992, 2000; Rasmussen, 1995), all orchids lack endosperm or, in a few cases, have a minimal number of cells forming the embryo.

The size, volume and weight of orchid seed indicate that the embryo volume tends to be very small compared to the testa surrounding it, (Barthlott, 1976; Arditti, 2000; Healey, 1980). This difference creates the so called "balloon effect". The air space, between the embryo and the testa endows the seed with an aerodynamic facility, which enables the orchid seed to remain airborne for a considerable time.

It is this aerodynamic facility, which provides one of the major features in the success of orchid long distance dispersal, (Arditti, 2000; Barthlott, 1976; Burgeff, 1936).

The large quantity of seed produced per pod and the seed volume to gross weight of each seed is typical of many other long distance seed dispersal plants, (van der Pijl, 1982; Nathan, 2002; Levin, 2003; Bullock, 2000).   Parasitic plants also produce large seed numbers and this has been suggested as a strategy to increase the potential of securing a host, (Teryokhin, 1982). In the case of orchids the necessity to expose the seed to a suitable mycorrhizal fungus is essential since the seed embryo requires additional nutrition that can supplement the storage resource of the lipid droplets the embryo contains, (Arditti, 2000; Burgeff, 1936; Withner, 1959; Quay, 1995; Richardson, 2000; Wilkinson,1997; Zettler, 1997).

No research of orchid seed transport to New Zealand has been investigated, although the evidence of long distance wind transport to and from various land masses within the Southern Hemisphere has often been reported, (Munoz, 2004; Sturman, 1997; Gandawijaja, 1983; Close, 1978).

This section of the thesis investigates the various aspects, important to the initial dispersal of *N. iridescens* seed, namely, plant peduncle elongation, dehiscence timing advantage, seed size, aerodynamic advantages and germination. Some characteristics of the *N. iridescens* seed testa, using the external and internal patterning of the testa could possibly be of taxonomic value, (Arditti, 1992, 2000; Rasmussen, 1993, 1995), and is briefly mentioned. An investigation comparing*: N. iridescens, N. papa, N. longipetalum* and *Singularybas oblongus* from within the genus *Nematoceras,* was also undertaken

Because of the long germination period found in the majority of temperate terrestrial orchids, (Rasmussen, 1993, 1995; Baskin, 1998), the time available for research and the time constraints imposed by thesis protocols, only some aspects can be presented in this thesis. A general lack of success has dogged previous researchers in their attempts to germinate temperate, terrestrial orchids of the Southern Hemisphere, (Batty, 2001; Rasmussen, 1995, 1998; Zettler, 1997; Quay, 1995; Collins, 1992; Clements, 1986; Warcup, 1971).

A1.2　　　Results

Mature pods, of *N. iridescens* occurred from late November – early February. The ambient weather appeared to "fine tune" dehiscence, if the weather was warm and had low humidity, the mature pods dehisced early. However, if the weather had a high humidity and temperatures were low the mature pods remained closed for a longer period, (*Pers.obs*).   The time taken, after fertilization, for three seed pods of *Nematoceras iridescens* peduncles to elongate to an average length of 153 mm to dehiscence was 63 ± 9 days. Initially the peduncle remained short, the base of the pod lying next to the adaxial leaf surface (See Fig.A1.1). The sole pod peduncle gradually lengthened, over a period of 57 days, to 0 – 20 mm followed by an exponential lengthening rate of 40 -100 mm over the final 10 days.(Fig. A1.2 and A1.3).

Figure A 1. 1 Early seedpod formation on a nursery *N. papa* specimen plant in September 2005.
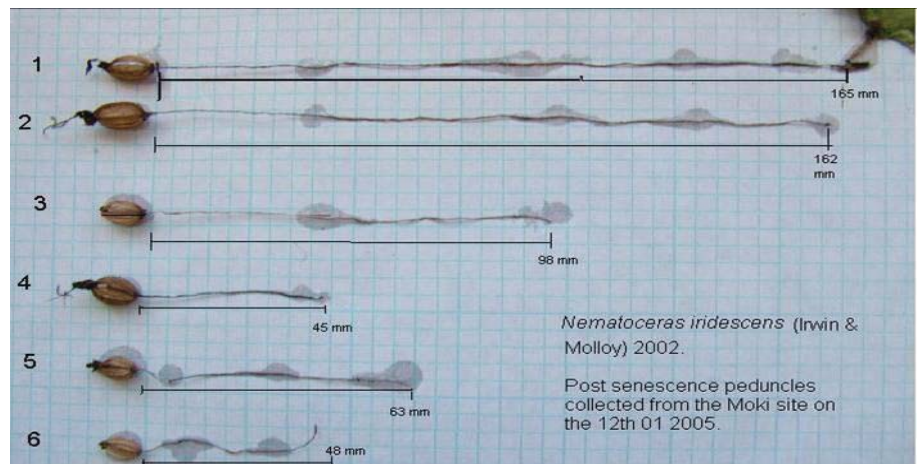
Figure A1. 2 Fully extended *Nematoceras iridescens* peduncles, together with their attached dehisced pods.
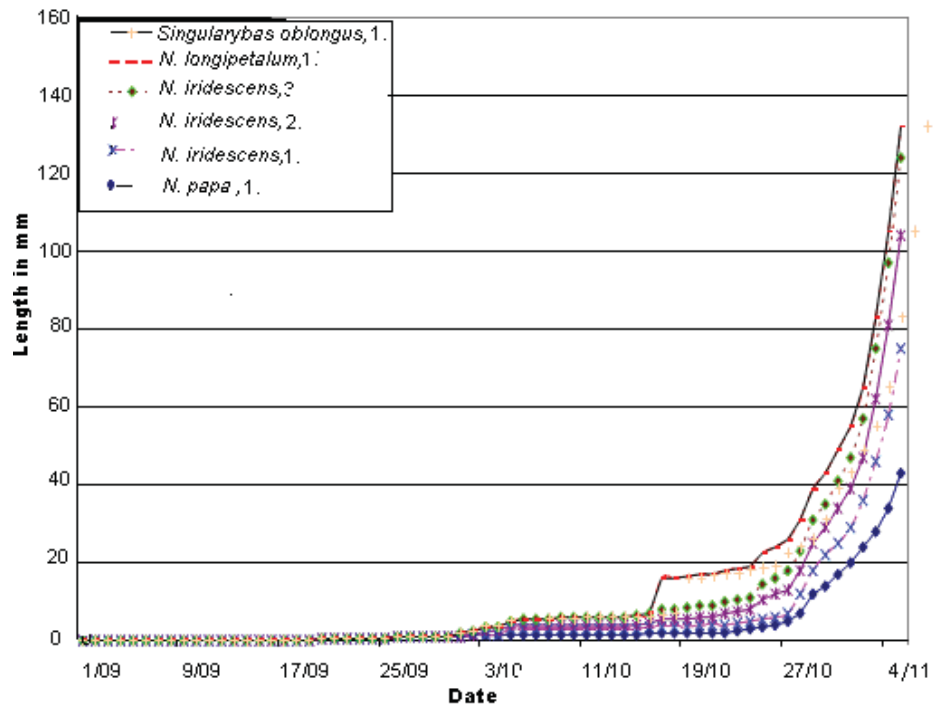
Figure A1. 3 Peduncle expansion rates of four species of the CA: *N. papa* (1), *N. iridescens* (1, 2 and 3), *N. longipetalum* (1) and *Singularybas oblongus* (1)

The first result was obtained by measuring the growth rate of the peduncle length every 9 days and recording this growth (Fig.A1.3.). The seed pod extends, in the 6 weeks prior to maturation, (Fig.A1.1 and A1.2) above the surrounding bryophyte vegetation and litter. Liberation of seed occurs at a climatically optimal time, distance, seasonal temperature and humidity, which provides an advantage of the thermal influx air currents that exist in forest gaps.

A1. 3   Pod oscillation

A process in which the pod dries out prior to releasing seed also occurs within *Nematoceras* (*pers obs*.) Once approaching maximum extension and dehiscence, the peduncles and pods show a degree of dehydration and lignification that reduces the dependence on turgidity to maintain an upright stance. The action of losing water when drying provides torsion to the external pod segments. When the ambient temperature and humidity is at optimal levels, an explosive pod opening occurs. This liberates much of the ripe seed, (Barthlott,1976; Baskin, 1998), into convection air currents created by the increase in environmental temperature However, much of the pod seed remains within the pod and these are shaken free by an ongoing oscillation of the peduncle (*pers obs.*)

The drying of the peduncle also confers a high degree of elastic periodicity to the stem of a seed disseminating pod, (Zebrowski, 1991).   Nodes and their septa provide an additional stiffening of the peduncle and act as a series of hinges, (Niklas, 1997).

All of the *Nematoceras* peduncles have three apparent nodes; one at the sessile leaf base and peduncle, the second in the middle section of the peduncle and the third at the bud – peduncle (Fig.A1.4), creating two long internodes. Septate nodes act as an internal spring hinge that can store, after wind bending, and release when the wind diminishes, to create strain energy, (Bruchert, 2003). This has a whip like effect on the free end of any protruding plant rods, (Spatz, 2002), in this case the peduncles.
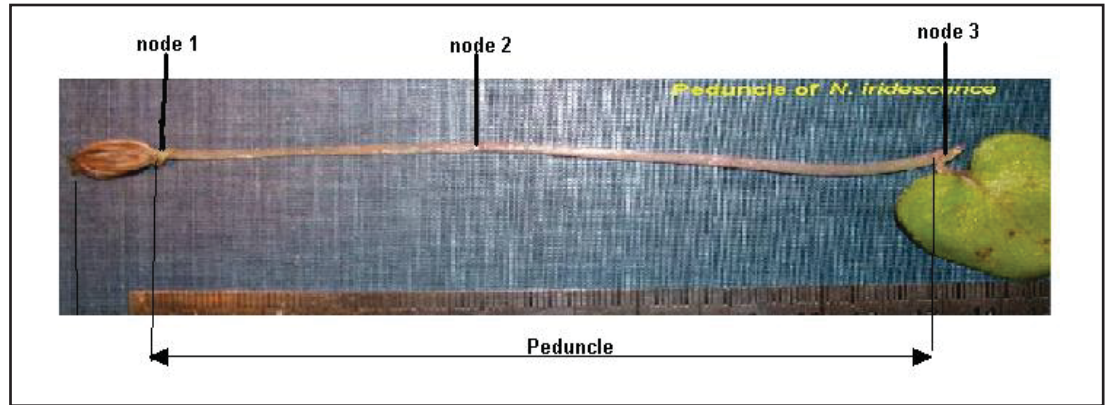
Figure A1. 4   A peduncle of a *N. iridescens* plant, showing the three nodes and the lignified peduncle. (Natural length)

Seed scattering from the open pod occurs at the completion of the recovery action of the peduncle when the stored energy initiates momentum to the pod and seed as a harmonic equilibrium is regained, (Niklas, 1997). The whip like "flick" of the open pod "throws" the "balloon "seed into the prevailing wind current (*Pers. obs*).

The minimal airflow initiating an oscillatory response in a *N. iridescens* pod and peduncle was examined. The mechanical solutions, of the degrees of oscillation are based on resonance, wind shear, tissue density, and elastic moduli, the solving of which is beyond the scope of this thesis, the theory of which is explained by, (Bruchert, 2003; Niklas, 1997; Spatz, 2002).

A series of oscillatory measurements, at 1mm from a vertical center point, occurred at wind speeds of 0.5 ms^{-1} and increased to 18 mm at wind speeds of 1.3 ms^{-1} (Figs. A1.5 and A1.6)
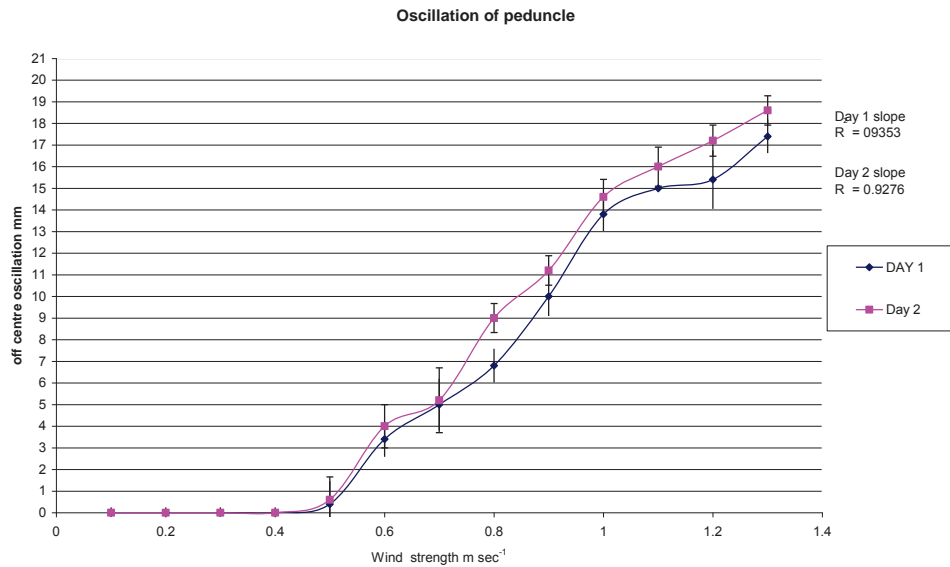
Figure A1. 5   Oscillations rate of *N. iridescens* seed pods on mature peduncles.
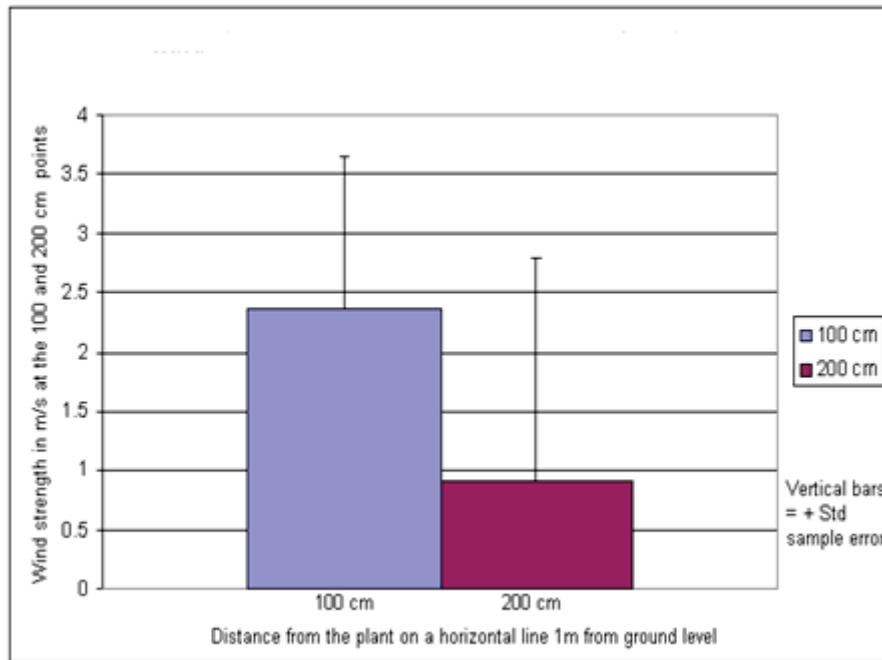
Figure A1.6   The average wind strengths at horizontally measured points from the *N. iridescens* plant.

This experiment was very simplistic and implemented to check the wind strength effectiveness upon dehiscent pods and the initial dispersal of the seeds of *N. iridescens.* Wind speeds of 1.3 ms^{-1}.appeared to be the minimal strength required to liberate seed from the pods. This was contrasted with local wind patterns and

strengths. The required wind strength to effectively dispense seed into the windflow is 1.3 ms^{-1}, the peduncle extension places the pod into the optimal wind strength flow of 2.36 ±1.87 ms^{-1}. Nowhere in the available literature, or in personal communications, does there appear any explanation of how seed, when released at pod dehiscence, moves from the point of release to enter the long distance wind flow.

A1.4   Seed mass

Seeds were weighed to obtain an average seed weight. Four pods from plants of *N. iridescens* provided seed samples. Each sample was placed in a small watch-glass and weighed on a Sartorius analytical balance, once the weight had been recorded the seeds were counted and an average seed weight calculated (see Table A1.1.)**.**

Sample #	Gross weight µg	Seed count	Weight /seed µg
1	200	23	8.7
2	1100	92	11.9
3	500	63	7.9
4	600	72	8.3
Totals	2400	250	**Avg** 9.6 ± 0.117

Table A1. 3   The average weights of *N. iridescens* seed.

The calculations gave a mean seed weight of 9.6 ± 0.117µg. The resultant seed weight for *N. iridescens* compares with a calculated range of 1.97 ± 0.06µg for *Cypripedium* (a northern hemisphere terrestrial orchid) and up to 8 µg for *Gymnademia* spp. another northern hemisphere terrestrial orchid, (Arditti, 2000).

However out of a total of 94 orchid species Arditti only references six individual species average seed weights. From this sparse comparison the seeds of *N. iridescens, that* were examined, are on the heavy side.

A1.5   The morphology of *N. iridescens* seed

While screening seed of *N. iridescens* for embryo changes, it was evident that the seed showed high levels of diverse morphology, especially in length, lacked an embryo, and had diseased seed and/or a degree of mechanical damage to the seed testa. To pre-empt a diagnostic check on the viability of each morphological group, an empirical analysis of the group and an allocation of total quantity of seed type to each group was made. There appears to be no reference to the variation in seed size in the available literature or to the seed viability in relation to size. Lack of viability in regard to seed without embryos or mechanical damage could realistically be presumed, based on the research conducted on other angiosperm seed, (Baskin, 1998).

To ascertain the natural viability of the "micro" seed would require a successful germination using symbiotic methods and comparing results with seed of normal morphology and visible embryos.

The texture and patterning of the seed testa could affect the aerodynamics of the seed and create a bias in the terminal dispersal sites. It would unfortunately be very difficult to ascertain the dispersion that clusters of "balloon" seed, of the two seed types, would have. Air flotation of orchid seed is dependent on the seed volume to mass ratio, (Arditti, 1991, 2000). This section measures seed to calculate volume

and relate this to the *N. iridescens* seed mass of 9.6 ± 0.117 µg, calculated previously, (Table A1.1).

Micrographs provide an example of each seed type (Fig.A1.7.). Measurements, based on the micrograph images, provided four very distinct seed morphologies (Table A1.2). The micro seed, as seen in (Fig A1.7 (2)), provided the largest average number of seed in the samples (65.47%) which is more than twice the number, 31.75 %, of "normal" seed (entire testa, fully formed embryo, fully expanded testa cells and no evidence of any pathology, see Fig.A1.7 (1). Those seed with no embryo and an existing pathology, (Fig.A1.7 (3), had a low total average count (1.66 ± 0.45) plus (Fig.A1.7 (4 5) 0.833 ± 0.083 and were only occasionally encounted.

	Seed "type"				
	normal + e	small + e	testa only	damaged	sample total
Sample from					
Pod 1	24	52	1	0	77
Pod 2	18	34	2	0	54
Pod 3	15	44	4	0	63
Pod 4	30	56	4	0	90
Pod 5	41	60	1	0	102
Pod 6	26	80	3	0	109
Pod 7	18	40	0	0	58
Pod 8	11	29	0	0	40
Pod 9	20	27	0	0	47
Pod 10	11	10	0	0	21
Pod 11	15	21	2	1	39
Pod 12	11	42	3	0	56
Total	240	495	20	1	756
n = 12	12	12	12	12	
Average	20	41.25	1.67	0.08	
sdev	8.91	19.95	3.23	3.32	
se	2.57	5.76	0.93	0.96	
%	31.7460317	65.47619	2.64550265	0.13227513	100

Table A1. 4   Distribution of seed forms found in seed pod samples of *N. iridescens*
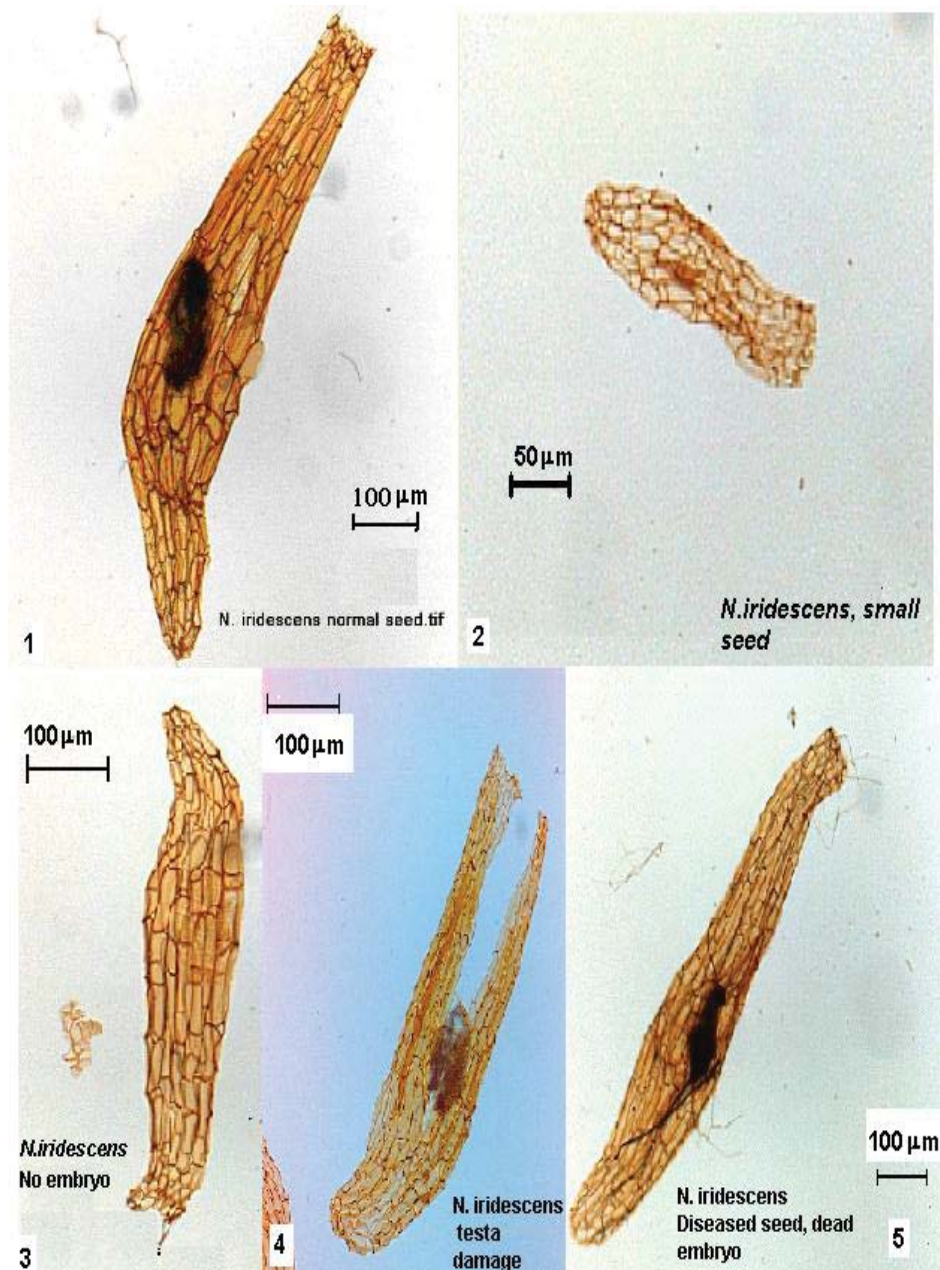
Figure A1.7 *N. iridescens* seed morphology: 1 = normal seed, 2 = small seed, 3 = normal seed without true embryo, 4 = testa damage, 5 = diseased seed with fungal hyphae attacking the embryo.

A1.5.1   Volume ratios

As mentioned earlier orchid seeds and their embryos are very small. The volume of air enclosed by the testa, less the volume of the embryo, provides the airspace. Seed flotation time has been calculated by Arditti, (2000), and found to be related to the seed weight / air space (Appendix 4).

Total Seed and embryo areas were calculated from a series of point measured micrographs (Fig.A1.8) and depth from z axis component (Fig.A1.9).
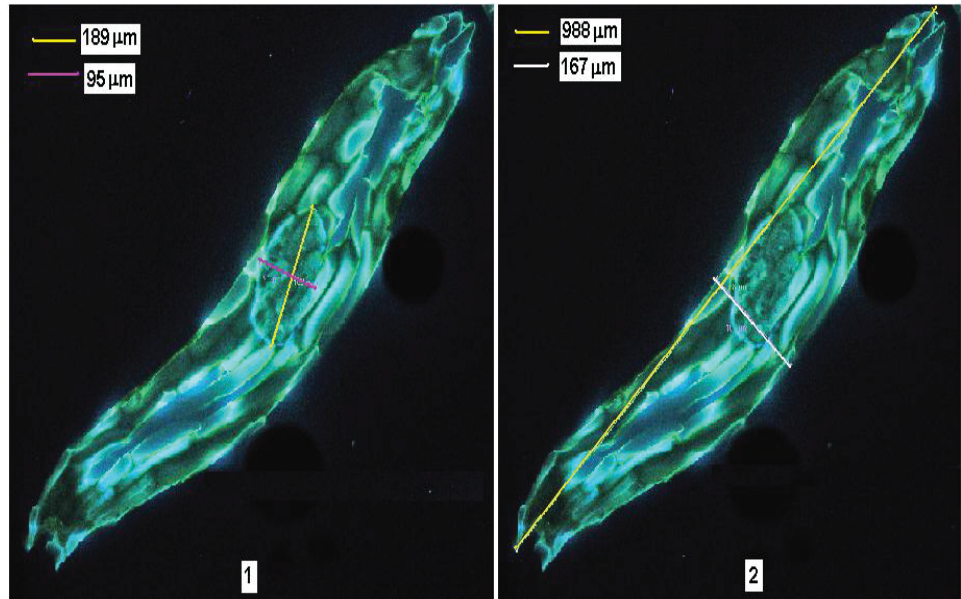
Figure A1.8 A seed of *N. iridescens* with measurement point lines. Micrograph 1 = measurements of the testa, 2 = embryo measurements,

Volume calculations are based on the assumption that a fusiform seed geometrically consists of two cones joined at their bases. The total volume was measured by using equation 1;

Eqn 1, for a fusiform volume = $2(\frac{\pi}{3} r^2 h) = 2(1.047 r^2 h)$
r = maximum testa radius
h = the ½ length of the longest testa axis.

Normal total seed volume = 2(1.047 r² h) = 2(1.047*9506.25*512.5) = 10201870 = $10.20.1000^3$ mm^3 =A

Micro total seed total volume = 2(1.047 r² h) = 2(1.047*1681.0*102.5) = 360801 = 3.6 mm^3 x 1000³ mm = A'

The embryo volume equates to a prolate spheroid represented by equation 2;

Eqn 2 , for an oblate spheroid volume = $\frac{4}{3} \pi a^2 b = 4.19 a^2 b$
a = ½ length of the embryo maximum axis
b = ½ length of the embryo minor axis.

Normal seed embryo volume = 4.19a2 b = 4.19 *2401*49
= 492949.3 = 0.493*1000^3 mm^3 =B
Micro seed embryo volume = 4.19a2 b = 4.19*462.25*18.5

$$= \; 35831.31 = \underline{0.036*1000^3 \, mm^3} = B'$$

The percentage free air space of the fusiform seed can be calculated by the formulae;

Eqn 3, Percentage volume of free air space $= \frac{(A-B)}{A} 100$

A = Equation 1 total volume of testa
B = Equation 2 total volume of embryo

Normal seed free air space = ((A-B)/A)*100 = ((10.20-0.493)/10.20)) *100 =

Normal   = 95.17% free air space

Micro seed free air space = ((A-B)/A)*100 =   ((3.6-0.036)/3.6)) *100 =

Micro 99.00 % free air space.

The calculation of *N. iridescens* seed, air volume ratio, for the two major forms (normal and micro) of seed revealed a small difference in the percentage of free air space and therefore the flotation time characteristics.   Using the seed mass measurements with the free air space percentage allows a comparison with other orchid seed as measured by, Arditti (2000), (see diagram A1.10).

The micrograph (Fig.A1.9) is from a CLSM using an inbuilt depth analysis function which further defines the embryo and testa morphology. A flattened profile was indicated for both the testa and embryo. This feature has not been incorporated in the seed volume calculations but it does provide a typical lenticular aerofoil profile, (Joseph-Wright, 2008). Further aerodynamic analysis is beyond the scope of this thesis, it would however, provide for a fascinating future study.
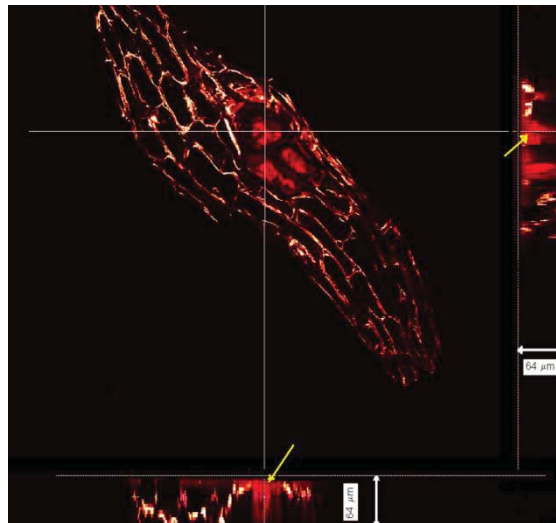
Figure A1.9   A *N. iridescens* seed. The cross hair indicates the embryo point targeted to create an image of the z axis (arrows) at both the y and x axis to provide a general embryo depth measurement ≈ 50µm.
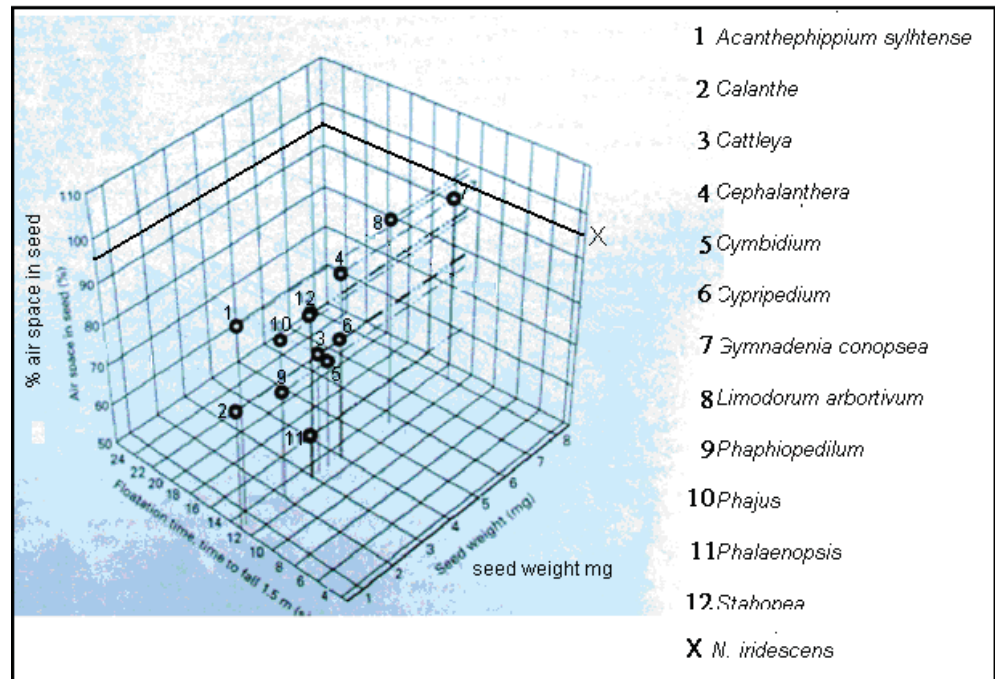
Figure A1.10. Air floatation times of a range of orchid seeds . The seed of *N. iridescens* have been marked with an X.

A1.5.2 The micromorphology of *N. iridescens* seed

Reviews of orchid seed, (Arditti, 2000; Rasmussen, 1993, 1995; Molvray, 1995) are comprehensive. Specific research concerning the micromorphology of orchid seed, conducted by, Molvray (1995) and Barthlott (1976), considered the periclinal walls, of orchid seed testa, provided sufficient evidence to justify the establishment of a morphological character, (Arditti, 1991). Recently, Gamarra (2007) used SEM to examine and compare the ultrastructure of *Neotinia* (Orchidaceae), a fusiform seed species, especially the internal markings of the anticlinal and periclinal testa walls. After identifying ornamentation patterns on the internal periclinal walls of the testa of *Neotinia*, Gamarra concluded there was sufficient evidence to differentiate species, which supported the molecular phylogenic study of the taxa by, Bateman, (2003).

This section of the thesis investigates the micromorphology of the seed of *Nematoceras* species, *N. iridescens, N. papa*, and *N. longipetalum*, in an effort to identify any differences that could assist as comparative morphological taxonomic characters.

Micrographs were examined for; external cell morphology, number, both seed width and long axis, anticlinal wall adhesion form and ornamentation of the periclinal walls.

A series of SEM micrographs, the first Fig A1.11(1) at 40 μm bar, the second Fig A1.11(2) at 200 μm bar, show a distinctive fusiform seed form with testa cells well imaged. The testa consisted of long rectangular, pavement cells with thin periclinal walls. The testa cells all exhibit a patternation of diagonal to vertical fine ridges with some having a lateral branching.
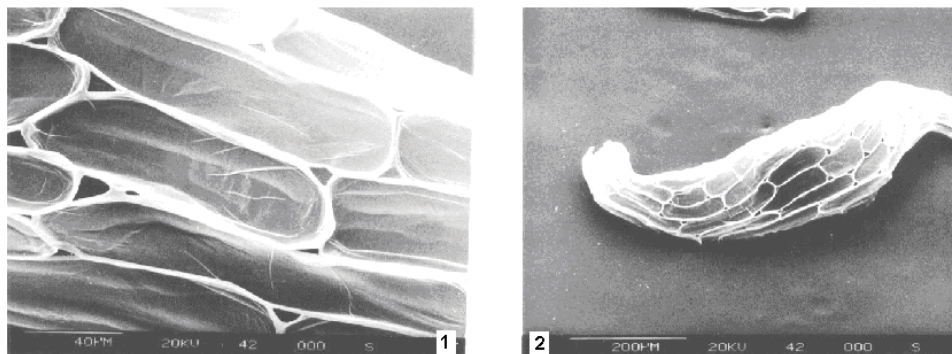
Figure A1.11   Reticulate testa patterns of *Nematoceras iridescence*.

Observed periclinal walls are very thin and transparent giving the seed a "caged embryo" appearance (Fig.A1.11 (2). Cells have a thick anticlinal wall that is relatively high with the apoplast often fissured and beaded. Gaps, of triangular form, appear in the apoplast at the edges of the adhesion points between three cells and are characteristic of the taxa.

Cell sizes (Tables A1.3 and 4) are generally consistent in form, being at their maximum around the medial aspect, become smaller, and taper down as they approach both the chalaza and apical ends

Nematoceras sp.	Medial axis μm	n	Length axis μm
N.iridescens	30.66 ± 1.542	6	107.83 ± 10.74
N.longipetalum	23.22 ± 2.107	9	85.00 ± 9.98
N.papa	28.88 ± 2.044	9	102.00 ± 7.80

Table A1.3. Cell measurements, medial section, of the testa cells of three *Nematoceras* species; *N. iridescens*, *N. longipetalum* and *N. papa*.
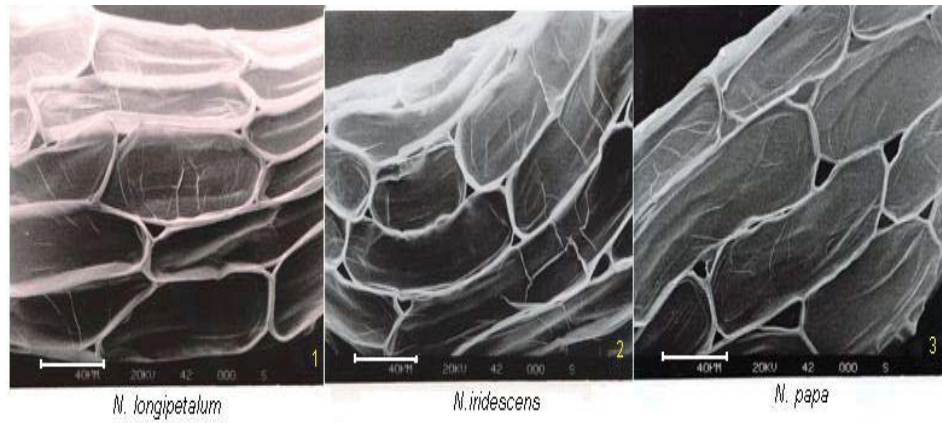
Figure A1.12 A comparison of the seed testa and apoplast cavities of; 1= *N. longipetalum*, 2= *N. iridescens*, 3= *N. papa*. Bars = 40 µm.

Cell # of SEM seed micrographs

	N. iridescens			N. longipetalum			N. papa	
	medial #	long #		medial #	long #		medial #	long #
1	6	11	1	5	10	1	5	11
2	6	12	2	6	10	2	5	10
3	6	13	3	5	9	3	4	10
4	5	11	4	6	11	4	4	9
5	6	12	5	6	12	5	5	9
6	6	10	6	5	12	6	5	8
7	5	10	7	5	8	7	6	11
8	5	10	8	6	10	8	5	11
9	5	10	9	4	9	9	6	10
Σ	50	99		48	91		45	89
Mean	5.555556	11		5.333333	10.11111		5	9.888889
SD	0.527046	1.118033989		0.707107	1.364225		0.707107	1.054093

	Sum of Squares	df	Mean Square	Fisher F- value	Significance (p)
Between Groups:	6.227	2	3.113	2.212	0.131
Within Groups:	33.775	24	1.407		
Total:	40.002	26			

Table A1.4. Cell measurements of the medial and long axis of the testa cells of three *Nematoceras* species; *N. iridescens, N. longipetalum* and *N. papa*. Analysis of variance (ANOVA) between the three species of *Nematoceras* produced a significance of $p = 0.131$.

A1.6   Germination trials of *N. iridescens* seed

Three basic germination methods were applied to *N. iridescens* seeds collected from sites as previously outlined. The methods; symbiotic germination, asymbiotic germination and field germination, have been used in this thesis in an endeavour to germinate *N. iridescens* seeds.   Early orchid enthusiasts had great difficulty in germinating orchid seed even after the obligate mycoheterotrophy of the Orchidaceae had been reported, (Burgeff, 1936).

Early success had been derived from cultivating sphagnum moss, clipping the strands into fine pieces, spreading a little orchid potting mix over the top and then sprinkling the particular orchid seed over the mix, (Veitch, 1887-1894).   Success, in the production of orchids by these means was exceptionally spasmodic and unreliable. A number of orchids were propagated by simply sprinkling the seed onto the potting mix in which plants of the same or closely allied species were growing.

These methods of producing orchids continued until, Burgeff (1936), proved the importance of the mycoheterotrophic relationship between fungus and seed germination and initiated the use of symbiotic germination of orchid seed in vitro, (Rasmussen, 1995).

A mycorrhizal fungus is obligate for the germination of orchid seeds, (Curtis,1939) however, the diversity of specific groups of fungi, found in terrestrial orchid plants has been only spasmodically researched.   Mycorrhizal fungi have been investigated in some epiphytic and terrestrial orchids, mostly in the Northern hemisphere, but mycorrhizal endophytes of the New Zealand orchids have been unidentified.

Bernard suggested that *Fusarium* fungus could stimulate seed germination, (Burgeff, 1936), however, the potential of *Fusarium* species was ignored since, when compared to more specific Rhizoctonia forms of orchid mycorrhizas, it is insignificant, (Vujanovic, 2000).

Terrestrial orchid seed are notorious in their difficulty to germinate and because of this difficulty, symbiotic germination is generally used when cultivation of hybrid seed, for breeding purposes, is required.   Virtually all research of orchid seed germination has occurred with orchids of commercial interest such as, Vanilla, *Cymbidium* spp., *Phalaenopsis* spp and *Cattleya* spp. Once a variety of orchid had been selected, as being potentially suitable for commercial release, tissue culture procedures would be initiated to bulk up marketable supplies.

The techniques of fungal culture, seed collection and germinating protocols have been described by, (Zettler, 1997; Porras-Alfaro, 2007), suggested that a specific orchid species can be found in an association with differing fungal endophytes.

The availability of a media that would initiate the germination of terrestrial orchid seed without inoculant fungi, asymbiotic germination, would provide a ready supply of orchid plants of both, threatened species and for conservational re-introduction. Unfortunately such is not the case; attempts to grow terrestrial orchid's asymbiotically have been fraught with disappointments, (Henrich, 1981; Kauth, 2008; Rasmussen, 1992, 1995; Yamazaki, 2006).

The provision of abiotic principals to germinate and provide continuing growth nutrition has proved successful when applied to commercial orchid propagation where ample finance is available.

Orchid seeds have varying degrees of mycoheterotrophism and any media used to artificially initiate germination and development must provide a similar nutritional

substrate and degree of nutrient absorption (Arditti, 1992; Rasmussen; 1992, 1993; 1995; Withner, 1959). The environmental parameters such as water, pH, humidity, atmosphere, temperature and light essentially remain similar for all methods of orchid seed germination and vary slightly in accordance with the genus and species, (Arditti, 1992; Rasmussen, 1995).

Prior to 1920 orchid seed germination, apart from natural scattering onto sphagnum media, was confined to the symbiotic method developed by, (Bernard, 1904, 1909; Burgeff, 1909). In most cases the pathogenicity of the symbiont overcame the seed prior to germination, Beardmore (1981). A method of overcoming this disadvantage was to develop a method of germination in which the potential mycorrhizal fungi was absent. Knudson, (1922) undertook an investigation of orchid seed nutrient requirements and produced a synthetic media formula that was successful in germinating the seed of a number of *Cattleya* species. Subsequent germination trials had variable results. Often seeds would germinate and then remain at a static phase, with no further development, until they finally expired. It was later found, that fluctuation of osmolality or secondary toxicity was the main cause of development termination. This was often due to the high concentrations of mineral salts in the early media formulae, Withner (1959). A current trend in asymbiotic orchid media lays emphasis on the reduction of mineral salts and an increase in the organic constituents, (Murashige, 1962; Arditti, 1992; Rasmussen, 1995). Contemporary asymbiotic formulae, formulated by specialist companies, are generally tailor made for specific species and are complexes of nutrients vitamins and various growth regulators in an endeavour to replicate the endophyte metabolic by-products, Kauth (2008). Three of these; BM1, T842 and M551 from Phyto Technology Laboratories LLC (See Appendix 2 for formulae) were trialled with *N. iridescens* seeds.

Because of their minute size and the length of time some orchid seeds take to germinate, natural germination in a field situation has been almost impossible to observe. Laboratory studies are by their very nature artificial and questions must be asked if *in vitro* studies duplicate those that apply in a natural environment? The mycorrhizal fungi that initiate germination have been found to rarely equate with those found in established seedlings and adult plants, (Hadley, 1970), and since the teleomorph has proved to be difficult to culture, most experiments relating to a fungal endophyte capable of germinating orchid seed have been very limited. In 1993 a technique was suggested to overcome the problem of dust seed observation by creating ultrafine mesh envelopes that could retain seed in a field situation for periods of up to two years and allow exposure of the envelope contents to a natural environment, (Bidartondo, 2008; Rasmussen, 1993).

In this chapter, an investigation of seed germination under natural field conditions were also analyzed, using the envelope technique. The extracted DNA of any seed, was amplified by PCR and subsequently sequenced to identify the endophyte (see Chapter 2 for methods). An endeavour was made to germinate seed of *N. iridescens* both symbiotically and asymbiotically using a variety of media and techniques.

A1.6.1 Symbiotic trial, series 1

Over the period, 23/03/06 to 20/04/06, all seeds showed adequate embryo imbibition over the first week of the trial, (see Fig. A1.13 left) in which embryos increased in size by 44.8% (see Fig. A1.13 right)
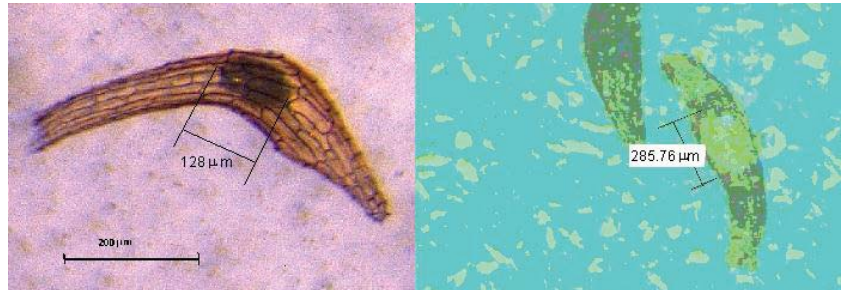
Figure A1.13.   A comparative measurement of the embryo of a *N. iridescens* seed after the first week of the symbiotic trial. Left figure is the embryo in a dry seed. Right figure is an imbibed embryo of a seed after one week of the trial.

At the completion, of the trial period the fungal inoculant had overwhelmed the seed in every case, except: Px 4 and 19; Ux 19; Tx 4 and 19; Nx 4 and 19. (Table A1.5)
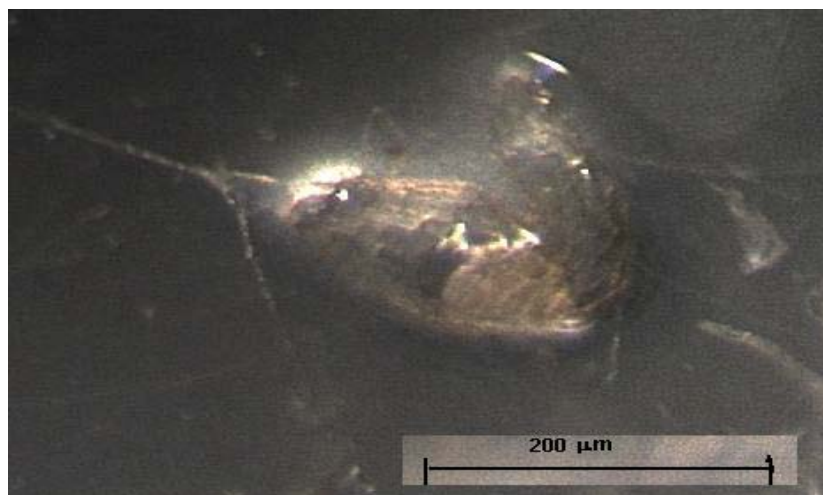
Figure A1.14. Fungal hyphae invading an *N. iridescens* seed.

Innoculant used   See Table 5a.3

Seed	Dates	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Px	8/02/2006	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	21/02/2006	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	2/03/2006	x	x	x	x	*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	*	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	23/03/06	+	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ux	8/02/2006	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	21/02/2006	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	2/03/2006	x	x	x	x	*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	*	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	23/03/06	+	+	+	+	+	+	+	+	+	+	+	+	x	+	+	+	+	+	+	*	x	+	+	+	+	+	+	+	+	+	+	+	+	+
Tx	8/02/2006	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	21/02/2006	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	2/03/2006	x	x	x	x	*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	*	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	23/03/2006	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nx	8/02/2006	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	21/02/2006	x	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	2/03/2006	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	*	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	23/03/06	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Key | = +ve imbibition   * = innoculant growth initiated   x = invasive innoculant   † = seed death

+++ = strong germination 75% <,   ++ 40% < germination,   + = 39% > germination.

Table A1.5.   Symbiotic germination trial results for *N. iridescens* seed inoculated with fungal isolate cultures.

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A1.6.2   Symbiotic trial, series 2

Light group	Plate #	Inoculant	25/1 2007	2.02 2007	Culture growth	8.02 2007	Culture growth	15/02 2007	Culture growth	1.03 2007	Culture growth	8/3 2007	Culture growth	14/3 2007	Culture growth	19/3 2007	Culture growth	28/3 2007	Culture growth
	L1A1	A1	24	0	w 9	0	w 28	0	w 60	0	w 80	†	w 85	†					
Light 12/24	L2A7	A7	18	0	b 30	0	b 43	0	b 54	-	b 90	†	b 90	†					
	L3N3	N3	15	0	g 10	0	bl 15	-	bl 40	-	bl 90	†	bl 90	†					
	L4T3	T3	30	0	bb 11	0	bl 20	0	bb 35	0	bb 90	†	bb 90	†					
	L1C	nil	32	0	nil	0	nil	0	nil	-	nil	-	nil	-	nil	-	nil	-	nil
	D5A1	A1	41	0	w 20	-	w 41	-	w 60	0	w 75	0	w 80	-	w 82	†	w 90		
Dark 24/24	D6A7	A7	26	0	b 27	0	b 51	0	b entire	-	b 90	i +	b 90	i ++	b 90	i ++	b 90	i ++	b 90
	D7N3	N3	18	0	g 10	0	g 20	0	g 20	-	g 20	-	g 25	i +	g 28	i ++	g 34	i ++	g 40
	D8T3	T3	11	0	bb 10	0	bb 23	0	bb 35	c	bb 35	ic	bb 35	ic	bb 35	†	bb 35		
	DC	nil	45	0	nil	0	nil	nil	0	-	nil	-	nil	-	nil	-	nil	-	nil
	L9A1	A1	20	0	w 20	0	bl 31	0	g 54	†	w , fb	†							
Light 24/24	L10A7	A7	11	0	b 35	0	b 47	-	b 80	c	b 90	†							
	L11N3	N3	15	0	g 11	0	g 32	0	bl.g 40	c	bl 45	†							
	L12T3	T3	11	0	w 18	0	bl 30	c	bl 36	c	bl 38	†							
	L2C	nil	37	0	nil	0	nil	-	nil	-	nil	-	nil	-	nil	-	nil	i	nil

Key :   w = white † = death c = contaminated   b= brown   g= grey   bb = blue black   bl= black   fb = fruit bodies

Table A1.6. Results of light and dark parameters on *N. iridescens* seed inoculated with four fungal cultures A1, A7, N3 and T3. L1C, DC and L2C representing controls without culture inoculants, all of which show imbibement.

All of the dark grown seed had a longer survival period (62 days) than that in 24/24 light (42 days) and in 12/12 dark and light groups (48 days). See Table A1.6. The cultures N3, a Sebacinaceae and T3, an *Ascomycete* spp. did not prove pathogenic and the seed embryos survived in the dark incubator group but not in either of the light groups eventually succumbed to the fungal inoculate.

A1.6.3 Asymbiotic germination trial

All seed had imbibed during the period 10/06/09 – 2/07/09 (see Table A1.7) Three plates: BM1 # 4, T842 # 4 and M551 #4 became infected with a smooth raised orange / yellow bacterial contaminate indicating a seed source infection in the old seed of the *N. iridescens* sample. The seed in BM1 #6 and M551 #6 became covered in a white fungal colony with divaricating hyphae that proved pathogenic in both media within a period of 57days (10 June – 6 August 2009) with the embryos of seed that were contaminated becoming distorted prior to death. The only other fungal colony appeared on plate T842 #5 as a black circular growth that over the course of the experiment did not prove to be pathogenic.

Of the media used T842 produced five complete embryos for the term of the experiment. Figure A1.16 shows both healthy imbibed embryos and four dead embryos. This micrograph of BM1 #7 portrays the embryos seen in all of the imbibed embryos of the other samples.   No seed survived for the term of the experiment in the BM1 media, five survived plated on T842 and a single survival in M551 #7.

An invasion by mites (see Fig.A1.15) occurred in sample M551 #1. The mites were first observed on the 30/07/09 a 50 day period from sowing the seed. The provisional identification was of a species within the Cryptostigmata family, (Luxton,1985). The Cryptostigmata mites feed on detritus, fungi and plant material, (Denmark, 1965). A question for further research, does this mite predate fungal mycorrhiza or destroy the actual seed?

Figure A1.15.   A predatory mite (Cryptostigmata family spp.) on *N. papa seed* in M551#1. Bar = 200 µm.

Media	#	seed spp	Date sown	2/07/09	30/07/09	6/08/09	24/09/09	26/11/09	10/12/09
BM1	1	N. papa	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	distorted embryos	†	†
	2	N. papa	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	distorted embryos	†	†
	3	N. papa	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	dead embryos	†
	4	N. iridescens (old)	10/06/2009	bacteria S. aureus	†	†	†	†	†
	5	N. iridescens (old)	10/06/2009	imbibed embryo	contaminated black	†	†	†	†
	6	N. iridescens (new)	10/06/2009	contaminated white	contaminated white	dead embryos	dead embryos	†	†
	7	N. iridescens (new)	10/06/2009	imbibed embryo	imbibed embryo	dead embryos	dead embryos	†	†
T842	1	N. papa	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo
	2	N. papa	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo
	3	N. papa	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo
	4	N. iridescens (old)	10/06/2009	bacteria S. aureus	†	†	†	†	
	5	N. iridescens (old)	10/06/2009	contaminated black	embryo bubble	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo
	6	N. iridescens (new)	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo
	7	N. iridescens (new)	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	dead embryos	†
M551	1	N. papa	10/06/2009	imbibed embryo	mites	mites	†	†	†
	2	N. papa	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	distorted embryos	†	†
	3	N. papa	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	imbibed embryo	dead embryos	†
	4	N. iridescens (old)	10/06/2009	bacteria S. aureus	†	contaminated black	†	†	†
	5	N. iridescens (old)	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	†	†	†
	6	N. iridescens (new)	10/06/2009	contaminated white	contaminated white	dead embryos	dead embryos	†	†
	7	N. iridescens (new)	10/06/2009	imbibed embryo	imbibed embryo	imbibed embryo	large size em	large size em	large size em

Table A1.7 Results of the asymbiotic germination trial of *N. iridescens* (old seed and fresh seed) plus a comparison of new *N. papa* seed. All seed was plated onto three different culture media; BM1, T842 and M551. Plates from each different media were incubated at 12°C in 24hrs darkness.

The symbol † = death of all observed embryos in that plate.
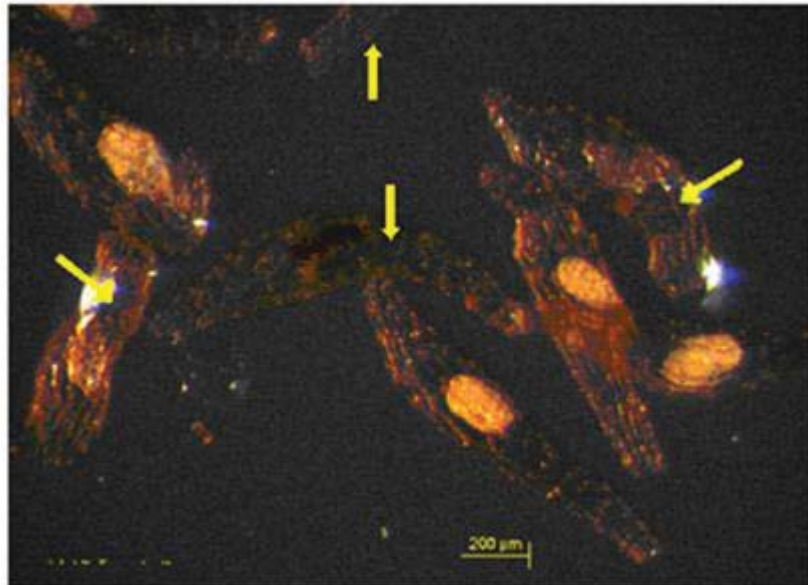
Figure A1.16 Imbibed embryos of *N. iridescens* (five orange ovals) growing on BM1 (#7) media. Four dead embryos (yellow arrows) can be seen.

A1.6.4 Field germination trial

Apart from embryo imbibition no results were recorded for any of the seed examined on the 15/12/2008 or the 20/05/2009. All seed appeared healthy and there was no evidence of fungal colonies within the seed samples (see Table A1.8)

Date	Field germination table of results		
	Site 1 adjacent *N. iridescens*	Site 2 adjacent *N. trilobum*	Site 3 adjacent *N. rivulare*
a 15/12/08	All embryos imbibed no evidence of fungi	All embryos imbibed no evidence of fungi	All embryos imbibed no evidence of fungi
b 20/05/09	No change	No change	No change
c 8/12/09	Appear to have dehydrated no viable embryos	Split testa protocorm emergence. Shows germination	No change

Table A1. 8 Record of seed germination in Field conditions at the Mangamingi site

A micrograph of the seed condition of the site 2 envelope on the 20/05/09 can be seen in Figure A1.17 with all of the seed still entire and embryos imbibed.

Figure A1.17   Field trial seed of *N. iridescens,* contained in the sample b site 2 on the (20/05/09).

All of the seed within the envelope at site 1 on the 8/12/09 examination were severely dehydrated. The envelope had been pulled out of the bank and was suspended, by its attaching nylon, in mid-air. There were obvious signs of either wild pig or feral goat damage to adjacent plant life. Site 2 seeds, at the conclusion of this experiment, had fungal hyphae accumulation at the hyphal end (Fig A1.18). Two seeds had successfully germinated to the protocorm stage (Figure A1.19) and others had split testa and very enlarged embryos.

Figure A1.18. Fungal hyphae accumulation in field trial seed of *N. iridescens.* Bar = 5 μm (15/12/09).
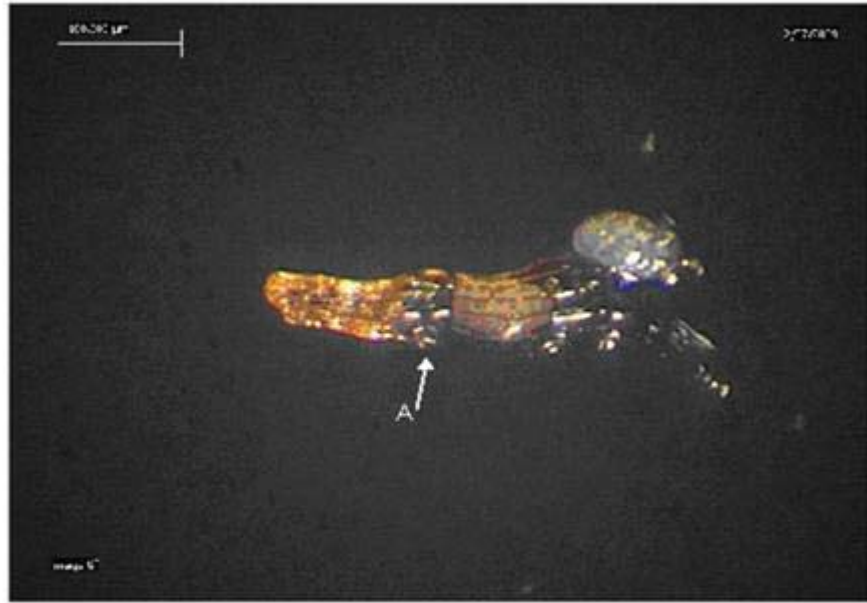
Figure A1.19   Seed germination initiated in a field trial seed, after one year.

Seed samples from site 2, adjacent to the germinated seed (Fig. A1.19) were investigated by CLSM and provided the first images of fungal hyphae within the embryo of an orchid seed (Fig A1.20).   The hyphal morphology shows a Rhizoctonia form.   Samples of similar seed from the envelope were taken for DNA sequencing and identification (see Molecular analysis).   Results from the sequencing showed that the hyphae resident within the embryo was a new strain of *Tulasnella calospora.*

The 2c seed sample with positive germination initiation came from a twelve month buried envelope (Table A1.8).   Germination was further confirmed by CLSM imaging of invasive hyphae (Fig.A1.18).
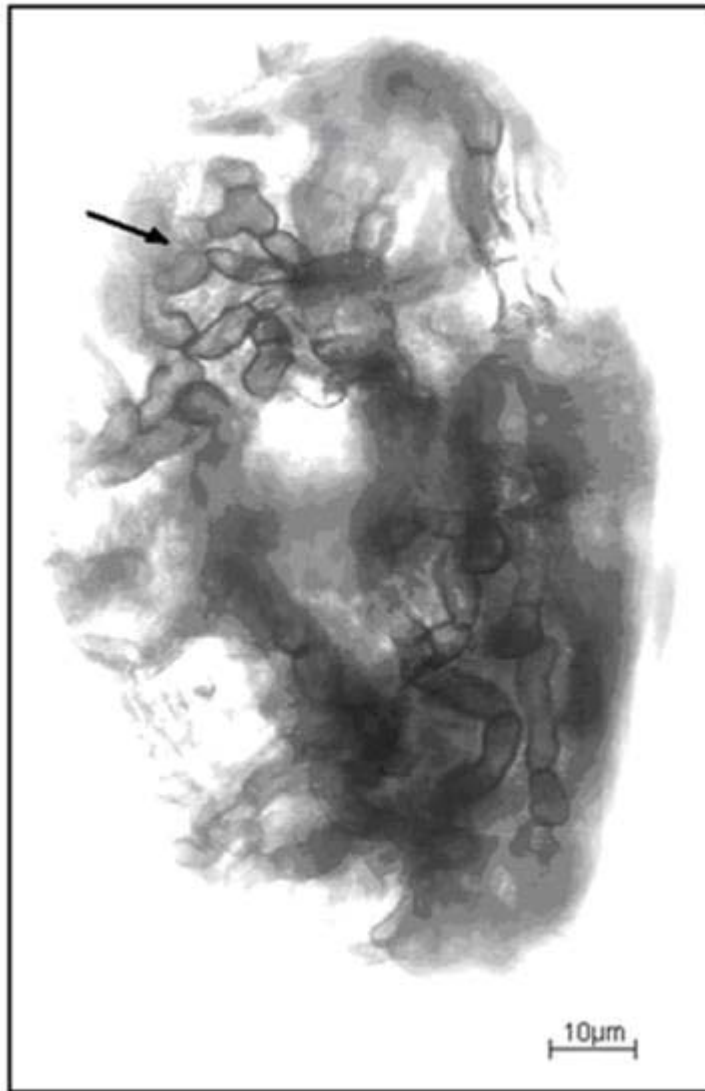
Figure A1. 20. The embryo interior with hyphae of *T. calospora*, strain (HM802310) arrow, after one year enveloped field trial.

A1.7 Discussion of Section A1

As explained in the introduction to this chapter *Nematoceras* orchid seed are produced in great numbers, the hypothesis being that a large number of seed provides a greater chance of survival in species that require a specific environment, host or is subject to transitory dispersal vectors, (Burrows, 1975; Howe, 1982; Nathan, 2006; Rasmussen, 1993; Soons, 2005; Teryokhin, 1982; Trakhtenbrot, 2005; van der Pijl, 1982). Various strategies to facilitate seed dispersal are apparent in *Nematoceras* spp.

Considering the seed mass to volume ratio and the average proportion of seed per pod, it would initially appear that the normal seed with viable embryos, amount to only 31.74% of total seed of an *N. iridescens* pod (Table A1. 5).

However it is possible, that the low seed mass to volume ratio (micro) seed (65.48%) are able to disperse to a greater distance ensuring a more complete coverage of downwind deposit sites, (Jersáková, 2007). Deposit shadow rings,

(Fig. A1.21), (Nathan, 2006), formed between micro seed, with viable embryos and normal seed volume mass, would ensure a far greater range of seed distribution and plant survival rate.
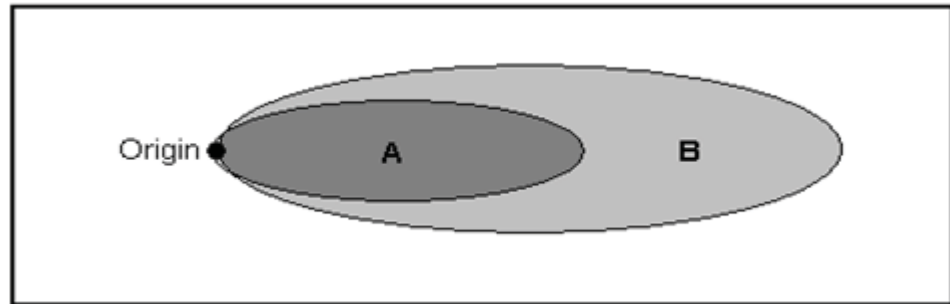
Figure A1.21 Seed deposit shadows; A = normal seed deposit shadow, B = micro seed deposit shadow

A major difficulty of long distance wind born seed dispersal encounted with terrestrial plants growing in forested areas is the resistance and air flow disturbance provided by the forest canopy to upward airflow, (Cain, 2000).

All of the Nematoceras spp. observed by the author were situated on banks or in forest gaps within open airflow zones   Extensions of the peduncles bearing the terminal seed pods, placed the pods into airflows of sufficient wind strength to shake the pods and free the "balloon" seed into valley wind flows.

Seed morphology is such that the Nematoceras seed surface simulates an aerofoil effect and having "Dust Seed" characteristics enables the seed to move over great distances, Burrows, (1975), provides formulae and methods for the calculation of primary trajectories for "Dust Seed", spores and pollen in unstable winds.

Valley wind currents tend to be strongest around mid-day and early afternoon, at the same period that vertical convection air currents occur, (Sturman, 1996). Any oscillation, with a wind speed greater than 1.3 ms-1, would enable the seed to be liberated into passing wind currents and then into the main valley wind currents.

Differences, between seed testa cell numbers, of N. iridescens, N. longipetalum and N. papa do not appear to be of taxonomic value. Testa shapes all conformed to a fusiform morphology with a large range of gross difference within and between each species.   This difference was not thought to be of value and was not calculated.   However, apoplast walls and their patternation presented visual interspecies differences.

Having minute seed, limits predation to a size relevance basis, (Harper, 1970). Very minute seed is predated by very small herbivores. The only case of seed predation the author observed was by a Cryptostigmata mite (Fig. A1.15).

Seeds within the pod, mature in the final weeks, become lignified, dry and loose moisture. A waxy cuticle covers the hardened seed testa and produces seed which are extremely difficult to wet. Contours in the testa of hardened orchid seed, along

with their minute size, produce extremely high water tension levels when the liberated seed is immersed in water, (Arditti, 2000; Rasmussen, 1993, 1995). This condition provides good flotation ability and a further dispersal advantage for orchid seed.

The results seen in the symbiotic germination section, although disappointing, reflect the results of many terrestrial orchid workers in this field, (Batty, 2001; Bonnardeaux, 2007; Henrich, 1981; Kauth, 2006; Quay, 1995; Rasmussen, 1998; Zettler, 1997). No formal research appears to have been conducted on NZ terrestrial orchid seed symbiotic germination.

Differences in orchid seed germination and seedling development could be attributed to a process of adaptation to changes in differing environments as, Kauth, (2008), has suggested.

The results, seen in the symbiotic series 1 trial used fungal cultures from the culture library, all of which had been derived from root tissue of *Nematoceras* spp. Culture 19 (N_3a1) was successfully sequenced and was nearest to an uncultured Sebacinaceae, Genebank accession number, AY634132. The Sebacinaceae are known endophyte mycorrhizal fungi of orchids, (Rasmussen, 1992; Zettler, 1997).

A comparison, between the immunity of successful orchid seed germination, to pathogenic fungi and bacteria, to that of the non-immune seed would provide a field of worthwhile study, especially since the period of orchid seed dormancy, in pathogen favourable environments, can extend for a year or more, (Arditti, 1992, 2000; Rasmussen, 1992; Yamazaki, 2006).

Based on the results reported in the Series 2, (Table A1.6), light regimes, play an important aspect in development of the fungi. Both continuous light, 24 / 24 and partial light 12 / 24, inhibited germination, apparently by affecting the fungal inoculant, rather than the seed, since the controls (seed alone) remained viable and imbibed in all of the light situations. The 24/24 dark group, A7 and N3, retained form, displayed imbibition and embryo colour throughout the experiment.

A mite found in the M551 #1 asymbiotic culture was provisionally identified as an *Orbiculata* mite member of the Cryptostigmata family. Most members of this group are fungal predators and so conjecture arises as to whether this species of mite is predating fungi or the orchid seed, (Luxton, 1985). All of the seed of M551 #1 had died by the 24/09/09.

The time taken for the final result of the field trial germination (Table A1.8) was one year from the initial sowing dates. This would indicate that the time allowed for two of the germination experiments, both symbiotic and asymbiotic could have been restrictive in the time allowed. Long-term seed dormancy could have inhibited germination. .

Vernilization, epicotyl dormancy (Kauth, 2006), could affect seed germination delay when consideration is accorded to the seasonal climatic variation of the sites in which plants of the *Nematoceras* species can be found

Of the three asymbiotic culture media, T842 supported 71% of embryos for the total term of the trial, BM1 supported 0% and M551 14.3%. A major difference between the three media was the activated charcoal and pineapple powder that T842 contained (see Appendix 3 Media). In hindsight (time taken for field trial germination to become apparent) a longer period of trial would have been valuable.

Chapter A2   Leaf characteristics of *N. iridescens*

A2.1   Introduction

Orchid leaves are typically monocotyledonous, exhibit parallel striate venation and possess an extended lamina. However, many terrestrial orchid genera display marked reticulation and a morphology more typical of a dicotyledon, (Arditti, 1992; Benzing, 1987; Clifford, 1974; Esau, 1953; Freudenstein, 1999; Pridgeon, 1994). The two general divisions of orchid leaves broadly delineate orchid leaf morphological classification; thin leaves tend to represent terrestrial and heavy shade epiphytes, whereas thick and fleshy leaves are borne by high light, drought prone, canopy epiphytes, (Arditti, 1992).

Field and nursery observations show that all *Nematoceras, Singularybas, Corybas, Anzybas, Molloybas*, (Hatch, 1951; Moore, 1970; Cooper, 1981; St George, 1996) and (*pers. obs.*), have a dormancy in association with dry weather.

Any adaptation, by single leaved plants such as CA species, to survive any change in levels of hydration, such as climatic change, could be expected to possess a number of specific characteristics:

1   a period of dormancy, covering the period of highest climatic stress,

2   thicker or more effective layers of cuticular wax,

3   water storage / retention mechanisms,

4   CAM or C4 carbon fixing pathways,

5   a reflective abaxial leaf surface,

6   sufficient stomata of proportional size to control homeostasis, and transpiration,

7   optimal niche environments (shade and water source),

8   antiherbivoury measures,

9   a supplementary nutritional source.

Generally, orchid leaves are arranged distichously (two ranks) with leaves alternating on opposite sides of the stem. Most of the CA genera exhibit single leaves arising from a stem or pseudobulb but scale leaves and sheaths maintain a distichous pattern, (Arditti, 1992; Dressler, 1981). Dressler (1993), considers a spiral arrangement of leaves about a stem as a primitive condition. Morphological taxonomists, consider the development of leaves and their folding to be an important character, with primitive characteristics exhibited as a rolled or convolute leaf development, (Arditti, 1992; Dressler, 1981, 1993). In a colder climate, the CA *Nematoceras* (Macquarie Island and high altitude CA specimens) retain the leaf cupping and folding. A tightly rolled early leaf form character possibly provides a form of cold temperature or wind insulation for the emerging flower bud, (Clements, 2007; Godley, 1989; St George, 1996).

The Acianthinae, in which the CA is nested, keys in the following leaf description for the taxa; leaves can be either achlorophyllous but usually chlorophyllous,

solitary leaved, petiolate on non-flowering stems, leaves short and broad, sessile on flowering stems, which Moore (1970), considers to be advanced characters.

The first recorded description of leaf stomata, in the orchid leaf epidermis, was by the German botanist, Möbius (1886), who compared the epidermal cells and the leaf stomata of 193 species in 95 genera of orchids. The orientation of the stomatal guard cells was in general found to be parallel to the long axis of the leaf, (Seib, 1903).

Further stomatal research of the Orchidaceae was sporadic until 1930 when, Solereder (1930), reported on studies concerning the size of various stomata, the histology of the stomatal complex and the presence of subsidiary cells in 73 orchid species, together with an updated review of the earlier work by Möbius. Most guard cells in the Orchidaceae are parallel to the long leaf axis even when the venation is semi-reticulate. In contrast, Zeigenspeck (1944) presented a list of European broad leaf orchids in which the stomata were not longitudinally oriented.

Reniform guard cell morphology is universal in the Orchidaceae and the circumference of the guard cell complex is generally elliptical, however, almost circular outlines appear in many species of *Bulbophyllum* and *Cirrhopetalum*, (Sprenger, 1904). A waxy cuticle covering over the leaf can obscure the stomatal appearance, especially the pore shape and orientation, (Solereder, 1930), which has created a level of skepticism about the early stomatal interpretation, (Rasmussen, 1981, 1987).

Orchid stomata show a high variation in polar axis length, ranging from 20µm in *Epidendrum equitans* to 75µm in *Trichoglottis soleraderi*, (Solereder, 1930). It was also noticed by Solereder, that the stomatal size could be correlated with habitat, with epiphytes tending to have smaller stomata and higher density mm^{-2} than the terrestrial species. Roth (1979) reported on the steadily increasing size of stomata and further refined this observation in all plant groups growing in a Venezuelan cloud forest. The smallest sized stomata were in crown canopy epiphytes and the largest stomatal size appearing in terrestrial species growing on or close to the forest floor.

An increase in stomatal size from canopy to forest floor was reported by, Pridgeon (1982). Another example being Vanilla, an aerial exposed epiphyte, with very small stomata, (Nayar, 1976). These examples contrast with the very large stomata, 81µm shown in SEM and light microscope studies of terrestrial *Paphiopedilum* species, (Rutter, 1979).

Subsidiary cells and their relationship to angiosperm stomatal complexes are taxonomically important and in many cases used in specific species identification, (Stace, 1989). The patterns of subsidiary cells, which differ from normal epidermal cells, surrounding the stomata are key features in many cases of specific plant leaf identification, Dilcher (1974), who classified 35 distinct patterns in vascular plants.

Monocotyledons generally have a leaf epidermis consisting of morphologically regular longitudinal cell files in which stomata-genesis occurs as a stomatal meristemoid developing from a small apical segregate, arising from an epidermal cell. A number of patterns are formed by the epidermal cell distribution and orientation as the apical segregate divides into two guard cells, (Strasburger,1866-67; Rasmussen,1987). Where only two subsidiary cells surround the stomata, the pattern is termed diacytic and found in a number of orchid spp. (Williams,1975). Where no subsidiary cells surround the guard cells, the condition has been termed anomycytic, (Solereder, 1930).

A tetracytic pattern, where four roughly equal sized subsidiary cells surround the stomatal guard cells, is commonly found in both epiphytic and terrestrial orchids,

(Solereder, 1930; Williams, 1975; Rasmussen, 1981, 1987). A pattern of more than four subsidiary cells surrounding a central pair of guard cells has been termed cyclocytic, (Pridgeon, 1979), and is thought to have developed from the tetracytic configuration.

If the pattern configuration of subsidiary cells that surround central stomata is a taxonomic character, the definition and recognition of such subsidiary cells requires elucidation. The criteria generally used are those of cell configuration and cell sizes, (Rasmussen,1987).

A wide range of subsidiary cell sizes occur. Rasmussen (1987), refers to the differences that exist between lateral and polar subsidiary cells and notes the disruptions that occur to the adjoining epidermal file cells can often highlight the subsidiary cells. This disruption of epidermal cells was used by, (Williams,1975, 1976, 1978), in a series of papers on orchid stomata and was subject to a critical review by, Rasmussen (1981), who suggested that Williams findings, based on file disruption identification of subsidiary cells, could be interpreted in many other ways. Subsidiary cells can display a range of topographic features that can visually isolate them from the surrounding epidermal cells and comprise:

1   enhanced surface projection, (Zornig, 1904),

2   cuticular patterns, (Sprenger, 1904; Zornig, 1904),

3   an elevation of the entire stomata above the epidermal plane, (Pridgeon, 1979, 1982),

4   a presence or lack of detectable cytoplasmic artifacts (when contrasted with the adjoining epidermal cells, such as calcium oxalate crystals, (Nayar,1976) and oil droplets, (Zornig,1904).

As early as 1895 starch was observed in *Corybas* guard cells, (Groom,1895), but no records have been found of CA guard cells containing oil droplets, a condition observed in the stomata of long-lasting evergreen leaves, (Rasmussen,1987).

Orchid species that have adapted to more xeric habitats can display a number of stomatal modifications, (Rasmussen, 1987), small sub-stomatal chamber size with limitation of mesophyll air passageways, (Tominsky, 1905) and thickening of guard cell walls, (Solereder, 1930; Tominsky, 1905). The development of stomata from meristemoids and the meristemoid development from protodermal cells were termed agenous stomatal development and this form of development is predominant in the Orchidoideae, (Rasmussen, 1981).

A further development of agenous stomatal development, in which the meristemoid again divides to produce a mesogene cell (a second order meristemoid) was also found to occur in the Orchidoideae, and was termed hemimesogenous patterning by, Rasmussen (1981). Hemimesogenous development was recorded in the Neottioideae by Williams (1975). The further development of mesogene cells in orchids is well described by (Rasmussen, 1981, 1987; Nayar, 1976; Singh, 1981; Williams,1975).

Stomata occur in all areas of the Orchidaceae except roots, even though many epiphytic orchids have photosynthesizing roots, (Willmer, 1983). Stomata can be found on both sides of the leaf, amphistomaty, although the most dominate distribution in the Orchidaceae is that found solely on the abaxial surface, hypostomaty, (Rasmussen, 1987). Amphistomaty is common in orchid species growing in arid habitats and rarely found in shaded humid forests, (Tominsky, 1905). Hypostomaty occurs mainly in mesophytic angiosperm species, (Parkhurst, 1978). Many orchids, especially those in areas of high light and xeric habitats, possess C4 or crassulacean acid metabolism (CAM), (Avadhani,1982) which

correlates with orchid species having thick leaves and related amphistomaty, (Mott, 1982).

Stomatal density tends to increase as the intensity of light increases, (Schoch, 1980), and decrease as the CO_2 concentration increases, (Woodward, 1987). In orchids, stomatal densities range from 18000 cm^{-2} (*Arundina graminifolia)* to 800 cm^{-2} in (*Thrixspermum calceolus)*, with an average of 5240 cm^{-2} taken over 20 species from 14 genera, (Arditti, 1992).

In solitary leaved orchids, such as seen in *Nematoceras* spp., survival is jeopardized if the single leaf is compromised. One would therefore hypothesize that any solitary leaved plant would have adapted to various strategies of protection to inhibit predation or pathogen attack, (Gibbs, 2003; Grime, 1979). While chemical anti-herbivoury adaptations are much cited in the literature, (Cheng, 2007; Grime, 1979; Pichersky, 2002; Roberts, 2006; Singer, 2003), an investigation of these is beyond the scope of this thesis. Instead, this section of the thesis investigates by SEM, E-SEM, CLSM and LM, some of the various physical strategies that *Nematoceras* spp., especially *Nematoceras iridescens*, has to protect its single leaf from possible predation.

Calcium oxalate crystals are widespread in flowering plants, including both dicotyledons and monocotyledons, (Prychid, 1999). Various structural forms of calcium oxalate crystals were recorded by Leeuwenhoek in the 17th century, (Frey, 1929).

The crystal shape allows relatively easy recognition especially the sharp, double pointed, elongated needle like calcium oxalate crystals termed raphides, (Franceschi, 1980, 2005).

Crystals, such as raphides and/or druses are present in the leaves and roots of most orchids, (Arditti, 1992). On assay, these crystals are composed of calcium oxalates of various levels of hydration. Raphides, composed of calcium oxalate monohydrate (wewelite), are the most commonly occurring form, (Prychid, 1999; Webb, 1999). Little is known of their function, but it has been assumed that; the crystals provide a calcium bank, conversely a calcium sequestration, (Prychid, 1999; Webb, 1999), or a sharp pointed, toxic, tissue defence mechanism, (Watson, 2005). Taxonomically the occurrence and distribution of raphides, and / or druses, have been used as characters in genus / species identification, (Pires, 2003). However, only very brief mention is given of calcium oxalate crystal presence in orchid leaf tissue and then mainly as an object appearing in histological drawings, (Arditti, 1992), or in respect of commercial worker dermatological sensitivity, (Morton, 1962).

In the more light exposed CA members, epicuticular wax can be clearly seen on SEM micrographs. These, epicuticular waxes are composed of a complex mixture of lipids, in an assembly process of wax molecules, that form a crystalline layer with nanostructures emerging from an underlying wax film, (Koch, 2004). The wax crystals grow by an extension of the tip, a process that can occur over a 24-hour period, often creating unique patternation, (Neinhuis, 2001).

The epicuticle layer is a multifunctional barrier between the plant leaf and the immediate environment contributing to protection and a reduction of water loss, (SchoÈnherr, 1976). Because of the hydrophobic quality of the wax, a water repellent surface is formed together with a cleaning effect, the Lotus effect, (Barthlott, 1997). A degree of protection against herbivory, and reflection of solar radiation can be attributed to some wax epicuticles, (Grant, 2003). Grant also reported that the filament structure provides a superior reflectance compared to single epicuticle wax sheets.

Leaves of the Corybas alliance species have, to date, had little, if any, investigation.

This section of the thesis aims to investigate:

1, the diversity of leaves, in a colony of *N. iridescens,* to show morphological variations,

2 the adaxial epidermis of leaves and compare differences between leaves of various species within the taxa,

3 to show a newly identified leaf margin cell "braiding",

4 the abaxial epidermis of leaves and compare differences between leaves of various species within the taxa,

5 the morphology of the stomata and their possible taxonomic potential,

6 aspects of anti-herbivoury, by the production and orientation of calcium oxalate raphide needles,

7 wax cuticle patterning on the adaxial epidermis of various members of the Corybas alliance,

8 predators of *Nematoceras iridescens* leaves.

Seasonal temperature increase, as a prime inducer of dormancy, was eliminated after a series of experiments, using two groups of nursery specimen plants, (*Nematoceras papa, N. triloba, N. iridescens* and *N. macranthum*) x2. These were held at ambient outdoor temperatures and light situations over the period June 2004 to February 2005. One group was thoroughly watered every second day and held in wet drip trays. The other group received only weekly watering. All water was from rain butts or the adjacent river (City water with constant chlorine levels could compromise soil fungi and orchid mycorrhiza, (Alexander, 1984). Leaves of plants in the dry location became necrotic and the plants assumed dormancy from the December – January period whereas leaves of the wet environment group were still turgid and healthy at the end of March 2005. This indicates a water availability response that could initiate plant dormancy. Field observations ratified this conclusion. Leaf colour varies and appears to be dependent on the light quality, being a dark green in heavy shade, to a light yellowish green, in areas subject to higher light (Fig. A2.1 (1). A deep maroon spotting around the leaf margins and along mid-ribs occurs in many species of the CA (Fig. A2.1 (3). The maroon spotting, presumably anthocyanin, (Gould, 1999; Harborne, 2001), appears scattered, in a colony with plants subject to sunfleck having more pigment than those in heavy shade (*Pers. Obs.*).

The abaxial surface appears silvery and pellucid with an entire laminar edge. All of the CA species investigated showed hypostomaty that is usual in terrestrial orchids, (Parkhurst, 1978; Rasmussen, 1987).

Variation in leaf morphology is high within the taxa, as can be seen in Fig. A2.1. Leaves of *N. iridescens* show diversity in morphology. These range, from a single large ovate leaf (Fig.A2.1 (2)), to a single occasionally pandurate form (Fig.A2.1 (3). Other leaf morphologies, as seen in the center, vertical row in Figure A2.1 (1) are possibly hybrids of *N. iridescens x N. trifoliata. N. trifoliata* has distinctive pandurate leaf blade morphology and colonies are often closely associated with *N. iridescens.*
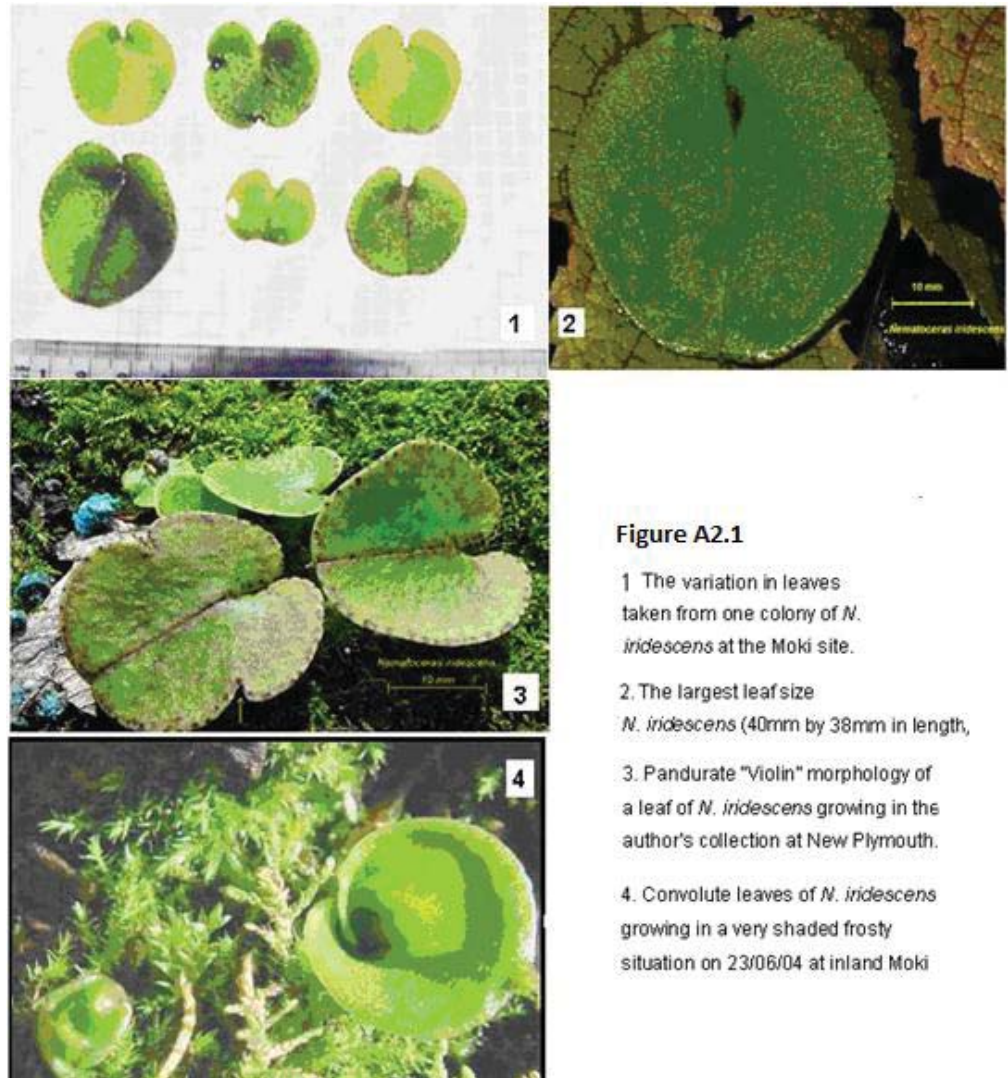
Figure A2.1

1. The variation in leaves taken from one colony of *N. iridescens* at the Moki site.

2. The largest leaf size *N. iridescens* (40mm by 38mm in length,

3. Pandurate "Violin" morphology of a leaf of *N. iridescens* growing in the author's collection at New Plymouth.

4. Convolute leaves of *N. iridescens* growing in a very shaded frosty situation on 23/06/04 at inland Moki

Many leaves produce a decurved apiculus, rounded and cordate at base; midrib grooved above, ridged beneath. This leaf form is similar to drip tip leaves of tropical rain forest plants in high rainfall areas, (Burd, 2007). *Nematoceras* species establish themselves in areas of high rainfall and mist. They have broad surface leaves with a waxy epidermis that is ideal for water droplet formation (Lotus effect). Water droplet run off provides a leaf cleaning function that would be an advantage in a solitary leaf situation close to the ground, (Koch, 2008; Neinhuis, 2001).

A2.2   Abaxial surface

A series of SEM and CLSM micrographs (60) were taken of seven fresh CA leaf samples; *Nematoceras rivulare, N. macranthum, N. triloba, N. iridescens, N. papa, N. orbiculatum* and *Singularybas oblongus*, to investigate the abaxial leaf surfaces and stomatal size and density. Stomatal density varied in the *Nematoceras* species investigated (Table A2.1).

All SEM micrographs, of the abaxial surfaces showed a distinctive patterned epidermis, hypostomaty and a low to medium density of stomata (range 16.36 stomata/mm^{-2} < 90.93 stomata/mm^{-2}, see Table A2.1, compared to the Orchidaceae overall (8-180 stomata mm^{-2}), Avadhani (1982). The lowest density recorded was for *Nematoceras rivularis*, 16.36 stomata mm^{-2}, a species found in very moist and generally heavily shaded situations. The highest density, 59.68 stomata mm^{-2}, occurred in *N. orbiculatum*, a light tolerant *Nematoceras* species

		A = 100 μm		B = 200 μm	
Genera	species	stomata / mm^2	micrograph #	stomata / mm^2	micrograph #
Nematoceras	*orbiculatum*	59.68	1443.32	55.57	1443.31
Nematoceras	*papa*	40.41	1443.27	36.34	1443.26
Nematoceras	*iridescens*	23.66	1443.21	23.66	1443.20
Nematoceras	*trilobus*	22.2	1443.16	24.92	1443.15
Nematoceras	*macranthum*	21.03	1443.11	18.69	1443.10
Nematoceras	*rivulare*	16.36	1443.07	16.36	1443.06
Singularybas	*oblongas*	41.68	1462.27	41.68	1462.28

Table A2.1   Average stomatal density / mm², of a selection of *Nematoceras* and a *Singularybas* sp.

The stomatal size and shape of *Nematoceras* tend to be limited to two basic forms; round and ellipsoid (Table A2.2).   Round forms of stomata are linked to those *Nematoceras* species (*N. iridescens, N. orbiculatum* and *N. trilobum*) which are situated in areas of higher light and of a drier environment than the elliptical forms of stomata (*N. macranthum, N. papa* and *N. rivulare*) that characterize very moist situations and heavier shade.
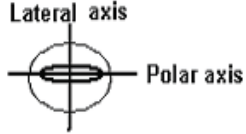
Specimen	Av.Polar axis in μm	Av.Lateral axis in μm	Form
Nematoceras iridescens	53.75 ± 1.16	57.50 ± 2.02	round
Nematoceras macranthum	69.67 ± 3.76	53.67 ± 3.48	ellipse
Nematoceras papa	73.25 ± 1.5	60.25 ± 2.78	ellipse
Nematoceras orbiculatum	57.67 ± 2.4	57.33 ± 1.67	round
Nematoceras rivulare	73.33 ± 3.18	63.00 ± 3.05	ellipse
Nematoceras trilobum	64.25 ± 1.32	63.50 ± 1.66	round
	Average	Average	
	65.32 ± 3.364	59.21 ± 1.54	

Lateral axis
Polar axis

Table A2.2 Stomata size, *Nematoceras* stomata giving a comparison of polar axis with lateral axis and form. The inserted Fig. defines the axis used.

Name	Form	Size μm²	Av. density /mm²
Nematoceras iridescens	round	247.25	38.38
Nematoceras macranthum	ellipse	299.14	23.66
Nematoceras papa	ellipse	353.07	23.56
Nematoceras orbiculatum	round	264.50	19.86
Nematoceras rivulare	ellipse	369.58	16.36
Nematoceras trilobum	round	326.39	41.68

Table A2.3 A comparison of stomata form, size and density from the previous tables.

The wet-mesophytic plants (*N. macranthum, N. papa, and N. rivulare*) with elliptical stomata also possess a lower stomata density with greater size than *Nematoceras* spp., *N. iridescens, N. orbiculatum, and N. trilobum*, in drier conditions (Table A2.3).
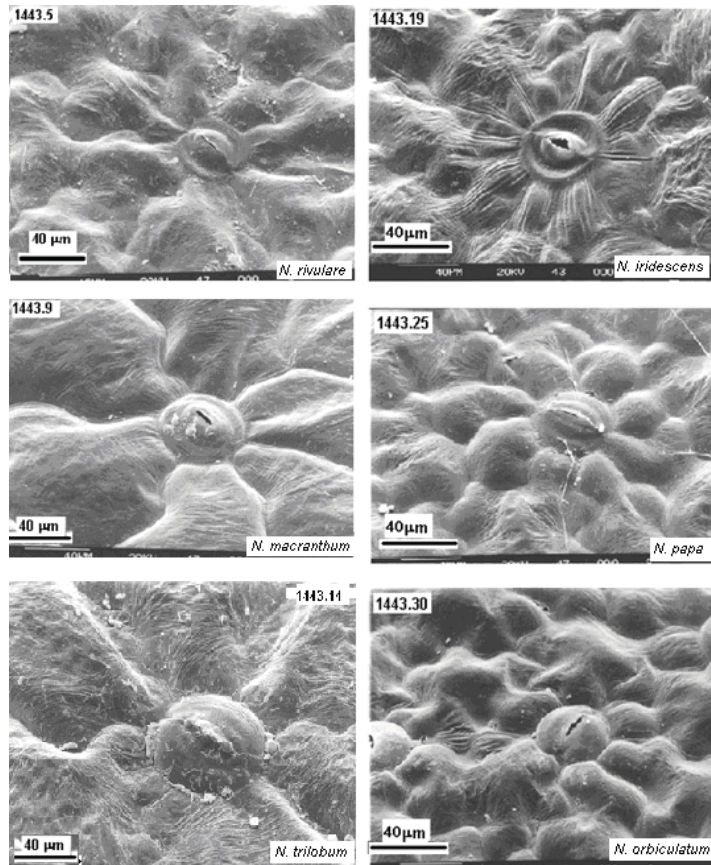
Figure A2.2 Abaxial stomata of six CA species; *N. rivulare*, *N. iridescens*, *N. macranthum*, *N. papa, N. trilobum* and *N. orbiculatum*.

The SEM micrographs displayed in Fig A2.2 and Fig.A2.3, show visible dissimilarity between the individual stomata. Patternation, of *N. iridescens* is distinctive when compared to the other images. Since the number of samples was limited (6 x 4) a significant variation was not completed. However, the difference between stomata would be worthy of further research.   Taxonomic characters, other than floral, would support speciation claims more fully, especially in identification of non-flowering *Nematoceras* specimens.

The topography of the CA, SEM abaxial epidermal micrographs, (Fig A2.2 and Fig. A2.3) is suggestive of either an:

1.   anomycytic condition (no subsidiary cells surround the guard cells,(Solereder, 1930) or,

2.   a tetracytic pattern, four roughly equal sized subsidiary cells surround the stomata guard cells or,

3.   a cyclocytic pattern, more than four subsidiary cells surrounding a central pair of guard cells, possibly derived from an earlier tetracytic condition, (Koch, 2009).
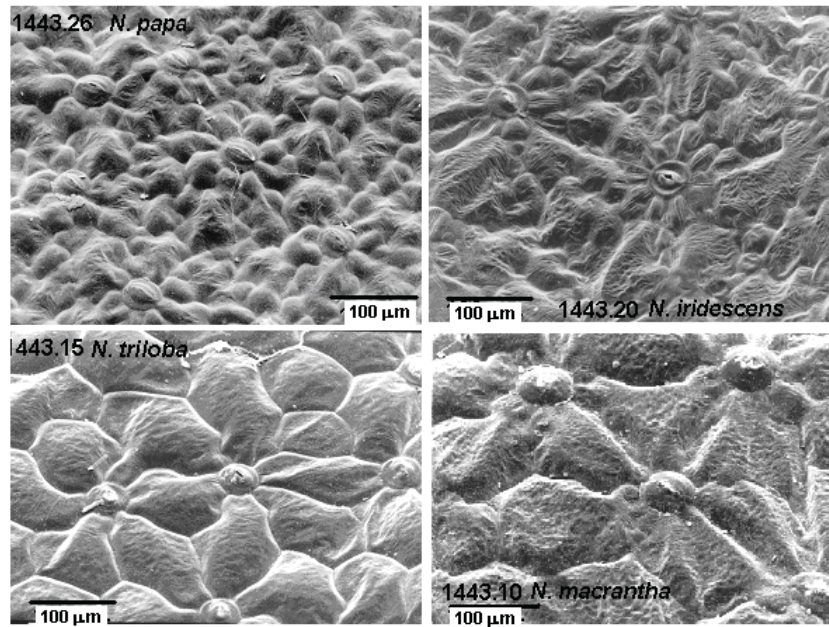
Figure A2.3   The Corybas alliance stomata of: *N. papa, N. iridescens, N. triloba and N. macranthum.*
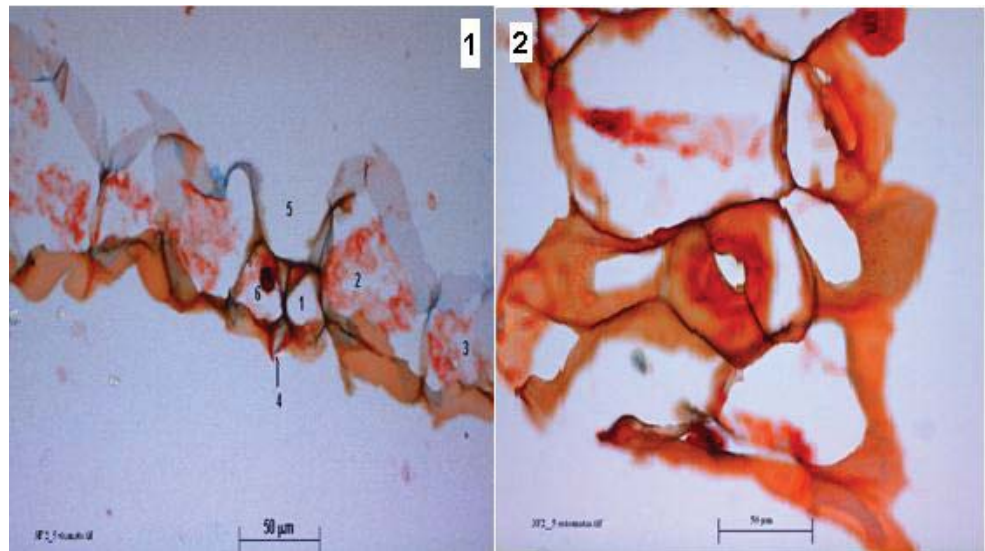
Figure A2. 4. 1= T/s of *N. iridescens* leaf stained with SafraninO and Alcian blue. At the center of this t/s micrograph a closed stoma can be seen. The base of the section image is the abaxial surface. Key 1 = guard cell, 2 = either a subsidiary or epidermal cell (see text), 3 = epidermal pavement cell, 4 = a closed stomatal ledge ("parrot beak" profile) 5 = substomatal chamber (note the thickened walls of the 2 guard cells' on the lumen side of the stomatal pore), 6 = unidentified object. Bar = 50 µm. **2=** A t/s of a *N. iridescens* leaf stained with SafraninO and Alcian blue. . Bar = 50 µm. Cells surrounding the central stomatal guard cells display large variations in sectional morphology. Guard cells are level with the epidermal surface (non-protruding).

All guard cells showed a thickened lateral wall, adjacent to the stomatal pore, which forms the exterior wall of the pore lumen. A stomatal ledge, Fig.A2.4 (1 #4), exists that, when the pore was closed, provided a external covering. Evidence of a prominent extended stomatal ledge is a xeromorphic trait, (Arditti, 1992).   The CLSM micrograph (Fig.A2.5) of stoma and the substomatal space reveals a triangular chamber profile with a depth of 16 ± 2µm and maximum sample breadth of 30 ± 3µm.
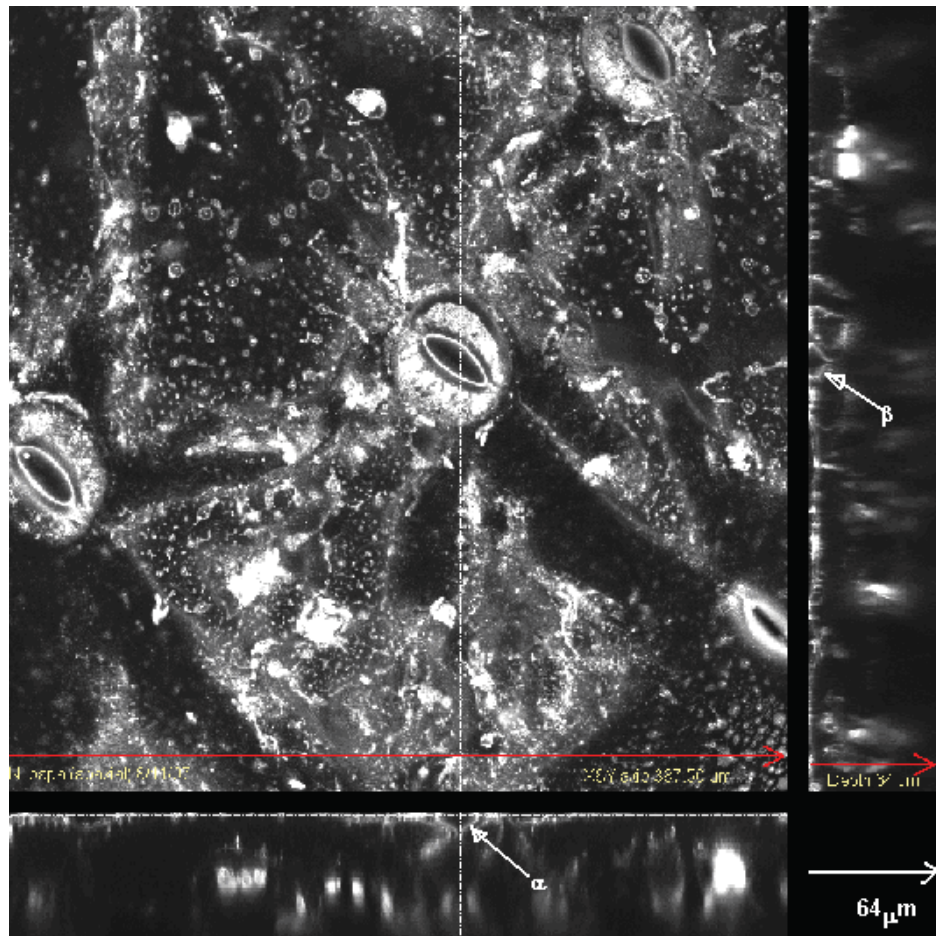
Figure A2.5   A multidimensional, CLSM micrograph, illustrating the topological features (x and y planes) together with a depth (z) field. The dotted line indicates the focus objective (stoma at the centre of the micrograph).   White arrows indicate the depth of the α, x z coordinates and the β, y z coordinates that image the sub-epidermal chamber walls of the sub- stomatal chamber. Sample = *Nematoceras papa* in grey / white imaging.

Orchid species that have adapted to more xeric habitats display a number of stomatal modifications, (Rasmussen, 1987), small sub-stomatal chamber size with limitation of apoplast passageways into the mesophyll, (Tominsky, 1905), and a thickening of guard cell walls, (Solereder, 1930; Tominsky, 1905).

The distance between stoma, Fig.A2.6, was considered not to be significant for use as a taxonomic character within the genus *Nematoceras*.   Variation of inter-stomatal distances within the same species ranged from 57µm < 347 µm, Fig A2.6. Interspecies difference was not sufficient to give a meaningful taxonomic identification.
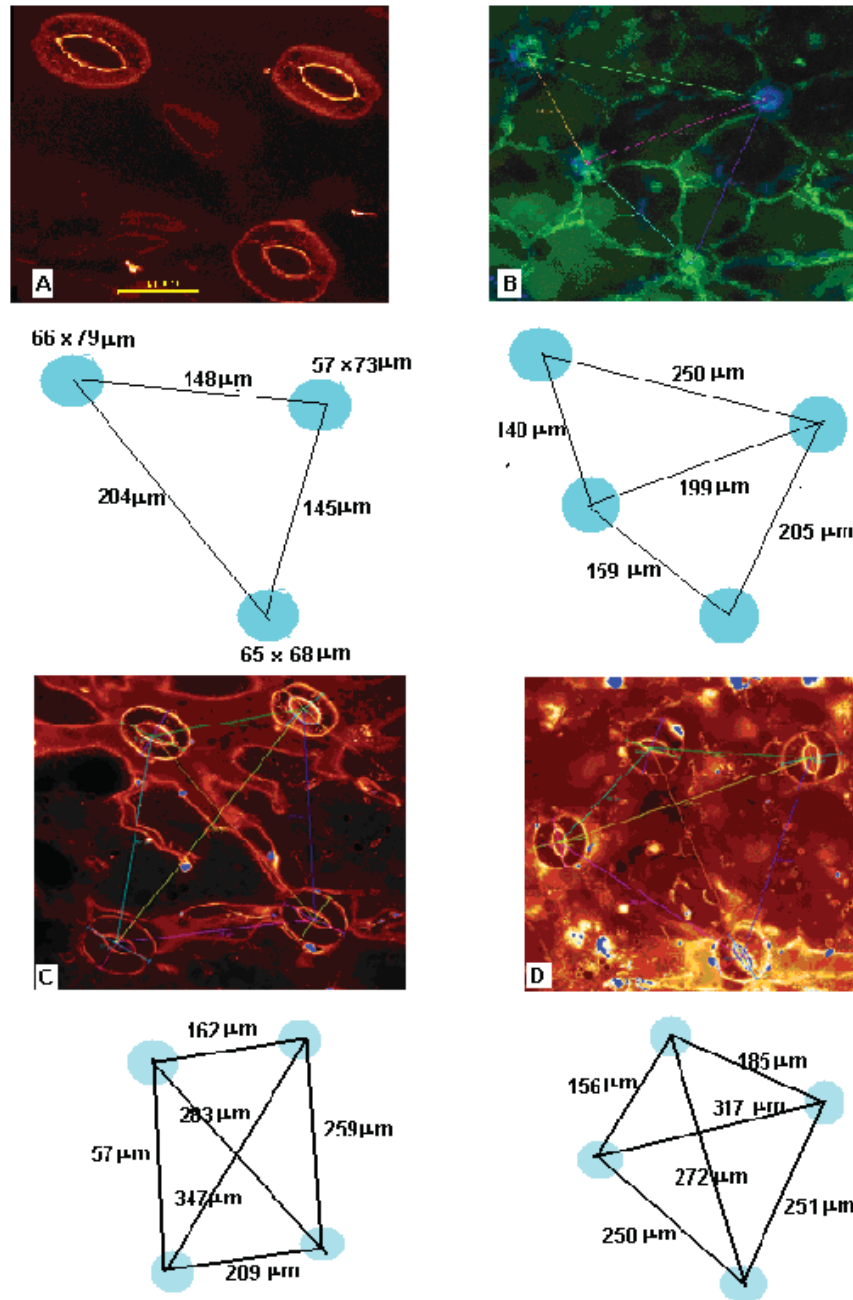
Figure A2.6 CLSM images of stomata: A = *N. papa* (elliptical form), B = *N. trilobum (*round form), C = *N. macranthum* (elliptical form) and D = *N. iridescens* (round form). Diagrams below each micrograph indicate µm distances between stomata.

The *N. trilobum* seen in Fig.A2.6 (B) displays cells adjacent to guard cells that are shared between neighbouring stomata guard cells. This form has been described as a post stomata genesis development, influenced by levels of CO_2, (Boetsch, 1996). Other researchers show that ethylene levels, at early stomatal development, create stomatal clustering with sharing of subsidiary cells, (Brett, 1979). Carbon dioxide concentration is generally high on tropical forest floors, (Holtum, 2001), which could partially account for the size and morphology of the stomatal apparatus.

A2.3   Adaxial surface

The heavy shade tolerant species, *Singularybas oblongus*, possesses a domed, papillose, adaxial cell surface, see Fig A2.7(6), very similar to the putative light focusing lens effect of epidermal cells found in extremely low light tolerant plants, (Vogelmann,1986,1993,1994,1996; Watkins, 2002).

Figure A2.7. A comparison of the adaxial epidermis of six CA species

1 = *N. iridescens*,

2 = *N.macranthum*,

3 = *N. papa*,

4 = *N. rivulare,*

5 = *N. trilobum*,

6 = *Singularybas oblongus*;

Bar =

1-5 = 200 µm,

6 = 100 µm.
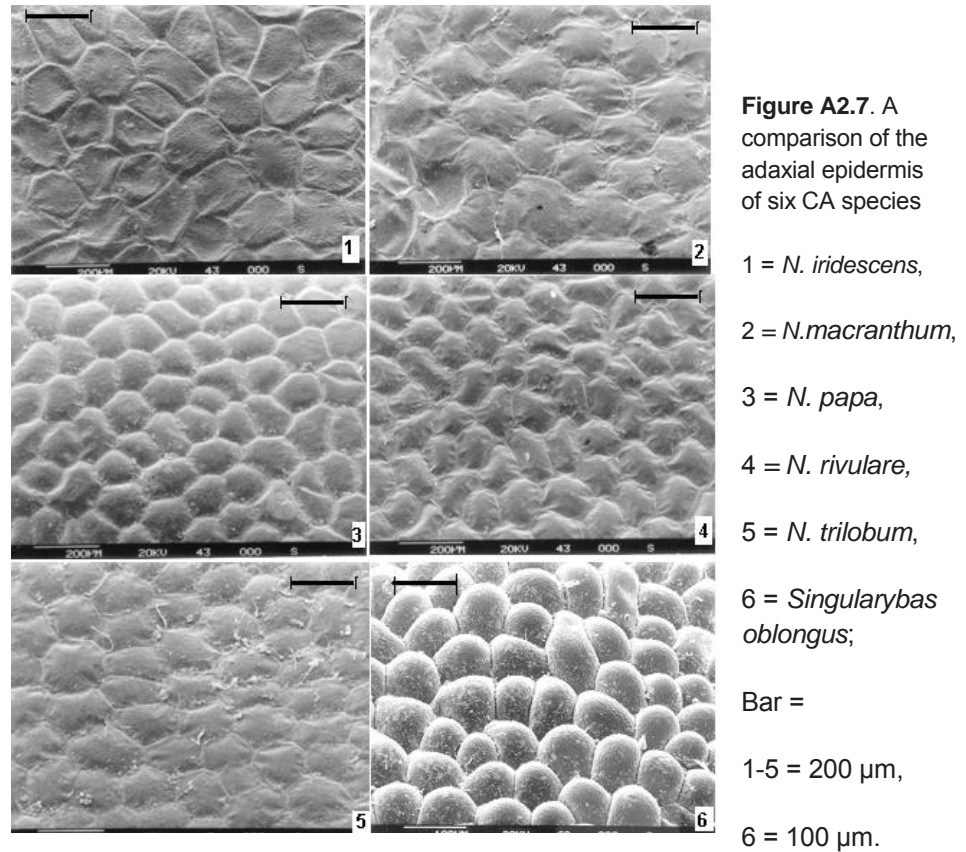
Of the adaxial leaf surfaces of the CA spp. Investigated, Fig A2.7 (1-6), all except the *Singularybas oblongus*, Fig A2.7 (6), exhibited a tabular isodiametric epidermis, (Esau, 1953), Fig A2.7 (1-5), with a patterning of similar form to the respective abaxial surface (Fig A2. 2 and A2.3.).

The *Singularybas oblongus* adaxial leaf surface is sufficiently different to be a definitive character of *Singularybas* taxonomy and is dissimilar to its abaxial aspect, unlike the *Nematoceras* spp. large adaxial surface epidermal cells (i.e. 90µm x 110µm, Fig.A2.8.
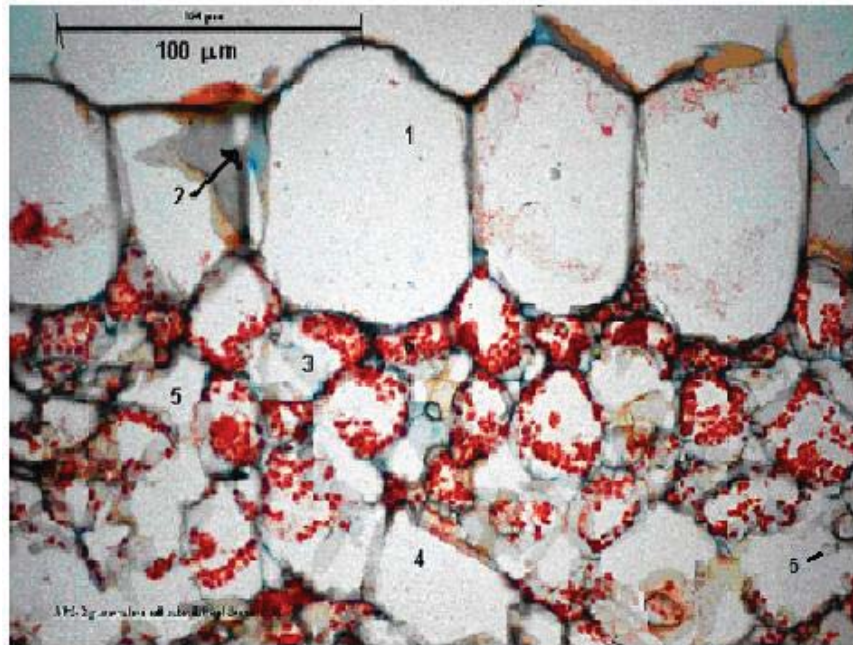
Figure A2. 8 An adaxial profile of a leaf section of *N. iridescens*, Bar = 100 μm. Note the large adaxial epidermal cells (1), (2 arrow) the non-viable stomata possibly a schizogenous development, (Esau, 1953), into a wax or oil duct. (3 and 5) are areas of palisade mesophyll with high chlorophyll content and (4) spongy mesophyll. Note the amyloplast (6)

A2.4 Laminar margin

The laminar margin of both the *N. iridescens* and *S. oblongus* leaf, imaged in SEM, shows rows of distinctive elongated cells see Fig A2.9 (1-2 and 4). A literature search found no reports regarding this feature which I have termed "marginal braiding", since it simulates plaited cell rows. Each cell, comprising the "braiding" is elongated (≈ 100 μm x 50 μm) and regularly shaped compared to the randomly shaped tabular isodiametric epidermis of the abaxial and adaxial surfaces Fig.A2.9 (4).

Each cell surface is covered with a waxy cuticle of longitudinal patterning (Fig.A2.9 (2). The braiding rows consist of four strings of adapted epithelial cells, each forming a serial strand around the leaf margin. Each strand lies in a straight row or, together, creating the appearance of a plaited row (Fig.A2.9 (1). The braided margin would function as reinforcement against leaf tear and help contribute to retention of form. The braided margin could also control the leaf blade orientation and form, with either contraction or relaxation, thus emulating a "purse string". A cell contraction, in dry conditions would tend to cup the leaf reducing the air envelope flow, assist in the retention of a higher degree of abaxial humidity and diminish surface evaporation, (Tsukaya, 2006). Relaxation of the edge cells, in humid conditions, could create a leaf expansion effect maximising light collection, water runoff and leaf photosynthesis, (Larcher, 2003).

The braiding rows consist of four strings of adapted epithelial cells, each forming a serial strand around the leaf perimeter Leaf edge change is thought to have been due to a change in the paleoclimate condition from warm humid forest environments to cooler environmental temperatures plus fluctuations in light and CO_2 levels, (Aizen, 2008).
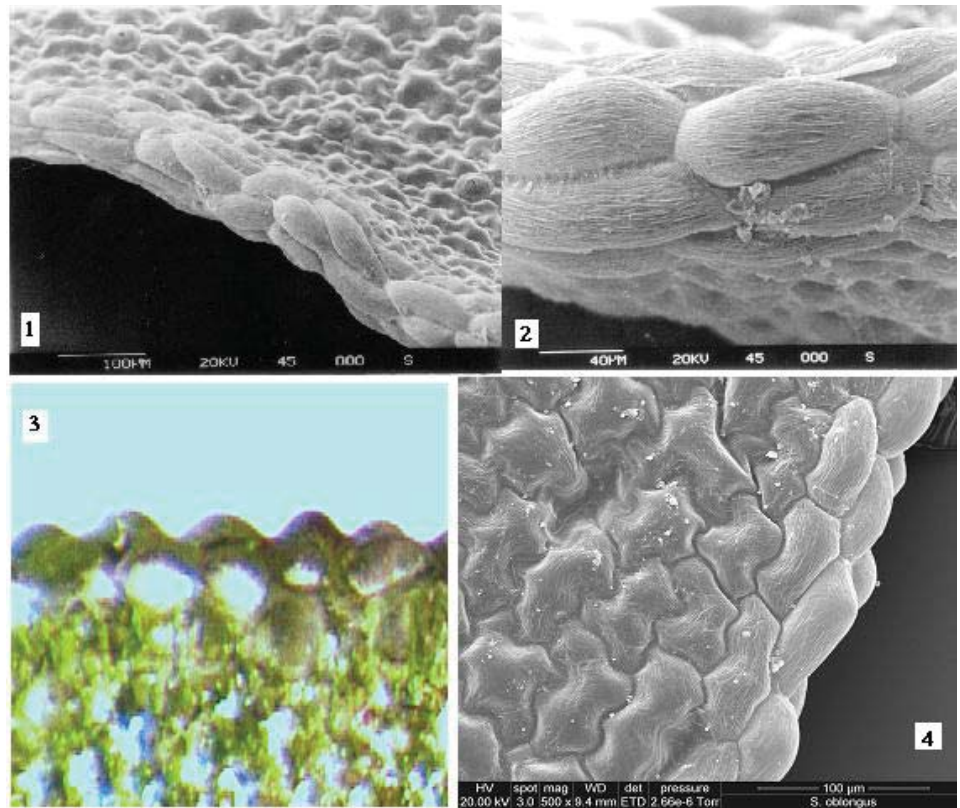
Figure A2.9. Laminar leaf margins of *N.iridescens*: 1 at 100 μm bar, 2 = 40 μm bar, 4 = *Singularybas oblongus*, bar 100 μm. All show elongated edge cells that have been termed laminar "braiding" by the author, as this appears to be the first reference to this characteristic in the Orchidaceae. The #3 is an l/s micrograph of the leaf laminar braiding of *N.iridescens*

The margin cell polarity, as illustrated in Fig A2.9 (1 and 2), would theoretically allow plasticity of the leaf conformity in response to environmental conditions a great advantage to a solitary leaved plant.

A2.5   Epicuticle

From the SEM micrograph of *Corybas cheesemanii* (Fig A2.10 (2)), the epicuticle has a thickness of ≈ 1μm - 2μm and is composed of sinuous structures.   Many stellate patterns, of presumably wax rod concentrations, are on each epidermal cell surface, (Buschhaus, 2007; Jeffree, 2006; Mariani, 2000; Post-Beittenmiller, 1996; Shiraishi, 1990; Stace, 1965). Each of these wax nodes has longer connections of wax rods that join up with the neighbouring wax nodes (Fig.A2.10 (1)). Laterally the "longer "wax connections extend down the cell sides, reminiscent of candle wax.
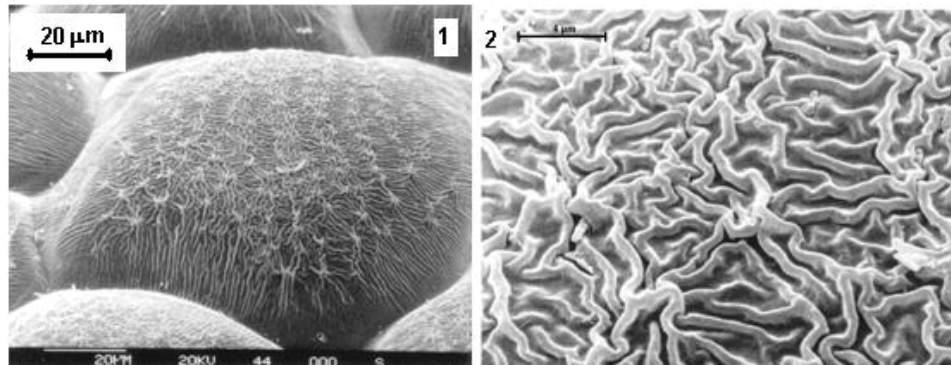
Figure A2.10 A waxy cuticle patterning covering the adaxial leaf side of *Corybas cheesemanii*, the most light exposed of the CA (Koch, 2009), (1) Bar = 20mm (2) Bar = 4mm.

In the *Anzybas*, (Fig.A2.11), the wax epicuticle has a similar but moderated pattern, compared to *Corybas cheesemanii*. Other members of the CA have waxy epicuticles but these are not as pronounced as that of *Corybas cheesemanii*. The leaf epicuticle on *N. iridescens* was very thin and not measured.
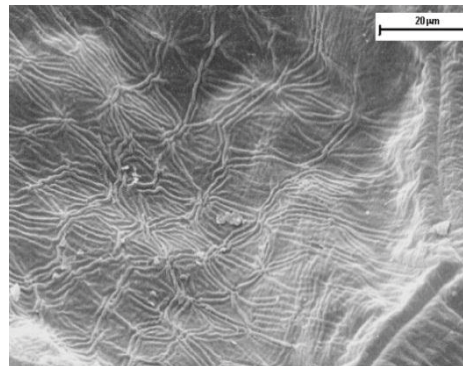
Figure A2.11. The adaxial wax patternation of the CA, *Anzybas rotundifolia,* epicuticle.

Wax depth and apparent quantity tends to diminish as the degree of environmental exposure reduces (Jeffree, 2006; Koch, 2008; Post-Beittenmiller, 1996). The high wax deposit that exists on the adaxial surface of *Corybas cheesemanii* corresponds to a drier more exposed location.

. These differences could provide sufficient generic difference to be of taxonomic use. *Anzybas rotundifolia* presents a shallow wax epicuticle deposit and inhabits moist, moderate light areas. *Nematoceras* species have very little apparent wax and generally grow in heavy shade while *Singularybas*, another heavy shade dweller, has a very different epidermis (Fig.A2.7 #6) in which the domed papillose cells are covered with a reticulate pattern of epicuticle wax

In dry conditions, the large epidermal adaxial cells dehydrate and shrink, thus the hydrophobic cuticle is compacted bringing the wax rods closer together. This increases the cuticle resistance to water vapour conserving water, (Larcher, 2003). In cooler or wet weather, the epidermal cells expand and increase the cell surface area thus reducing the cuticle wax density and decreasing the cuticle resistance to water vapour. The patternation of the cuticle wax would appear to be ideally suited

as a compression / relaxation dynamic. Errors in interpretation need to be considered since potential lipid / wax solvents are used in SEM dehydration steps. The micrographs of the epicuticular wax (Fig.A2.10 -11) from the CRI SEM in 2004 were obtained prior to the installation of the ESEM that does not require the use of wax solvents in specimen preparation.

A2.6   Crystal inclusions

Raphides occurred in the majority of the Nematoceras micrographs taken with the SEM, ESEM, and CLSM microscopes throughout this research project. They appeared as bundles of narrow, elongated needle-shaped crystals (acicular), usually in a similar orientation, with both ends very sharply pointed. Numbers of crystals varied widely in each bundle. They are initially contained within crystal idioblasts occurring in the sub-epidermal cortex of roots (Fig.A2.13) and in leaf spongy mesophyll tissue (Fig.A2.12). Possibly they exist as Rosanoffian crystals, (bundled crystals within a membrane or specialist cell). (Figure A2.12 and Figure. A2.20 #1), (Rosanoff, 1865; Frey-Wyssling, 1981).
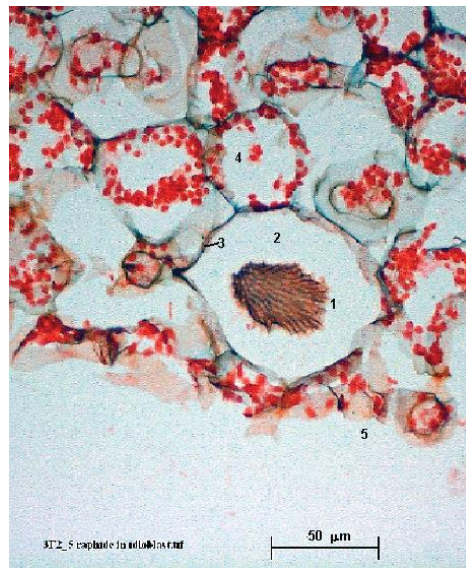
Figure A2.12.   A young leaf t/s of *N. iridescens* with an idioblast containing a developing raphide bundle of calcium oxalate crystals (1), a crystal idioblast cell (2), the thickened wall of the idioblast (3), the spongy mesophyll layer (4) and abaxial epidermis (5). At this point of genesis, the raphides measure 50 µm in length, fully developed they change orientation to lie parallel to the long axis of the idioblast and measure ≈ 100 µm.

Raphides occurred in the majority of the *Nematoceras* micrographs taken with the SEM, ESEM, and CLSM microscopes throughout this research project. They appeared as bundles of narrow, elongated needle-shaped crystals (acicular), usually in a similar orientation, with both ends very sharply pointed. Numbers of crystals varied widely in each bundle. They are initially contained within crystal idioblasts occurring in the sub-epidermal cortex of roots (Fig.A2.13) and in leaf spongy mesophyll tissue (Fig.A2.12). Possibly they exist as Rosanoffian crystals, (bundled crystals within a membrane or specialist cell). (Figure A2.12 and Figure. A2.20 #1), (Rosanoff, 1865; Frey-Wyssling, 1981).

Figure A2.13. A *N. iridescens* root with; 1 a sub epidermal cortex idioblast with raphide crystals scattered within, 2 a developing bundle of raphide crystals (blunt and chisel ended), 3 endophyte hyphae. Bar = 100 µm.

Figure A2.14. Scattering of raphide crystals on the abaxial surface of an *N. iridescens* leaf, after it had been lightly abraded and stained with solophenyl flavine. Bar = 100 µm.

No raphides were evident in the stem. Mechanical traumas sufficient to rupture cells, such as sample preparation, caused raphide needles to deploy over an extensive area, see Fig.A2.14.

Micrographs, taken with the ESEM, exposed crystal inclusion bodies within the root cortex of *N. iridescens* (Fig.A2.15) illustrate a diversity of idioblast crystals. A number of recognised forms of raphides, Fig.A2.15 (1) and a very occasional druse crystal Fig.A2.15 (3) were observed. One unrecognised form, Fig.A2.15 (4) consisting of a "trellis" like cylinder comprising diagonal straps measuring approximately 1μm wide x 8μm in length, was occasionally observed. The" fan" like formation as shown in Fig.A2.15 (2) was apparent in all leaf LM micrograph sections. The base was clustered with rough textured, flat, blunt ended projections radiating out at an approximate 70° arc.
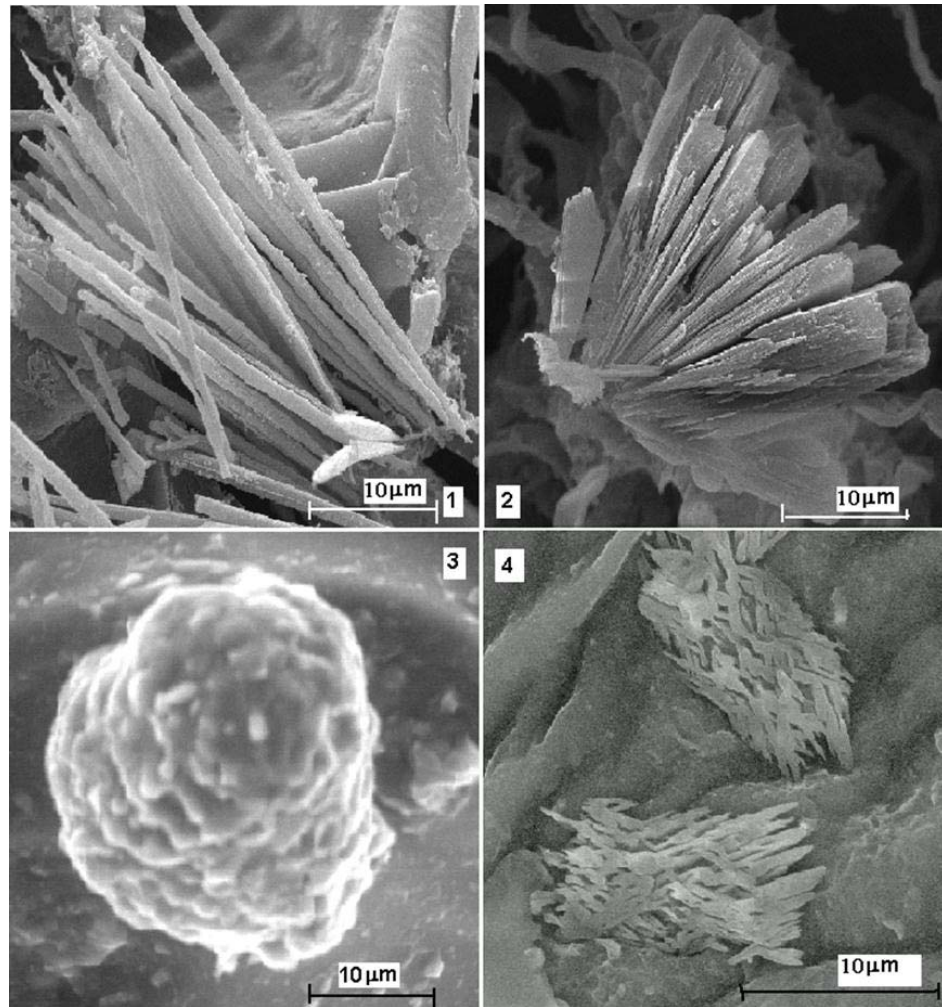
Figure A2.15 ESEM's of crystal inclusion forms found in *N. iridescens* young root sections*;* 1 = scattered raphides 2 = early forming raphide bundle, 3 = Druse and "crystalline sand" (right background) and 4 an unknown form within an idioblast. All Bars = 10μm
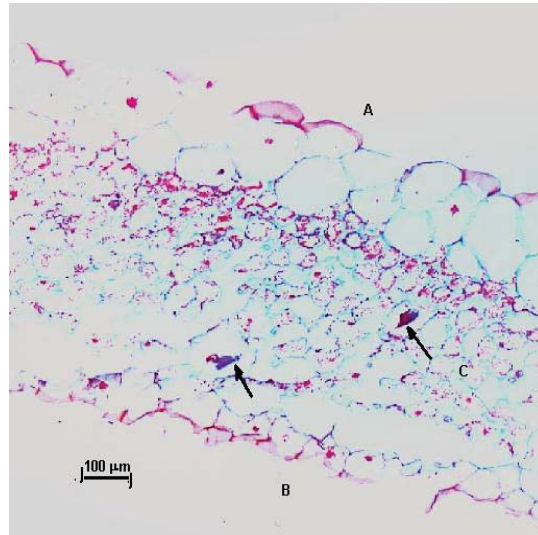
Figure A2.16. Two idioblasts, each containing raphide bundles. Idioblasts with similar contents are interspersed throughout the entire leaf, in a regular pattern, within the spongy mesophyll layer A = Adaxial surface   B = Abaxial surface.

Bar = 100 μm.
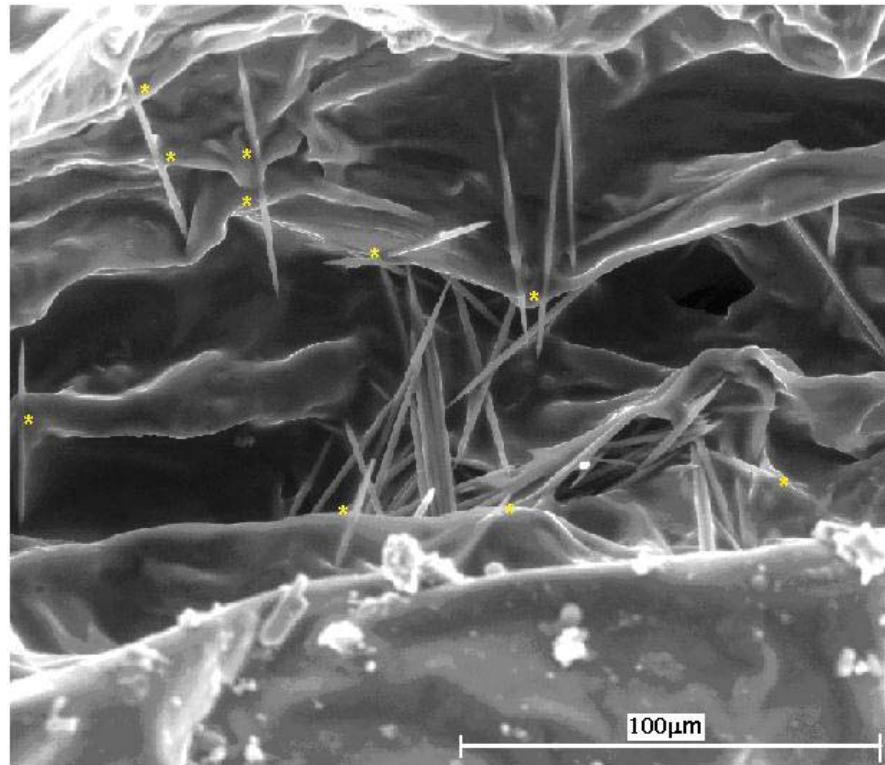
Figure A2.17   ESEM of a *N. iridescens* root with raphides penetrating (asterisks) root cell walls.

During development, each raphide initially becomes covered in lamellae and surrounded by mucilage, (Kausch, 1983). To verify the identity of the crystal inclusions, selected crystals were analysed with the E-SEM EDAX facility. For verification the resultant graph (Fig A2.18) was compared with that of known raphide crystal EDAX results, (Franceschi, 2005; Jáuregui-Zúñigaa, 2003; Perera, 2006). Similar peaks and coordinates of calcium, support calcium oxalate as the observed crystal species.
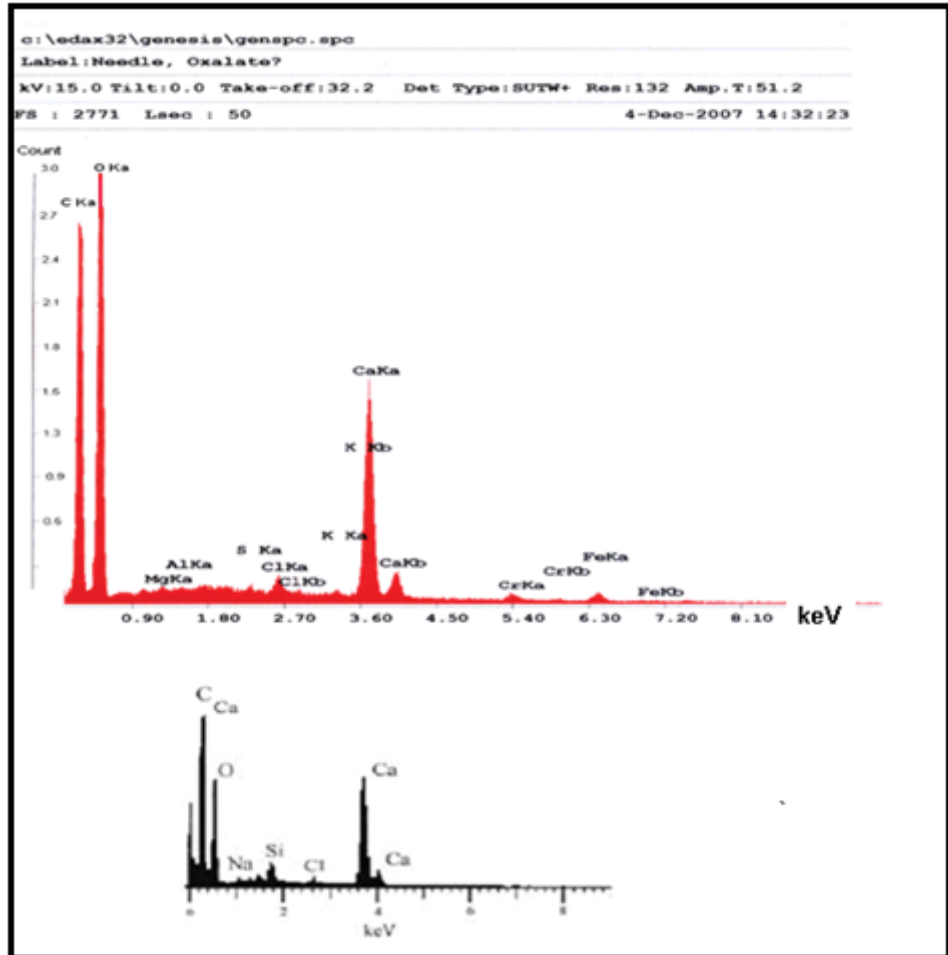
Figure A2.18. EDAX analysis of a raphide crystal. The uppermost graph is an analysis of a needle crystal within the root of *N. iridescens* producing a spike at 3.6-3.8 keV. The lower graph represents a chemically assayed raphide needle crystal,(Jáuregui-Zúñigaa, 2003).

A brief study, over a chronological scale of two hours, to investigate if production of crystals, after trauma, would increase needle numbers at the trauma site, showed that numbers on the initial E-SEM image did not increase over this period.

The raphide morphology was within the parameters described by, (Jáuregui-Zúñigaa, 2003; Keil, 2009; Prychid, 1999; Webb, 1999). Their research, showed the morphology of various crystal raphide structures that occurred in a range of plants.
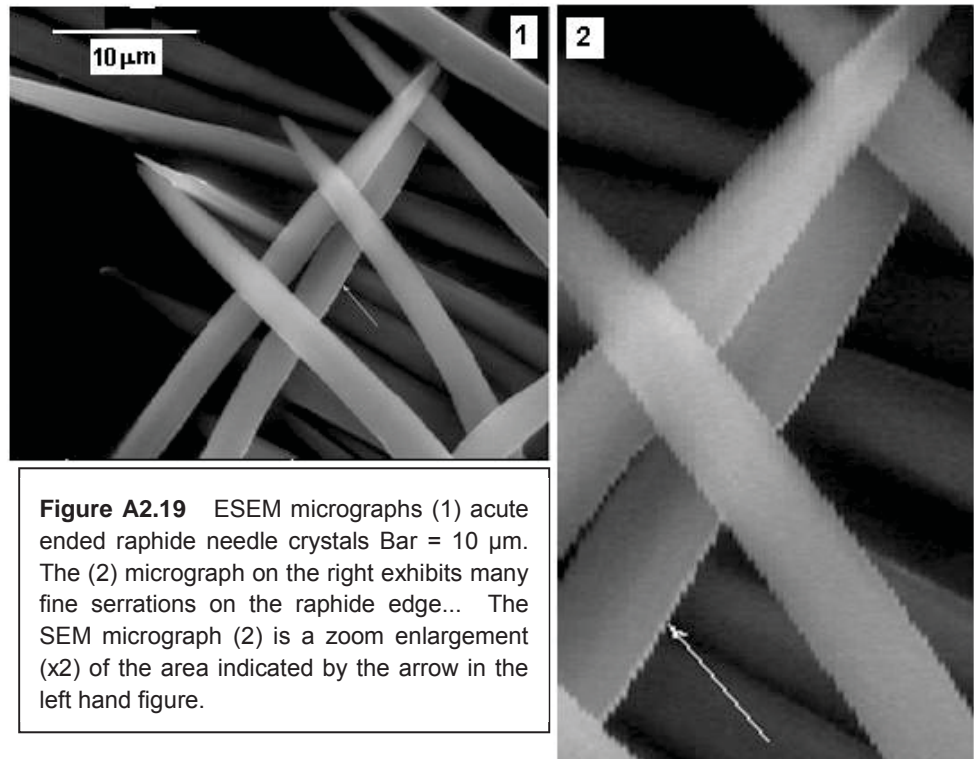
Figure A2.19 ESEM micrographs (1) acute ended raphide needle crystals Bar = 10 μm. The (2) micrograph on the right exhibits many fine serrations on the raphide edge... The SEM micrograph (2) is a zoom enlargement (x2) of the area indicated by the arrow in the left hand figure.

E-SEM's (Fig.A2.19 (1).) taken of mature *N. iridescens* raphides were consistent in form; acicular crystals of between 80μm – 100μm in length x 3μm in width, both ends possessing very acute points some of which were barbed, elliptical cross section (x/s) form, shallow striations parallel to the long axis, some having an equatorial notch. A new feature, not previously reported elsewhere, is the discovery of fine serrations along the edges of raphide needles, (Fig.A2.19 #2). The serrations are set at approximately 45° to the antero-posterior axis of the raphide crystal, with each side having an opposite set *i.e.* one side angled to face the anterior and the opposite side angled to face the posterior of the crystal. Physically, this would provide both a ratchet effect, a series of barbs and a greater surface area for the crystal.

In early raphide formation, the individual crystals occur in the tonoplast of an idioblast vacuole forming bundles of blunt ended crystals (Fig.6A2.20 (1)) surrounded by lamellae during mucilage accumulation, (Kausch, 1983).

The mucilage appears to bind the crystals, tightly initially and decreasingly with maturity, in a fascis like (bundle) Fig.A2.20 (2).
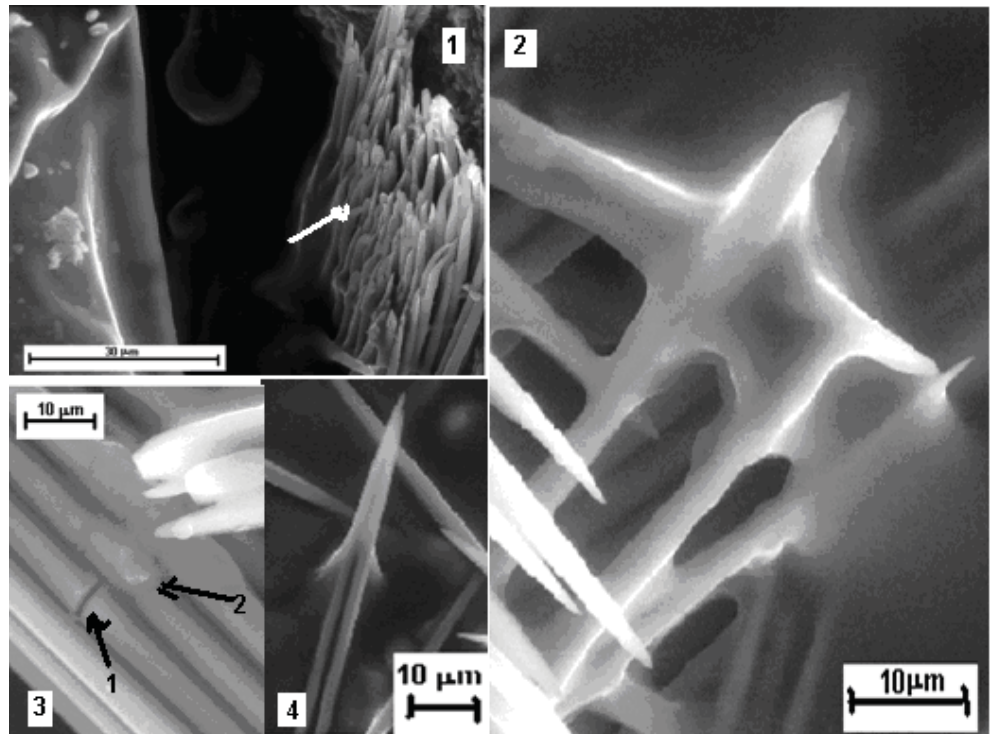
Figure A2. 20. ESEM micrographs of early raphide development; 1 = fascis of blunt raphide crystals (arrow), 2 = raphides bound together with mucilage, 3 = middle cleft (arrow 1) and "daubing" of neighbouring clefts (arrow 2), 4 = arrow barbing of raphide needle.

In the middle of many raphide crystals, a cleft was apparent, Fig.A2.20 (3), with both butting ends of the crystal having a slight swelling. Twin crystals have been reported, (Arnott, 2000; Ishii, 1992; Singh,1987), some of the raphide crystals display a rough "daubing" of material Fig.6.20 (3 #2) as though the cleft had been repaired. Arnott (2000) describes raphide crystals of *Vitus vinefera* with pointed and bidentate ends that are distributed equally at both ends of a bundle, which indicates that raphides form in either one of two orientations within the cell. No free bidentate ends were observed in any of the micrographs or with light microscopy in this project. Arrow like barbs were apparent at the ends of some raphide crystals (Fig.A2.20 (4)).

Many reports of a toxic reaction, occurring with workers in trades that are associated with raphide bearing plants have been recorded. With the ornamental tropical foliage plant, *Dieffenbachia picta,* severe skin rashes occurred, which was found to be caused by a proteinase (dumbcane) intra-cutaneously introduced by the calcium oxalate raphides present in the *Dieffenbachia* tissue, (Walter, 1972). Vanilla orchids and Kiwifruit have also been found to possess the same raphide irritant factor, (Perera, 1990).

A2.7 Leaf predation

Established colonies of *N. iridescens* show little predation however, newly emerging plants can be devastated by slugs and snails (*Pers. Obs.*). A tray of *N.*

triloba, well established the previous season, had the total new growth of 18 emergent leaves predated over a nine-hour period.
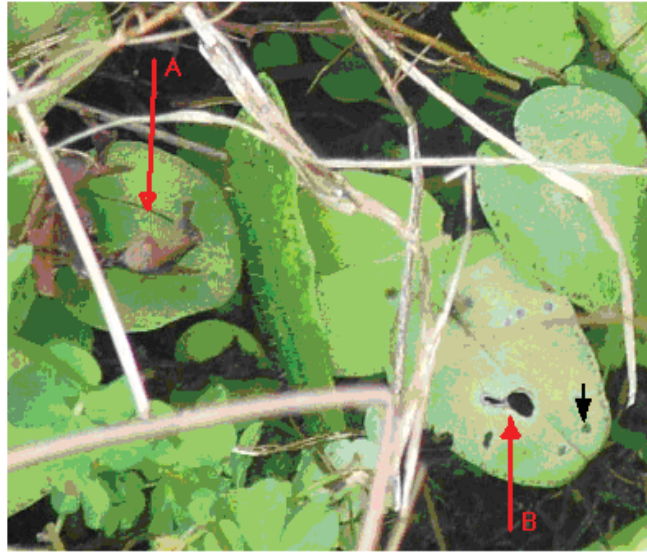
Figure A2.21 A snail (A) traversing a leaf of *N.iridescens* at the Tarata site showing some evidence of leaf damage that can be seen (B) however, the damage was old and on a leaf that was approaching senescence. Small arrow indicates tentative predation.

Neighbouring pots of *N. iridescens*, *N. rivulare* and *N. macranthus* treated with Baysol® (Methiocarb (mercaptodimetur)) 20g/kg, Calcium sulphate 75 g/kg, butylated hydroxy toluene 2 g/kg and fillers) a molluscide, were untouched and bodies of snails were regularly found on the potting mix surface. None of the other leaves showed evidence of predation.

Once the plants of the *Nematoceras* species had achieved maturity (fully expanded leaf and emergent florescence), snail / slug, predation diminished although, in the field, snail mucous tracts and partial injury (tentative herbivory) were apparent on leaf surfaces (Fig.A2.21). A heavily predated leaf of *N. iridescens* found at the Tarata site revealed bundles of frass with a caterpillar and webbing on the abaxial surface(Fig.A2.22).

Figure A2.22   A heavily predated leaf of *N. iridescens* found at the Tarata site on the 12/12/2007. The abaxial aspect contains the larvae, its frass and webbing (fine arrows)

The larva, Fig A2.23 (1), was unidentifiable by the author, so was retained for pupation and identification of the emergent moth.   The emergent moth, Fig.A2.23 (3), was identified from the Landcare, Lepidoptera slide collection, (Dugdale, 1988) and verified, by holotype comparison, Fig.A2.23 (2), as a New Zealand endemic species, *Scoparia ustimacula,* refer to Appendix 1f 1875:pl.135:17.   Its natural habitat is moist forest and moss areas, a very similar habitat to the *Nematoceras* and other CA genera.   The life cycle of the moth, *S. ustimacula, parallels* that of *N. iridescens.* Larvae produced during optimal leaf development pupate within a frass web at the onset of leaf senescence. Adults emerge as the newly produced leaves of *N. iridescens* commence maturation.

Leaves of *N. iridescens*, the most common *Nematoceras* species in the Taranaki sites, were predated at the leaf centers with leaf edge predation not as commonly observed. Predation occurs in areas of minimal raphide presence, Fig.A2.24. The leaf edges all possess high concentrations of raphides.
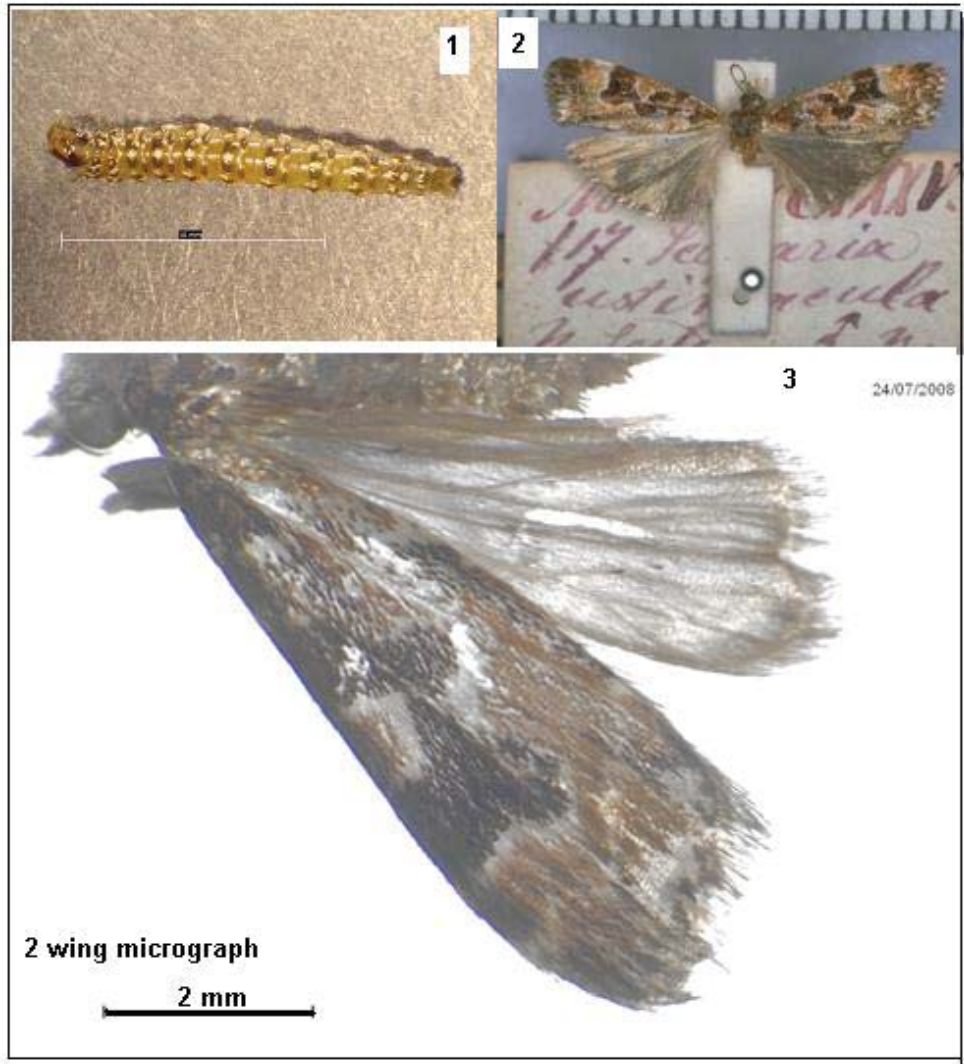
Figure A2.23. The herbivorous leaf predator found on an *N.iridescens* leaf: 1 = larvae, 2 = holotype of *Scoparia ustimacula* (Felder and Rogenhofer, 1875) 3 = mature adult hatched from the pupae of the larvae (1), identified as *Scoparia ustimacula* (Boot wing moth) after the "boot" pattern on the wing. (Photo #2 ex. Landcare catalogue)

Figure A2.24. Leaf center damage to the solitary leaf of *N. iridescens* with the peripheral margin untouched.

The response to herbivory of both endo and ectomycorrhizal fungi has been researched which has resulted in a number of papers (33) which have recently

been reviewed by, Barto (2010). Their review suggested mycorrhizal colonization was not significantly reduced after herbivory and they challenged the carbon limitation hypothesis that had implied that a carbon reduction would proportionally limit or diminish the degree of mycorrhizal colonization

A2.8 Diatoms
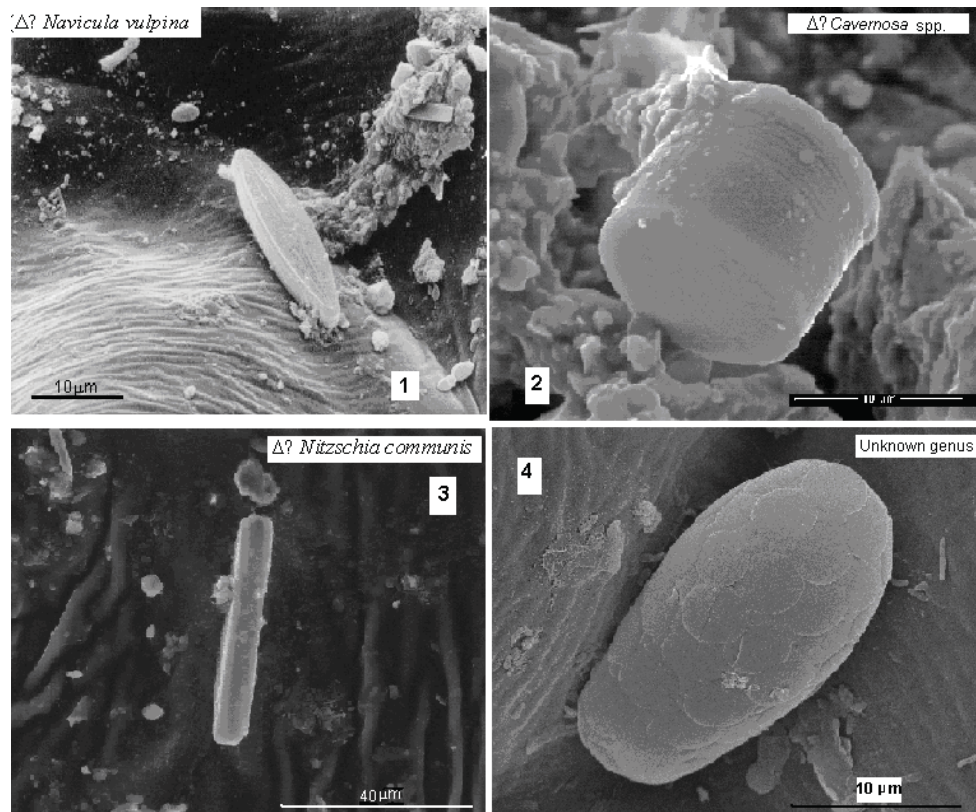
Figure A2.25 Four diatoms found on adaxial leaf surfaces of *N. iridescens*. Plants were located in a very moist area of the Tarata tunnel (South). Micrographs 1-3 are definite diatoms (provisionally 1= *Navicula vulpina*, 2= *Cavernosa* possibly *kapitiana*, 3= *Nitzschia communis*). Micrograph 4 represents a currently unknown organism covered in disks. Identification of these specimens was made with referral to, Foged (1979), Cassie (1984) and Stidolph (2002).

While investigating SEM adaxial micrographs of N. iridescens leaves, evidence of diatoms became apparent. The leaves were from a particularly wet position on the south side of the Tarata tunnel site and three of the diatom species were identified. One organism (Fig.A2.25 (4)) was unable to be identified and two SEM micrographs and an informal request for genus identification were made to the Cawthron Institute, Nelson, who were also unable to identify it. Diatoms are used in forensic pathology, (Auer, 1991) for site location analysis. Possibly this technique, with adaptations, could be of value to Bio-security agencies in the identification of plant origin since many plants are CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) protected.

A2.9 Discussion

The range of leaf morphology within a Corybas alliance species can be varied, even with plants in the same colony, and any identification of a species, using a vegetative key, (Jane, 2001), must be done with caution. As an example,

Nematoceras acuminata (Syn. *Corybas acuminatus*), was found at Waikaremoana exhibiting leaf size and shape variation within the same colony at the same date, some as an oval broad smooth leaf and others display elongated sagittate, veined and undulate leaves, (Scanlen, 1996).

Leaves also adopt a number of strategies, as an adaptation to various environmental situations, especially temperature, (Ledford, 2008). Leaf twisting (early emergence) and cupping of the leaf (pre and during anthesis) to conserve heat is one such strategy of the Macquarie Island, *Nematoceras dienema*, (Jones, 2002), one of two orchids in the Sub-Antarctic region, (Clements, 2007). The average annual temperature on Macquarie Island is below $5^{o}C$ with a mean daily temperature range of 3.5 °C, which is relatively constant through the year. Proximity of the sea creates a relative humidity of 87% (Aust. Bureau of Meteorology, 2004)

Possibly the unrolling leaf form is just an expansion from a sub-soil / moss penetrating tip. Considering the congested nature of the bryophytes and Hepatophytes together with surface rooting ferns and general litter the leaf must penetrate to gain light access it would seem that the tight convolute form is an essential adaptation.

The morphology of the stomatal apparatus, as seen in SEM micrographs, Figs.A.2 .3-6, displays distinct visual differences in surface pattern, shape and abaxial distribution. This would have potential merit in pre-flowering *Nematoceras* spp. identification. Obviously, a greater range of sampling, specimen numbers and allowance for variation would be required to ratify this conclusion. Prominent pore characteristics could also be used Visual differences were obvious in the SEM micrographs and would be sufficient to differentiate *Nematoceras* spp. Adaxial epidermal surfaces Fig.A2 .7 (#1-5) in a small range of *Nematoceras* spp. and the sole *Singularybas* spp. Fig.A2.7 (#6) illustrate sufficient image difference to apply as morphological characters in taxonomic identification.

The "marginal braiding" found in micrographs of *N.iridescens* and *Singularybas oblongus* has not been previously reported, the samples taken from only two CA species and in a limited geographic area inhibits conjecture as to whether this character is represented in all New Zealand CA species. However, the physiological importance of marginal braiding and its impact on leaf function would be of future interest.

Wax deposits on the epicuticle of the orchids imaged by SEM, Figures A2.10-11 show differences in depth and pattern. Based on observations of, (Jeffree, 2006; Koch, 2004; Post-Beittenmiller, 1996) Epicuticular wax is deposited in response to the ambient temperature at the period of the highest environmental risk. The small number of SEM micrographs taken, indicate that exposed CA plants of *Corybas cheesemanii* possess the heaviest wax depth of 1mm compared to the low light exposure plant *N. iridescens* in which the cuticular wax is minimal.

These visually apparent differences would require wide geographical verification and statistical analysis to confirm the epidermal / cuticle patternation in answer to the question "do patterns differ between sites and species"? (Pridgeon, 1994; Stern, 1997), and is there significant difference between CA species to provide taxonomic characters?

Crystals, of calcium oxalate monohydrate have been used in the systematic identification of a number of plant genera and species; Arecaceae, (Barfod, 2001), *Phaseolus*, (Grimson, 1982), *Actinidia*, (Perera, 2006)   in drier conditions *Prosthechea, Encyclia* and Orchidaceae, (Pires, 2003), Monocotyledons (Prychid, 1999) and *Dieffenbachia,* (Walter, 1972).   The ratios of crystal raphides, druse and crystalline sand were used to distinguish between *Araceae* spp., (Prychid, 2008).   A comparison of calcium oxalate crystals in CA species, when more species are collected, would be an interesting exercise.   The presence of raphides in *Nematoceras iridescens* and their potential for antiherbivoury, initiated the investigation of their properties.   Dispersion of barbed, needle like, toxic, (Dogigey, 1991; Watson, 2005), crystals in the leaf and the pattern of herbivoury on the leaf, Figs A2.22 and Fig.A2.24, suggest that in areas of raphide accumulation herbivoury is absent.   Apart from young plant predation by snails and slugs, very little damage occurs on mature leaves.

An interesting discovery made during the raphide investigation was the serrated edge effect, seemingly reported for the first time. Planes of calcium oxalate could cause the serrated edges, (Frey-Wyssling, 1981).

All known diatoms are polyunsaturated aldehyde producers (PUA), (Barreiro, 2011). Following cell trauma, diatoms liberate volatile biologically active toxins such as 2, 4-decadienal, which has various degrees of affect, depending on concentration, on fertilization, embryogenesis, teratogenisis and biological fitness on the larvae of leaf herbivores, (Caldwella, 2005). In situations of high water run-off, shade and humidity, such as where many *Nematoceras* species inhabit the association of meso-hydrophytes with diatoms would assist in single leaf protection.

Section B   The peloton endophyte

Chapter B1   **The fungal mycorrhiza of *N. iridescens***

The known orchid mycorrhizal fungi exist in a relatively narrow phylogenic clade of which individual species are often specific to individual orchid genera and species (Fig.B1.1). This chapter of the thesis will concentrate on the identification of the fungal endophyte resident in the pelotons of *N. iridescens*.
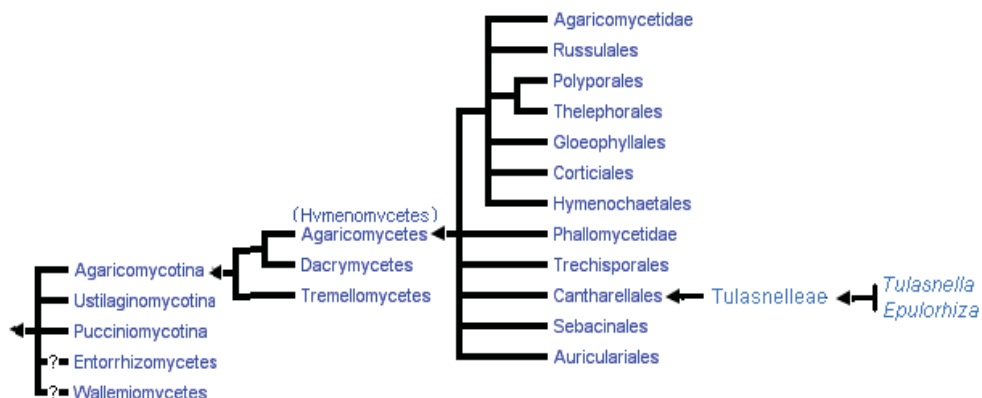
Figure B1.1   A phylogenic tree, based on the Basidiomycota, of a major orchid mycorrhizal genus, *Tulasnella* (teleomorph) / *Epulorhiza* (anamorph).   Many groups, within the Agaricomycetes, also possess mycorrhizal affinities with a wide range of host plants. Adaptation based on the phylogenetic tree of (Weiß and Oberwinkler, 2001).

B1.1   Mycoheterotrophy in New Zealand orchids

Fungal ectophytes in New Zealand have been subjected to significant levels of research, particularly in the areas of crop development, pasture production, forestry production, conservation and disease control, comprehensively reviewed by, (Cooper, 1976; Orlovich, 2004).   However, orchid endophyte mycorrhizal associations in New Zealand have received very little detailed study. In 1910, Lancaster presented a paper to the Wellington Philosophical Society on the fungi of N.Z epiphytic orchids, (Lancaster, 1911). Lancaster reported his observations of hyphal masses occurring in the cortex cells of *Dendrobium cunninghamii* Lindl, (Now *Winika cunninghamii*, (Lindl.) Clem. Jones and Molloy), *Earina mucronata* Lindl. (Now *Earina autumnalis* (G.Forst.) Hook.f.), and *Earina suaveolens* Lindl. and. *Earina autumnalis* (G.Forst.) Hook.f.).

Lancaster commented on the texture, morphology, size and colour changes of the hyphae within the host cells, and concluded that possibly many different species of fungi were being hosted. The enlarged nuclei occurring in the host parenchyma cortex cells were described. Possible degradation of vacuolarized hyphae was described as being of a granular character. No attempts to culture and identify the fungi were made. The results and detail that Lancaster achieved, with the light microscope technology then available, were commendable for the period, considering that Bernard (1904 and 1909), had only just published his seminal research on orchid endophytic fungi.

Curtis (1917), published an anatomical review of six epiphytic New Zealand orchids. A mention of root cortex fungal hyphae was made in his review of *Earina mucronata* (Lindl.), (now *Earina autumnalis*). Curtis described the hyphal entry point into the velamen and its progress, via the ectoderm passage cells, through to

the cortex apoplast. The hyphae enter into the outer cortex parenchyma where the pelotons are formed with the concurrent commencement of nuclear enlargement. According to Curtis's summary, pelotons were found in all of the orchids he examined, *E. mucronata* (Lindl.)*, E. suaveolens* (Lindl.) both now (*Earina autumnalis* (G.Forst.) Hook.f.) *Dendrobium cunninghamii* (Lindl.), (now *Winika cunninghamii* (Lindl.) M.A.Clem., D.L.Jones et Molloy), Bulbophyllum *pygmaeum* (Sm.) (now *Ichthyostomum pygmaeum* (Sm.) D.L.Jones, M.A.Clem. and Molloy), *Bulbophyllum tuberculatum* (Colenso), *Sarcochilus adversus* (Hook.f., Fl. Nov.-Zel), all now *Drymoanthus adversus* (Hook.f.). All of the orchids described are epiphytes. No further research on N.Z. orchid mycorrhizal associations appeared over the intervening, 1917-1960, period. Baylis (1961), mentioned mycorrhizal associations with N.Z. orchids but considered any experimental work on the taxa unimportant.

(Campbell, 1962,1964), presented two papers on the relationship of the Basidiomycete *Fomes mastoporus* (Bracket fungus) as a possible ectomycorrhizal associate with the achlorophyllous N.Z. orchids: *Gastrodia sesamoides* (R.Br.)*, G. cunninghamii* (Hook.f.) and *G. minor* (Petrie), including their mycorrhizal co-association with the Australian adventist, *Acacia melanoxylon*. Campbell discovered a further association between the N.Z. tarairi, *Beilschmiedia tarairi* and the N.Z. achlorophyllous orchid, *Yoania australis* (Hatch) (now *Danhatchia australis* (Hatch) Garay and Christenson) co-associated with the saprophytic "Puff Ball" fungus, *Lycoperdon perlatum*, (Campbell, 1970). This paper reported Campbell's observations on hyphal morphology and data concerning the fungal hyphal dimensions. The paper provides a large variation in observed hyphal morphological data; hyphal widths ranging from 1.7µm to 52µm, networks, external to roots and rhizomes, arbuscular evidence (pelotons), differing hyphal colours, hyphal septa variation and both clamped and clampless hyphae. Such a range in observational detail would suggest that a number of fungal genera and species were present, representing both ectomycorrhizal and endomycorrhizal forms.

A number of papers have more recently been published regarding the diversity of both endophytic and ectophytic fungi, (Hadley, 1970; Van der Heijden,1998, 2003; Leake, 2001; McCormick, 2004; Orlovich, 2004; Vogelsang, 2006; Bougoure, 2007).

A more detailed investigation was carried out by Campbell (1972), on the achlorophyllous mycoheterotrophic NZ endemic terrestrial orchid, *Corybas cryptanthus,* Hatch (1956), (now *Molloyabas cryptanthus*, Jones and Clem (2005).
Campbell, commented on the putative ectomycorrhizal association between *Corybas cryptanthus* (Hatch) and both *Leptospermum scoparium* and *Nothofagus solandri* (Hook.). This study utilized both light (LM) and scanning electron microscopy (SEM) on orchid mycorrhiza and investigated some aspects of the spatial orientation and the histology of the fungal hyphae, root entry points into the host plant and emergent hyphae from the associated plants of *Nothofagus* and *Leptospermum* species. The endomycorrhizal fungi of *Nematoceras iridescens* have not previously been researched in New Zealand and no research appears to have been directed at identifying the genus and species of endophytic fungus within the NZ orchids.

This section of the thesis will aim to record the anatomy of the mycorrhizal fungi found within the pelotons of the study orchid *Nematoceras iridescens* and identify it to species level

The overall aims are to:

1   Identify to genus and species level, the fungal endophyte resident within the pelotons of *N. iridescens*,
2   Record the penetration points of endophyte fungi into the root and cortex cells of *N.iridescens*.
3   Chronologically investigate the peloton stages for evidence of; onset of hyphal lysis and seasonal variation in hyphae.
4   Investigate the likelihood of endophytic fungal hyphae, other than peloton forming species, being present within the root of *N. iridescens*.
5   Identify infective hyphal pathways and cell wall penetration.

B1.2   Results and discussion

Correct identification of the mycorrhizal fungi within the orchid peloton has long been plagued with difficulties, (Currah, 1992; Rasmussen, 1995, 2002). Firstly was the difficulty of culturing the fungal mycorrhiza from the peloton source, (Kristiansen, 2001), with the certainty that the cultured fungus is the actual mycorrhizal symbiont and not an associated or pathogenic endophyte. Secondly the Rhizoctonia form, representing the greatest bulk of the orchid endophyte mycorrhiza, (Rasmussen, 1995; Taylor, 2002), appears to be ubiquitous in its anamorphic form, (Burgeff,1959); Hadley, 1970; Rasmussen,1995, 2002; Warcup,1982). It is difficult to induce a transformation into its teleomorphic state to enable a classical morphological examination of the basidia to ascertain its taxonomic status, (Warcup, 1967, 1981; Sneh (1991).

The current concept of Rhizoctonia spp. has been initially derived from the work of, Parmeter (1970), who stipulated that isolates of *R. solani* possess a high proportion of the following characteristics:

1   a shade of brown hyphal pigmentation,
2   branching close to a distal septum of cells in young vegetative hyphae,
3   constrictions of hyphae combined with septa close to the point of lateral   origin,
4   dolipore septa,
5   and multinucleate cells.

Other characteristics of the genus Rhizoctonia can include, and often present are:
1   monilioid cells,
2   sclerotia,
3   hyphae >5µm in diameter,
4   rapid growth rates,
5   plus a high degree of pathogenicity.

The preceding taxonomic classification was further revised by, Ogoshi (1972) and Parmeter (1970), who both elaborated on a range of morphological characters that were never present: in Rhizoctonia forms:

1   clamp connections,
2   conidia,
3   differentiation of sclerotia into rind and medulla,
4   rhizomorphs,
5   and pigmentation of the culture in shades other than brown.

In root sections of *Nematoceras iridescens, N. trilobum* and *N. papa*, Rhizoctonia forms were present, identified from specific characteristics; 90° branching hyphae

with pinched branch collars (Fig.B1.2), intercellular septae and binuclear hyphal cells that are typical of Rhizoctonia form fungi, (Sneh, 1991).
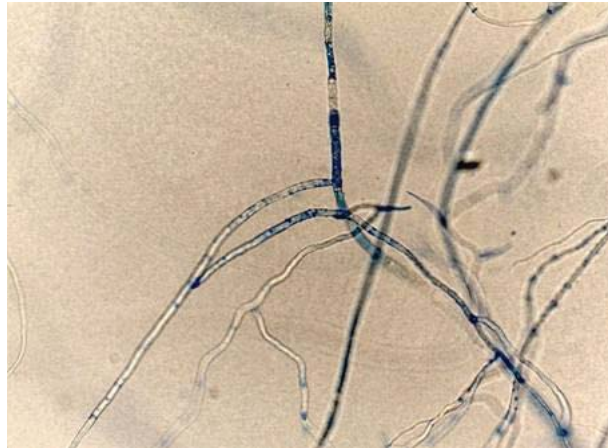
Figure B1.2   A typical Rhizoctonia hyphae, x400, stained with 0.05% w/v trypan blue in lactoglycerol.

The unique morphology of the teleomorph phase is taxonomically definitive.   Yet it has been difficult, especially with temperate terrestrial orchids, to grow isolate cultures and even more difficult to induce the teleomorph phase to produce reproductive bodies, as was found by previous researchers, (Andersen, 1996; Rasmussen, 1995, 2002; Sneh, 1991; Warcup, 1966, 1967, 1981).

Hyphal distribution and *N. iridescens* root histology are described by using an enhanced, colour inverted, SEM micrograph (Fig B1.3) of an entire transverse section (t/s) of a young root from a plant collected at the Tarata site in June 2008. This micrograph shows the stele (st) in the centre of the root surrounded by a single cell layer of endodermis (end) with thickened, suberinised, cell walls. No evidence of fungal intrusion, peloton formation or individual hyphal filaments was observed within the stele in this micrograph or in any other root sections observed during this project. This is in accord with all observations made to date, (Arditti, 1992; Burgeff, 1959; Hadley, 1970, 1971; Pridgeon, 1995; Rasmussen, 1995, 2002; Stern, 1997; Warcup, 1981).

External to the stele is the root cortex. The inner cells of the cortex, the largest measuring approximately 75μm x 30μm, form a 1 - 2 cell layer. These elongated cells are compressed anticlinally, exhibiting only an occasional hyphal presence. The mid cortex consists of 4-5 layers of large, thin walled parenchyma cells between 50μm -100μm in width. Three layers of these large cells lie interiorly to the sub-epidermal cortex and show heavy infestations of fungal hyphae and peloton vacuoles. The sub-epidermal cortex lies interiorly, adjacent to a single layer of epidermal cells and consists of 1-2 layers of small parenchyma cortex cells between 30-55 μm in width.

When compared to the large mid cortex cells, the smaller sub-epidermal cortex shows a lighter hyphal presence, without the consolidated hyphal appearance that is apparent in the large mid cortex cells (Figs.B1.5 and Fig.B1.6).

Externally to the epidermis, the root has a cuticle through which emerge trichoblasts, (Esau, 1953), containing multisereate trichomes which are typical of the Corybas alliance, (Pridgeon, 1995), see strands emanating from the trichoblasts, (Fig B1.4 #1).
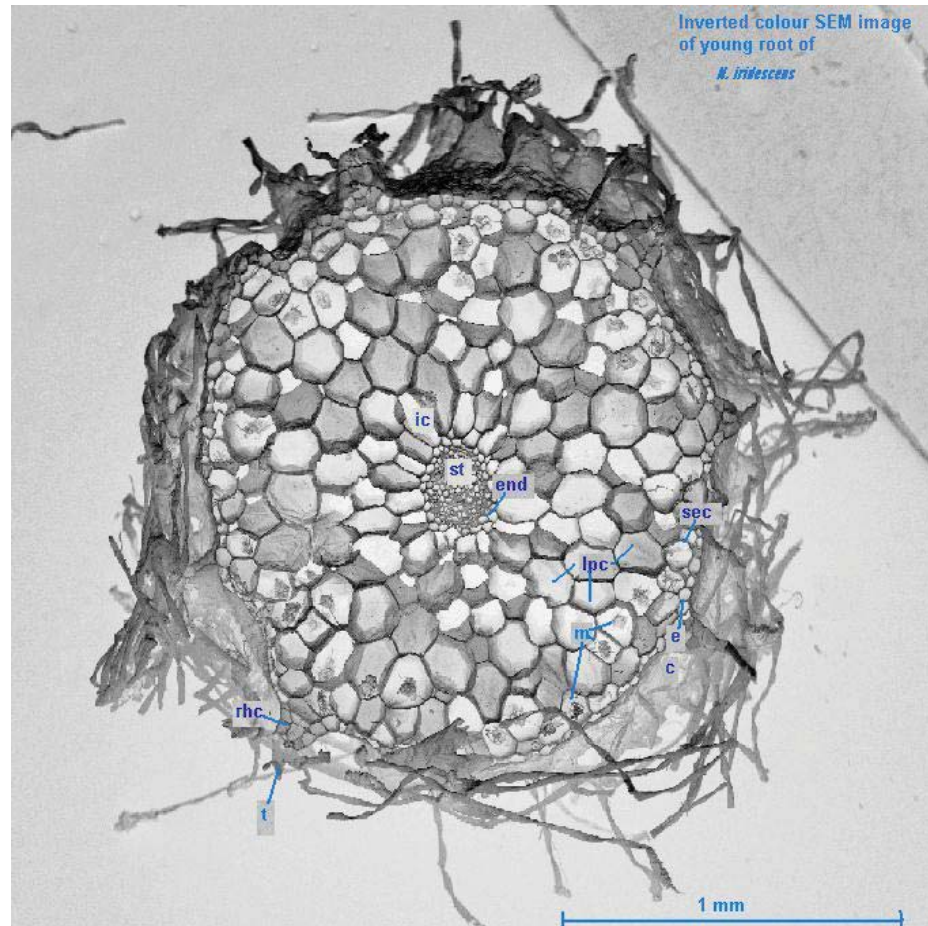
Figure B1.3 TS of a young root of *Nematoceras iridescens*
Key: c = cuticle, e = epidermis, sec = sub epidermal cortex, mc = mycorrhizal pelotons, lpc = large parenchymal cells, end = endodermis, st = stele, rhc = trichoblast
t = trichome.

The trichoblast, comprises a cone of epidermal cells surrounding a core of sub-epidermal cortex cells, Fig.B1.4 (1and 3). Up to 3-4 trichomes emerge from the edges of the trichoblast crater, Fig.B1.4 (1), comprising of at least two histological differing cells (epidermal cells and outer parenchyma cortex cells, Fig.B1.4 (3).
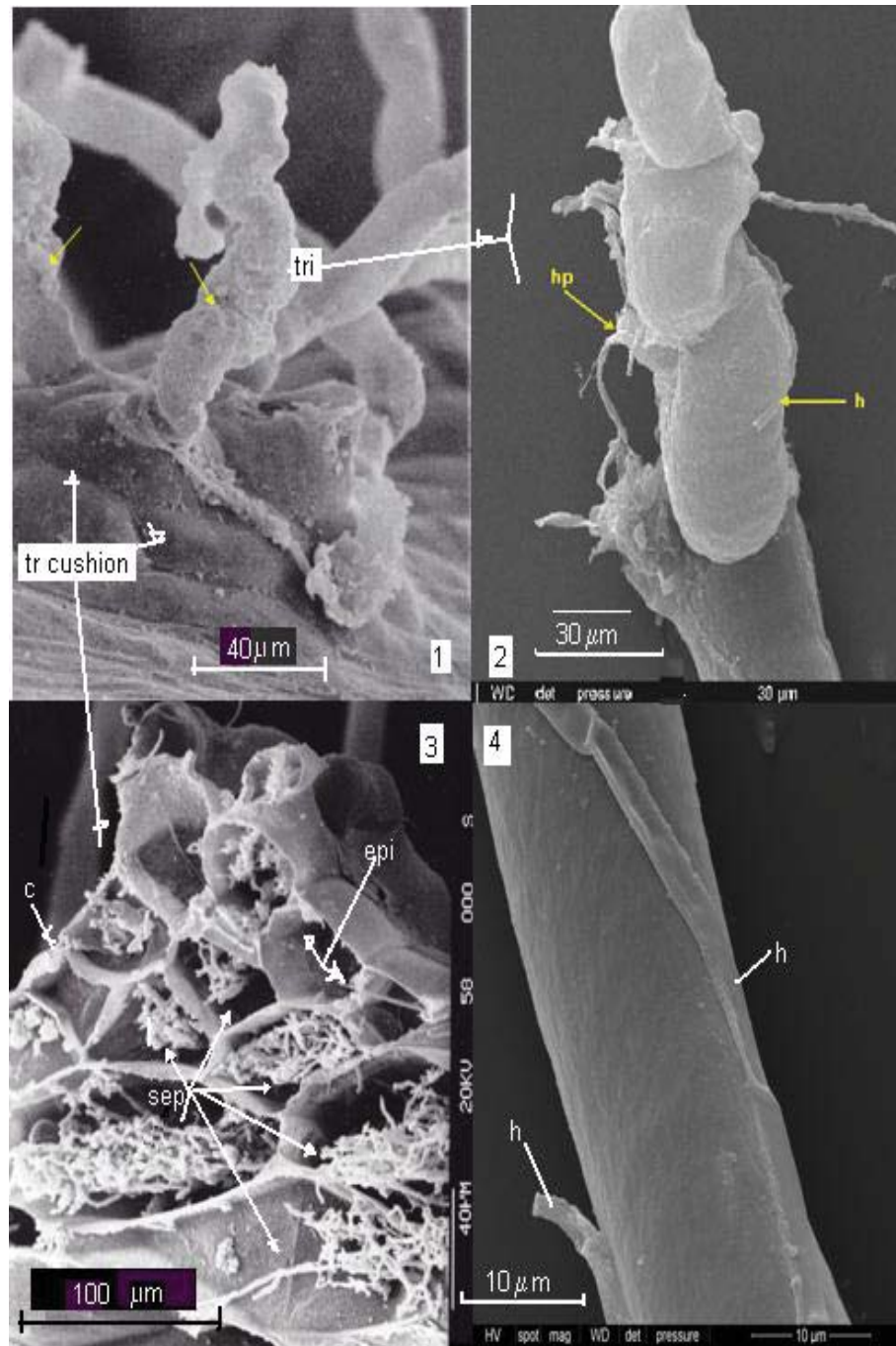
Figure B1.4　　**1** Four trichomes (tri) emerging from a trichoblast, yellow arrows point to hyphal entry points midway up the trichome, not the tip. **2** A trichome filament showing cell serialization creating a multisereate trichome plus a hyphal entry (hp) into the anastomosis point between the basal trichome cell and the second trichome cell. The tip, not seen in this micrograph copy, is free of hyphal filaments. **3**　A trichoblast showing external cuticle (c), single layer of epidermal cells (epi) all with non-proliferative hyphal content, a cone of small sub-epidermal parenchyma cortex cells (sep. arrows).　　Below the cone are larger parenchyma cortex cells with young pelotons (p). **4**　　A trichome base with (h) external septate hyphae.

Multiple round external hyphae, of between 2.0μm -2.5μm in diameter, enter the plant's multisereate trichomes, Fig.B1.4 (2), at a mid-point between an external joining of the two base cells of the trichome cell wall. These hyphae enter at the side rather than at the tip as previously reported in other orchid species, (Arditti, 1992; Rasmussen, 1995). No other external hyphae entry points into the root were observed. What attracts the hyphal tip to these particular points? Aspects have

been reviewed by, (Gadkar *et.al.,* 2001; Parniske, 2004; Arkowitz and Bassilana, 2011).

Hyphae progress from an origin in the trichome, which can be seen in Fig.B1.4 (3). The epidermal cells that comprise the first trichoblast show hyphae in loose bundles. In all other images of the epidermis only those cells involved in the trichoblasts show any presence of convoluted hyphae.
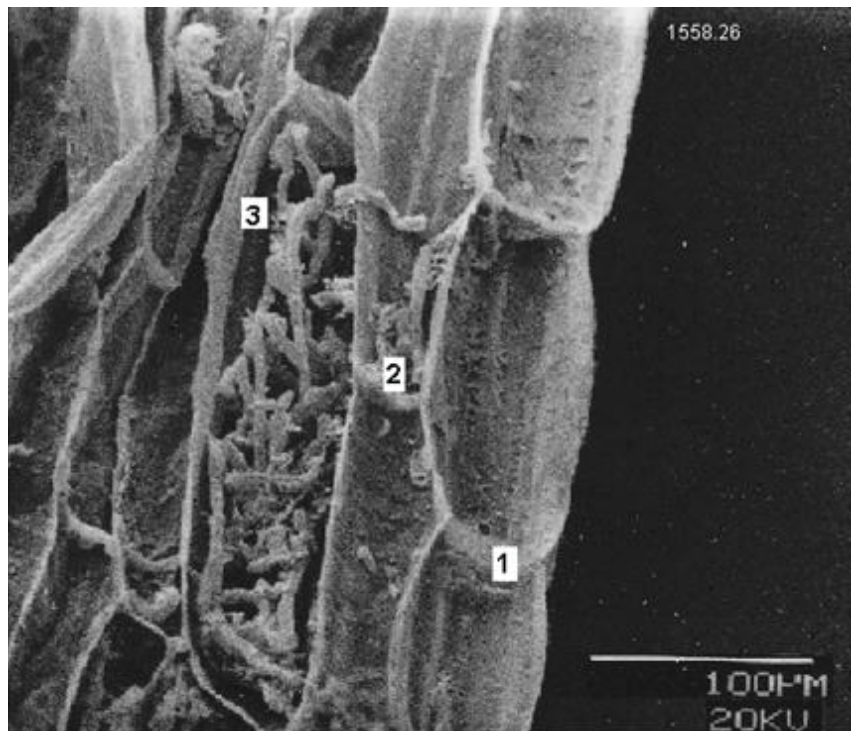
Figure B1.5   A *Nematoceras iridescens* root the degree and location of hyphal penetration can be observed. Key; 1 = epidermal cells with no hyphae, 2 = sub epidermal cortex some hyphae evident, 3 = mycorrhizal peloton within a large   cortex parenchyma cell.

The hyphae enter the sub-epidermal area cells, proximal to the entry point, with long minimally divaricating hyphae (Fig.B1.6).. In the outer mid cortex cells the peloton formation is congested and consolidated (Fig. B1.6). The mid-cortex cells also display an entire repertoire of hyphal stages, from newly formed to final lysis stages and would justify the title of "main peloton digestion cells", (Dangeard, 1898; Rasmussen, 1995), Fig.B1.6 e and d. From the advanced state of hyphal lysis in the pelotons of many of the large mid-cortex cells, it is logical to assume that the initial hyphal accumulation occurred in these cells.

Results, from the light microscopy sections of the root of *N. iridescens,* show the endomycorrhizal development (Fig.B1.6 and Fig.B1.7).   The trichoblast, in (Fig.B1.6) shows the distal section, the two arrows indicate the base of the trichoblast. The endophyte hyphae are shown in the proximal epidermal cells (layer a). The only epidermal cells containing hyphae are situated immediately below the trichome sites. The sub-epidermal cortex cells show an open hyphal arrangement and lack of any hyphal consolidation. The red stained clumps in the centre of these outer cortex cells are interpreted as nuclei (Fig.B1.6 (b) and Fig.B1.14 (n)), rather than hyphal consolidation.

Within the large (<80+ µm in diameter) parenchyma cells of the mid-cortex, large hyphal pelotons are in various stages of development (see Fig.B1.6 and Fig.B1.7). Throughout this project all microscopy confirmed this pattern of hyphal distribution. The usual pattern of mature pelotonized hyphae occurs within the three cell layers of the mid cortex area. Between the mid cortex and the endodermis the smaller parenchyma cells appear to lack developed pelotons (Fig.B1.5).
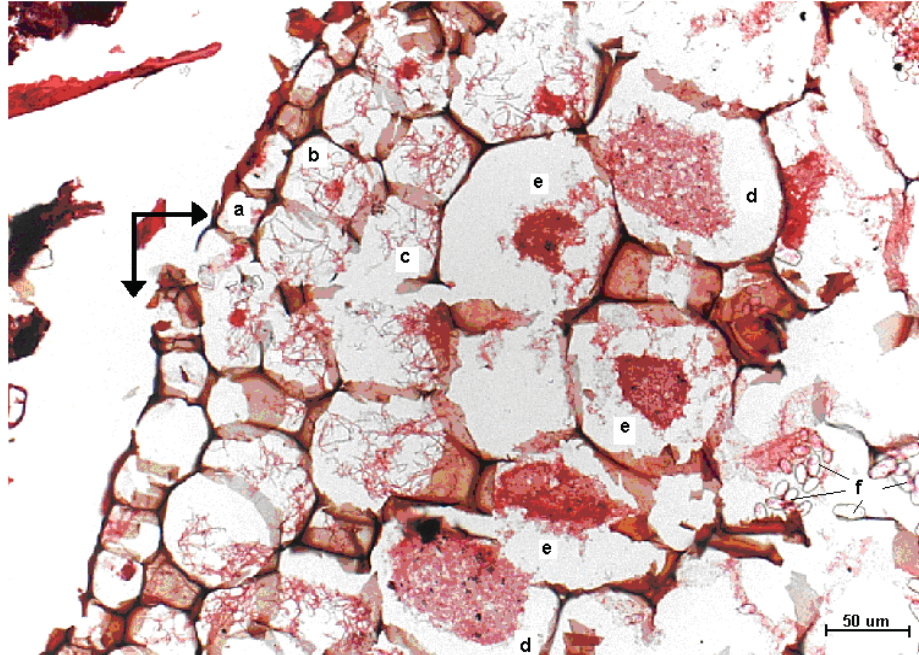
Figure B1. 6 A t/s of a new seasons *Nematoceras iridescens* root taken from an <u>early flowering plant, July 2007</u>. Key; a = epidermis (arrows indicate a trichome site), b = sub-epidermal cortex, c = small cortex cells, d = mid cortex mega-cells with mature pelotons, e = advanced lysis of hyphae within a peloton, f = amyloplasts.

The older plant sections, (Fig. B1.6), were taken during the period August – September, 2008, and illustrate the degree of endophyte "pelotonization" (Fig.B1.6 (c)), advanced peloton lysis (Fig.B1.6 (d)) and heightened accumulation of amyloplasts (Fig. B1.6 (a) compared to July (Fig. B1.5.).

Limited hyphal lysis occurs in the mid-cortex mega-cells (June-July, Fig.B1.6) culminating over August-September (Fig.B1.7) and is completed by late November December (Fig. B1.8) Amyloplast numbers adjoining the infested "digestion" cells of the mid cortex (Fig.B1.6 #f) and Fig.B1.7#a)) show an increase over this period.

Figure within the micrograph labeled: Mycorrhizal arrangement 3E2 root.tif   50 µm
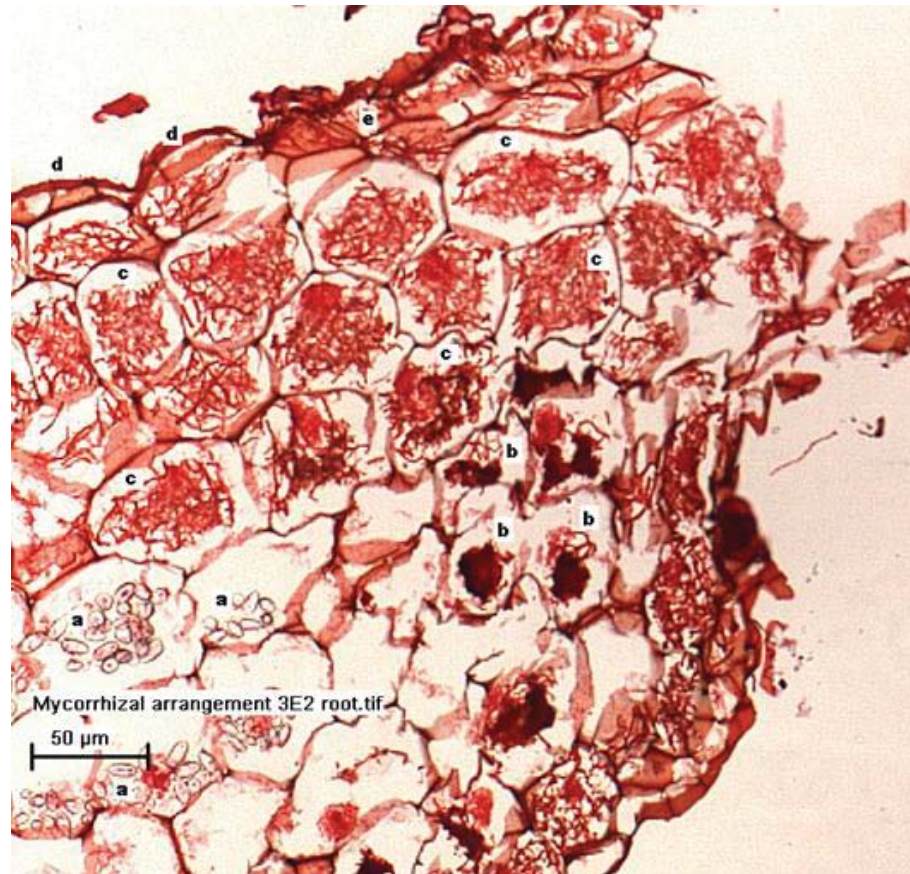
Figure B1.7. A *N. iridescens* root section incorporating all phases of mycorrhizal distribution. This micrograph is typical of a mature phase (August-September). Key: a = amyloplasts in cells of inner cortex, b = cortex cells with advanced lysis of peloton hyphae, c = cortex parenchyma mega cells with active pelotonized hyphae, d = cuticle, e = epidermal cells. Section stained with Safranin O and Alcian blue. Bar = 50µm

Some of the inner cortex cells in the more mature plants are full of amyloplasts (Fig.B1.9) that measure up to 30 µm along their polar axis (Fig B1.9, Fig B1.10) and Fig.B12.2 #2). This evidence appears to contradict comments made by, (Dangeard and Armand, 1898; Burgeff, 1909; Rasmussen, 1995), who commented on the disappearance of starch from cortex cells of other orchid genera as soon as hyphae entered through the plasmalemma.
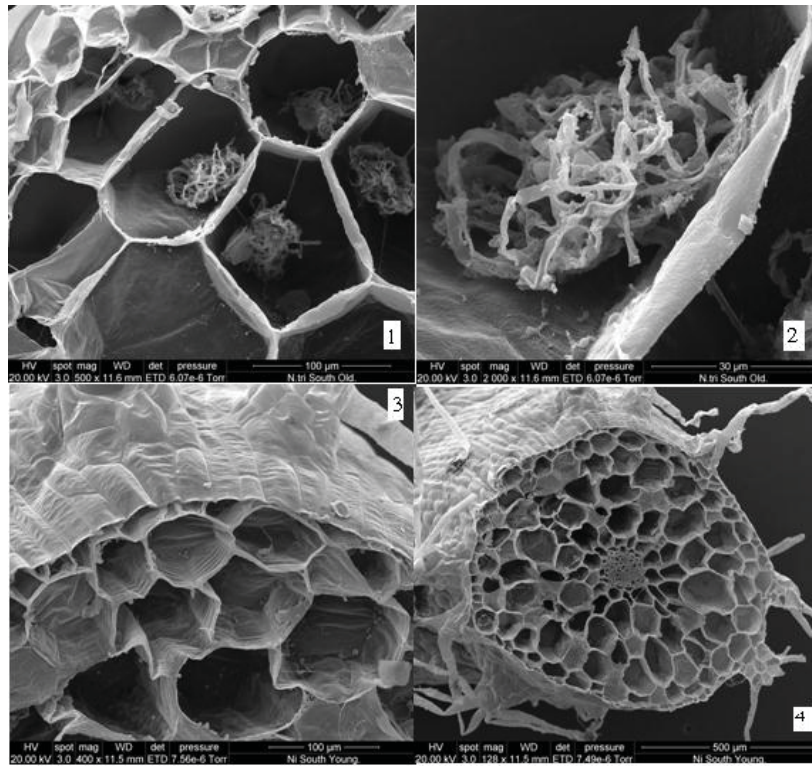
Figure B1.8. Four older *N. iridescens* root sections (late November 2008.) (1) A major diminishment of pelotons within the "digestive" cells of the root cortex, the few pelotons that remain (2) appear to be composed of hyphal "shells" and inactive, exhibiting the "tape like" morphology As the plant gets older the cortex and trichome cells appear to divest themselves of viable hyphae see (3 and 4).
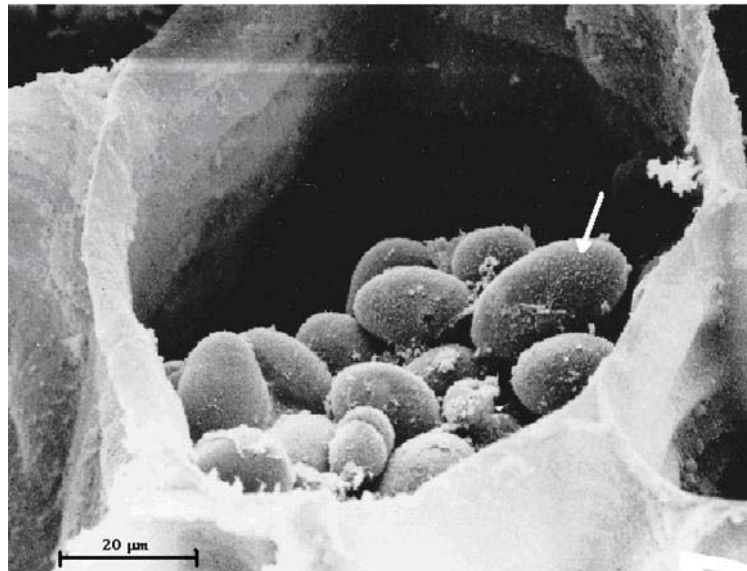
Figure B1.9. An inner cortex cell of *N. iridescens* containing amyloplasts, the largest indicated with an arrow.

Amyloplasts in a root section (2D1.1) of *N. iridescens*
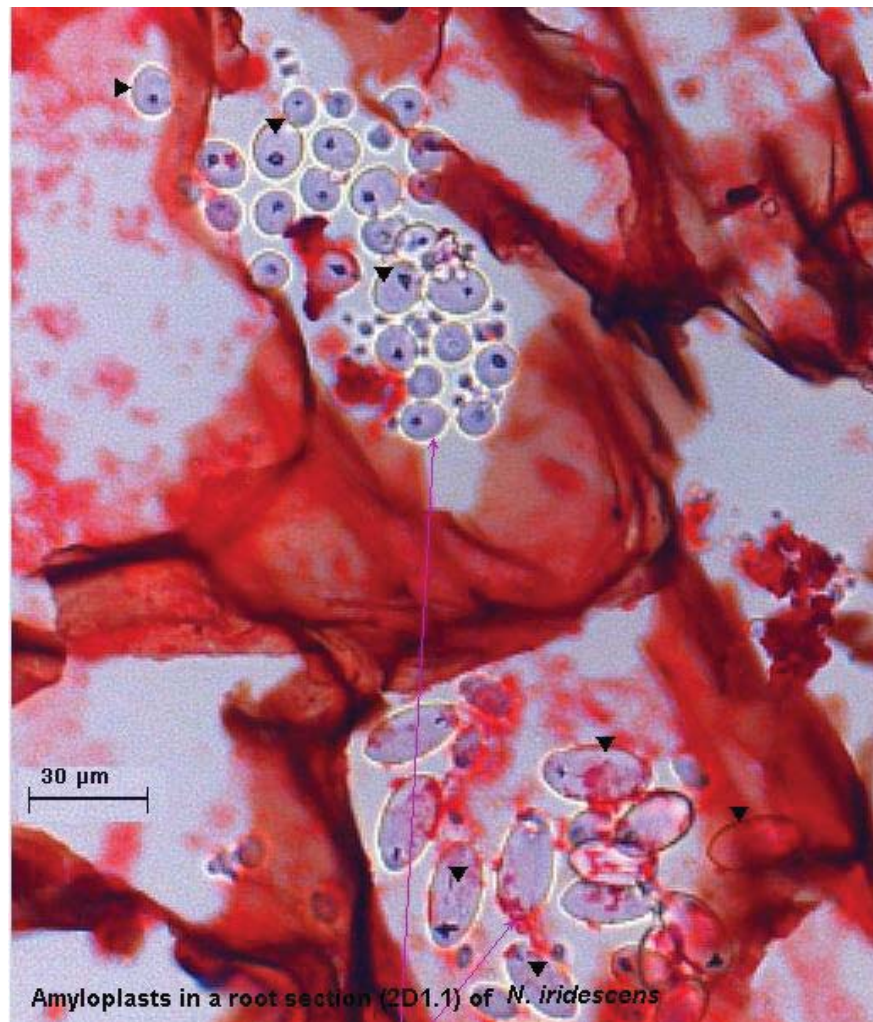
Figure B1.10 A late season (Feb. 2007) *Nematoceras iridescens* root.
Arrow heads indicate some of the many amyloplasts. Bar = 30µm
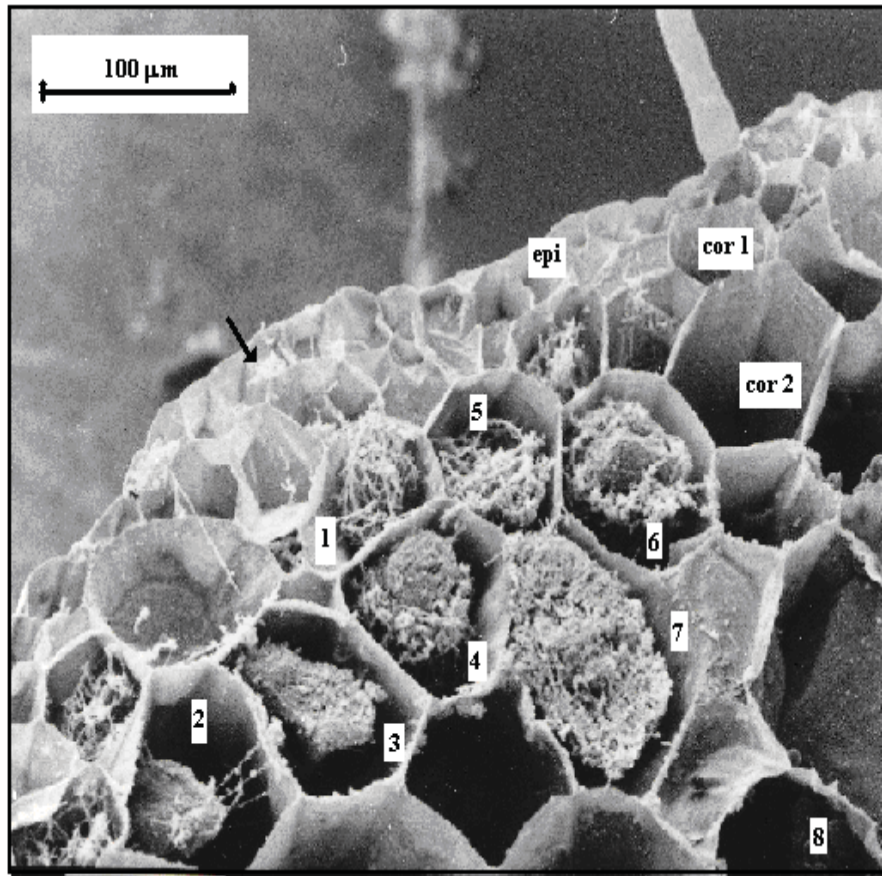
Figure B1.11   An external mature root section taken from a young plant of *N. iridescens* in July.   Key = epi = epidermis, cor 1 = sub-epidermal cortex, cor 2 = giant parenchyma cells of the mid cortex, 1 = sub-epidermal cortex cell with unconsolidated hyphae, 2 = mid-cortex with pelotonized hyphae and "transmission[1] hyphae", 3-4 = congested pelotonized hyphae undergoing lysis, 5-6 = early peloton formation, 7 = fully mature peloton and 8 = mid-cortex parenchyma cell without evidence of any hyphae

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[1] Authors term for non-divaricating hyphae that progress across cell interiors to enter adjacent cells
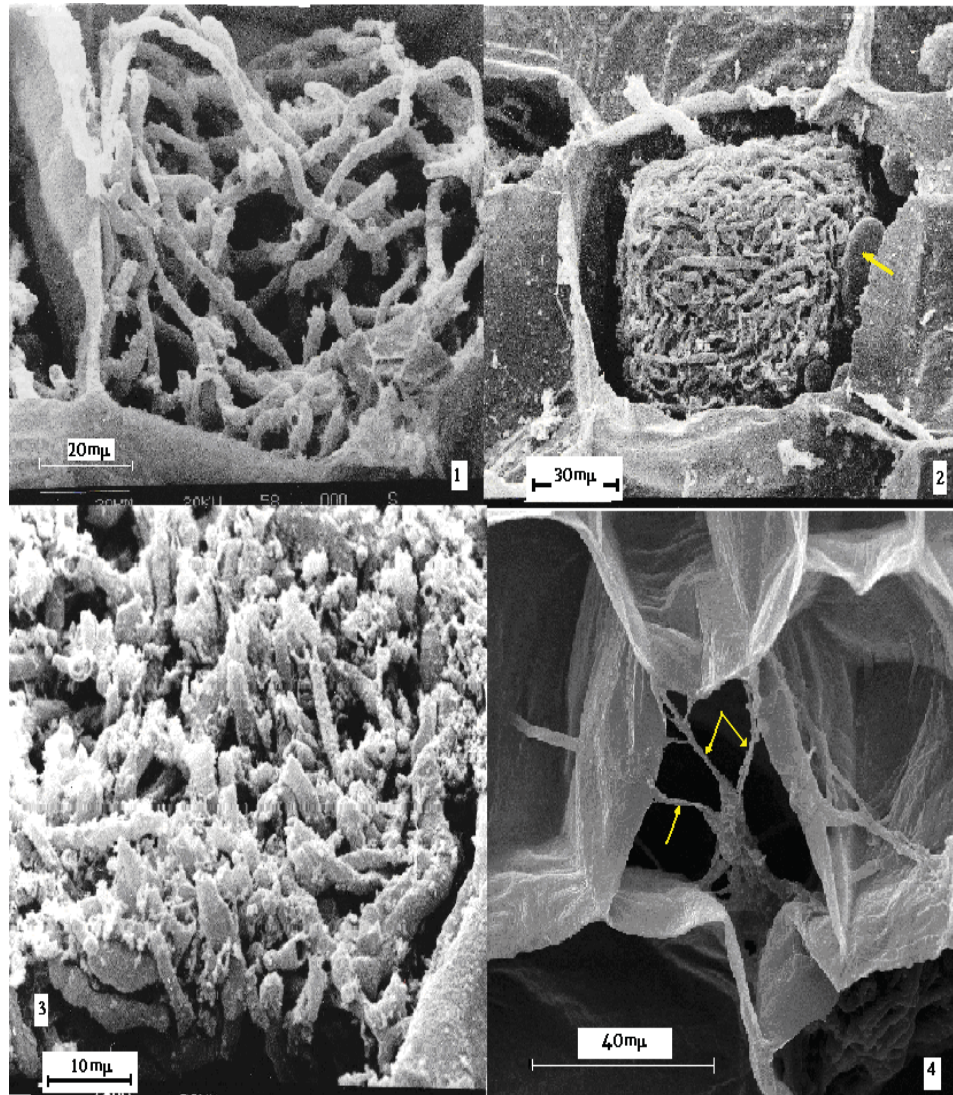
Figure B1.12 Four stages of peloton formation and degradation. (1) Early formation of hyphae, tip branching has not yet occurred. (2) Tip branching has developed and peloton has reached its pre-lytic size. Note the amyloplasts in the peloton containing cell, see arrow. (3) Lysis of peloton hyphae is well developed. (4) Peloton has fully lysed and collapsed, the yellow arrows point to what I assume are either threads of cortex cell plasmalemma that made up the outer wall of the peloton vacuole and also provided a suspension apparatus for the peloton, or are remnant hyphae.

As can be seen, in both (Fig.B1.13 (A), the SEM micrograph of a *N. iridescens* stem and in (Fig.B1.13 (B), a L.M micrograph of a *N. iridescens* leaf X/S) no evidence of endophyte intrusion occurs. None of the above ground sections, the author has taken from the *Nematoceras* samples, exhibit hyphae. Root tubers from *N. iridescens* did not contain any observable fungal hyphae. These observations, of terrestrial chlorophyllous orchids, are in accord with others, in that no evidence of hyphae appear in the leaves, stems or tubers, (Arditti, 1992; Hadley, 1970; Rasmussen, 1995 , 2002; Zelmer,1995).
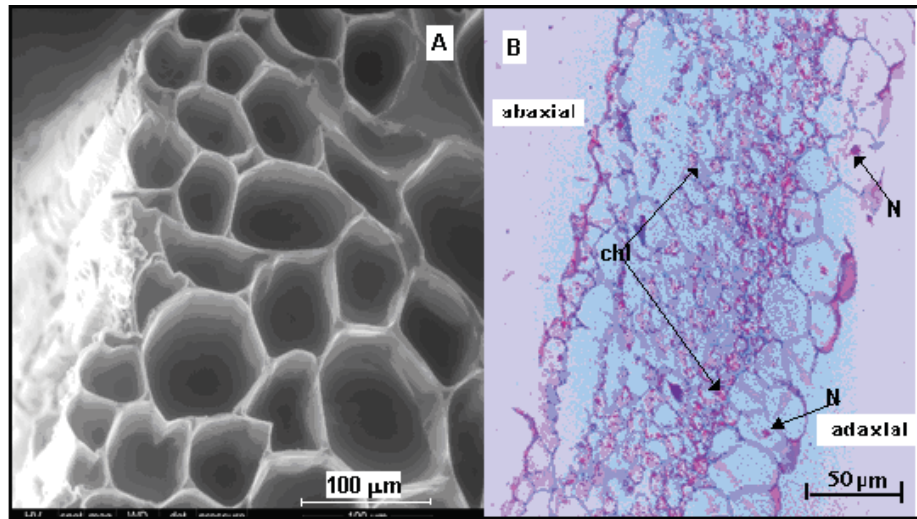
Figure B1.13   T/s of (A) he stem and (B) a leaf section). The small cerise stained particles (chl mesophyll area) in B are chlorophyll plastids. N arrows are nuclei within large adaxial epidermal cells. (Note the size difference between these nuclei and the mega-nuclei of the peloton containing mid cortex cells shown in Fig B1.14)

One difference observed between similar histological cells, one hosting a peloton the other with no peloton, is the size of the relevant nuclei. The nuclei have an average diameter of 28µm in peloton filled cells (Fig.B1.14) compared to 11.42µm in leaf cells (Fig. B1.13; A and B), a 2.5: 1 ratio. This variance has been reported in a number of papers; in which the ratios range from x8 to x64 from the normal nuclei volume of the uninfected cell size, (Williamson, 1969; Rasmussen, 1990).

The increase in nuclei size has been attributed to large increases in metabolic activity, especially RNA enzyme synthesis, (Kogel, 2006; Barosso, 1990). Comparing the hyphae, for differences in morphology, no changes in the diameter of hyphae were apparent, with measurements of between 1.8µm and 2.5µm, that is in accord with, Sneh's (1991), morphological range for orchid Rhizoctonia types.
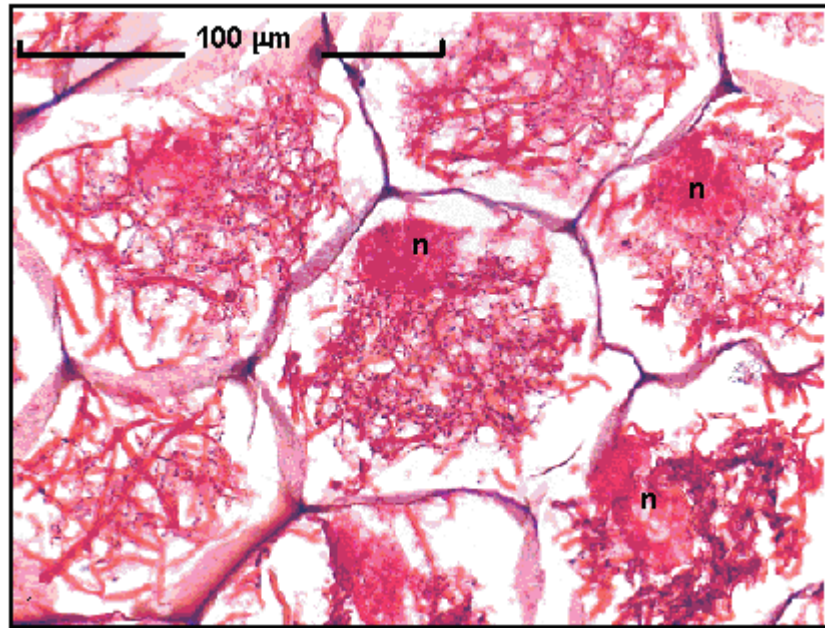
Figure B1.14. Large cortex cells from a root of *N. iridescens* with mega-nuclei (n) in peloton filled cells (Safranin O and Alcian blue staining).

When very young, (July), sections of the *N. iridescens* root were observed, some of the individual hyphae had a "tape like" appearance (see left micrograph Fig. B1.15), the hyphal width of 2.5 ± 0.5µm is similar to other hyphae, 1.8 ± 0.2 µm. A tube like morphology is apparent in the micrograph (Fig. B1.15, right).

Distinct differences in hyphal distribution are discernible between the peloton hyphae, in the more open forms of the sub-epidermal cortex, compared to the very congested hyphal conglomerates exhibited in the pelotons of the large cortex cells (Fig.B1.6, a, b and c compared to (Fig.B1.6, d and e).

Hyphal structure also shows variation between older hyphae and young hyphae. The hyphae in the younger plants (July, flowering) have a high degree of ribbon effect while the hyphae in the more mature plants (October, pod and seed maturation) appear as distinct tubes

A change in hyphal morphology in the mature, post inflorescence plants is also displayed in many of the large cortex cells (Fig. B1.6 (d and e)), that occur in up to three layers interiorly to the sub-epidermal cortex tissue (Fig.B1.7 (c)). These cells have been termed digestive cells, (Dangeard, 1898), since lysis of the peloton hyphae takes place within them. A number of hyphae show morphological and route changes and are apparent in (Fig.B1.11). The hyphal route, (Fig. B1.16 (1, yellow arrow)) originates from a trichoblast base and penetrates through a number of cortex cells and their walls to the endodermis. This type of hypha has limited divarication and does not appear to form pelotons, even though the large mid-cortex digestive cells are present and penetrated. The hyphae produce lateral branches (Fig.B1.16 (2)) and swollen hyphal tips, (Fig.B1.16 (3)), that appear to be in an early stage of differentiation. Early hyphal peloton development is shown in (Fig. B1.16 (4)). In this micrograph, the lateral tips produce rapid divarication and development of a peloton that can almost fill the entire cell.
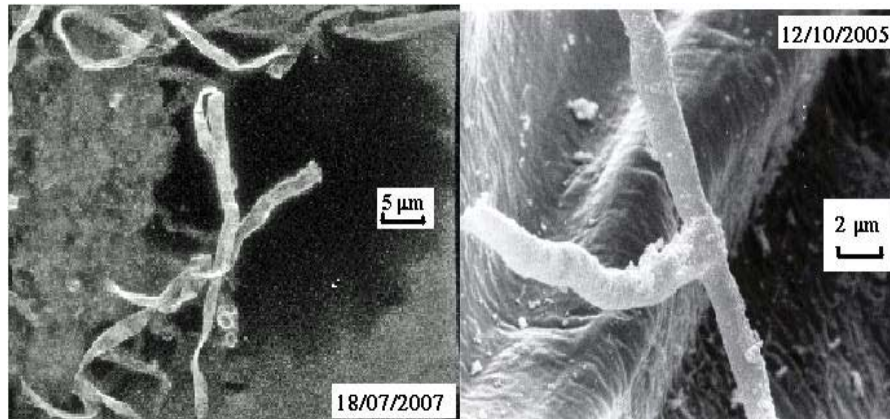
Figure B1.15 Cortex hyphal forms; left a flattened, 2 µm wide septate form of hyphal morphology and protruding from the cell three severed ends showing thick hyphal walls, of between 0. 5 < 0. 8 µm; on the right are circular 1.8 < 2.0 µm wide septate tube like hyphae with a 90° lateral branch typical of a Rhizoctonia form.

Two different stages of hyphae morphology were apparent over the active growth period of *N. iridescens*: a rounded tubular type and a flat "tape like form. The tubular type has two phases: a long, minimal lateral branching form and a multiple divaricating form. The long, minimal lateral branching form appears as a primary cell entry hypha ranging from the trichome entry point through to the cortex (see Fig. B1.16 (1 - 2)). This form introduces the peloton forming hyphae into the digestion cells of the cortex. There is evidence of multiple hyphae penetrating the same cell as seen in, (Fig. B1.16 (1 and 3), this phase occurs early, June – July, and develops the resident pelotons within the large cells of the cortex.

As the plant ages some hyphae continue to divaricate until a congested maximum cell mass is attained. Once this point is reached, the congested pelotons are subject to lysis (Fig. B1.11 (2-3)). Amyloplasts form during the lytic process, (Fig B1.12 #2, yellow arrow). When lysis is complete, the cell lumen of the "digestive" cortex cells empty (Fig. B1.8 (1-4) or retain peloton hyphae of the "tape" variety, (Fig B1.12 #4) and (Fig B1.15 (left). The tape form can also be found in cortex cells throughout the host-growing season and is often accompanied by the tubular form.
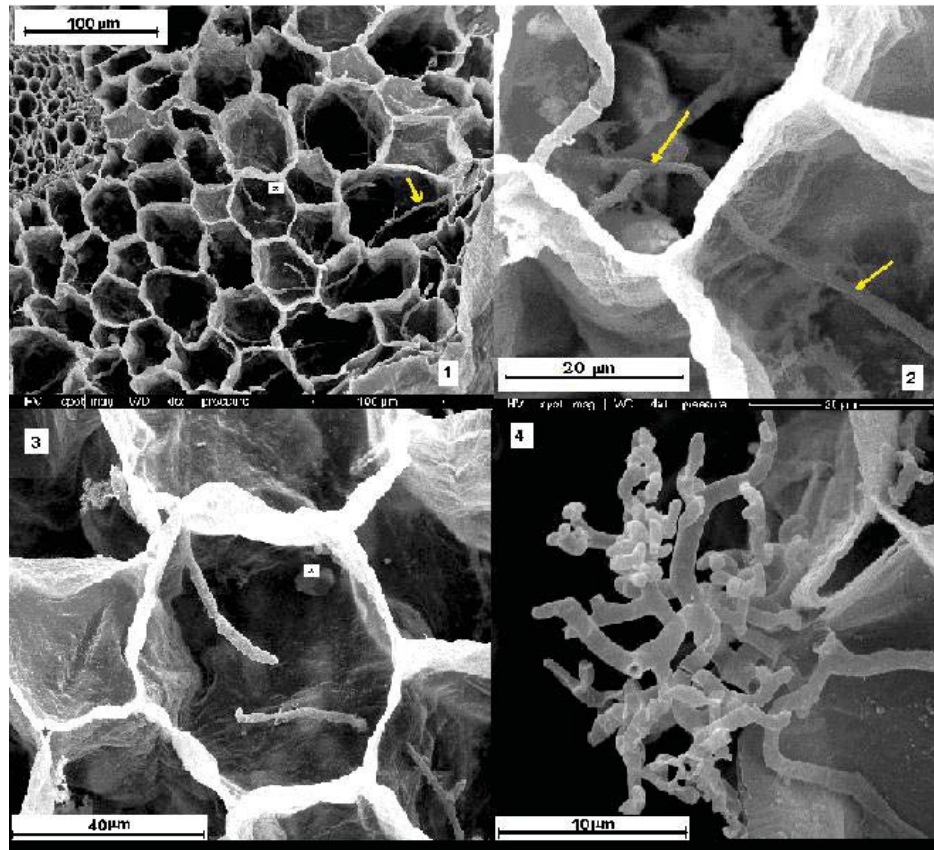
Figure B1.16 Fungal endophyte hyphae penetrating cell walls and producing adaptive morphology. 1. Fine non peloton forming hyphae arising from the trichoblast and travelling through cell walls up to the endodermis, the asterisk * area has been magnified and is shown in micrograph (3). 2. Shows a single hyphae penetrating two cell walls, it has a typical 90° Rhizoctonia form lateral branch with a neck restriction. 3. Shows cell wall penetration by two separate hyphae exhibiting engorged terminal ends. 4. Shows a rapidly divaricated hyphal mass with possible production of monilioid cells or other morphological differentiation (18/09/08).
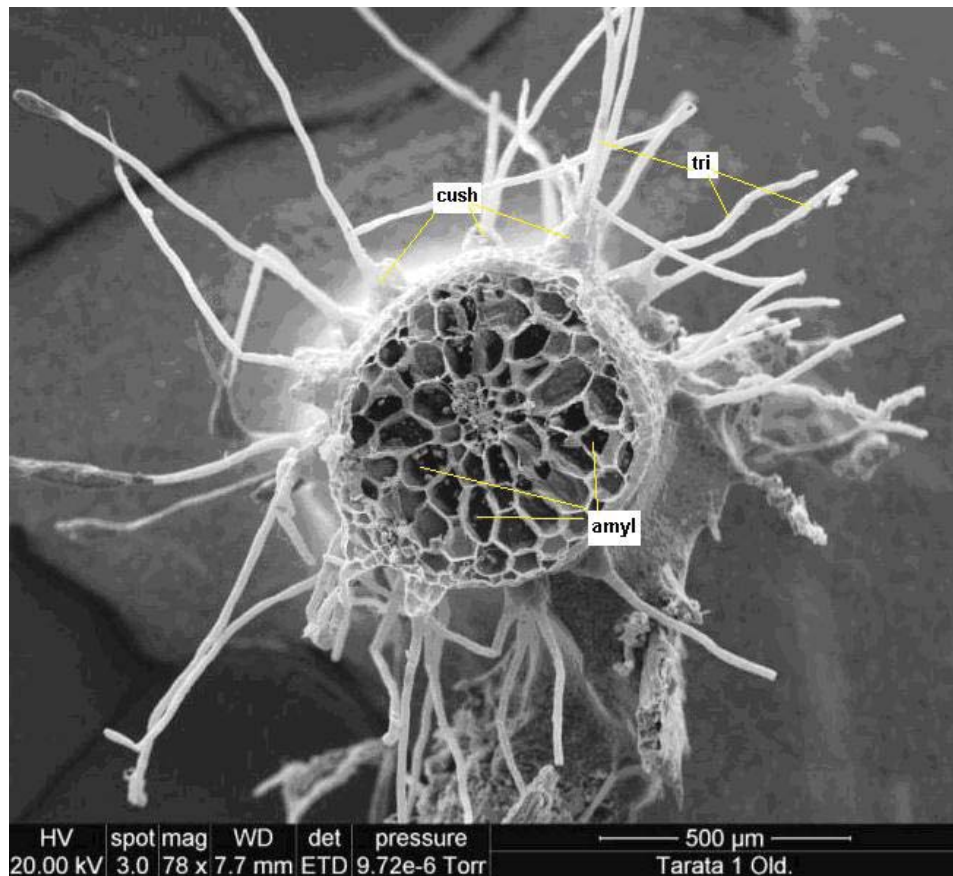
Figure B1.17. An accumulation of amyloplasts (amyl) in the large mid-cortex parenchyma cells that were previously occupied with hyphal pelotons. (An old root from *N. iridescens,* Tarata site).

Examination of pelotons by CLSM, after fluorophore staining with solophenyl flavine 7G 500, shows an elongated peloton (Fig.B1.18). That has multiple hyphal cell wall penetrations. The cell wall penetrating hyphae continue to proliferate in adjacent cells. The hyphae reaching the large, mid cortex parenchymal cells (digestive cells) divaricate rapidly, the peloton almost entirely filling the host cell.

Initially, tubular external hyphae, of between 2.0 µm to 2.5 µm in width, enter the plant trichomes through the external cell wall membrane, at a mid-point between an external joining of the two base cells of the trichome cell walls (Fig. 4a.3. (1, 2 and 3) rather than at the tip, which has previously been reported in other orchid spp., (Arditti, 1992; Rasmussen, 1995). No other external hyphae entry points into the root were observed. What attracts the hyphal tip to these particular points is unknown and further investigation would be required. The trichomes are on the trichoblast made up of at least two histological differing cells, epidermal cells and parenchymal outer cortex cells (Fig. 4a.3 (3)
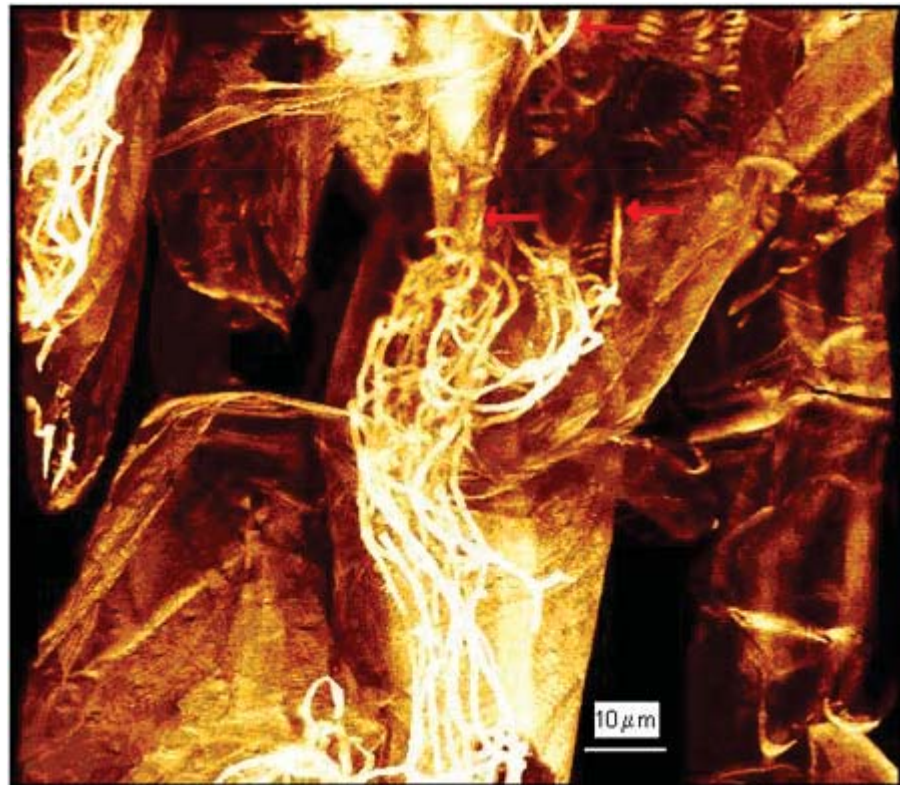
Figure B1.18. CLSM micrograph image of the hyphae within a peloton of *N. iridescens.* Arrows indicate penetrations, by hyphae, into adjacent cells. Tissue stained with solophenyl flavine. Bar = 10μm.

These primary hyphae travel through the epidermis with little divarication and follow a path from the root epidermal cells to the cortex cells. Only those epidermal cells involved with the trichoblast are affected, the remainder of the epidermis presented no evidence of hyphal invasion (Fig. B1.4). Once a cortex parenchyma cell is reached and penetrated the hyphae produce lateral hyphae which divaricate extensively forming a peloton within the "digestive" cells of the mid cortex. Divaricating hyphae produce some non-divaricating lateral branches that have a relatively direct path, similar to the primary penetrative hyphae, to an opposite internal cell wall which is then penetrated, (Fig B1.16 #s 1, 2 and 3), (Fig B1.18 and Fig B1.19). This penetrating hypha reverts to a primary form passing through cell walls and its laterals forming divaricating peloton masses.

The primary hyphae appear to be dispersive with the secondary divaricating hyphae producing a maximum surface area which, when the peloton becomes congested hyphal lysis commences and amyloplasts are produced (Fig B1.19).
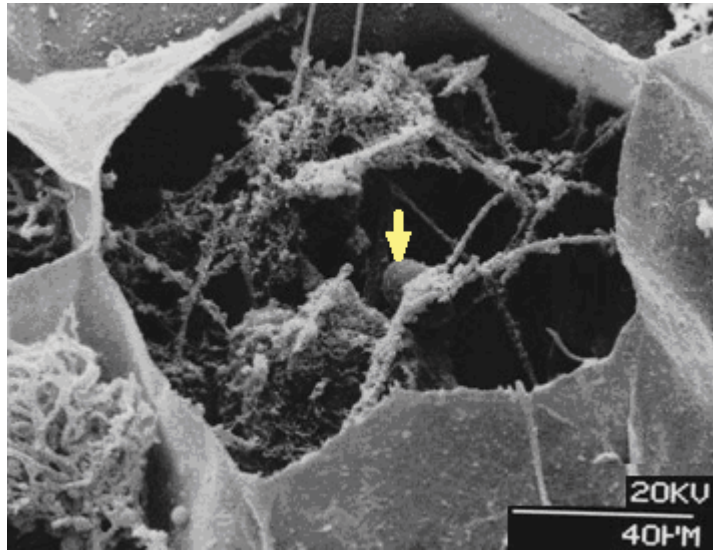
Figure B1.19 Late lysis of the peloton hyphae within a giant cortex cell of *N. iridescens.* Only a few areas of active lysis remain. The "long" intercellular hyphae, without heavy lytic encrustation, are readily seen penetrating cell walls. The arrow indicates an amyloplast.

A seasonal pattern of peloton aggregation followed by lysis and amyloplast accretion is associated with aestivation, (Larcher, 2003).

What changes the hyphae from the long tube to the tightly divaricating form in only the large parenchyma cortex cells? Three phases of hyphal morphology are apparent in the microscopic imaging: early tape form, tubular thick walled and late tape form. Interestingly plant defensins have been reported that reduce hyphal elongation and are able to induce hyper-branching, both with and without morphological change, (Broekaert, 1995; van der Weerden, 2008).

Is the hyphal pattern of the mycorrhizal fungi, hosted by *N. iridescens,* controlled by defensins or do the hyphae possess a specific signal

Chapter B2   The ultra-structure of the fungal septae

B2.1   Introduction

Transmission electron microscopy (TEM) was used to identify the genus of the fungi observed within the pelotons of, *N. iridescens,* by comparing the septal pore apparatus (SPA) of the resident peloton hyphae with selected examples from the higher Basidiomycota.

While molecular sequencing has the ability to identify endophytes to the species level, identification as to the *in situ* mycorrhizal fungi can remain problematic. Earlier investigations by others, of the septal pore morphology in peloton hyphae, was found to provide a reasonable taxonomic identity to genus level of the resident mycorrhizal fungus, (Andersen, 1996; Moore, 1985, 1987; Suárez *et al.,* 2006; Tu and Kimbrough, 1978; Williams and Thilo, 1989). This, together with using the molecular evidence from the same sample, would corroborate the endophyte as to genus and allow for an appropriate selection of primers for polymerase chain reaction (PCR) amplification to assist in the identification to species level.

B2.2   The septal pore

Two phyla of the kingdom Mycota, the Ascomycota and the Basidiomycota, possess many features in common thus pointing to a common ancestry. One of these features is having septa which can be distinguished from each other by the septal pore and the pore "plug". The second is that all known orchid mycorrhizal fungi spp. are to be found within these two phyla.

In the Ascomycota, the septa are essentially simple without having the swelling surrounding a relatively large pore (0.05 < 0.5µm in diameter) and possessing adjacent Woronin bodies that have the ability to block the central pore, whereas the Basidiomycota septal pore apparatus (SPA) have septae with swollen edges, dolipores, a smaller central pore (100 < 150nm in diameter) and septal pore caps, the parenthesomes, which appear to serve a similar function analogous to the Woronin bodies.

Pores in the septum of the septate fungi were first investigated by, (De Bary, 1887; Wahrlich, 1893), both of who described the septal disk, in the fungal hyphae, as being similar to an internal cell wall with a simple central pore, which essentially divides the hyphae into numerous cells.

It was not until, Buller, (1933), that the structural architecture and importance of the septal pore was more fully elaborated.

The taxonomic importance of the ultrastructure of the SPA has been explained by, (Currah and Sherburne, 1992; Currah and Zelmer, 1992; Khan and Talbot, 1976; Moore, 1985,1987; Tu and Kimbrough, 1978), the results of their research present a good correlation between vegetative morphological characteristics and the relevant septal ultra-structure in the fungal taxa investigated.

Currently the hyphal SPA is considered to be a highly specialized dynamic structure that controls the hyphal integrity, homeostasis and intercellular migration of various organelles and vesicles within the hyphal filament, (Moore and McAlear, 1962; Orlovich and Ashford,1994). The filaments of the fungi have a coenocytic hyphal cytoplasm with only the "valve like" septa interrupting the overall continuity. A constant protoplasmic streaming is also controlled by the septa, (Bracker and Butler, 1964; Müller *et al.*, 2000). The pore also acts as a homeostasis control mechanism after hyphal trauma.

The septa of the Agaricomycotina is characterized by a swelling (flare) proximal to the pore, this swelling forms a suspended elongated torus termed the dolipore (Figs. B2.1 and Fig. B2.2). Both openings of the dolipore have electron dense caps that appear to act as, gate keepers, to this aperture. It is this septal pore cap, the parenthesome, which constitutes a major factor in the taxonomic ultra-structure interpretation of the Basidiomycota, (Moore, 1985,1987; Sneh, Burpee and Ogoshi, 1991; Tu and Kimbrough, 1978). The author can find no references in the literature as to there being any significant difference between septal pore morphology in the anamorph as compared to the teleomorph phases of the various species of the Basidiomycete.

There is, however, reference to basal basidia dolipore septae not forming in the teleomorph phase, (Tu and Kimbrough, 1978).

The degree of controversy regarding the teleomorph / anamorph taxonomy of the Basidiomycota, especially within the Rhizoctonia, *sensu lato*, is confusing and well beyond the scope of this thesis. The taxonomic interpretation as used by, (Moore, 1985,1987; Tu and Kimbrough,1978), will be used henceforth.

Illustrated in Moore's Diagram, (Fig.B2.1), is a summary of hyphal pore structures that are outlined to show the orientation and characteristics of the various septal pore components and their taxonomic relationships.

Moore cites examples of the various combinations of septal pore structures from the Homobasidiomycetidae sub-order of Basidiomycotina. In the (Fig.B2.1) various combinations of the morphological characters are used to identify the SPA as to genus, for example an O1P1 amalgamation characterizes the majority of "Jelly Fungi" in having; O1 simple granular occlusions and P2 imperforate parenthesomes

The genesis of these structures has largely remained a mystery. However, recent work has shown that the fungal septa of the Agaricomycotina orders of Hymenochaetales and Cantharellales, which display both perforate and imperforate parenthesomes respectively, involves a protein matrix which appears to be derived from the endoplasmic reticulum (ER). The encoding gene SPC18 expresses a septal pore cap (SPC) protein, which was also found to reside in the pore plug occlusion material; this protein contains both a signal peptide to direct it to the ER, and an N-glycosylation motif (van Driel, *et al.,* 2008). The Woronin bodies of the Ascomycota contain the peroxisome HEX-1 protein, (Zekert, Veith and Fischer, 2010), and appear to act in an analogous fashion to the parenthesomes of the Homobasidiomycetidae, (Jedd, 2007).
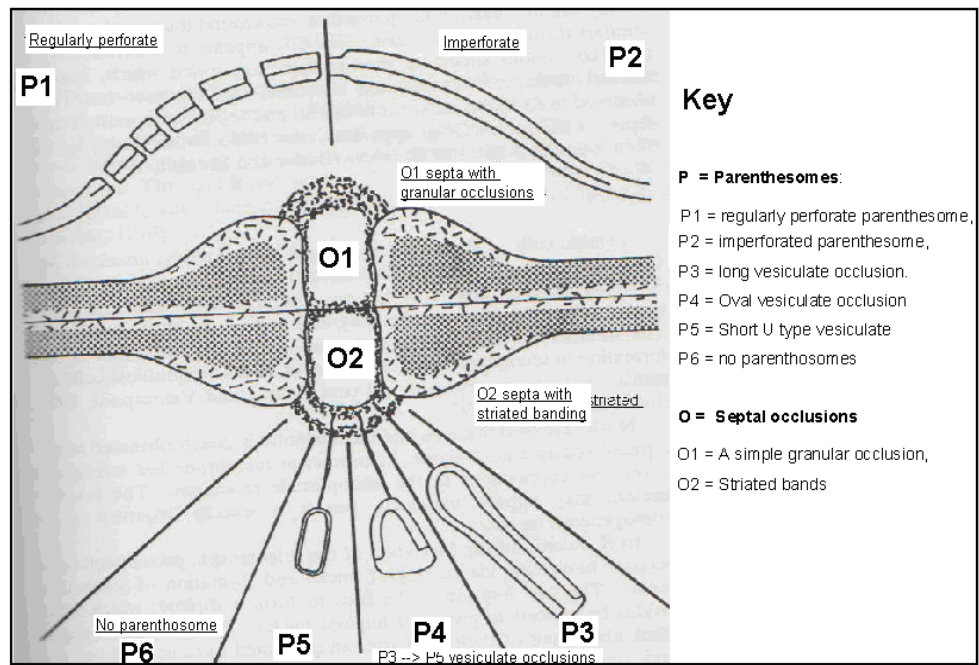
Figure B2. 1 An adaptation of Moore's illustration of septal pore ultrastructure which indicate the anatomical morphology that differentiates the various genera, (Moore, 1985).
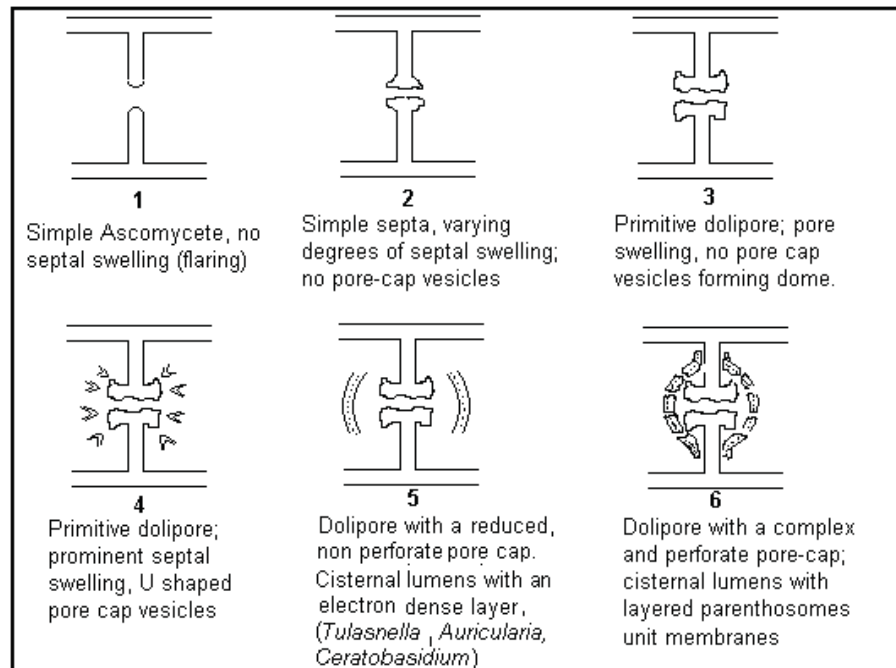
Figure B2. 2 An adaptation of the fungal SPA's characteristic of various filamentous fungal genera. Adapted by the author from, (Tu and Kimbrough, 1978).

An initial key to septal pore structures, (Fig.B2.2), as taxonomic characters was proposed by, Tu and Kimbrough (1978), who separated the ultrastructure detail of the Basidiomycetes into their respective ability to plug or constrain flow through the septa, (Jedd and Chua, 2000). Moore proposed, that fungal septal pore ultrastructure and its teleomorphic taxonomic characters, could be correlated sufficiently and be distinct enough to establish a taxonomy within the Rhizoctonia complex, (Moore, 1987).

Moore assigned various members of the complex into Ascomycetes, Ustomycetes, Holobasidiomycetes and Heterobasidiomycetes. Parenthesome ultrastructure detail is also promoted as a major distinguishing character at the genus level within the Rhizoctonia (*sensu lato*), (Sneh, Burpee and Ogoshi,1991), in their seminal reference "The identification of Rhizoctonia Species".

The TEM micrographs below (Figs.B2.3 - B2.9) show the comparative ultrastructure of the SPA in a range of Basidiomycota and an Ascomycota as illustrated by, (Currah and Sherburne, 1992; Wells and Bandoni, 2001).
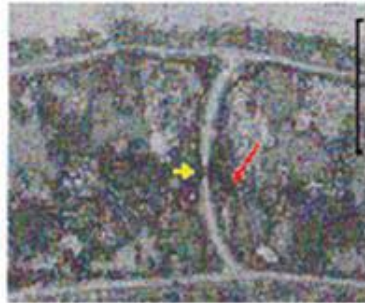
Figure B2.3 A micrograph of a typical *Ascomycete* septum characterized with a simple single pore, centrally located (see yellow arrow) and with electron dense Woronin bodies (see red arrow) on either side . Bar = 1um. From Currah,(1992a) Typified by item 1 in Figure 4.20 above
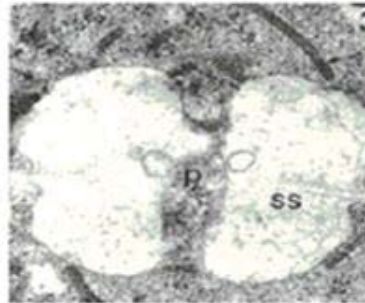
Figure B2.4   A micrograph of *Ceratobasidium cornigerum* showing a median section of a septal pore apparatus (SPA).
SS = septal swelling.
P = pore
Arrow shows portion of a perforated parenthesome
Bar = 0.2 µm Wells (2001)
Typified by item 6 in Figure 4.20 above.
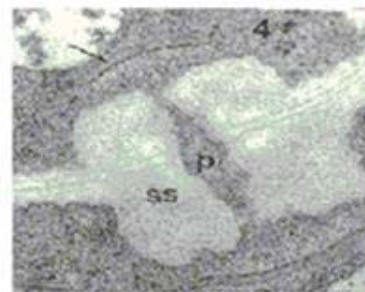
Figure B2.5 A micrograph of a *Tulasnella* sp. Showing a median section of a SPA.

SS = septal swelling.
P = pore
Arrow shows portion of a straight bar like imperforate parenthesome
bar = 0.1 µm
Typified by item 5 in Figure 4.20 above Wells (2001)
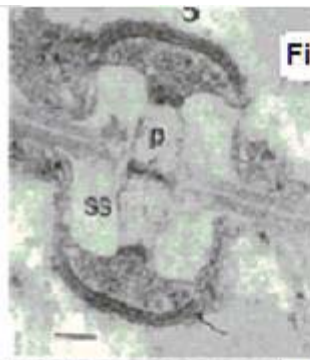
Fig B 2.6 Typical Dactramycetale (*Cerinomyces altaicus*) showing a median section of an SPA.
SS = septal swelling
P = pore
Arrow shows a portion of an imperforate parenthesome. Note the curved character of the bilayered parenthesomes and electron dense pore occlusion plugs typified by item 5 in Diagram 2 above. Bar 0.1 μm Wells (2001).
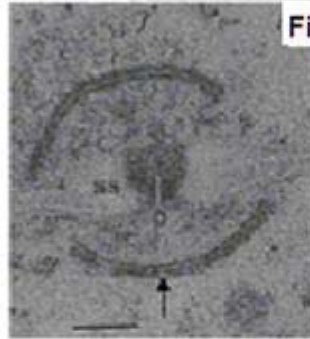
Fig B 2.7 A micrograph of a median section of the Tremulaccea; *Exidiopsis plumbescens* showing the typical SPA morphology.

SS = reduced septal swelling.
P = pore
Arrow shows portion of an imperforate parenthesome. Note the curved character of the bilayered parenthesomes Bar 0.2 μm. Wells (2001).
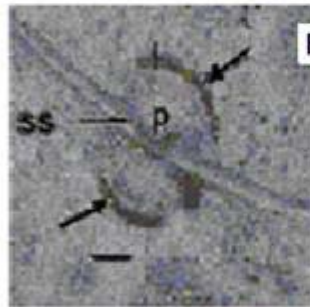
Fig B 2.8 Showing a median section of a *Auricularia* sp. SPA. Arrows show two sections of the domed parenthesome.
SS = very reduced septal swelling.
P = pore
Bar 0.2 μm Wells (2001).
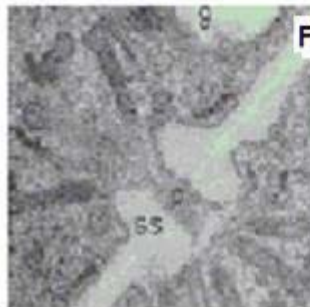
Fig B 2.9 A median section SPA of *Tremella globospora* "Jelly fungi".

SS = septal swelling
P = pore
Arrow indicates a sacculate portion of the parenthosome see Fig 4.20 (4) These structures are termed long vesiculate parenthosomes and have no obvious pore occlusions.
Bar = 0.10μm Wells (2001).

B2.3   Results and discussion

Figure B2.10 is a TEM micrograph showing a portion of a peloton resident hypha, 1.1 µm to 1.4 µm in width with distinct septa and a central pore. All the septa show a distinct swelling surrounding a central pore of varying width (50 – 125 nm) and an approximate pore axis of 550 nm. At either end of the dolipore are electron dense, bar like parenthesome caps with "elbow" like, 50° bends adjacent to the "top" hyphal wall, both of which point away from the septal pore. The parenthesomes measure 500nm (p labeled (left hand) parenthesome) and 470 nm (right hand unlabeled parenthesome).
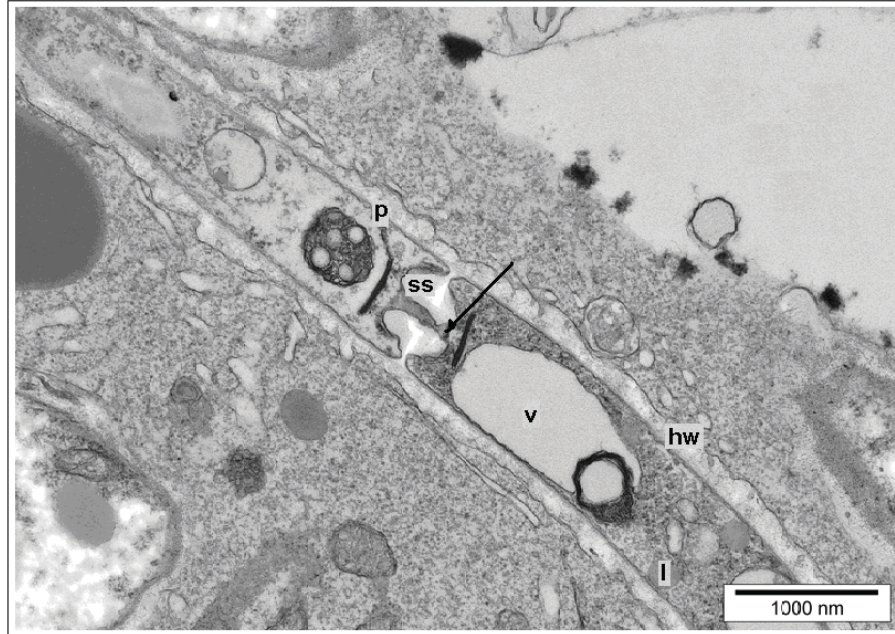
Figure B2.10   A peloton hyphae extracted from a young root of the NZ Terrestrial orchid *N. iridescens.*
Key : ss = septal swelling, p = parenthesome The arrow points to one of two electron dense dolipore plugs (occlusions) at both ends of the dolipore, v = vacuole. Hw = hyphal wall with slime bodies   Bar = 1000 nm
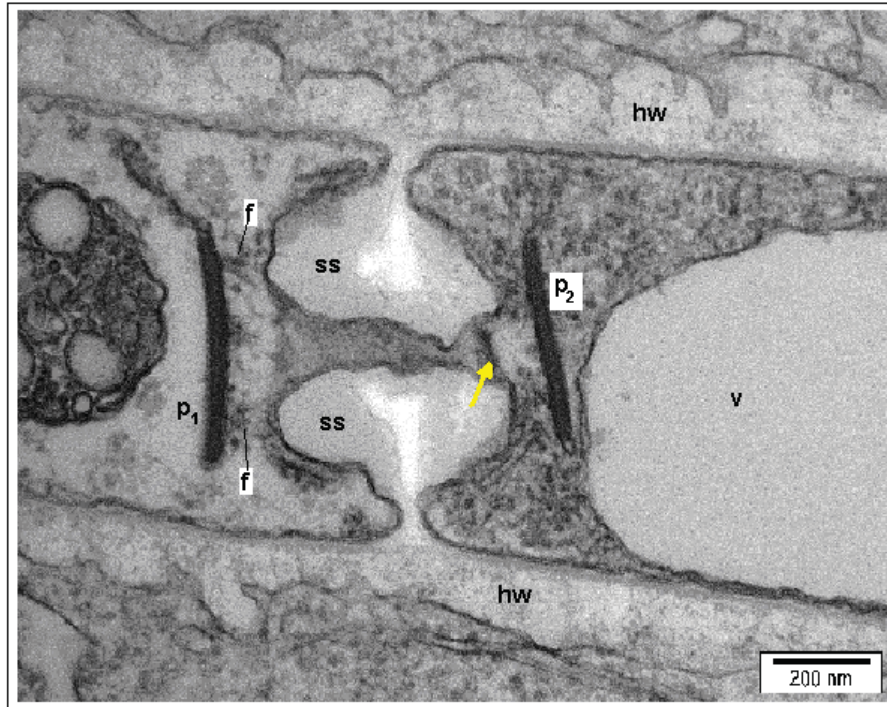
Figure B2.11 The hyphal septa of peloton hyphae from a young root of *N. iridescens.* Key, ss = septal swelling, p1 and p2 are parenthesomes, arrow points to an electron dense dolipore plug (occlusion) between the vacuole and the end of the dolipore, v = vacuole, hw = hyphal wall, d = dolipore lumen, pl = plasmalemma shows as electron dense interior edge to the hyphal lumen and surrounding the interior of the dolipore plus septal swelling, f = possible filaments connecting the parenthesome to the septal swelling . Bar = 200nm.

At a higher magnification, (Fig.B2.11), the micrograph shows the parenthesomes as slightly bowed rods of 450 nm and 420 nm lengths respectively and the previous "50° elbow" appearing as an electron dense area, linking the P_1 parenthesome with the hyphal wall plasmalemma.

Between the parenthesome p1 and the dolipore in Fig.B2.11, connections can be observed (f) which could be contractile filaments, that give a degree of control of the parenthesome, (Orlovich and Ashford, 1994). The angle of these filaments suggests a slight screwing of the parenthesome in contraction rather than a perpendicular pull.
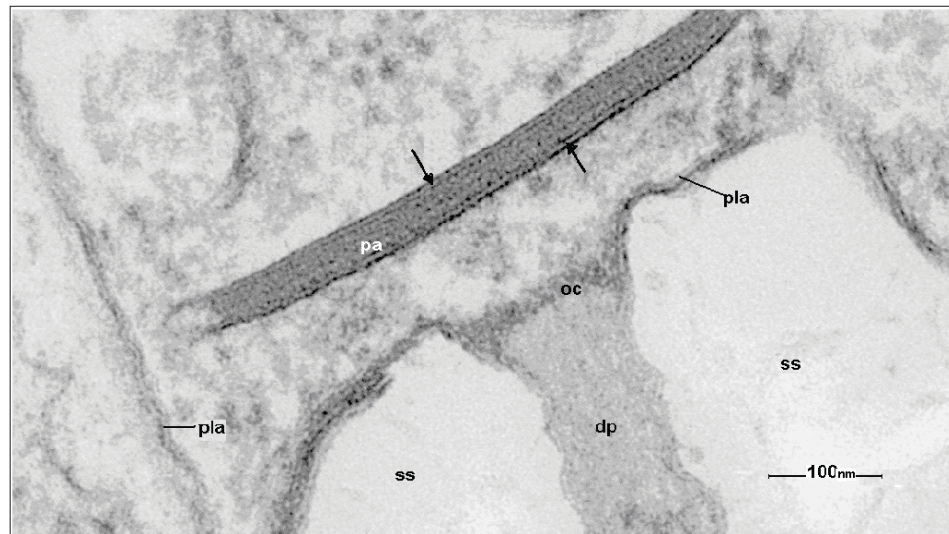
Figure B2.12   The electron dense parallel layers (arrows) making up the parenthesome body (pa).   The plasmalemma (pla) is indicated at the edge of the septal swelling (ss).   The dolipore (dp) is uniform in width (100nm) with the pore orofice being encircled with a granular product (oc). Bar = 100nm

The enhanced image, (Fig.B2.12) of the SPA shows that the parenthesome consists of an outer membrane layer that encloses parallel layers of electron opaque material. The occlusion body, shown in this image, links the outer plasmalemma enclosing the septal pore swelling. The parenthesome measurements are 570 nm in length and have a width of 40 nm.

All the parenthesomes observed in the sampled sections were imperforate and slightly curved, appearing to be end-linked with endoplasmic reticulum (ER) similar to that reported by, (van Driel, Boekhout, Wösten, Verkleij and Müller , 2007; van Driel, *et al.,* 2008).

No Woronin bodies, pore-cap vesicles or cisternal lumens were found in any of the section micrographs studied.

The SPA, in all the resultant micrographs showed dolipore forms characterized with major septal swelling and adjacent to parenthesomes without doming, significant curvature, or perforations.
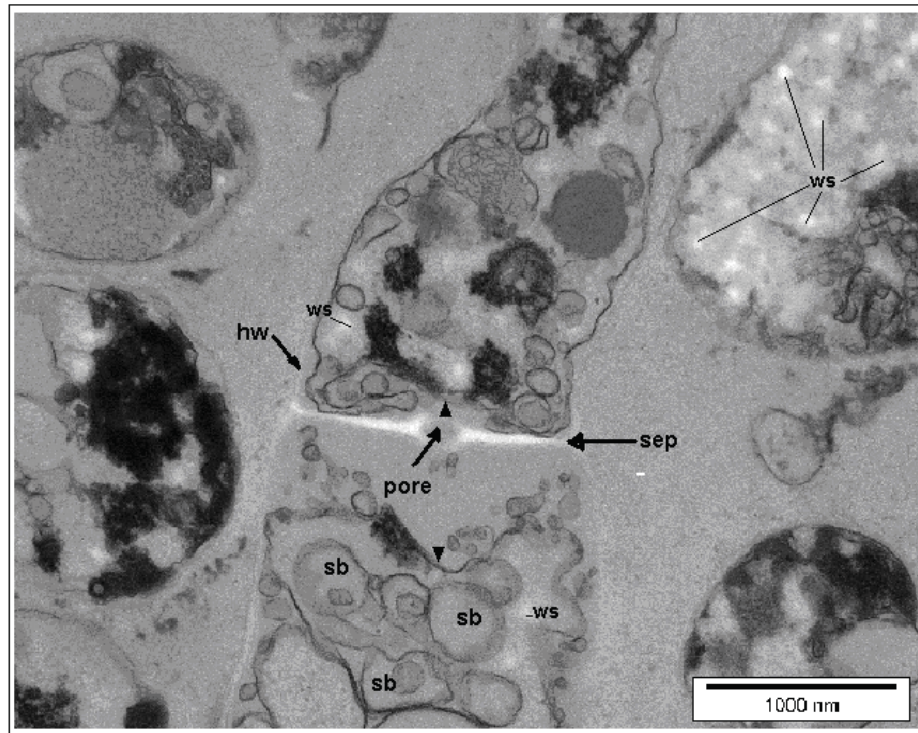
Figure B2.13 A peloton hyphae from an older plant (November) of *N. iridescens* in which hyphae are starting to degrade. The septa of this hyphae can be seen as an intense white "slash" (sep) in the center of the image. The dolipore (pore) is disintegrating. Storage bodies (sb) seen within the lower central hyphae have formed and cell detritus is building up within the hyphae. It is too early to observe whether lysis is occurring, although the white spotting (ws) can be indicative of lysis (Isaac, S 1992). The parenthesomes (▼) are degrading. Bar = 1000 nm

None of the TEM micrographs in this study showed evidence of Woronin bodies but all septa had narrow pore size, 50 – 125 nm typical of dolipore swellings, thin hyphal walls and parenthesomes. The absence of Woronin bodies suggests that the fungi within the pelotons of *N. iridescens* are not Ascomycota. Dolipores capped with imperforate parenthesomes indicate that the fungi are all Heterobasidiomycetes. The micrographs portray a typical Basidiomycota form. Showing swollen septal pore characteristics, dolipores with small diameter and a long pore axis length plus the presence of slightly curved imperforate parenthesomes lacking bowing, doming or vesiculate morphology and having external membranes, indicating the peloton fungus to be a species of the teleomorphic genus *Tulasnella* or the anamorphic form, genus *Epulorhiza*. (See Micrographs Figs B2.10 and 11 and compare the micrograph in Fig B2.5. *Tulasnella*.
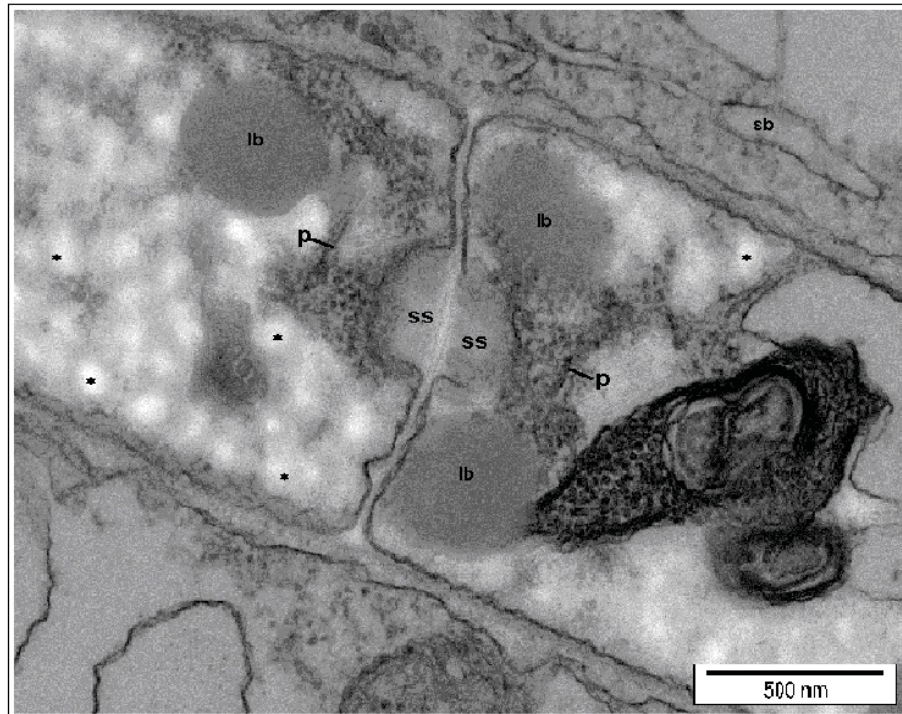
Figure B2.14 The parenthesomes are disinteragating (p) and the central dolipore has been almost eliminated. However the parenthesome image could be an image of a non-central section, cut either above or below a mid-axis line. The septal swelling (ss) remains entire but loses previous definition. Cytoplasm indicated by asterisks (∗) shows the translucent spotting spreading over a wider area than that in Fig. 4c.14. ☐Bar = 500 nm.

Chapter B3 aims to identify, by molecular methods, which species of the genus *Tulasnella – Epulorhiza* complex, forms the peloton endophyte The restriction of identification, by septal pore analysis, is limited to genus and species identification is absent. It does however; assist species identification by indicating primer specificity for molecular sequencing, as will be seen in Chapter B3 Molecular identification. In the case of this thesis, it showed that *Tulasnella* spp were present in the peloton hyphae of *N. iridescens,* even though the first series of PCR amplification and later sequencing proved negative for the endophyte.

Chapter B3   The molecular analysis of the fungal endophyte

B3.1   Introduction

Prior to the advent of fungal molecular analysis, (Gardes *et al.,* 1991; Gardes and Bruns,1993,) the taxonomic identification of mycorrhizal fungal endophytes was a long and laborious process. Which involved culturing, often on specialized media and with specific incubation protocols, to convert anamorphic fungi to the teleomorphic phase. This process, when successful, induced sexual reproductive bodies that could be microscopically analyzed to identify the specific species from their comparative reproductive morphology, (Warcup and Talbot, 1966, 1967; Warcup, 1971). Also available was TEM analysis of hyphal septal pore apparatus (SPA) which could distinguish specimens to genus level, (Moore and McAlear; 1961,1962; Tu and Kimbrough, 1978; Moore, 1985), (see Chapter B2). TEM requires expensive apparatus, expert technique, detailed sample preparation and is not species conclusive.

Gardes and Bruns, (1993), developed a number of fungal specific primers that enabled amplification of the internal transcribed spacer (ITS) regions of the nuclear ribosomal repeat. The ribosomal RNA locus is a highly conserved, tandemly repeated region within the Basidiomycete genome which encodes the three types of rRNA: 18S, 5.8S and the 28S, separated by species variable ITS regions.

The use of the polymerase chain reaction (PCR) amplification, (Mullis and Faloona , 1987), of the ITS of the nuclear ribosomal DNA, with fungal specific primers, has revolutionized the identification of fungi. Generally the ITS region is accepted as the most effective locus, to species level, for endophyte fungus, (Gardes and Bruns, 1993; Sen *et al.*, 1999; McCormick, 2004; Martin and Rygiewicz, 2005; Calonje *et al.*, 2008; LeeTaylor and McCormick, 2008). A more detailed description of the ITS region and mycorrhizal fungal endophyte specific primers is given later in this chapter.

The sequencing and fingerprinting of mycorrhizal fungi, to assist in the identification of specific orchid symbionts, is now well established, (Taylor and Bruns, 1997; Bruns *et al.*, 1998; Sen *et al.*, 1999; Kristiansen *et al.*, 2001; Gronberg *et al.*, 2003; Landeweert *et al.*, 2003; McCormick *et al.* 2004; Bougoure *et al.*, 2005; Martin and Rygiewicz , 2005; Lee Taylor and McCormick, 2008). Copies of the ITS1 and ITS4 primer pair that were initially used for the identification of the endophytes isolated were, at first, used in this project in a similar way.

The two marker regions first used, have previously been successful in the molecular analysis and phenology of the Basidiomyceteae, (Hibbett *et al.*, 1997; Bruns *et al.*, 1998); Bidartondo *et al.*, 2002; Rasmussen, 2002; Landeweert *et al.* ,2003; Chapela and Garbelotto;2004).

The methodology and results sections are presented in two parts, Series 1 and Series 2. This has been due to the initial primer selection, Series 1, yielding few amplicons. Series 2, in this research project, was initiated after reading that fungal mycorrhiza belonging to the Tulasnellaceae, one of the most ubiquitous of orchid mycorrhizal fungi, was not being amplified with the broad-

spectrum Basidiomycete primers, ITS1, ITS4 and ITS5. It was suggested, (Binder *et al.*, 2005; Moncalvo *et al.*, 2006), that the accelerated evolution of the nuclear ribosomal operon was the cause. A specific set of *Tulasnella* primer pairs, ITS4-Tul / ITS-5, was developed by, Lee Taylor and McCormick, (2008), and it was this primer pair that was subsequently used in a new series of PCR amplifications and in re-screening of the earlier DNA extracts from Series 1.

This section, (Chapter B3), aims to identify to species level, for the first time, the hyphal peloton endophyte of the N.Z. endemic terrestrial orchid, *Nematoceras iridescens* and in so doing establish a molecular protocol to facilitate further study of the New Zealand orchid endophytic mycorrhiza. Also included in this section are two further aims, to identify, to species level, the embryonic endophyte, present in field exposed seed of *N. iridescens* and to ascertain if differences occur in the mycorrhizal endophytes of *N. iridescens* between selected geographical growing locations.

B3.2   Results

B3.2.1 Series 1

A summary of the results for the first Series is presented in Tables B3.1 and Table B3.2 (series 1 and 2). For each site, a number of plants were selected – e.g. T1 is the first plant from the Tarata site, U5 is the fifth plant from the Uruti site.   Only roots were extracted, some young and some old and this is indicated by a (young) or b (old).   Tubers are indicated by tub.   Root or rhizome sampling is indicated in the "Tissue" column of the tables 4e.1 and .2. Forty-nine DNA extractions were made in total, from 6 sites: Moki, Uruti, Tarata, Pukeiti, Matemateonga and the authors nursery collection (See Chapter 3 for site locations and description).   The samples represented 3 different orchid genera, (*Nematoceras, Molloybas* and *Singularybas*) all New Zealand CA endemics.   The Hepatophyte (*Aneura* spp.) was included in these DNA extractions to enable an investigation as to the identity of the hyphae present in its tissue, as observed with SEM images and their close association with observed CA plants.

#1	Table of Seies 1 Results					
Code	Plant	Library #	Source	Tissue	Molecular Id	Accession #
1a	Niridescens		Moki	Root	nil	
1b	Niridescens		Moki	Rhizo	nil	
2a	Niridescens		Moki	Root	nil	
2b	Niridescens		Moki	Rhizo	nil	
3a	Niridescens		Moki	Root	nil	
3b	Niridescens		Moki	Rhizo	nil	
4a	Niridescens		Moki	Root	nil	
4b	Niridescens		Moki	Rhizo	nil	
5a	Niridescens		Moki	Root	nil	
5b	Niridescens		Moki	Rhizo	nil	
M1a1	Niridescens	27	Moki	Root	Penicilium	FJ008997
M2a	Niridescens	31	Moki	Root	nil	
M3b1	Niridescens	34	Moki	Root	Neonectria	FJ481038
M3b4	Niridescens	33	Moki	Root	nil	
M4b	Niridescens	30	Moki	Root	nil	
M5b1	Niridescens	29	Moki	Root	nil	
M5b2	Niridescens	R5	Moki	Root	Cryptosporiopsis	AY853167
T2a1	Niridescens		Tarata	Root	nil	
T3a1	Niridescens	A5	Tarata	Root	Ascomycete	AY568066
T3b1	Niridescens	25	Tarata	Root	nil	
T3b2	Niridescens	28	Tarata	Root	Leptodontidium	
T3b4	Niridescens	26	Tarata	Root	Penicillium	FJ008997
U1b	Niridescens		Uruti	Root	Tulasnella	DQ925664
U1tub	Niridescens	9	Uruti	Tuber	nil	
U2a1	Niridescens	16	Uruti	Root	Trichoderma	AJ279483
U2a3	Niridescens	R1	Uruti	Root	Tulasnella	AY634130
U3b	Niridescens	36	Uruti	Root	nil	
U3b1	Niridescens	A8	Uruti	Root	Neonectria	DQ132846
U3b3	Niridescens	15	Uruti	Root	nil	
U3b1numa	Niridescens		Uruti	Root	nil	
U41.2	Niridescens	11	Uruti	Root	Mucor	AY625074
U4a1	Niridescens	14	Uruti	Root	nil	
U4b1	Niridescens	13	Uruti	Root	Rhodotorula	AB026033
U4b2	Niridescens	12	Uruti	Root	nil	

Table B3. 2 (Series 1) One of two tables (#1 and #2) showing the results of the investigation to find the identity of the endophyte within the peloton of *N. iridescens* and other CA species.

#2	Table of Seies 1 Results					
Code	Plant	Library #	Source	Tissue	Molecular Id	Accession #
N112tub	N.iridescens	18	Nursery	Tuber	Trichoderma	AJ279483
N11tub	N.iridescens	19	Nursery	Tuber	Trichoderma	AJ279483
N13tub	N.iridescens	17	Nursery	Tuber	nil	
N14tub	N.iridescens	A3	Nursery	Tuber	Trichoderma	AF218788
N2b1	N.iridescens	24	Nursery	Root	nil	
N2b2	N.iridescens	23	Nursery	Root	Penicillium	FJ008997
N2b4	N.iridescens	22	Nursery	Root	Penicillium	FJ008997
N3a1	N.iridescens	A11	Nursery	Root	Sebacinaceae	AY634132
N3a1	N.iridescens	R3	Nursery	Root	Leptodontidium	AF486133
N3b1	N.iridescens	21	Nursery	Root	nil	
Mollyalba	Mollybas cryptanthus (alba		Matemate	Root	nil	
Mollyred	Mollybas cryptanthus (red)	A7	Matemate	Root	nil	
Mollyred	Mollybas cryptanthus (red)		Matemate	Root	Neonectria	AY295325
Mollyred	M. cryptanthus (red form)		Matemate	Rhizo	nil	
singVR2	Singularybis	A12	Pukeiti	Root	Sebacinaceae	DQO68978
	Aneura		Pukeiti	Root	Basidiomycete	AM260847

Table B3.2   (Series 1)   A continuation of the earlier table B3.1

From the 49 extractions, 22 PCR products were obtained and sequenced. The sequences were compared to sequences in GenBank using the BLAST algorithm from the NCBI website and the default settings, (http://www.ncbi.nlm.nih.gov).   The results from the BLAST search are summarised (Tables B3.1 and B3.2) in the accession column. Twenty-two products produced readable sequences, but only a small proportion of these (four samples; N3a, SingVR2, Ub1 and U2A3, match known orchidaceous mycorrhiza and only two of these, Ub1 and U2A3, match to the genus *Tullasnella*. Matches to *Ascomycete, Sebacinaceae* and *Neonectria* were also made; these fungi have been suggested as being possible ectomycorrhizal orchid symbionts by several authors, (Roy *et al.*, 2009; Stark *et al.*, 2009), but this is still to be confirmed.   *Neonectria* spp are also recognized plant pathogens, (Mantiri *et al.*, 2001).

Further investigation, of the presence *of Trichoderma* spp., would be warranted for future research, since some species of this genus are known to inhibit other filamentous fungi and are often utilized as fungicidal controls, (Verma *et al.*, 2007) An association between a mycoheterotroph and a *Trichoderma* could have the possibility of producing a potential threat to the mycorrhizal endophyte, or conversely, a protection for the orchid against external pathogenic fungi

B3.2   Series 2

The most successful PCR for Series 2 was conducted with the primer pair ITS-4Tul (*Tulasnella* specific primer) and ITS5 (Table B3.3) on DNA that had been extracted using the Bashing Bead technique.

Primer	Target clade	Sequence	Paired 1°	Annealing (°C)
ITS1-OF	All Basidiomycota	Mix these two primers) AACTCGGCCATTTAGAGGAAGT AACTTGGTCATTTAGAGGAAGT	ITS4-OF	60°C
ITS4-OF	All Basidiomycota	GTTACTAGGGGAATCCTTGTT	ITS1-OF	
ITS4-Tul	*Tulasnella* specific	CCGCCAGATTCACACATTGA	or ITS1 ITS5	54°C

Table B3.3. Primers used in the second series of PCR amplifications

4/11/2008	Using Roche MagNA Lyser with Bash*ing Beads* and ITSx / ITS4-OF primer pair				60°C anneal PCR#	Gel
Location	**S p e c i e s**	**Tissue from**	**1°**	**#**		
Tarata, S.East	*N. iridescens*	Old roots	1b	1	1A1	---
Tarata, S.East	*N. iridescens*	Young roots	4a	2	1A2	---
Tarata S East	*N. trilobum*	Old roots	7a	3	1A3	---
Tarata S East	*N. trilobum*	Young roots	8a	4	1A4	---
Tarata North	*N. iridescens*	Young roots	8b	5	1A5	---
Tarata S East	*N. iridescens*	Old roots pestle grd.	9a	6	1Ac1	---
Tarata S East	*N. iridescens*	Young roots pestle gr.	9b	7	1Ac2	---
Control						---

Table B3.4 PCR gel results using MagNA Lyser and the ITS mixed / ITS-OF primer pair with a 60°C annealing temperature.

A series of trials to compare the two (series two) primer pairs and two forms of tissue disruption was undertaken. The results of this trial were recorded (Fig. B3.1, Tables B3.4 and B3.5).

4/11/2008		Using Roche MagNA Lyser with Bash*ing Bead*s and ITS Tul4 / ITS5 primer pair				54°C anneal PCR#	Gel result
Location	S p e c i e s	Tissue from	1°	#			
Tarata, S.East	N. iridescens	Old roots	1b	1		2A1	+++
Tarata, S.East	N. iridescens	Young roots	4a	2		2A2	++++
Tarata S East	N. trilobum	Old roots	7a	3		2A3	+
Tarata S East	N. trilobum	Young roots	8a	4		2A4	++++
Tarata North	N. iridescens	Young roots	8b	5		2A5	+++++
Tarata S East	N. iridescens	Old roots pestle grd.	9a	6		2Ac1	+++++
Control							---
Tarata S East	N. iridescens	Young roots pestle gr.	9b	7		2Ac2	++

Table B3.5

PCR gel results using MagNA Lyser and the ITSTu1-4 / ITS 5 primer pair with a lowered annealing temperature of 54°C

Row 1

Row 2

1 = 1 kb ladder.
2 = N. iridescens B. bead.
3 = N. iridescens B. bead.
4 = N. trilobum   B. bead.
5 = N. trilobum   B. bead.
6 = N. iridescens B. bead.
7 = N. iridescens Pestle
8 = control
9 = N. iridescens Pestle
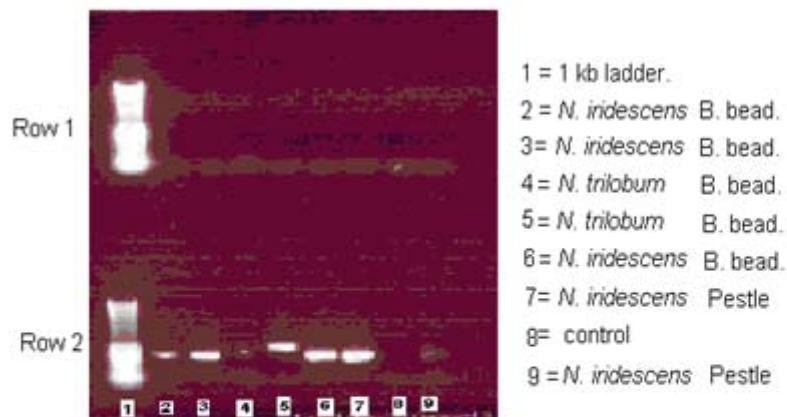
Figure B3. 1 Gel Doc (BIORAD gel electrophoresis of the PCR produced after two different primer pair protocols (ITSx / ITS-O4 Row 1) and (ITS4-Tul / ITS5 Row 2). The Gel Doc image also shows a comparison of the two tissue processing methods, "Basher Bead" tissue disruption v. hand pestle N_2 treatment

PCR products were visualised, as before, and sequenced at MGS. Forward and Reverse sequences were edited against each other and a consensus made using the program Sequencher (GeneCodes TM GCG). All of the sequences were compared to sequences in GenBank using the BLAST algorithm from the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cg) using the nucleotide settings.

The results for Series 2 are shown in Tables B3.4, B3.5, B3.6, B3.7 and B3.8, from a total of 38 samples 15 sequences were obtained (RW01-RW15). In contrast to the results from Series 1, all of these second sequences matched to the genus *Tulasnella*.

Series 1 PCR Repeats				
Code	Genus and spp.	Source	Tissue	Sequence
1R	*S. oblongus*	Puketi	Root	
2R	*S. oblongus*	Puketi	Root	DNS
3R	*S. oblongus*	Puketi	Root	RW15
svar1R	*S.oblongus* (var. single)	Puketi	Root	RW09
svar2R	*S.oblongus* (var. double)	Puketi	Root	RW09
M5b1.1	*N. iridescens*	Moki	Root	DNS
M5b2.2	*N. iridescens*	Moki	Root	RW02
1aMiro	*N. iridescens*	Moki	Root	RW06
1bMiro	*N. iridescens*	Moki	Rhizo	DNS
2aMiro	*N. iridescens*	Moki	Root	DNS
2bMiro	*N. iridescens*	Moki	Rhizo	DNS
3aMiro	*N. iridescens*	Moki	Root	RW01
3bMiro	*N. iridescens*	Moki	Rhizo	DNS
4aMiro	*N. iridescens*	Moki	Root	
4bMiro	*N. iridescens*	Moki	Rhizo	DNS
5aMiro	*N. iridescens*	Moki	Root	
5bMiro	*N. iridescens*	Moki	Rhizo	RW04
N1a	*N. iridescens*	Nursery	Root	RW03
N1b	*N. iridescens*	Nursery	Root	DNS
N2a	*N. iridescens*	Nursery	Root	
N2b	*N. iridescens*	Nursery	Root	
N3a	*N. iridescens*	Nursery	Root	
N3b	*N. iridescens*	Nursery	Root	DNS
N3c	*N. iridescens*	Nursery	Root	
N4b	*N. iridescens*	Nursery	Root	DNS
N5b	*N. iridescens*	Nursery	Root	DNS
N13.5	*N. iridescens*	Nursery	Root	
N14.7	*N. iridescens*	Nursery	Root	
N1b18	*N. iridescens*	Nursery	Root	
N2b19	*N. iridescens*	Nursery	Root	
T3b24	*N. iridescens*	Tarata	Root	RW05
U2a3	*N. iridescens*	Uruti	Root	RW08
Uhi	*N. iridescens*	Uruti	Root	RW07

Table B3.6 Results obtained from retained DNA samples from the 1st series and using the ITS4-Tul / ITS5 primer pair with the PCR amplification **DNS=**Did not sequence

Only one of the seed samples (Seed 1, Table B3.7) produced a faint PCR product; this product failed to sequence initially due to insufficient concentration of the product. This product was then used as template in a second round of PCR with an internal primer (ITS5 and ITS4-Tul.) which gave a short (434bp) product of sufficient quantity to sequence. The sequence was identical to sequence RW03, (*T.calospora*).

2nd series extractions				
Code	Genus / spp.	Source	Tissue	Sequence
2T2A	Culture plug		culture	
M26a	*N.iridescens*	Moki	Root	DNS
M26b	*N.iridescens*	Moki	Root	
M26c	*N.iridescens*	Moki	Root	
M27a	*N.iridescens*	Moki	Root	
M27b	*N.iridescens*	Moki	Root	
M27c	*N.iridescens*	Moki	Root	
M30a	*N.iridescens*	Moki	Root	RW14
M30b	*N.iridescens*	Moki	Root	RW14
M30tub	*N.iridescens*	Moki	tuber	
Ma1tub	*N.iridescens*	Matemateonga	tuber	
Ma2a	*N.iridescens*	Matemateonga	Root	
Ma2b	*N.iridescens*	Matemateonga	Root	
T1a	*N.iridescens*	Tarata	Root	RW13
T1b	*N.iridescens*	Tarata sth	Root	RW11
T1b	*N.iridescens*	Tarata sth	Root	RW11
T1c	*N.iridescens*	Tarata sth	Root	RW11
T2a	*N.iridescens*	Tarata	Root	RW13
T2c	*N.iridescens*	Tarata sth	Root	RW11
T31a	*N.iridescens*	Tarata	Root	
T31b	*N.iridescens*	Tarata	Root	RW14
T3a	*N.iridescens*	Tarata	Root	RW13
T5b	*N.iridescens*	Tarata nth	Root	RW10
U28a	*N.iridescens*	Uruti	Root	
U28b	*N.iridescens*	Uruti	Root	
U29a	*N.iridescens*	Uruti	Root	RW13
U29b	*N.iridescens*	Uruti	Root	RW13
Ttri	*N.trilobus*	Tarata	Root	RW12
Ttri	*N.trilobus*	Tarata	Root	RW12
Macheese	*C.cheesemanii*	Matemateonga	Root	
Umetz	Metzgeriales	Uruti	Root	
T29metz	Metzgeriales	Tarata	Root	
T30metz	Metzgeriales	Tarata	Root	
T31metz	Metzgeriales	Tarata	Root	
Aneura	*Aneura* spp.	Matemateonga	Root	
from seed				
Seed 1	*N.iridescens*	Matemateonga	Seed	RW03 (short)
Seed 2	*N.iridescens*	Matemateonga	Seed	
Seed 3	*N.iridescens*	Matemateonga	Seed	

Table B3.7   Table of the 2nd series of extractions from root and seed tissue using BasherBead preparation and incorporating the ITS4-Tul / ITS5 primer pair with the PCR

Accession #	identity	host	geog.locatio	ref
TULCAL				
AJ313445	Epulorhiza B1	*Spathoglottis*	Singapore	Ma et al
AJ313446	Epulorhiza sp	*Diplocaulobium*	Singapore	Ma et al
AB369931	Epulorhiza sp SO 035	*Cypripedium macranthos*	Japan	Shimura et al (2009)
AY373270	uncult Tul 241	*Goodyera pubescens*	USA	McCormick et al (2004)
AY373298	culture Tulcal CBS 326.47		USA	McCormick et al (2004)
AY634123	uncult Tul 6009	*Epipactis gigantea*	USA	Bidartondo et al
AY643806	Fungal sp AP2	*Acianthus*	Qld, Australia	Bourgoure et al
DQ178089	uncult Tul 7.20.4	*Stelis concinna*	Ecuador	(ACF)Suarez et al (2006)
DQ178097	uncult Tul 9.8	*Stelis concinna*	Ecuador	(ACF)Suarez et al (2006)
DQ178099	uncult Tul C2.1.1	*Pleurothalis lilijae*	Ecuador	(ACF)Suarez et al (2006)
DQ178113	uncult Tul 1.1	*Stelis hallii*	Ecuador	(ACF)Suarez et al (2006)
DQ178114	uncult Tul 1.6	*Stelis hallii*	Ecuador	(ACF)Suarez et al (2006)
DQ178115	uncult Tul C3.MN4	*Stelis superbiens*	Ecuador	(ACF)Suarez et al (2006)
DQ388041	culture TulcalMAFFP305801 J.H. Warcup 07	*Acianthus exsertus*	Australia	(ACF)Suarez et al (2006)
DQ388042	culture TulcalMAFFP305802 J.H. Warcup 0388	*Diuris maculata*	Australia	(ACF)Suarez et al (2006)
DQ388043	culture TulcalMAFFP305803 J.H. Warcup 0584	*Thelymitra aristata*	Australia	(ACF)Suarez et al (2006)
DQ388044	culture TulcalMAFF P305804 J.H. Warcup 0638	*Thelymitra sp.*	Australia	(ACF)Suarez et al (2006)
DQ388045	culture TulcalMAFFP305805 J.H. Warcup 0689	*Thelymitra sp.*	Australia	(ACF)Suarez et al (2006)
EF393621	TulcalShenCF07	*Cymbidium*	China	unpub Yang et al
FJ594913	Epulorhiza sp C0401-2	*Cymbidium*	China	unpub Li et al (2009)
FJ613217	TulcalC0103-23	*Cymbidium*	China	unpub Wang et al
FJ613255	Tulcal C0103-6	*Cymbidium*	China	unpun Wang et al
FJ613180	Tulcal H0402-5	*Cymbidium*	China	unpub Yang et al (2009)
GU166429	Tulcal isolate Ps-AT-0-2	*Paphiopedilum*	Thailand	
DQ368717	uncult Tul Q21b	*Richardii sp*	Ecuador	unpub Kottke et al
AY634130	uncult Tul 4065	*Dactylorhiza majalis*	USA	Bidartondo et al
AM711618	uncult fungus 6OMt76	*Orchis militaris*	Hungary	unpub Ouanphanivanh et al
DQ178071	uncult Tul 1.19b	*Stelis hallii*	Ecuador	(ACF)Suarez et al (2006)
DQ178073	uncult Tul 1.19	*Stelis hallii*	Ecuador	(ACF)Suarez et al (2006)
DQ178072	uncult Tul 1.21	*Stelis hallii*	Ecuador	(ACF)Suarez et al (2006)
DQ388047	Tul asym MAFF P305808 Warcup & P.H.B.Talbot	*Thelymitra epipactoides*	Australia	(ACF)Suarez et al (2006
DQ388048	Tul asym MAFF P305809 J.H. Warcup 0591	*Thelymitra epipactoides*	Australia	(ACF)Suarez et al (2006
DQ388046	Tul asym MAFF P305806 J.H. Warcup 085	*Thelymitra luteocilium*		
DQ520101	Tul asym AFTOL-ID 1678 MAFF 305807 Warcup & P. H. B. Talbot"		Australia	Garnica,S. and Weiss,M.
DQ178099	uncult Tul C2.1.1	*Pleurothallis lilijae*	Andes, Ecuado	(ACF)Suarez et al (2006)
DQ178115	uncult Tul C3 MN4	*Stelis superbiens*	Andes, Ecuado	(ACF)Suarez et al (2006)
AY373270	Tul sp 241	*Goodyera pubescens*	USA	McCormick et al (2004)
AY373295	Tulasnella pruinosa	specimen voucher DAOM 17	USA	McCormick et al (2004)
AY373303	Tulasnella violea	specimen voucher DAOM 21	USA	McCormick et al (2004)
AY373293	Tulasnella eichleriana	specimen voucher KC 852	USA	McCormick et al (2004)
EU218889	Tulasnella irregularis	type strain CBS 574.83	USA	Taylor and McCormick (08)

Table B3.8   GenBank accessions used in this study.

Code	Host		GB Accession #
RW01	*N.irridescens*	NZ	HM802313
RW02	*N.irridescens*	NZ	HM802318
RW03	*N.irridescens*	NZ	HM802310
RW04	*N.irridescens*	NZ	HM802314
RW05	*N.irridescens*	NZ	HM802317
RW06	*N.irridescens*	NZ	HM802319
RW07	*N.irridescens*	NZ	HM802320
RW08	*N.irridescens*	NZ	HM802321
RW09	*N.irridescens*	NZ	HM802322
RW10	*N.irridescens*	NZ	HM802315
RW11	*N.irridescens*	NZ	HM802316
RW12	*N.irridescens*	NZ	HM8023233
RW13	*N.irridescens*	NZ	HM802311
RW14	*N.irridescens*	NZ	HM802312
RW15	*N.irridescens*	NZ	HM802324

Table B3.9. *Tulasnella* NCBI acession numbers from sample sequences gathered in this project

B3.3 Phylogenetic methods

In order to understand how the strains isolated here relate to others of the *Tulasnella* genus, a dataset was prepared that contained all determined sequences and a selection of sequences, identified as highly similar in BLAST analyses (see Tables B3.6-B3.9). Sequences were first aligned by eye in the program Se-AL, (Rambaut, 2002). The *Tulasnella* genus is genetically very diverse; the sequences aligned well at the 5' and 3' ends where the primer binding sites lie, and across the 5.8S region, as was expected, but the internal transcribed spacer regions ITS1 and ITS2 could not be aligned over all sequences, as they were too variable. So the first dataset (dataset 1) includes all the sequences (57) and was trimmed to the region (5.8S) that could be confidently aligned (157bp). Although this a short fragment, it is similar in length to that used successfully by, Suárez, *et al.,* (2006), and its analysis will enable a comparison between their results and those of this study to be made.

Three other datasets (datasets 2, 3,and 4) were also constructed in Se-Al. These were subsets of more closely related accessions from the 57 taxa dataset for which longer regions of the ITS locus could be aligned.: dataset 2:   (8 sequences, 371bp) dataset 3: (28 sequences, 404 bp) dataset 4: (6 seq, 650bp). Neighbour-Joining trees using p-distances were constructed with SplitsTree 4.

The finding that the genetic diversity among *Tulasnella* precludes the alignment of ITS1 and 2 for many taxa has been previously reported , (Weiß and Oberwinkler, 2001; Taylor et al., 2003; Suárez et al., 2006).

The Neighbour-Joining tree (Fig. B3.2) reconstructed from dataset 1 shows that the mycorrhiza strains isolated in this study, fall into four groups 1, 2, 3 and 4. The relationships within the four groups are described below.
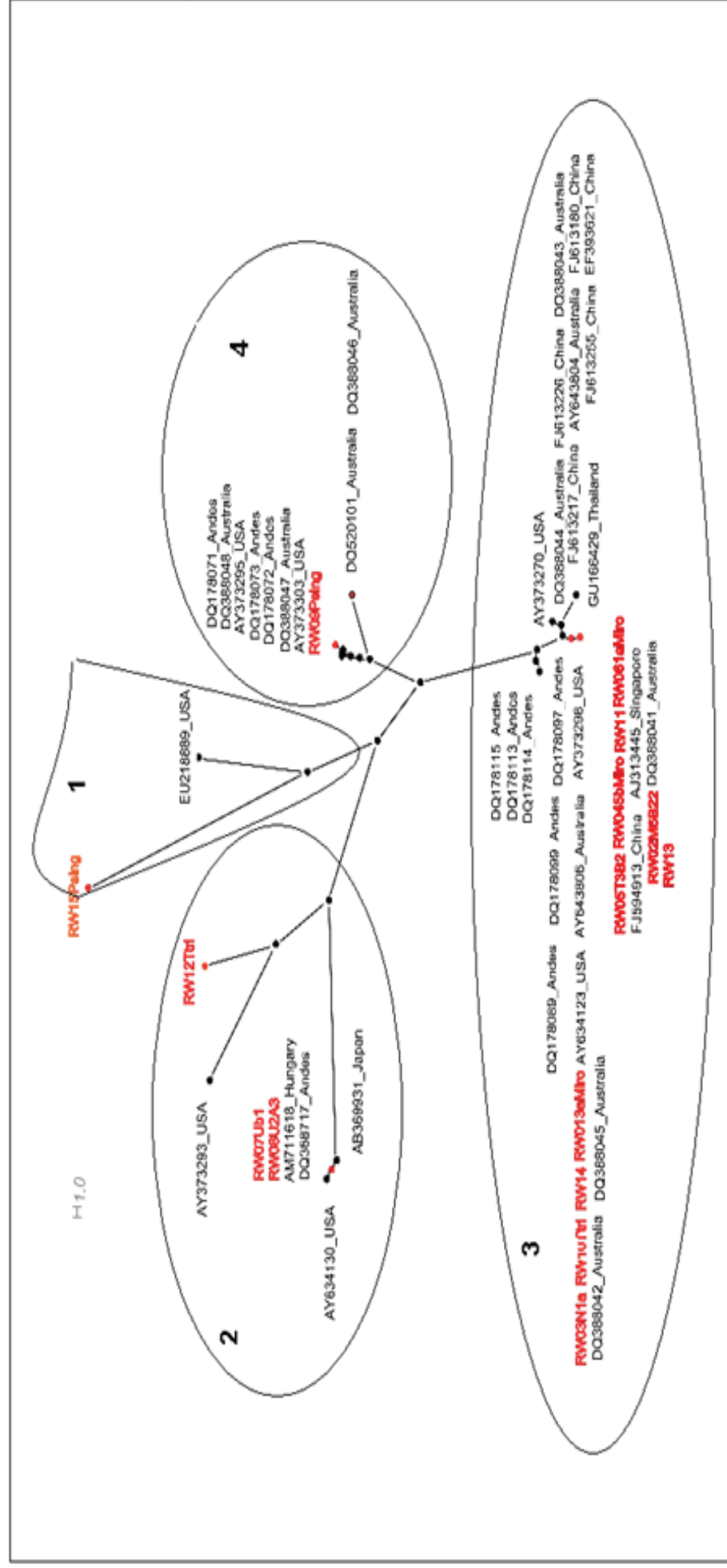
Figure B3.2 Neighbour-Joining tree using dataset 1 (57 taxa, 157bp aligned) showing the great diversity within the genus Tulasnella.

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B3.3.1   Group 1   (Fig. B3.3) contains the single sequence RW15, (HM802324), the endophyte isolated from the orchid Singularybas oblongus from the Pukeiti site. This fungal strain is quite different from any other reported strain, with the closest relative being *Tulasnella irregularis* isolated from the orchid *Dactylorhiza majalis* from Australia by, Taylor and McCormick, (2008).
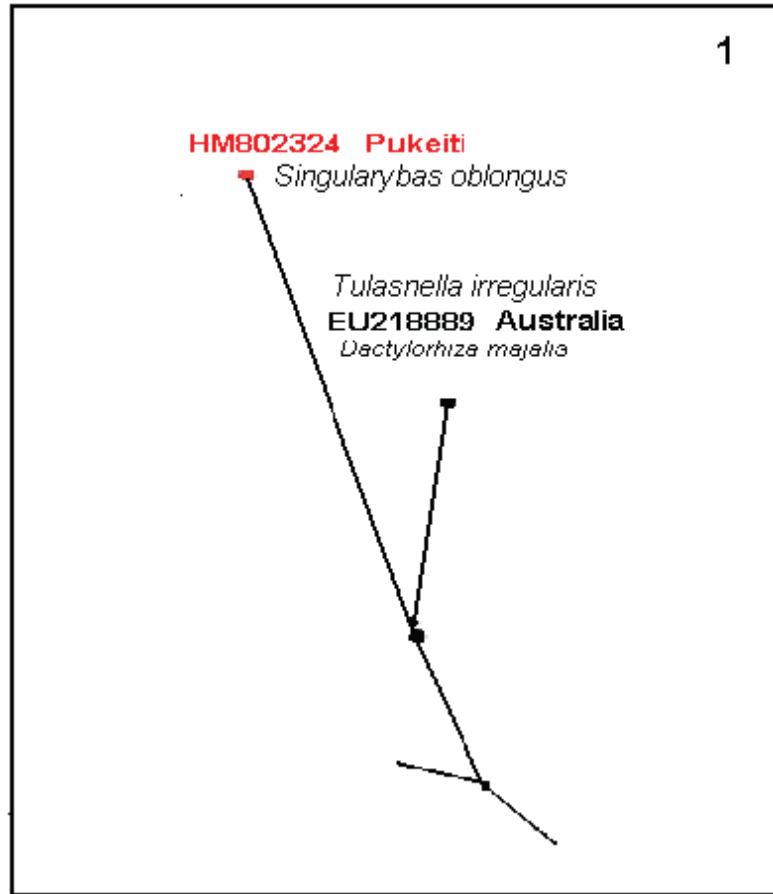
Figure B3. 3 Group 1 from the phylogenic tree

B3.3. 2   Group 2

(Figure B3.4) contains 3 sequences from this study, RW07   (HM802320), RW08 (HM802321) and RW12 (HM802323). RW07 and 08, were isolated from *Nematoceras iridescens* orchids from the Uruti site; these strains match closely to a strain isolated from the Hepatophyte, Riccardia DQ368717, from the South American Andes, (unpublished data, Kotke et al).   An uncultured mycorrhiza of Tulasnellales, RW12 is more unique, this strain was isolated from *Nematoceras trilobus* from the Tarata site and the closest relative for this strain is the cultured type strain *Tulasnella violea* from the USA (Bidartondo, *et al.*, 2004).
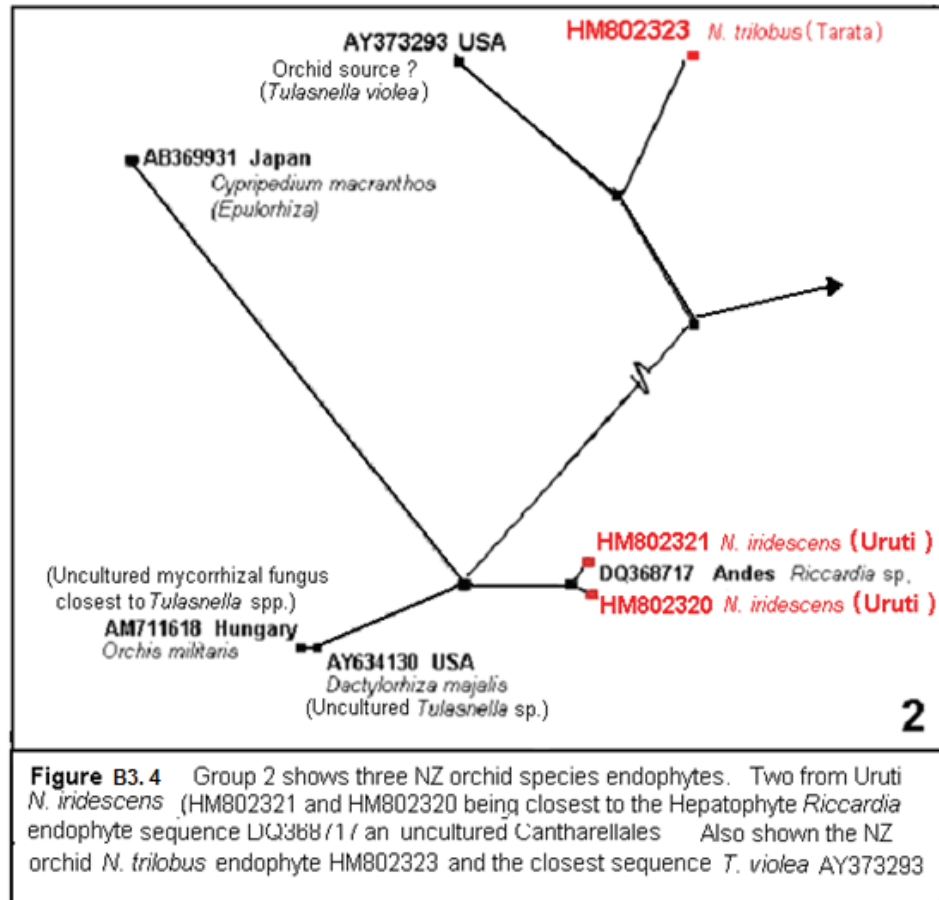
Figure B3. 4   Group 2 shows three NZ orchid species endophytes. Two from Uruti *N. iridescens* (HM802321 and HM802320 being closest to the Hepatophyte *Riccardia* endophyte sequence DQ368717 an uncultured Cantharellales   Also shown the NZ orchid *N. trilobus* endophyte HM802323 and the closest sequence *T. violea* AY373293
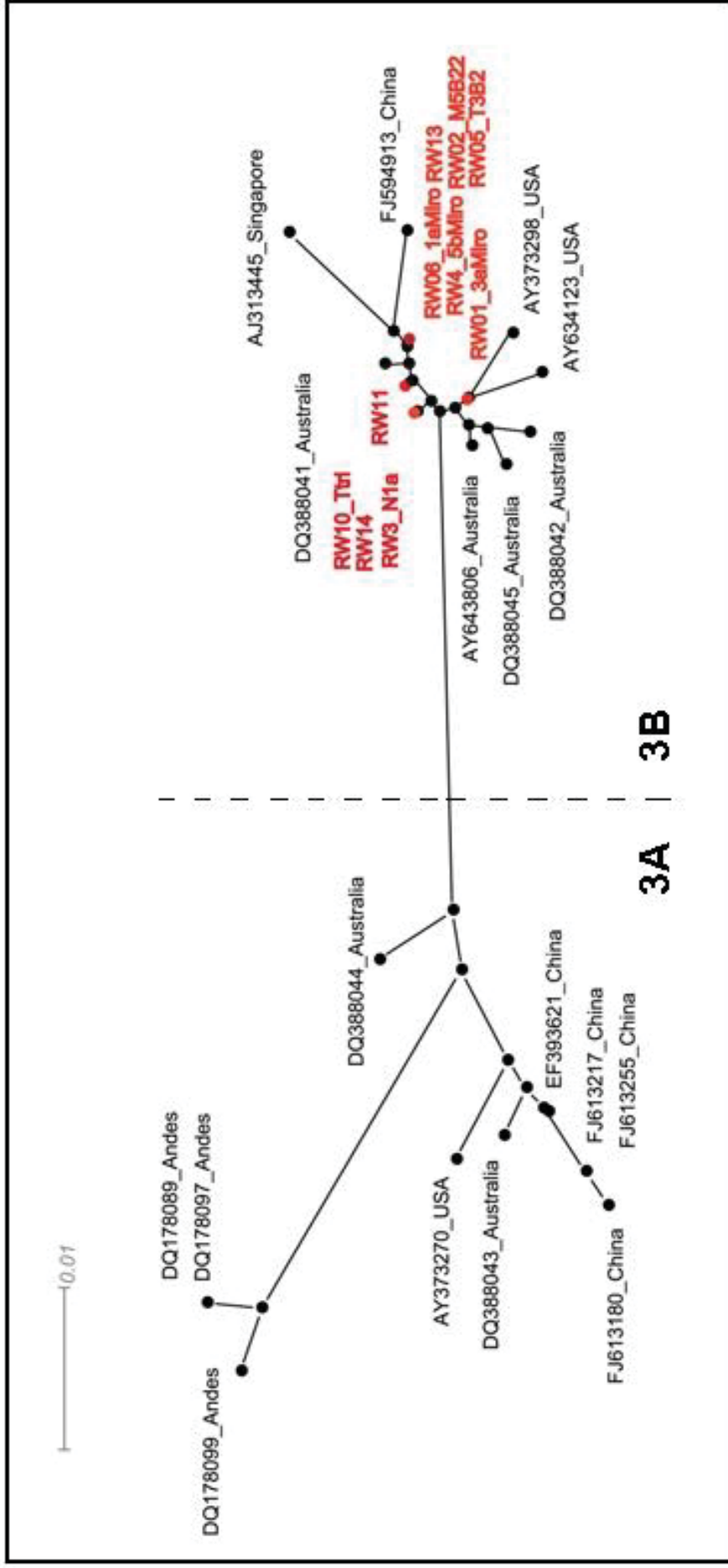
Figure B3. 5 Neighbour-Joining tree using dataset 3 (28 taxa, 404bp aligned).

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B3.3.3

Group 3 was further sub-divided into two groups, 3a and 3b which reflects genetic diversity, geographical and host species differences.

Most of the sequences determined in this study fall into Group 3, (Fig. B3.2 and Fig. B3.5 –Dataset 3), these sequences are very closely related, differing only in a few base pairs from each other and from GenBank strains identified as *Tulasnella calospora*   (eg. AY373298, DQ388041, see Table B3.7). All sequences in Group 3 were isolated from endophytes growing in *Nematoceras iridescens* orchids or seed. The sequence RW03 was found twice – from a plant grown in the nursery and from seed.

RW11 was found four times, in plants from the Tarata site. RW13 was found five times, twice in plants from the Uruti site and three times in plants from the Tarata site. Sequence RW14 was found three times, twice in plants from the Moki site and once in a plant from the Tarata site. The New Zealand sequences are most closely related to sequences from *Tulasnella* strains from a variety of orchids (*Epipactis gigantea*, *Acianthus* sp, *Diunis maculata*, *Spathaglotis plicata* and *Cymbidium* spp) from Singapore, China, Australia and USA   (Fig 4e7). Overall, Group 3 contains species that are closely related yet extend over a wide host and geographic range including strains from the orchids *Pleurothallis lilijea* and *Stelis concinna* from the Andes, *Thelymitra* spp and *Goodyera pubescens* from Australia and *Cymbidium* spp from China.

B3.3.4

The 3A group   (Fig.B3.6) contained orchid species from Ecuador   (3), USA (1), Australia   (2) and China   (2). All extracted fungal sequences from Ecuador, submitted to NCBI BLAST, were of uncultured *Tulasnella* their closest neighbouring sequences were from the Chinese *Cymbidium* spp. Mycorrhizal fungi *Tulasnella calospora* isolates (EF393621, FJ13217, FJ13255 and FJ613180). The Australian orchid species genus *Thelymitra*, also represented in NZ , provided fungal sequences close to the *T. calospora* strains MAFF P305804 and MAFF P305804" . The Andean cloud forest paper, Suárez, *et al.* , (2006), cited these strain sequences in their review of orchid mycorrhiza in Andean orchids (DQ178089, DQ178097). No NZ orchid fungal sequences were included in Group 3a..

3A

Pleurothallis lilijae (Tulasnella uncultured)
DQ178099 Andes

DQ178089 Andes
Stelis concinna (Tulasnella uncultured 7.20.4)

DQ178097 Andes
Stelis concinna (Tulasnella uncultured 9.8)

AY373270 USA
Goodyera pubescens (Tulasnella sp. 241)

DQ388043 Australia
Thelymitra aristata
(Tulasnella calospora strain MAFF305803)

FJ613180 China   Cymbidium faberii (Tulasnella calospora)

EF393621 China   Cymbidium floribundium (Tulasnella calospora)

FJ13217 China   Cymbidium goeringii (Tulasnella calospora)

FJ13255 China   Cymbidium goeringii (Tulasnella calospora)

DQ388044 Australia
Thelymitra sp.
(Tulasnella calospora strain MAFF P305804)

Figure B3. 6   Group 3A Neighbour-Joining tree using dataset 3   (8 taxa, 404bp aligned

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3B

AJ313445 **Singapore**
Spathaglotis plicata (Epulorhiza sp. B1)

DQ388041 **Australia**
Acianthus exsertus (Tulasnella calospora MAFF P305801)

Cymbidium (Epulorhiza sp. CO401-2)
FJ594913 **China**

HM802319 **Moki**
HM802314 **Moki**
HM802313 **Moki**
HM802317 **Tarata**
HM802318 **Moki**
HM802311 **Tarata**

HM802315 **Tarata Nth.**
HM802312 **Tarata Sth.**
HM802316 **Tarata Sth.**
HM802310 **Seed Matemateonga**

AY373298 **USA**
Orchid host not recorded *(Tulasnella calospora)*

AY634123 **USA**
Epipactis gigantea (Tulasnella uncultured)

AY643806 **Australia**
Acianthus pusillus (Fungal sp. AP2)

DQ388045 **Australia**
Tulasnella calospora MAFF P305805)

DQ388042 **Australia**
Tulasnella calospora uncultured MAFF P305802)

Epiphytic orchid ? *(Tulasnella calospora)*

Diuris maculata (Tulasnella calospora uncultured)

Figure B3.7   Group 3B Neighbour-Joining tree using dataset 2   (8 taxa, 371bp aligned).

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B3.3.5   Group 3b   (Fig.B3.7) contained the bulk of the NZ *Nematoceras iridescens* orchid fungal sequences (HM802311 –> HM802319) plus the germinated seed sample (HM802310). Fungal sequences within this group have been assigned to *Tulasnella calospora* (DQ388045, DQ388042 and AY634123) and to *Epulorhiza* (AJ313445, FJ594913) which is the anamorph of *Tulasnella calospora.* The sequences from NZ appear to consist of unique strains of *T. calospora*. The reference isolates have been isolated across a wide geographic range   (Asia, Australia and USA).
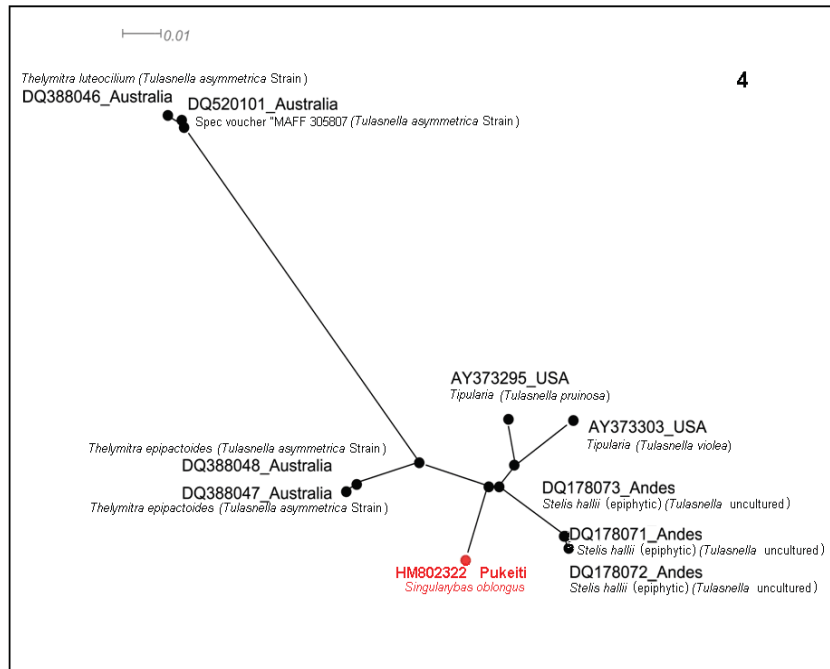
Figure B3. 8 Neighbour-Joining tree using dataset 3 (28 taxa,404bpaligned).

B3.3.6   Group 4   (Fig. B3.2 and Fig. B3.8) contains a single sequence RW09 from a strain isolated twice, from the orchid *Singularyabas oblongus* from the Puketi site. Close phylogenetic relatives are *T. pruinosa, T. violea and T. asymetrica* isolated from a variety of orchids (*Stelis, Thelymitra, Tipularia* species) from Australia, South America and USA (see Fig. B3.8).

Group 4 has the highest diversity of cultured and uncultured *Tulasnella* species including   (*T. asymmetrica, T. pruinosa* and *T. violea.* None of the host orchids in Group 4, apart from *Singularybas*, are members of the Acianthinae in which the entire CA taxa is nested..

In their study of mycorrhiza isolated from epiphytic orchids in an Andean rainforest, Suarez et al., (2006), identified 7 clades (A-G) in their data analyses. The diversity in clades A-C is represented in analyses of this data   (Group 3). Their clade E occurs in Group 4. Sequences from Groups 1 and 2 fall outside the diversity of clades identified in Suarez et al.

B3.4   Discussion

The main objective of this thesis was to identify the endophyte forming the peloton of *N. iridescens* in New Zealand. We show here that most isolates were found to be closely related to *Tulasnella calospora*.

Identification of orchid seed mycorrhiza, has to date, relied on either the culture of the mycorrhizal fungus followed by molecular analysis or the prior identification of an adult plant mycorrhiza which is then cultured and seed introduced to a culture plug (symbiotic germination); the eventual germination of the seed being associated with the introduced fungal culture.

Orchid seed has an obligate mycoheterotrophic requirement, (Clements, 1981; Warcup, 1981; Rasmussen and Whigham,1998; Arditti and Ghani, 2000; McKendrick *et al.*, 2000; Esitken *et al.,* 2005; Stewart and Kane, 2006; Bidartondo and Read, 2008), that differs from that of the mature plant in the majority of cases, (Clements, 1981; Peterson *et al.*, 1998; Otero *et al.*, 2002; Rasmussen, 2002; Brundrett, 2004; Bonnardeaux *et al.*, 2007).

Imaging, by CLSM of the orchid seed mycorrhiza in situ (Fig. A1.20) and the molecular analysis of the germinating seed confirmed that the mycorhiza was a strain of *T. calospora*   (RW03). The seed was germinated in a field trial envelope at Matemateonga, after 12 months in situ.

From our result, using seed, we propose that a strain of *T.calospora* is involved in the initiation of *Nematoceras iridescens* seed germination; however this hypothesis needs to be tested with characterisation of further samples.

The mycorrhiza *Tulasnella* is a very diverse genus and the "Tul- specific" primer pairs ITS4-Tul / ITS5 amplify very different ITS sequences, some, in fact, so different that they can only be aligned over the conserved 5.8S region. It has been noted by other authors that there are taxonomic problems with the naming of the species in this genus, (Roberts, 1999; Bidartondo *et al.*, 2004; Suarez *et al.*, 2006; Warcup, 1981). For example, in our tree, strains named *T .violea* appear in two well separated groups (AY373303, group 4, AY373293 group 2 Fig. B3.2).

It has already been suggested that the *T.calospora* group (Group3) may be several species, (Shefferson and Simms, 2007; Yukawa *et al.,* 2009) and that the full diversity of these endophytes has yet to be sampled, (Suarez *et al.,* 2006; Shefferson *et al.,* 2007). Our study adds 15 unique sequences and while most are closely related to known strains, two are quite different   (RW12 and RW15, Fig B3.2). The mycorrhiza *T. calospora* appears to be a very diverse species with a proliferation of strains and would appear to require a thorough taxonomic review, (Cruz *et al.*, 2010).

It is a tantalising result that the sequences isolated from strains from the *Singularybas* orchid and from *Nematoceras trilobus* (RW12, RW15, and RW09) are so different. The *N. trilobus* plants were taken from the same site as *N.iridescens* plants that carried a *T.calospora* endophyte   (RW11, RW13, RW14, Tarata).

Specificity of mycorrhizal fungi to a host species has been researched as early as 1970, Hadley (1970). This author concluded there was no apparent specificity of mycorrhizal orchid fungi after investigating the development of mycorrhiza from thirty-two introduced *Rhizoctonia* spp. Isolated from orchids. One sample, *Tulasnella calospora* in particular, provided a high degree of mycorrhizal induction in a large range of orchids and was suggested as a possible universal orchid symbiont.

Strains of the same sequence type were found at different sites (RW14 – Moki and Tarata, RW13 – Uruti and Tarata) and matched closely to sequences from strains from other countries (Australia, China, USA, South America, (Figs. B3.2 – B3.6), reflecting the known cosmopolitan nature of the *Tulasnella* genus in soils, (Tupac *et al.*, 2002; Diez, 2007).

Most of the mycorrhiza from *Nematoceras iridescens* are *T.calospora*, however two strains, represented by the sequences RW07 and RW08, both from the Uruti site seem to be something different, matching most closely to a strain from the Metzgeriale Aneuraceae *Riccardia* (DQ368717) an Ecuadorian liverwort, (Kottke and Nebel, 2005; Preußing *et al.*, 2010).

Orchid mycorrhizas have been shown to occur in the common ancestor of the Orchidaceae and appear to have shifted from the Glomeromycota to a Basidiomycota clade associated with tulasnelloid, ceratobasidioid, and sebacinoid fungi, (Shefferson *et al.*, 2005). Several unique mycorrhizal characteristics may have contributed to the diversification of the family. However, the origin of and the diversity of orchid mycorrhiza associated with orchids remains unclear, (Yukawa *et al.*, 2009).

As mentioned earlier, the liverwort, *Aneura orbiculata*, has been ubiquitous in its association with all *Nematoceras* spp. samples collected for this thesis. Efforts to sequence fungal samples of *Aneura* were unsuccessful although SEM images of *Aneura* showed hyphae very similar to those found in the *Nematoceras iridescens* root cortex. In series one, tissue of *Aneura orbiculata* was cultured and isolates were sequenced with the closest sequence being a Basidiomycota (AM260847 see Table B3.2).

Species of *Tulasnella* have a much longer evolutionary history as mycorrhizal endophytes of the Hepatophyte orders of Jungermanniales and Metzgeriales, (Bidartondo, 2003; Kottke, 2005).

The first Hepatophyte-like land plants, in the early Devonian (400 million years ago), had fungal associations resembling vesicular–arbuscular mycorrhizas (VAM) before the evidence of true roots, (Brundrett, 2002; Selosse, 2005). Evidence of mycorrhizal endophytes in the Metzgeriales and Jungermanniales in Ecuador and Europe gave evidence of *Tulasnella* spp. Mycorrhiza in *Aneura* and other thallose liverworts, (Preußing *et al.*, 2010). The Hepatophyte taxon, in these trees, is represented by the Metzgeriale *Riccardia* Figure 4e.7 (DQ368717) which had a fungal sequence that was similar to the fungal sequence found in *N. iridescens* plants collected at Uruti, (HM802320 and HM802321).

Mycorrhizal diversity appears to be lower in achlorophylous orchids, (McCormick, *et al.*, 2004). In the NZ CA, *Mollybas cryptanthus* is the sole representative. Only

one sample from three, in series 1, was sequenced in this project. The closest sequence match was *Neonectria* (AY295325). A further analysis on the threatened *Mollybas cryptanthus* would be justified using the series 2 protocols with the ITS4-Tul / ITS5 primer pair.

In photosynthetic orchids, mycorrhizal fungal diversity was found to be higher in adult orchids than in germinating seed and protocorms, (McCormick, *et al.,* 2004).

Further research would be a valuable aid in the conservation of threatened orchid species by utilising techniques such as Next Generation Sequencing, (Baker, 2010), of soils to identify potential fungal mycorrhiza, (Bräutigam and Gowik, 2010).

Once identified and co-related to symbiotic seed germinators, mycorrhizal species locations for establishment could be ascertained or soil samples containing proven mycorrhizal endophytes could be transported to suitable geographic positions for re-establishment of threatened orchid species.

Section C   Summary of the Thesis

This thesis supports the overall statement that orchids, due to their ability to adapt to their environment, have been one of the most successful of the plant families, (Arditti, 1992). However, with specialization added risks become apparent and diverse techniques of conservation are required, (Larcher, 2003).

Long distance seed dispersal of CA, from Australia or Papua New Guinea, is unsubstantiated and lacks empirical evidence, since the tracking of identified "dust seed" over very long distances is impractical. A wide biogeographic footprint for the CA taxa is apparent and supported by taxonomic and botanical collections. Very diverse, remote and largely unexplored high altitude areas within the biogeographic footprint suggest that there are many, as yet undiscovered, CA species, (van Royen, 1983; Dransfield, *et al.,* 1986). However, molecular sequencing, (Jones, *et al.*, 2002; Lockhart, 2004), could provide evidence of origin.

It would appear that the New Zealand CA could have originated from founder events arising from "dust seed" via wind distribution from Australia and / or Papua New Guinea. Vectors of distribution for CA seed, such as wind pattern coordination between source, carriage and deposition have analogs in long distance transport, of other plant species, dust particles, small animals and fungal spores from Australia, (Tomlinson, 1973; Close *et al.,* 1978; McKenzie, 1998; Viljanen-Rollinson *et al.,* 2002). Conditions that are required for the successful establishment of CA, such as; suitable climate, ample hydration, suitable environment, and effective pollinators are present along the west coast, and high range areas in New Zealand. This thesis, identifies four systems that could provide avenues for effective dispersal of the seeds of the CA orchids. Local microclimate winds provide the initial dispersal, both localised and uplift, into the hill valley winds. Both Australian and Papua-New Guinea valley winds provide access to stronger trajectory winds with a long distance capacity and finally in high country impact winds which can unload seed both directly and in rain droplets onto land sites (Palmer, 1942; Sturman, 1983; Viljanen-Rollinson, *et al.,* 2002; Felicísimo, *et al.,* 2004; Munoz, *et al.,* 2004; Joseph-Wright *et al.*, 2008).

The lignified testa and the balloon effect of the seed provide flotation, (Arditti, 1992; Arditti and Ghani, 2000). The hydrophobic nature was apparent in the difficulty experienced in hydrating the seed during this, and others research. The ability to support the seed in water creates a further land dispersion avenue via water run off and streams, (Rasmussen, 1993, 1995).

Adaptations by *Nematoceras* spp., such as peduncle extension of the pod into suitable wind currents, aerodynamically advantaged seed produced in large numbers and in suitable seasonal periods, enable the provision of an efficient long distance seed dispersal system.

The presence of suitable symbiotic mycorrhizal fungi is necessary to satisfy the obligate mycoheterotrophic orchid seed germination requirements. Did the fungus travel with the seed or independently via spores carried by similar wind vectors or were mycorrhizal fungi already present in New Zealand?

Hepatophyte presence, such as Metzgeriales, have been found in fossils from the Devonian era, (Krings, *et al.,* 2007), and fossil evidence shows the presence of arbuscular mycorrhiza as endophytes within these early hepatophytes, (Remy, *et al.*, 1994). The extant species of Metzgeriales inhabit the tropics, subtropics, and temperate regions of the Southern Hemisphere, with centres of diversity occurring in South America and Australasia, (Schuster, 1984). Many of the hepatophytes are represented in the Gondwana paleology, (Feldberg, *et al.*, 2007). Research shows similar tulasnelloid mycorrhizal fungi are present in the cosmopolitan thalloid liverwort *Aneura,* (Ligrone, et al., 1993; Preußing, et al., 2010), and in the *N. iridescens* plants collected at Uruti (HM802320 and HM802321). Most of the mycorrhiza from *Nematoceras iridescens* are *Tulasnella calospora*, however, two strains, represented by the sequences RW7 (HM802320) and RW8 (HM802321), both from the Uruti site, seem to be something different, matching most closely to a *T.calospora* strain from the Metzgeriale Aneuraceae *Riccardia* (DQ368717) an Ecuadorian liverwort, (Kottke and Nebel, 2005, Preußing, *et al.,* 2010). Both *Aneura* and *Riccardia* are represented in New Zealand, (Allison and Child, 1975), within similar growing situations as many CA species. This would suggest that a range of suitable fungal endophytes were already established in NZ and able to initiate germination of mycoheterotrophic orchid seed.

Conservation of *Nematoceras* species in New Zealand would be enhanced by introducing plugs, or seed, of threatened *Nematoceras* species into areas supporting those Metzgeriales in which Tulasnellaceae endophytes are present.

Based on the definition of the term mycorrhiza, should the endophyte be termed "a mycorrhizal fungus"? The generally accepted definition of the term, mycorrhizal fungus, is, "a fungus which grows in association with the roots of a plant in a symbiotic or mildly pathogenic relationship, (Beentje, 2010). While the term "association" is applicable, as seen in the micrographs presented in this thesis, no evidence, as to the fungal entity being symbiotic or parasitic, is presented. The group Rhizoctonia incorporates many fungal pathogens, (Agrios, 1988). Cell necrosis has not been in evidence within the "host" plant but hyphal mycophagy, (Leveau and Preston, 2008), is in evidence within the digestive cells of the cortex, as seen in the hyphal lysis micrographs imaged from SEM and LM microscopy. In this case, parasitisation of the fungal hyphae by the orchid occurs without the hyphae receiving any obvious benefit. A chronological nutrient exchange between the endophyte and "host" plant could be worthy of further study. The fungi appear, from this research, to provide for the plants germination and nutrition, apparently without receiving any apparent benefit.

Three different hyphal morphologies occur in the SEM micrographs examined; an early tape like form, a tubular thick walled and a late tape form. Some suspicion of the sample preparation for SEM and whether the fixing and dehydration processing has any significant effect on the morphology of hyphae, should be considered in the hyphal micrograph interpretation, (O'Connell and Carzaniga, 2001; Hoppert, 2003).

The tubular hyphae appear to have two phases: one, a minimal branching form and two, a multiple divaricating form. Type one hyphae, are present in the initial trichome cell penetration and in intercellular cortex cell wall invasion. Type two hyphae, commence divarication from a swollen hyphal tip and assume a congested mass with short branch internodes. It is these hyphae that, after achieving a volume of high consolidation, proceed to lyse.

A recent paper, Lee and Lu, (2011), suggests that plasmodesmata are a prime entry point for fungi and other pathogens into plant cells and that plants have a defensive mechanism to control the spread of infections. Unfortunately, the infected plasmalemma, of *Nematoceras* species cells, were not examined for hyphae entering the plasmodesmata portals into adjacent cells, and this remains a field for further research. External hyphae enter the *N. iridescens* root through the trichome at a specific point, to verify this specificity would require many more observations, since only one micrograph was taken.

Cells, in certain areas of the CA plants examined, resist hyphal penetration thus creating distinct areas of infection. Infected areas occur in the Inner cells of the trichome cushion, both epidermal and cortex, outer cortex and mid cortex large parenchyma cells. No evidence of hyphal presence occurs in, the leaf, stem, peduncle, endodermis, stele, epidermis (except cells immediately below the trichome cushion), and inner cortex cells immediately adjacent to the endodermis. Multiple type one hyphae can penetrate the same cell with lateral branches divaricating and a branch continuing to penetrate an opposite internal wall. When the bulk of the hyphae are lysed the long type one hyphae could provide a vegetative reproductive phase during *Nematoceras* dormancy.

As hyphal lysis progresses, amyloplast development becomes apparent and accumulates within the digestive cells. Amyloplast development occurs when starch production is high in association with increased hyphal lytic activity. Presumably amyloplasts act as an energy reserve for the plant (Preiss, 2009). Highest levels of amyloplasts occur just prior to dormancy, diminishing during the early plant growth phase and reappearing after lysis has commenced. Amyloplasts were not evident in the stem or leaf in the *Nematoceras* plants examined.

All of the hyphae in the CA roots examined have Rhizoctonia type morphology, (Warcup and Talbot, 1967,1971); Parmeter and Whitney, 1970). Identification of the orchid endophyte can be made by culturing, isolating and inducing a sexual phase in the hyphae extracted from pelotons. The classic identification relies on the morphology of the basidia and basidiospores, (Warcup and Talbot, 1967). Identification of the fungal species by basidia and basidiospore morphology, while definitive, has many drawbacks, mainly in obtaining fruiting bodies from the orchid endophyte culture isolate, (Kristiansen, *et al.*, 2001; Rasmussen, 2002; Kottke and Suárez, 2009), and length of time to acheive a result and the degree of expertise required in the taxonomic identification of the basidia and basidiospores, (Warcup and Talbot, 1967).

A series of TEM micrographs, of the septal ultrastructure character, has shown that the putative mycorrhizal fungus, contained within the observed hyphae of the samples investigated is a member of the Tulasnellaceae family identified to genus level as a *Tulasnella* spp. or the anamorph form *Epulorhiza* spp. Refining identification of the hyphal identity, to species level, is faster and more practical with molecular sequencing.

Imaging of the hyphal septal pores by TEM has been an interesting exercise, creating a number of hypotheses on the dynamics of parenthesomes and dolipores that could be worthy of further research. Importantly for this thesis it provided an indication that a *Tulasnella* species was present in spite of the minimal molecular sequencing results obtained in the series 1 experiment.

Poor results were obtained with the initial series of primers and tissue preparation methods. This forced a change in primer selection from series 1 using ITS1-F / ITS4 or ITS1-F / ITS5.primers to the *Tulasnella* specific primer combination ITS4-Tul / ITS5 which produced positive results

One of the main aims of this thesis was to identify the endophyte, taken from the root cortex from a selection of New Zealand CA, to species level, and this has been accomplished, for the first time, in these orchids.

The closest sequences to the samples were:

N. iridescens	=	*Tulasnella calospora* strains,
N. trilobus	=	*Tulasnella violea*
Singularybas oblongus	=	*Tulasnella irregularis*

The work in this thesis further extends the degree of how widespread *Tulasnella* are. Unique strains of *T. calospora* were found throughout the *N. iridescens* plants sampled, as well as being allied with: GenBank accessions from the epiphytic orchids in the Andean cloud forest, the Australian, *Acianthus* (CA genus), and within the USA, Europe, China and Asian orchids.

Tulasnellales are cosmopolitan in distribution, (Wells and Bandoni, 2001). This wide diversity of *Tulasnella* species would indicate a long evolutionary period with a high probability of speciation and strain development, (Taylor, *et al.,* 2000). The phylogenic diversity of *T. calospora* suggests that a reclassification and possible speciation of the species is warranted.

Considering the broad spectrum of generalist Basidiomycete primers and the specificity of the ITS4-Tul / ITS5 it is likely that early researchers failed to obtain *Tulasnella* spp. sequences. In more recent sequencing, only the 5.8S rDNA locus was consistently amplified for all of these tulasnelloid mycorrhizal samples.

Differentiation of *Tulasnella* strains between sites is apparent, as was the comparison between strains of the *N. iridescens* seed endophyte (HM802310) and endophyte strains of *N. iridescens* adult plant, cultivated at the nursery site. Both samples recorded close sequences compared to sequences derived from other mature *N. iridescens* plants. The dissimilarity often displayed between same species of germinating orchid seed and mature plant endophytes were reported by, (Hadley, 1970; Rasmussen,1995).

The series 2 protocols would appear to form a basis for future *Tulasnella* endophyte identification in the Orchidaceae. Further research, utilising techniques such as Next Generation Sequencing (NGS), (Shendure and Ji, 2008; Baker, 2010; Glenn, 2011), of soils to identify potential fungal mycorrhiza, (Bräutigam and Gowik, 2010), would be a valuable aid in the conservation of threatened orchid species. Once identified and co-related to mature orchid plant and symbiotic seed germination the mycorrhizal species location for establishment of new colonies could be ascertained. Soil samples of proven mycorrhizal endophytes could be transported to suitable geographic positions for re-establishment of threatened orchid species or placed in the required location, along with orchid seed envelopes.

An interesting result, arising from the series one basidiomycete primers, produced a sequence allied to two *Trichoderma* spp. (AJ279483 and AF218788), close sequences of *Trichoderma viride* and *Hypocrea viridescens*. Many *Trichoderma* are mycoparasitic on pathogenic fungal species, (Elad, *et al.,* 1982; Verma *et al.*, 2007; Kubicek, *et al.*, 2011). Could this *Trichoderma* have implications to the mycorrhizal endophytes, either as a protection against pathogenic fungi or as a mycoparasite, (De Jaeger, *et al.*, 2011), affecting the mycorrhizal endophyte? This question would provide an interesting field for future research, which could supply information on seed germination and mature plant perturbations.

Conservation of plants is reliant on their reproductive success and this is apparent in the range of strategies that plants have acquired. Orchids possess a wide repertoire of devices that ensure their continued survival. This thesis has explained a possible route and vectors, pertinent to long distance seed dispersal, by association with similar sized objects, whose providence is detailed and that have been transported over a long distances..

A number of strategies are evident in the *Nematoceras* examined; large numbers of seed are produced, 32.65% fully formed and 67.35% microseed. This difference allows for a wider range of distribution through variations in seed mass and air volume, wide shallow balloon seed which maximise aerodynamic features, (Burrows, 1975; Nathan, *et al.*, 2002). Seeds are hydrophobic with a high water tension surface created by the many exposed ridged cell boundaries comprising the testa pattern, (Harper, *et al.*,1970). This allows water transport and prevents seed anoxia. Hyphae congregate at the seed micropyle which possibly enables future water access, (Prutsch, *et al.*, 2000; Yoder, *et al.*, 2000), allowing for imbibition and entry for the myco-endophyte that is necessary for germination.

Plant peduncle elongation, from fertilization to dehiscence, occurs over a period of 63 ± 9 days (September to November / December period). Peduncle elongation is slow over the first fifty-seven days and becomes exponential in the final 10 days until explosive dehiscence occurs together with oscillation of the peduncle at minimal wind strength speeds of 1.3 ms^{-1} which is required to liberate seed from the pods. The peduncle elongation places the pod into a localised wind current that is not evident closer to the plant leaf.

Conservationally it would be important for local body councils to preclude roadside mowing and earthwork activity during this period, in areas in which CA plants exist. However roadside bank clearing in overgrown shaded rainforest areas would provide future sites for CA establishment, since the taxa appears to be a perennial colonizer following bryophyte establishment.

Seed germination experimental results, both asymbiotic and symbiotic, were disappointing and reflected previous difficulties, by others, in propagating terrestrial orchids from seed, (Henrich, *et al.*, 1981; Quay, *et al.,*1995; Rasmussen, 1995; Zettler, 1997; Rasmussen and Whigham,1998; Batty, *et al.,* 2001; Kauth, *et al.*, 2006; Bonnardeaux, *et al.*, 2007).

Field seed germination trials proved more successful and presented a possible reason for the lack of success in other seed germination experiments. The time elapsed, one year prior to proof of germination, active endophyte and visual

identification, suggests a dormancy period. Dormancy types, (Baskin and Baskin, 1998; Finch-Savage and Leubner-Metzger, 2006), were not investigated and would be a priority in any future CA conservation research. A one-year dormancy period suggests that the time allowed for the laboratory in vitro germination trials was insufficient.

Seed envelopes of *N. iridescens*, after being in situ for twelve months, gave positive evidence of inducing germination and that this technique could be used to provide a conservation method for *N. iridescens* and presumably other CA species. Envelopes of pre-germinated seed could be transported to suitable sites for reestablishment.

Asymbiotic germination experiments could be repeated, based on the positive parameters observed in the initial *N. iridescens* seed experiments;

1. selected media T842 (Terrestrial (Cypripedium) Orchid Medium,

2. initial temperature of 18 - 25 ^{o}C for six months,

3. lowering of temperature to 5-8 ^{o}C for three months,

4. temperature of 10 - 15 ^{o}C for the final three months,

5. dark conditions over the entire period.

Symbiotic germination experiments could also be repeated using a *T. calospora* strain of endophyte identified and collected from the germinating seed of the CA species. The solitary single leaf, possessed by the majority of CA plants, is the "Achilles' heel "of the genera. While the "mycorrhizal" fungi assist in the overall nutrition the leaf also produces a large proportion of plant nutrients. This mycoheterotrophic arrangement creates a situation that allows these terrestrial orchids to occupy heavily shaded environments, (Bidartondo, *et al.*, 2004). In low light, herbivory may constrain plant functional responses to shading and could explain the low plant tolerance of herbivore damage in the shade, (Salgado-Luarte and Gianoli, 2011).

Diversity of leaf morphology in *N. iridescens* is indicative of plasticity and adaptive phenotypic change, presumably to enhance plant performance, which is fundamental for plant success in shaded and sunflecked communities, (Vogelmann, *et al.*, 1996). Moisture levels play an important part in *Nematoceras* species leaf form, (Ledford, 2008), and timing of seed dehiscence and aestivation. This is an important consideration in reestablishment of CA colonies for conservation purposes.

Survival of the plant, from herbivoury, is an essential component in any conservational strategy, especially plants with only a single leaf, such as the CA. Two physical constraints are proposed, in this thesis, as natural deterrents to the predation damage of *N. iridescens* leaves.

Crystals of calcium oxalate monohydrate, raphides, are contained within crystal idioblasts. These occur in the sub-epidermal cortex of roots and in the spongy mesophyll tissue of the leaf. The needle like architecture of raphides present a tissue penetrating form, some barbed, some with serrated edges (first reported in this work) many with longitudinal ridging and all with acutely sharp pointed ends. Numerous reports linking raphides to antiherbivory and animal contact

dermatitis, (Morton, 1962; Finley, 1999; Arnott and Webb, 2000; Saltz and Ward, 2000; Watson, *et al.*, 2005; Korth, *et al.*, 2006; Perera, *et al.*, 2006), support the efficacy of raphide crystals.

Predation site locations in the leaves of *N. iridescens* indicate a herbivore preference for areas with immature raphide bundles (young emerging plants are heavily predated) or the scarcity of idioblasts within the inner leaf interior of mature plants. Raphide locations appear strategically placed in leaf areas which, on trauma, are deployed by a scattering over a much wider area than that of the original idioblast raphide encapsulation. Acute toxicity, in rats and humans, causing renal failure due to calcium oxalate monohydrate, has been reported, (Guo and McMartin, 2005). This factor could possibly act as an additional deterrent in protection against herbivoury, (Dogigey, 1991; Watson, *et al.,* 2005).

Leaf cuticle waxes have a significant effect on the prepenetration phase of a number of pathogenic fungi and the effects of damaging UV radiation, (Mariani and Wolters-Arts, 2000). The length of the wax derived very-long-chain aldehydes, (Buschhaus and Jetter, 2010), in *Blumeria graminis hordei* appears to control eventual infection of maize. Long chain aldehydes enhance the fungal production of prepenetration cursors, whereas absence of the very-long-chain aldehydes, such as in the maize mutant glossy 11, devoid of very-long-chain aldehydes, totally inhibits the development of prepenetration cursors, (Hansjakob, *et al.*, 2011).

Cuticle wax can attenuate ultraviolet radiation at wave-lengths < 400 nm with maximum attenuations of UV radiation at 300nm, (Krauss, *et al.*, 1996). Cuticular wax thickness and leaf patternation was exemplified in this thesis when a comparison between *Corybas cheesemanii* (thick waxy cuticle, high light with autumn-winter dormancy) and the heavy shade tolerant *Nematoceras* spp. (very light waxy cuticle, summer-autumn dormancy) was made.

A previously unreported feature in CA leaves is the marginal lamina edge "braiding". Micrographs from SEM images of an *N. iridescens* leaf show interwoven epidermal cells forming a plait like edge. Although no testing of attributes was performed, speculation suggests that such an edge would create a resistance to tearing in a similar fashion to the hemming of fabric. The long axis orientation of the "braiding" cells are at right angles to the pavement cells of the epidermal leaf plane and could present a barrier to the leaf edge predation. Investigation of and comparison to other orchids would be of taxonomic and anatomical interest. An investigation, into the physical effects occurring to the leaf, with alteration in the "braiding" edge structure associated with changes in water potential, a "draw string" hypothesis that could alter the leaf symmetry and change the air envelope would be worthy of further research.

Leaf micrographs, from SEM of a range of *Nematoceras, Corybas* and *Singularybas,* display sufficient visual differences, in the abaxial surface patterning distribution and shape of stomata, to create taxonomic characters. A greater range of geographical CA leaf sampling, and allowance for variation would be required to answer the question "do patterns differ between sites and species?" (Pridgeon, 1994; Stern, 1997).

A contrast in stomata between the *Nematoceras (N.. macranthum, N. papa,* and *N. rivulare*), resident in very wet situations with almost constant water over the

growth period and with the heaviest shade, posses elliptical stomata with lower stomata leaf surface density and with greater size compared with *Nematoceras* (*N. iridescens, N. orbiculatum,* and *N. trilobum*) growing in drier and less shady situations have smaller, round stomata in a higher density.

Observed predation of *Nematoceras* leaves was traced to molluscs (snails and slugs) which can totally destroy newly emerging seedlings. These are controlled by regular application of a suitable molluscide in and around colonies that are to be conserved, seasonal timing being essential as only young plants appeared to suffer predation.

An endemic Lepidopteran larvae, *Scoparia ustimacula* , was identified, by the author, as a predator of mature *Nematoceras* leaves, its eating pattern apparently conforming to raphide distribution and braided edge boundaries. Indications that the moth – plant association is not a recent one is apparent in the larvae conformity to the *Nematoceras* seasonal changes. Speculatively the moth eggs are laid at or just prior to flowering. The larvae develop as the leaves mature and pupate immediately prior to plant senescence, emerging as the young plants redevelop, after an autumn aestivation period.   Predation of *Nematoceras* colonies was not excessive and does not appear to pose a conservation problem.

Overall the initial aims of the thesis have been accomplished. Firstly a protocol for the molecular identification of and recognition of the "mycorrhizal" endophyte has been established.   The identification, of the endophyte of *N. iridescens* and another two NZ species within the CA, has been accomplished for the first time, as has the obligate seed endophyte. An investigation of the spatial organisation of the endophyte has revealed morphological differences and areas of concentration within the root cortex.   Seed dispersal methods and seed germination have been investigated with success in the field envelope germination trials.   The leaf abaxial and adaxial surfaces suggest that, after greater sampling of the CA, a taxonomic character identification could be established. Three predators (slugs, snails and a moss moth) of *Nematoceras* leaves have been identified along with natural protection agents. A number of original observations are recorded in this thesis as a result of this investigation

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Appendix 1

Appendix 1a Botanical description of *Nematoceras iridescens*

Nematoceras iridescens (Irwin & Molloy) Molloy, D.L.Jones and M.A. Clements. Described by Irwin and Molloy New Zealand J. Bot. 34 (1)-5,f.1 (1996) as a Summer-green, terrestrial, glabrous, tuberous herb forming extensive colonies.

Leaf solitary, fleshy, shortly-petiolate; usually spreading and held flat to the ground surface; lamina 20-40 x 15-35 mm, sometimes larger, ovate-oblong or broadly wedge-shaped, often pandurate, squared and apiculate at tip, with apiculus decurved, rounded and cordate at base; midrib grooved above, ridged beneath; leaf colour dull dark green above and often purple-spotted on margins, on midrib, or overall, silvery and pellucid beneath, Petiole 3—5(—10) x 1.5—3 mm, winged. Flowers usually solitary, rarely two, 12—20 mm long, dark redgreen, dominated by the labellum, dorsal sepal, and long filiform petals and lateral sepals, on a peduncle 4-5 mm long. Ovary 4—8 mm long, green or whitish and purple flecked, curved, subtended by two unequal floral bracts, the smaller 3—5 mm long, linear-subulate, terete, the larger equal to or exceeding the ovary, 7—10 mm long, lanceolate, green with purple flecks. Dorsal sepal extending well past labellum, 20—35 x 8—12 mm, narrowly ovate in outline when flattened, concave, cucullate and arching over labellum tube, with tip often recurved, green with purple spots and striations; veins ridged. Lateral sepals 50-70 x 0.5 — 1.0 mm at widest point, filiform, greatly exceeding labellum, whitish with purple striations, suberect to erect and projecting forwards, channelled, twisted, and connivent at expanded base. Petals 40-60 x 0.5— 1.0 mm at widest point, usually shorter than lateral sepals but not greatly so, filiform, greatly exceeding labellum, whitish with purple striations, horizontal to suberect and projecting forwards and outwards, channelled, auriculate on the base of the column. Auricles short, projecting downwards and forwards, with apertures 1.5— 2.5 mm across.

Labellum conspicuous, dark red almost black throughout, sometimes greenish streak at front, strongly iridescent when wet; labellum tube 5—7 mm long, erect, then abruptly deflexed through 160-180° and expanding into the lamina, with a prominent bead-like callus in throat of tube at the bend; lamina 10—20 x 10—15 mm, broadly ovate to orbicular; upper margins folded inwards, joining or overlapping; lower surface spreading, deflexed against ovary, with margins erose-papillose, and a long median apiculus; inner surface with dense, minute, retrorse papillae and ridged veins; throat of labellum tube high on lamina. Column 3—4 mm long, broadest and ridged at base, inclined backwards, minutely winged. The Stigma is shield-shaped to oblong, c. 1.0 mm across, concave with margins fimbriate. Anther c. 1.0 mm, obtuse, purple-flecked, with papillose margins; pollinia 4, united in pairs, c. 1.0 x 0.7 mm, oblong, mealy, yellow; viscidium c. 0.5 mm across, oblong to orbicular, concave with thickened edges, at first translucent white, later light brown. Capsule 12—15(—20) x 5- 7

mm, elliptic, at first pale green, later brown, on a greatly elongated peduncle. Tuberoids globose to ellipsoid on extended roots.

Flowering: August to October. Plants in the wild and in cultivation flower annually though they vary in intensity from year to year. Pollination appears to be wholly insect-dependent. Fungus gnats (*Mycetophila* spp.), some with pollen attached to the thorax, have been observed entering and leaving flowers of *Corybas iridescens* grown in cultivation (Fuller 1980, 1994; B. P. J. Molloy pers. obs.), and some of these flowers have subsequently developed seed capsules. However, the pollination biology of *C. iridescens* needs to be confirmed experimentally (see especially Godley 1979).

Fruiting: September to November. The seed capsules develop rapidly after fertilisation. Capsules are carried upwards on greatly elongated peduncles as they approach maturity. Most mature capsules examined were well filled with apparently sound seed that is dispersed by wind, water, or gravity following capsule dehiscence.

Chromosome number: In = 36 This somatic number is regarded here as diploid, with an effective base number of 18, Dawson (2007).

Appendix 1b Consent from Te Atiawa tribal authority,

Te Atiawa Iwi Authority

Level 1 Education House, 45 Eliot Street New Plymouth
P O Box 1018, New Plymouth

Phone / Fax (06) 758 9777   E-mail taia@xtra.co.nz

Chairperson   Secretary
Wikitoria Keenan   Chargn Keenan
Mobile 0272 906 027

21 September 2005

ROGER WATKINS
413 DEVON ST EAST
NEW PLYMOUTH

LETTER OF SUPPORT

Thanks again, Roger, for your recent consultation with Te Atiawa Iwi Authority (TAIA) about the content of your PhD project and any possible concerns with your method of collection and the host gathering area.

I understand that consultation with TAIA was suggested by Massey University through its Kai Arahi Maori, Nick Roskruge, and we acknowledge that advice.

I appreciate the time you took to explain your project and its genesis. Although I am very much an amateur in such matters, your explanation was such that I was able to understand both your project and some of the scientific processes.

As advised during your consultation, I confirm that TAIA has no concerns with your method of collecting plants (from roadside embankments) or with your particular collection areas.

Your work is very much appreciated as it will contribute both to the wider body of scientific and Iwi knowledge about native flora and fauna and, given the survival risk to many native plants, will provide a powerful tool for ongoing conservation.

Thanks again Roger – good luck with your project.

Naku noa
na

Wikitoria Keenan

Figure 1   The letter of support for this research and plant collection, by Te Atiawa   tribal authority, where it occurred on ancestral lands, is acknowledged l   .

Appendix 1c NZ orchid collection data

Date	Genus	Species	Plate index	Site	Collec	Herbarium voucher
24/10/2006	Anzybas	rotundifolius			MM	
27/07/2004	Bulbophyllum	pygmaeum	Bupy1	Moki	JD	
27/07/2004	Bulbophyllum	tuberculatum	Butu1	Napier	JD	
27/07/2004	Bulbophyllum	tuberculatum	Butu2	New Plymouth	JD	
27/07/2004	Chiloglottis	trapeziformis	Chtr1	Waiwarapa	JD	
17/04/2004	Corybas	cheesemanii	Coch1	Ahititi	EC	
16/05/2004	Corybas	cheesemanii	Coch2	Te Henui	JD	*MP*N28330
27/07/2004	Corybas	cheesemanii	Coch3	Te Henui	JD	
27/07/2004	Drymoanthus	adversus	Drad1	Moki	JD	
27/07/2004	Drymoanthus	adversus	Drad2	Napier	JD	
26/10/2004	Drymoanthus	adversus	DrΔ?	Koru Pa	RW	
10/03/2004	Earina	autumnalis	Eaau1	413 slab	BW	
27/07/2004	Earina	autumnalis	Eau2	Te Henui	JD	
27/07/2004	Earina	destardii	Eade1	Nth Egmont	JD	
10/03/2004	Earina	mucronata	Eamu1	413 slab	BW	
10/03/2004	Earina	mucronata	Emu2	413 basket	BW	
25/06/2004	Earina	mucronata	Eamu3	Maikai bra	RW	
27/07/2004	Earina	mucronata	Eamu4	Te Henui	JD	
27/07/2004	Microtus	unifolia	Miun1	Te Henui	JD	
10/03/2004	Nematoceras	iridescens	Cir1	Moki	RW	
25/06/2004	Nematoceras	iridescens	Cir2	Maikai	RW	
10/10/2004	Nematoceras	longipetala	Nelo1	Matemateonga	RW	
10/10/2004	Nematoceras	macrantha	Nema	Matemateonga	RW	MPN 28703
27/07/2004	Nematoceras	oblongus	Neob1	Te Henui	JD	
9/2004	Nematoceras	oblongus	Neob2	Kiri Stream, Kaita		
0/2004	Nematoceras	orbiculata	Neor1	Matemateonga		
10/03/*2004*	Nematoceras	papa	Cpa	Moki	BW	
7/08/2004	Nematoceras	papa	Cpa1	Whangamomona	RW	MPN 28643
10/03/2004	Nematoceras	rivularis	Cri1	Te Henui	JD	
27/07/2004	Nematoceras	rivularis	Neri2	Te Henui	JD	
6/09/2004	Nematoceras	rivularis	Neri2	Te Henui	*J*D	MPN 28700
28/09/2004	Nematoceras	rivularis	Neri3	Te Henui	*J*D	
26/10/2004	Nematoceras	rivularis (Whiskers)	NeriW	Koru Pa	*R*W	MPN 28702
27/07/2004	Nematoceras	trilobus	Netr	Te Henui	JD	
7/08/2004	Nematoceras	trilobus	Cotr1	Moki	RW	
27/07/2004	Orthoceras	novae-zeelandiae	Ornz1	Te Henui	JD	
9/06/2004	Pterostylis	aff. Montana	Ptmo	413 potted	BW	
27/07/2004	Pterostylis	alobula	Ptal1	Moki	JD	
27/07/2004	Pterostylis	trullifolia	Pttr1	Waitakeres	JD	
11/10/2004	Singularybas	oblongus	Siob1	Pukemahoe	RW	MPN 28701
27/07/2004	Spiranthes	sinensis	Spsi1	Ruapehu	JD	
27/07/2004	Thelymitra	longifolia	Thlo1	Te Henui	JD	
27/07/2004	Thelymitra	pauciflora	Thpa1	Te Henui	JD	
10/03/2004	Winika	cunninghamii	Wicu1	413 basket	BW	
27/07/2004	Winika	cunninghamii	Wicu2	413 basket	BW	

Appendix 1d

Distribution of Corybas alliance spp. within the N.Z. botanical ecoregion areas
Shaded areas indicate regions with greater than 6 spp.

Ecoregion	Corybas alliance genera						
	Acianthus	*Anzybas*	*Corybas*	*Cyrtostylis*	*Molloybas*	*Nematoceras*	*Singularybas*
1 Kermadec							
2 Three Kings	1				1		1
3 Te Paki	1	1	1	1		3	1
4 Aupouri	1	1	1	1		2	1
5 Western Northland	1	1	1	1		5	1
6 Eastern Northland	1	1	1	2	1	5	1
7 Poor Knights	1			1		1	1
8 Kaipara	1		1	1		2	
9 Auckland	1	1	1	1		1	
10 Coromandel	1		1	2		5	1
11 Waikato	1	1	1	1		6	1
12 Tainui	1		1	2		8	1
13 Northern Volcanic Plateau	1		1			6	1
14 Whakatane	1					1	
15 Western Volcanic Plateau	1			1		4	1
16 Central Volcanic Plateau	1		1	1		6	1
17 Eastern Volcanic Plateau			1			3	1
18 Tongariro	1				1	9	1
19 Raukumara	1					2	1
20 East Cape	1		1	1		3	1
21 Urewera	1					5	1
22 Wairoa	1			1		5	1
23 King Country	1					6	1
24 Taranaki	1		1		1	8	1
25 Egmont	1		1		1	8	1
26 Moawhango						3	
27 Kaimanawa						3	
28 Ruahine						2	
29 Hawkes Bay				1		3	
30 Rangitikei						7	1
31 Manawatu	1		1	1		7	1
32 Manawatu Gorge						1	
33 Pahiatua						3	1
34 Eastern Hawkes Bay						1	
35 Eastern Wairarapa	1		1	1		5	
36 Wairarapa Plains	1		1	1		5	
37 Aorangi	1		1	1		7	1
38 Tararua	1		1	2	1	7	

39 Sounds - Wellington	1		1	1	1	5	1
40 Richmond	1		1	1		5	1
41 Wairau						3	1
42 Inland Marlborough						3	
43 Molesworth						3	
44 Clarence						1	
45 Kaikoura						3	1
46 North-West Nelson	1		1			8	1
47 Nelson	1		1	1	1	9	1
48 North Westland	1		1		1	6	1
49 Spenser						6	1
50 Whataroa						6	1
51 Aspiring						3	1
52 Lowry							
53 Hawdon						3	
54 Puketeraki							
55 Canterbury Foothills					1		
56 Canterbury Plains						1	
57 Banks						3	
58 D'Archiac						1	
59 Heron							1
60 Tasman						1	
61 Pareora						3	
62 Wainono							
63 Mackenzie							
64 Waitaki							
65 Kakanui							
66 Lakes						3	
67 Central Otago						2	
68 Lammerlaw						2	
69 Otago Coast						3	1
70 Catlins						2	1
71 Olivine						2	1
72 Fiord						4	1
73 Mavora	1					2	
74 Waikaia						2	
75 Gore					1		
76 Southland Hills							
77 Te Wae Wae						5	1
78 Makarewa						1	1
79 Rakiura						5	1
80 Chathams	1	1	1			6	1
81 Bounty							
82 Antipodes						1	
83 Auckland Islands						2	1
84 Campbell						2	1
85 Macquarie						2	

Appendix 1e

Kew Gardens; notification of nomenclature changes to orchids within the Corybas alliance.

Anzybas abditus (D.L.Jones) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 443 (2002): *Corybas abditus.*

Anzybas carsei (Cheeseman) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 443 (2002): *Corysanthes carsei. Corybas carsei.*

Anzybas fordhamii (Rupp) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 443 (2002): *Corysanthes fordhamii.*

Anzybas montanus (D.L.Jones) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 443 (2002): *Corybas montanus.*

Anzybas rotundifolius (Hook.f.) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 443 (2002): *Nematoceras rotundifolia.*

Anzybas unguiculatus (R.Br.) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 443 (2002): *Corysanthes unguiculata.*

Corysanthes dentata (D.L.Jones) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 447 (2002): *Corybas dentatus.*

Corysanthes despectans (D.L.Jones & R.C.Nash) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 447 (2002): *Corybas despectans.*

Corysanthes expansa (D.L.Jones) D.L.Jones & M.A.Clem. in *Orchadian*

Gastrosiphon aundensis (P.Royen) M.A.Clem. & D.L.Jones in *Orchadian 13(10): 447 (2002):*

Gastrosiphon ekuamensis (P.Royen) M.A.Clem. &

13(10): 447 (2002): *Corybas expansus.*

Corysanthes hispida (D.L.Jones) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 447 (2002): *Corybas hispidus.*

Corysanthes incurva (D.L.Jones & M.A.Clem.) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 447 (2002): *Corybas incurvus.*

Corysanthes limpida (D.L.Jones) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 447 (2002): *Corybas limpidus.*

Corysanthes ×miscella (D.L.Jones) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 447 (2002): *Corybas ×miscellus.*

Corysanthes recurva (D.L.Jones) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 447 (2002): *Corybas recurvus.*

Gastrosiphon (Schltr.) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 447 (2002): *Corysanthes* sect. *Gastrosiphon.*

Gastrosiphon aduncus (Schltr.) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 447 (2002): *Corysanthes adunca.*

Gastrosiphon arfakensis (J.J.Sm.) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 447 (2002): *Corysanthes arfakensis.* D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corybas ekuamensis.*

Gastrosiphon fenestratus (P.Royen) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corybas fenestratus*.

Gastrosiphon fornicatus (Blume) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Calcearia fornicata*.

Gastrosiphon gibbiferus (Schltr.) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corysanthes gibbifera*.
Gastrosiphon karkarensis (P.Royen) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corybas karkarensis*.

Gastrosiphon klossii (Ridl.) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corysanthes klossii*.

Gastrosiphon paleariferus (J.J.Sm.) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corysanthes palearifera*.

Gastrosiphon ramosianus (J.Dransf.) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corybas ramosianus*.

Gastrosiphon royenii (Kores) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corybas royenii*.

Gastrosiphon schlechteri M.A.Clem. & D.L.Jones in *Corybas aundensis. Orchadian* 13(10): 448 (2002): *Corysanthes gastrosiphon* Schltr.

Gastrosiphon scutellifer (J.B.Comber & J.Dransf.) M.A.Clem. & D.L.Jones in *Orchadian* 13(11): 502 (2002): *Corybas scutelliferus*.

Gastrosiphon stenotribonos (J.B.Comber & J.Dransf.) M.A.Clem. & D.L.Jones in *Orchadian* 13(11): 502 (2002): *Corybas stenotribonos*.

Gastrosiphon subalpinus (P.Royen) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corybas subalpinus*.

Gastrosiphon urikensis (P.Royen) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corybas urikensis*.

Gastrosiphon ventricosus (J.J.Sm.) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corysanthes ventricosa*.

Gastrosiphon vespertilionis (P.Royen) M.A.Clem. & D.L.Jones in *Orchadian* 13(10): 448 (2002): *Corybas*

Molloybas cryptanthus (Hatch) D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 448 (2002): *Corybas cryptanthus*

Nematoceras acuminatum (M.A.Clem. & Hatch) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Corybas acuminatus*.

Nematoceras dienemum (D.L.Jones) D.L.Jones, M.A.Clem. & Molloy in *Orchadian* 13(10): 449 (2002): *Corybas dienemus*.

Nematoceras hypogaeum (Colenso) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Corysanthes hypogaea*.

Nematoceras iridescens (Irwin & Molloy) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Corybas iridescens*.

Nematoceras longipetalum (Hatch) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Corybas macranthus* var. *longipetalus*.

Nematoceras orbiculatum (Colenso) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Corysanthes orbiculata*.

Nematoceras panduratum (Cheeseman) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Corysanthes rotundifolia* var. *pandurata*.

Nematoceras papa (Molloy & Irwin) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Corybas papa*.

Nematoceras papillosum (Colenso) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Corysanthes papillosa*.

Nematoceras rivulare (A.Cunn.) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Acianthus rivularis*.

Singularybas oblongus (Hook.f.) Molloy, D.L.Jones & M.A.Clem. in *Orchadian* 13(10): 449 (2002): *Nematoceras oblongus*

From "Transactions and proceedings of the Royal Society of New Zealand. Vol 17, 1884.

Art. XI.—*Descriptions of New Zealand* Micro-Lepidoptera.

By E. Meyrick, B.A.

[*Read before the Philosophical Institute of Canterbury, 7th August,* 1884.]

IV.—SCOPARIADÆ.

This family occupies an unusually prominent position in New Zealand, and the principal genera attain here their maximum of development. The development is however mainly specific, and there is no large number of peculiar genera, as in some other groups. The family is undoubtedly of very ancient type, and the food of the larvæ, which probably consists wholly of mosses, will allow of a possible origin earlier in time than the appearance of flowering plants. It is probably due to this persistence of habit that the type has undergone so little generic modification; specific change being sufficient to allow of all the adaptation required. The distribution of the family seems chiefly limited by the suitability of the climate for the growth of their food-plants; hence they are found principally in cool temperate latitudes, or at considerable elevations.

Although of universal distribution, this genus is little developed except in temperate latitudes, hardly occurring in the tropics except at a considerable elevation. Over thirty European species are known, and scattered forms are found in most other regions. Australia possesses at present sixteen, which number will be considerably increased, especially from the Tasmanian mountains. In comparison with these the development of the genus in New Zealand is extraordinary, forty-two species being here given, and it is unquestionable that the actual number is much larger, as each mountain seems to possess peculiar species. *Scoparia* is in fact the largest genus of *Lepidoptera* in New Zealand.

Forehead vertical. Ocelli present. Tongue well developed. Antennæ moderate, $\frac{2}{3}$ of forewings, in male filiform, evenly ciliated ($\frac{1}{4}$–$1\frac{1}{2}$). Labial palpi moderate or long, straight, porrected, second joint beneath with long dense projecting scales, terminal joint moderate, exposed or resting in scales of second. Maxillary palpi rather long, triangularly dilated. Posterior tibiæ with outer spurs half inner. Abdomen moderate. Forewings with vein 11 rather oblique. Hindwings from somewhat broader to nearly twice as broad as forewings; 3 remote from 4, 4 and 5 stalked or from a point; lower median naked; discal area above it without hairs; internal area

30. *Scop. ustimacula*, Feld.

(Scoparia ustimacula, Feld., Reis. Nov., pl. cxxxv., 17; *Scoparia conifera*, Butl., Cist. Ent., ii., 493.)

Male.—21 mm. Head and thorax ochreous, mixed with white, reddish-fuscous, and black; patagia margined with white. Palpi 2½, ochreous-fuscous, basal joint white. Antennæ fuscous; ciliations 1½. Abdomen light grey. Legs ochreous-white, tibiæ and tarsi banded with black. Fore-wings rather elongate, triangular, costa gently arched, apex rounded, hind-margin somewhat sinuate, oblique; rather bright ochreous; costa and inner margin irrorated with black and white; base suffused with dark fuscous; a slender obscure whitish line near before first line; first line slender, white, posteriorly irregularly black-margined, not curved, rather oblique, thrice sinuate; a broad fascia-like oblique blackish streak from costa immediately beyond first line, white-margined, reaching submedian fold, apex rounded; a similar inwardly oblique broad streak from costa beyond middle, reaching middle of disc, broadly bifurcate at apex, anterior branch almost coalescing with first streak; second line slender, white, margined anteriorly on costa, submedian fold, and inner margin by three small blackish spots; terminal space somewhat suffused with dark fuscous, tending to form streaks on veins; subterminal line cloudy, whitish, almost marginal throughout; a terminal row of white dots: cilia grey-whitish, with dark grey anterior and lighter posterior lines. Hindwings 1⅓, whitish-grey; central lunule, postmedian line, and hindmargin darker grey; cilia as in forewings.

Also a very distinct and handsome species, characterized by the two conspicuous black fasciæ from costa to disc, and unusually long antennal ciliations, the longest in the genus. Butler's description is hardly recognizable, but is intended for this species, as I have seen his type in the British Museum.

Castle Hill (Mr. J. D. Enys) and Dunedin (Mr. A. Purdie), probably amongst bush; I have not met with the species myself; three specimens.

Appendix 2: Media Formulae.

2a Media preparation for mycology cultures

Bonnardeaux (As per Bonnardeaux 2007)

NH_4NO_3	0.4g
KH_2PO_4	0.0136g
$MgSO_4$	0.61g
NaCl	0.058g
$CaSO_4$	0.861g
FeEDTA (Na)	0.073g
Agar (Bacto) Difco	10.0g
DPA	10.0g
Water RO	1000 ml
Plates marked V	

Cornmeal Agar,

CMA (Difco)	6.8g
RO water	400ml

FIM (Fungal initiating media), Clements (1986).

$Ca(NO_3)_2$ $4H_2O$	0.25g
KH_2PO_4	0.1g
KCl	0.05g
$MgSO_4$. $7H_2O$ `	0.05g
Yeast extract (GibcoSRL)	0.05g
Sucrose	2.5g
Agar	10.0g
Water RO	500ml

Autoclave above for 15min. When cooled to $50^{o}C$ add: Novobiocin Na (N 1628), 0.025g / 500ml of H2O Sterile MilliQ. Bring to a pH = 5.8.

Modified Melin-Norkrans agar (MMN) fungal media, Marx (1969).

Glucose	5g
Malt extract	2g
Yeast extract	1g
Potassium phosphate monobasic	0.5g
Ammonium phosphate dibasic	0.25g
Magnesium sulphate	0.15g
Calcium chloride 1%sol	(5 ml aliquot)
Sodium chloride 1%sol	(2.5 ml aliquot)
Ferric chloride 1%sol	(1.2 ml aliquot)
Agar	15 g.

Make up to 1000 ml with distilled water and autoclave at $121^{o}C$ for 30 min, Taylor and Bruns (1997), also included 50 µg ml^{-1} of streptomycin
in sterile water added to the poured plates just prior to the media gelling

Oatmeal + PDA (Clements, 1986) modified by (Rasmussen, 1990a) For symbiotic cultures.

$Ca(NO_3).4H_2O$	120 mg
KCl	60 mg
KH_2PO_4	120 mg
$MgSO_4.7H2O$	60 mg
Yeast extract (Difco)	60 mg
Glucose	1.200 g
Ground oatmeal	1.800 g
PDA	14.400g
Water RO 1000ml	

PDA (Difco; Potato dextrose agar)

Potato Dextrose Agar, PDA (Difco)	23.4.6g
RO Water	600ml

Pumpkin + PDA

Pureed pumpkin	10g
PDA	19g
Water RO	600ml

Water Agar

Water Agar (Difco Bacterial Agar)	6g
RO water	400ml.

2b Media used for Orchid seed culture

Stock solution of Microelements

$MnSO_4.7H_2O$	7mg
$ZnSO_4.7H_2O$	1 mg
H_3BO_3	1 mg
KI	0.01 mg
$CuSO_4$	0.03 mg
$AlCl_3$	0.03mg
$NiCl_2.6H_2O$	0.03 mg
Distilled H_2O	1000 ml.

Fast's terrestrial media, (Fast, 1974), Media 2 (Terrestrial orchid seed media)

$CaNO_3.4H_2O$	0.05g
NH_4NO_3	0.10g
KH_2PO_4	0.05g
KCl	0.10g
$MgSO_4\ 7H_2O$	0.05g
$FePO_4$	50mg
Sucrose	7.0g
Peptone	1.0g
Fructose	3.0g
Fresh Brewers yeast	5.0g
Nicotinic acid	0.05mg
Biotin	0.006mg
Agar	5.0g
Coconut water	30ml
Distilled water	600ml
Trace from microelement stock	2ml
H_2SO_4	pH to 5.5 with

N3f , (Burgeff, 1936), amended by, (Withner, 1953).

Water (distilled)	1000ml
Citric acid	90mg
K_2HPO_4	250mg
$MgSO_4\ 7H_2O$	250mg
KCl	250mg
$FeSO_4$	20mg
$CaNO_3.4H_2O$	1000mg
$(NH_4)2SO_4$	250mg
Glucose	10gm
Laevulose	10gm
Agar	12gm

Dissolve the ingredients, listed above, sequentially to avoid precipitation.

N3f, Burgeff, amended, (Rasmussen, 1995).
The Withner (1953) amendment plus 40 gm/L of fresh mashed banana Vacine and Went (Clements modification) Orchid seed germination media.

$Ca_3(PO_4)_2$	200mg
KH_2PO_4	250mg
KNO_3	525mg
$MgSO_4.7H_2O$	250 mg
$(NH_4)2SO_4$	500mg
Ferric tartrate	28mg
$MnSO_4.4H_2O$	7.5mg
Sucrose	20g
Agar	8g

Make up to 1000 ml with distilled water and autoclave at 121°C for 30 min

Proprietary Media used
Phyto Technology Laboratories, LLC.

BM-1 Terrestrial Orchid Medium
Boric Acid 10mg
Cobalt Chloride•6H$_2$O	0.025mg
Cupric Sulfate•5H$_2$O	0.025mg
Na$_2$ EDTA•2H$_2$O	37mg
Ferrous Sulfate•7H$_2$O	28mg
Magnesium Sulfate, Anhydrous	100mg
Manganese Sulfate•H$_2$O	25mg
Molybdic Acid (Sodium Salt) •2H$_2$O	25mg
Potassium Phosphate, Monobasic	300mg
Zinc Sulfate•7H$_2$O	10mg
Agar	5000mg
D-Biotin	0.05mg
Casein, Enzymatic Hydrolysate	00mg
Folic Acid	0.5mg
L-Glutamine	100mg
Glycine (Free Base)	2mg
myo-Inositol	100mg
Nicotinic Acid (Free Acid)	5mg
Pyridoxine•HCl	0.5mg
Sucrose	20g
Thiamine•HCl	0.5mg

Make up to 1L with RO water
Media strength = 26.22 g/L of RO water.
pH = 5.25 – 6.00

M551 Malmgren Modified Terrestrial Orchid Medium

Calcium Phosphate, Tribasic	5mg
Na$_2$ EDTA•2H$_2$O	37mg
Ferrous Sulfate•7H$_2$O	28mg
Magnesium Sulfate, Anhydrous	98mg
Manganese Sulfate•H$_2$O	1.54mg
Potassium Phosphate, Monobasic	75mg
Activated Charcoal	1000mg
Agar	7g
D-Biotin	0.05mg
Casein, Enzymatic Hydrolysate	400mg
Folic Acid	0.5mg
Glycine (Free Base)	2mg
myo-Inositol	100mg
Nicotinic Acid (Free Acid)	5mg
Pineapple Powder	20g
Pyridoxine•HCl	5mg
Thiamine•HCl	10mg

Media strength = 28.84 g/L of RO water to pH = 4.0 – 4.5
Make up to 1L with RO water

T842 Terrestrial (Cypripedium) Orchid Medium

Ammonium Citrate	19mg
Boric Acid	0.5mg
Calcium Nitrate	600mg
Cupric Sulfate•5H_2O	0.025mg
Ferric Ammonium Citrate	25mg
Magnesium Sulfate, Anhydrous	97.69mg
Manganese Sulfate•H_2O	1.54mg
Molybdic Acid, Sodium Salt•2H_2O	0.02mg
Potassium Chloride	100mg
Potassium Iodide	0.1mg
Potassium Nitrate	200mg
Potassium Phosphate, Monobasic	200mg
Zinc Sulfate•7H_2O	0.5mg
Agar	6g
Casein, Enzymatic Hydrolysate	200mg
D-Glucose	20g

Make up to 1L with RO water
Media strength = 27.44 g/L of RO water. at pH = 4.75 – 5.75

Appendix 3 Stains used in this thesis.

Calcofluor white M2R (C. I. 40622) with Potassium hydroxide

Solution A; Potassium hydroxide reagent.
Dissolve the KOH in water and add glycerol.
Potassium hydroxide 10 g
Glycerin 10 ml
Distilled water 90 ml

Solution B; Calcofluor white reagent.
Dissolve Calcofluor white powder in the distilled water by gentle heating.
Calcofluor white 0.1 g
Distilled water 100 ml

Method for making microscopic mounts:
1. Mix one drop of each solution on the centre of a clean microscope slide.
2. Place the specimen in the solution and cover with a coverslip, squash the
preparation with the butt of the inoculation needle and then blot off the excess fluid.
3. Gently heat the slide and examine microscopically for the presence of fungal
elements that fluoresce a chalk-white or brilliant apple green colour, depending on the
filters used.

Comments: This is a very rapid and sensitive method; however a fluorescence
microscope fitted with filters to give an excitation with ultraviolet light below 400 nm
wavelength is required.
Further reading (Reinhard, R., Martin, B., Björn, T., and Hartmut, L., 2001; Hageage and Harrington in Laboratory Medicine, (1984,15:109-112).

Safranain / Alcian Blue
1% Alcian Blue
1g Alcian blue (Aq)
2.5ml Acetic acid (glacial)
0.2ml Formalin 40%
to 100 ml Water (deionised)
Premix the acetic acid - formaldehyde solution in the water. Add the Alcian Blue with
stirring. Stir until homogenous.
1% Safranin O
1g Safranin O
100ml 50% isopropyl alcohol

Staining Schedule

Bring sections to water. (After dewaxing, place slides in two changes of absolute
alcohol (5mins each), followed by 5 minutes each in 75%, 50%, 25% alcohol, and
finally 1 minute in water.)
Stain for 30 minutes in the Alcian blue
Wash in water to remove excess stain 3 or 4 changes of water in a coplin jar
Stain for 10 mins in 1% Safranin solution
Wash in water as above.
Differentiate in 50% isopropyl alcohol until the section shows a clear blue in parts
Dehydrate 100% alcohol I (dip and reverse)
Dehydrate 100% alcohol II Dehydrate 100% alcohol I (dip and reverse)
Clear in Xylene I Dehydrate 100% alcohol I (dip and reverse)
Clear in Xylene II Dehydrate 100% alcohol I (dip and reverse)
Mount in Shears Mounting media
Further reading, Battersby (2004) and Ruzin (1999).

Solophenyl Flavine 7GFE 500
(2-[4-[1-[(2-methoxyphenyl)carbamoyl]-2-oxopropyl]diazenyl-3-sulfo-phenyl]-6-methylbenzothiazole-
7-sulfonic acid from Ciba Specialty Chemicals NZ Ltd.) kindly supplied as a powder sample courtesy of Mr. Chris McGregor of Huntsman Textile Effects NZ Branch.
Solophenyl Flavine 7GFE 500 is a stilbene-based fluorophore dye which was prepared for use as a 0.01% (w/v) stock solution in pH 9.2 buffered (one BDH, Ph 9.2 Buffer tablet 100 mL-1 Milli-Q).
The stock solution was further diluted to a working strength of 0.001% Solophenyl Flavine 7GFE 500.

The effectiveness of the chitin binding fluorescent stain, solophenyl flavine 7GFE, Hoch (2005), used in this thesis for hyphal definition with CLSM, has been reported in a paper specifically mentioning this fluorophore stain, Knight (2011), and as a component in the patent application, IPC8 Class: AC12Q106FI .

Trypan Blue 0.05% w/v in lactoglycerol, Sneh (1991) and Burpee (1978).
A stock solution of Trypan Blue 0.05% w/v in lactoglycerol was available that had been made up from the standard formula.

Lactoglycerol is a 1:1:1 mixture of lactic acid, glycerol, and water. To prepare about
105ml of this reagent mix together 35 ml lactic acid, 35 ml glycerol, and 35 ml distilled water.

To prepare a 0.05% w/v solution of Trypan Blue (TB) in lactoglycerol dissolve 50mg of TB in 100 ml of lactoglycerol.

Appendix 4, Seed data

Data of "seed form", of seed taken from 12 *N. iridescens* seedpods.

size / status	1.0 x 0.2 mm normal + embryo	0.25 x 0.1 mm small + embryo	Testa only 1.0 x 0.2 mm	small - embryo 0.25 x 0.1 mm	n
L1	24	52	1	0	77.00
L2	18	34	2	0	54.00
L3	15	44	4	0	63.00
L4	30	56	4	0	90.00
	87	186	11	0	284.00
%	30.63	65.49	3.87	0.00	100.00
L9	41	60	1	0	102.00
L10	26	80	3	0	109.00
L11	18	40	0	0	58.00
L12	11	29	0	0	40.00
	96	209	4	0	309.00
	31.07	67.64	1.29	0.00	100.00
D5	20	27	0	0	47.00
D6	11	10	0	0	21.00
D7	15	21	2	1	39.00
D8	11	42	3	0	56.00
	57	100	5	1	163.00
	34.97	61.35	3.07	0.61	100.00

Data of *N. iridescens* viable seed type

size	1.0 x 0.2mm	0.25 x 0.1 mm
status	normal + embryo	small + embryo
	24	52
	18	34
	15	44
	30	56
	41	60
	26	80
	18	40
	11	29
	20	27
	11	10
	15	21
	11	42
total	240	495
sample size n	12	12
average	20	41.25
sd	5.656854249	20.68287335
standard error of sample	± 1.63	± 5.97

Percentage of micro / normal seed /pod sample count

normal seed	micro seed		
240	495	735	Pod sample totals
32.65306	67.34694		Percentage of normal + micro seed

Pod collection sites, code and status of pods

Site	Pods collected	Code	State
Tarata	1	T1	entire
Tarata	2	T2	entire
Uruti	1	U1	entire
Uruti	2	U2	entire
Matemateonga	1	M1	entire
Matemateonga	2	M2	entire
Matemateonga	3	M3	dehisced
Nursery	1	N1	dehisced
Nursery	2	N2	entire
Mangamingi	1	V1	entire
Mangamingi	2	V2	entire
Mangamingi	3	V3	entire

Germination of seeds inoculated and subjected to either; dark (24 hrs), half light (12/12 hrs), light (24 hrs).
Incubator temperature = 12°C

	seed #	imbibed	Culture width	colour	Seed germination
			2/02/07 9 days after incubation		
L1A	24	all	9 mm	white	nil
L2A7	18	all	30	brown	nil
L3N3	15	all	10	grey	nil
L4T3	30	all	11	blue/black	nil
D51A	41	all	20	white	nil
D6A7	26	all	27	brown	nil
D7N3	18	all	10	grey	nil
D8T3	11	all	10	blue/black	nil
L91A	20	all	20	white	nil
L10A7	11	all	35	brown	nil
L11N3	15	all	11	grey	nil
L12T3	11	all	18	white	nil
L1A	8/02/07	14days after incubation		white	nil
L2A7			43 mm	brown	nil
L3N3			15	black	nil
L4T3			20	black	nil
D51A			41	white	nil
D6A7			51	brown	nil
D7N3			20	grey	nil
D8T3			23	blue/black	nil
L91A			31	black	nil
L10A7			47	brown	nil
L11N3			32	grey	nil
L12T3			30	black	nil
L1A	15/02/07	21 days after incubation		white	nil
L2A7			54 mm	brown	nil
L3N3			40	black	nil
L4T3			35	black	nil
D51A			60	white	nil
D6A7			90	brown	nil
D7N3			20	grey	nil
D8T3			35	black	nil
L91A			54	black	nil
L10A7			80	brown	nil
L11N3			40	black	nil
L12T3			36	black	nil
L1A	01/03/07	35 days after incubation		white	nil
L2A7			62 mm	brown	nil
L3N3			45	black	seed destroyed
L4T3			40	black	nil

D51A		75	white	media hydrolysis necrosis
D6A7		90	brown	embryo swollen
D7N3		25	grey	nil
D8T3		38	black	bacterial contam
L91A		62	black	bacterial contam
L10A 7		90	brown	seed infected
L11N 3		45	black	seed infected
L12T 3		40	black	seed infected
L1A	08/03/07 42 days after incubation		white	nil
L2A7			brown	nil
L4T3			black	nil
D6A7			brown	2 seed with lrg embryos
D7N3			grey	media hydrolysis
D8T3			black	media hydrolysis
L91A			black	seed destroyed
L10A 7			brown	contaminated mites
L1A	14/03/07 48 days after incubation		white	seed infected
L2A7			brown	seed destruction
L3N3			black	seed destroyed
L4T3			black	nil
D51A			white	seed destruction
D6A7			brown	3 seed with lrg embryos
L91A			black	seed destroyed
L10A 7			brown	contaminated mites
L11N 3			black	static
L12T 3			black	static
	19/03/07 53 days after incubation		white	seed destruction
L4T3			black	no seeds evident
D6A7			brown	same as
D6A7	28/03/07 62 days after incubation		brown	3 seed with lrg embryos

Oscillation of *Nematoceras iridescens*

Wind speed	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	1.1	1.2	1.3
Oscillation													
a	0	0	0	0	0	5	5	8	10	15	15	16	20
b	0	0	0	0	0	2	5	5	10	12	15	15	16
c	0	0	0	0	2	5	6	8	12	15	15	15	18
d	0	0	0	0	0	2	5	5	9	15	15	15	16
e	0	0	0	0	0	3	4	8	9	12	15	16	17
Avg	0	0	0	0	0.4	3.4	5	6.8	10	13.8	15	15.4	17.4
SDg	0	0	0	0	0.894427	1.516575	0.707107	1.643168	1.224745	1.643168	0	0.547723	1.673320
SE	0.25	0.25	0.25	0.25	1.0574	0.812	1.1892	0.7801	0.9036	0.7801	0	1.3512	0.7731
Oscillation													
f	0	0	0	0	1	5	6	10	10	12	15	15	15
g	0	0	0	0	0	3	5	10	10	15	16	18	18
h	0	0	0	0	0	3	5	10	10	16	18	20	20
i	0	0	0	0	0	4	5	5	11	15	16	17	20
j	0	0	0	0	2	5	5	10	15	15	15	16	20
Avg	0	0	0	0	0.6	4	5.2	9	11.2	14.6	16	17.2	18.6
SDg	0	0	0	0	0.894427	1	0.447214	2.236068	2.167948	1.516575	1.224745	1.923538	2.19089
SE	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	1.0574	1	1.4953	0.6687	0.6792	0.812	0.9036	0.721	0.6756

Appendix 5 Culture of *N. iridescens* root cortex tissue

Key 1st letter = location, 1st number = plant, 3rd letter = (a = root section b = rhizome section) section quadrat 1/4's

Location	Media	culture #	Code 1°culture	cult detail	sub-cultured	DNA Library	Sequence analysis
Moki 1	PDA	M1a1	M1a1	contaminated			
Moki 1	PDA	2	M1a2	contaminated			
Moki 1	PDA	3	M1a3	nil			
Moki 1	PDA	4	M1a4	nil			
Moki 1	PDA	b 1	M1b1	ontaminated			
Moki 1	PDA	2	M1b2	nil			
Moki 1	PDA	3	M1b3	nil			
Moki 1	PDA	4	M1b4	nil			
Moki 2	PDA	a 1	M2a1	nil			
Moki 2	PDA	2	M2a2	+	17/08/05	24/08/05	6/09/05 nil product
Moki 2	PDA	3	M2a3	nil			
Moki 2	PDA	4	M2a4	nil			
Moki 2	PDA	b 1	M2b1	contaminated			
Moki 2	PDA	2	M2b2	nil			
Moki 2	PDA	3	M2b3	nil			
Moki 2	PDA	4	M2b4	nil			
Moki 3	PDA	a 1	M3a1	contaminated			
Moki 3	PDA	2	M3a2	nil			
Moki 3	PDA	3	M3a3	nil			
Moki 3	PDA	4	M3a4	nil			
Moki 3	PDA	b 1	M3b1	nil			6/09/05 nil product

Location	Medium	Code	Sample	Result	Dates
Moki 3	PDA	2	M3b2	nil	6/09/05 nil product
Moki 3	PDA	3	M3b3	+++	17/08/05 24/08/05 27/09/05 nil product
Moki 3	PDA	4	M3b4	+	24/08/05 13/09/05 27/09/05 nil product
Moki 4	PDA	a 1	M4a1	contaminated	
Moki 4	PDA	2	M4a2	nil	
Moki 4	PDA	3	M4a3	nil	
Moki 4	PDA	4	M4a4	nil	
Moki 4	PDA	b 1	M4b1	+	24/08/05 13/09/05 20/09/05 nil product
Moki 4	PDA	2	M4b2	nil	
Moki 4	PDA	3	M4b3	nil	
Moki 4	PDA	4	M4b4	nil	
Moki 5	PDA	a 1	M5a1	contaminated	
Moki 5	PDA	2	M5a2	nil	
Moki 5	PDA	3	M5a3	nil	
Moki 5	PDA	4	M5a4	nil	
Moki 5	PDA	b 1	M5b1	+	6/09/05 13/09/05 20/09/05 nil product
Moki 5	PDA	2	M5b2	+	6/09/05 13/09/05 20/09/05 nil product
Moki 5	PDA	3	M5b3	nil	
Moki 5	PDA	4	M5b4	nil	
Tarata 1	PDA	a 1	T1a1	nil	
Tarata 1	PDA	2	T1a2	nil	
Tarata 1	PDA	3	T1a3	nil	
Tarata 1	PDA	4	T1a4	nil	
Tarata 1	PDA	b 1	T1b1	nil	
Tarata 1	PDA	2	T1b2	nil	
Tarata 1	PDA	3 T	1b3	nil	
Tarata 1	PDA	4	T1b4	+	6/09/05
Tarata 2	PDA	a 1	T2a1	+	20/09/05 27/09/05 04/10/05 nil product
Tarata 2	PDA	2	T2a2	nil	
Tarata 2	PDA	3	T2a3	nil	
Tarata 2	PDA	4	T2a4	nil	

Sample	Medium	Rep	Code	Result	Notes
Tarata 2	PDA	b 1	T2b1	nil	
Tarata 2	PDA	2	T2b2	nil	
Tarata 2	PDA	3	T2b3	nil	
Tarata 2	PDA	4	2b4	nil	
Tarata 3	PDA	a 1	T3a1	+	20/09/05 Slow, brown, cushion 27/09/05 25/10/05 *Ascomycete*
Tarata 3	PDA	2	T3a2	nil	
Tarata 3	PDA	3	T3a3	nil	
Tarata 3	PDA	4	T3a4	nil	
Tarata 3	PDA	b 1	T3b1	+	0/09/05 Slow, white, cushion 27/09/05 18/10/05 nil product
Tarata 3	PDA	2	T3b2	+	20/09/05 27/09/05 04/10/05 nil product
Tarata 3	PDA	3	T3b3	nil	
Tarata 3	PDA	4	T3b4	+	20/09/05 V slow, radiate,white 27/09/05 18/10/05 nil product
Tarata 4	PDA	a 1	T4a1	nil	
Tarata 4	PDA	2	T4a2	nil	
Tarata 4	PDA	3	T4a3	nil	
Tarata 4	PDA	4	T4a4	nil	
Tarata 4	PDA	b 1	T4b1	contaminated	
Tarata 4	PDA	2	T4b2	nil	
Tarata 4	PDA	3	T4b3	nil	
Tarata 4	PDA	4	T4b4	contaminated	
Tarata 5	PDA	a 1	T5a1	contaminated	
Tarata 5	PDA	2	T5a2	nil	
Tarata 5	PDA	3	T5a3	contaminated	
Tarata 5	PDA	4	T5a4	nil	
Tarata 5	PDA	b 1	T5b1	nil	
Tarata 5	PDA	2	T5b2	nil	
Tarata 5	PDA	3	T5b3	nil	
Tarata 5	PDA	4	T5b4	nil	

Site	Media	Code	Isolate	Result	Dates	Notes
Matemateonga 1	PDA	1a 1	Ma1a1	+	20/09/05 27/09/05 27/10/05 nil	
Matemateonga 1	PDA	2	Ma1a2	nil		
Matemateonga 1	PDA	3	Ma1a3	nil		
Matemateonga 1	PDA	4	Ma1a4	nil		
Matemateonga 1	PDA	1 b 1	Ma1b1	nil		
Matemateonga 1	PDA	2	Ma1b2	nil		
Matemateonga 1	PDA	3	Ma1b3	nil		
Matemateonga 1	PDA	4	Ma1b4	nil		
Matemateonga 2	PDA	a 1	Ma2a1	nil	17/08/05 24/08/05 + 13/09/05	Rhizoc form 27/09/05
Matemateonga 2	PDA	2	Ma2a2	nil		
Matemateonga 2	PDA	3	Ma2a3	nil		
Matemateonga 2	PDA	4	Ma2a4	nil		
Matemateonga 2	PDA	b 1	Ma2b1	nil		
Matemateonga 2	PDA	2	Ma2b2	nil		
Matemateonga 2	PDA	3	Ma2b3	nil		
Matemateonga 2	PDA	4	Ma2b4	nil		
Matemateonga 3	PDA	a 1	Ma3a1	nil		
Matemateonga 3	PDA	2	Ma3a2	nil		
Matemateonga 3	PDA	3	Ma3a3	nil		
Matemateonga 3	PDA	4	Ma3a4	nil		
Matemateonga 3	PDA	b 1	Ma3b1	nil		
Matemateonga 3	PDA	2	Ma3b2	nil		
Matemateonga 3	PDA	3	Ma3b3	nil	17/08/05 24/08/05 + 13/09/05	Rhizoc form 27/09/05
Matemateonga 3	PDA	4	Ma3b4		24/08/05 13/09/05 fast	Rhizoc form 27/09/05
Matemateonga 4	PDA	a 1	Ma4a1	nil		
\Matemateonga 4	PDA	2	Ma4a2	nil		
Matemateonga 4	PDA	3	Ma4a3	nil		
Matemateonga 4	PDA	4	Ma4a4	nil	24/08/05	
Matemateonga 4	PDA	b 1	Ma4b1			

Location	Media	Code	Sample			Dates
Matemateonga 4	PDA	2	Ma4b2		nil	
Matemateonga 4	PDA	3	Ma4b3		nil	
Matemateonga 4	PDA	4	Ma4b4		nil	
Matemateonga 5	PDA	a 1	a5a1	nil		
Matemateonga 5	PDA	2	Ma5a2		nil	
Matemateonga 5	PDA	3	Ma5a3		nil	
Matemateonga 5	PDA	4	Ma5a4		nil	
Matemateonga 5	PDA	b 1	Ma5b1		nil	
Matemateonga 5	PDA	2	Ma5b2		nil	
Matemateonga 5	PDA	3	Ma5b3		nil	
Matemateonga 5	PDA	4	Ma5b4		nil	
Uruti 1	PDA	a 1	U1a1		nil	
Uruti 1	PDA	2	U1a2		nil	
Uruti 1	PDA	3	U1a3		nil	
Uruti 1	PDA	4	U1a4		nil	
Uruti 1	PDA	b 1	U1b1		nil	
Uruti 1	PDA	2	U1b2		nil	
Uruti 1	PDA	3	U1b3		nil	
Uruti 1	PDA	4	U1b4		nil	
Uruti 1	PDA tuber 1		U1tub1		+	13/09/05 18/10/05 25/10/05 nil product
Uruti 1	PDA tuber 2		U1tub2		+	13/09/05 27/09/05 25/10/05 nil product
Uruti 1	PDA tuber 3		U1tub3			
Uruti 2	PDA	a 1	U2a1	+		20/09/05 18/10/05 25/10/05 nil product
Uruti 2	PDA	2	U2a2			
Uruti 2	PDA	3	U2a3	+		20/09/05 27/09/05 04/10/05 nil product
Uruti 2	PDA	4	U2a4			
Uruti 2	PDA	b 1	U2b1	no b roots		
Uruti 2	PDA	2	U2b2	no b roots		
Uruti 2	PDA	3	U2b3	no b roots		
Uruti 2	PDA	4	U2b4	no b roots		
Uruti 3	PDA	a 1	U3a1	+		27/09/05

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Location	Media	Rep	Code	Result	Notes
Uruti 3	PDA	2	U3a2	nil	
Uruti 3	PDA	3	U3a3	nil	
Uruti 3	PDA	4	U3a4	nil	25/10/05 nil product
Uruti 3	PDA	b 1	U3b1	+	20/09/05 04/10/05
Uruti 3	PDA	2	U3b2	++	rown
Uruti 3	PDA	3	U3b3	+	20/09/05 04/10/05 25/10/05 nil product
Uruti 3	PDA	4	U3b4	nil	
Uruti 4	PDA	a 1	U4a1	+	20/09/05 04/10/05 25/10/05 nil product
Uruti 4	PDA	2	U4a2	nil	
Uruti 4	PDA	3	U4a3	nil	
Uruti 4	PDA	4	U4a4	nil	
Uruti 4	PDA	b 1	U4b1	+	20/09/05 04/10/05 25/10/05 nil product
Uruti 4	PDA	2	U4b2	+	20/09/05 04/10/05 25/10/05 nil product
Uruti 4	PDA	3	U4b3	nil	
Uruti 4	PDA	4	U4b4	nil	
Uruti 5	PDA	a 1	U5a1	nil	
Uruti 5	PDA	2	U5a2	nil	
Uruti 5	PDA	3	U5a3	nil	
Uruti 5	PDA	4	U5a4	nil	
Uruti 5	PDA	b 1	U5b1	nil	
Uruti 5	PDA	2	U5b2	nil	
Uruti 5	PDA	3	U5b3	nil	
Uruti 5	PDA	4	U5b4	nil	
Nursery 1	PDA	a 1	N1a1	nil	
Nursery 1	PDA	2	N1a2	nil	
Nursery 1	PDA	3	N1a3	nil	
Nursery 1	PDA	4	N1a4	nil	
Nursery 1	PDA	b 1	N1b1	nil	
Nursery 1	PDA	2	N1b2	nil	double sequence 22/11/05

Nursery	Media	Rep	Sample	Result	Notes
Nursery 1	PDA	3	N1b3	nil	*Trichoderma 22/11/05*
Nursery 1	PDA	4	N1b4	nil	*Trichoderma 22/11/05*
Nursery 1	PDA t	tuber	N1tub1		+ 13/9/5 20/09/05 27/09/05 nil product
Nursery 1	PDA	tuber	N1tub2		+ 13/9/5 20/09/05 27/09/05 nil product
Nursery 1	PDA	tuber	N1tub3		+ 13/9/5 20/09/05 27/09/05 nil product
Nursery 1	PDA	tuber	N1tub4		+ 13/9/5 20/09/05 27/09/05 nil product
Nursery 2	PDA	a 1	N2a1	nil	
Nursery 2	PDA	2	N2a2	nil	
Nursery 2	PDA	3	N2a3	nil	
Nursery 2	PDA	4	N2a4	nil	
Nursery 2	PDA	b 1	N2b1	+	13/9/5 20/09/05 27/09/05 nil product
Nursery 2	PDA	2	N2b2	+	13/9/5 20/09/05 27/09/05 *Trichoderma 22/11/05*
Nursery 2	PDA	3	N2b3	nil	
Nursery 2	PDA	4	N2b4	+	13/9/5 20/09/05 27/09/05 nil product
Nursery 3	PDA	a 1	N3a1	+	13/9/5 20/09/05 18/10/05 *Sebacinaceae*
Nursery 3	PDA	2	N3a2	nil	
Nursery 3	PDA	3	N3a3	nil	
Nursery 3	PDA	4	N3a4	+	13/09/05 27/09/05
Nursery 3	PDA	b 1	N3b1	+	13/09/05 27/09/05 18/10/05 nil product
Nursery 3	PDA	2	N3b2	nil	
Nursery 3	PDA	3	N3b3	nil	
Nursery 3	PDA	4	N3b4	nil	
Nursery 4	PDA	a 1	N4a1	nil	
Nursery 4	PDA	2	N4a2	nil	
Nursery 4	PDA	3	N4a3	nil	
Nursery 4	PDA	4	N4a4	nil	
Nursery 4	PDA	b 1	N4b1	contaminated	
Nursery 4	PDA	2	N4b2	nil	
Nursery 4	PDA	3	N4b3	nil	
Nursery 4	PDA	4	N4b4	nil	
Nursery 5	PDA	a 1	N5a1	nil	
Nursery 5	PDA	2	N5a2	nil	
Nursery 5	PDA	3	N5a3	nil	

Nursery 5   PDA   4   N5a4   nil
Nursery 5   PDA   b 1   N5b1   nil
Nursery 5   PDA   2   N5b2   nil
Nursery 5   PDA   3   N5b3   nil
Nursery 5   PDA   4   N5b4   nil

Singularybas oblongus   PDA   plain leaf   1   Sing1L   15/11/05 nil product
Singularybas oblongus   PDA   plain leaf   2   Sing2L   15/11/05 nil product
Singularybas oblongus   PDA   plain leaf   3   Sing3L   15/11/05 nil product
Singularybas oblongus sngl flwr   PDA var. leaf 1flwr
S. oblongus Var   PDA   1 L   15/11/05 nil product
Singularybas oblongus dbl. flwr   PDA var. leaf 2 flwr
SingVar   PDA   2 L   15/11/05 nil product

Singularybas oblongus   PDA root section 1 Sing1R 15/11/05 nil.
Singularybas oblongus   PDA root section 2 Sing2R 15/11/05 *Gyoerffyella* spp. Ascomycete
Singularybas oblongus   PDA root section 3 Sing3R 15/11/05 nil product
Singularybas oblongus sngl flwr   PDA root section 4 SingVar 1 R 15/11/05 nil product
Singularybas oblongus dbl. flwr   PDA root section 5 SingVar 2 R 15/11/05 *Sebacinaceae*

CULTURES 18/10/2005 21 deg C dark

PDA   1   T3b1   slow white
PDA   2   T3b4   42mm   white fluffy
PDA   3   U2a1   8 mm   white concentric rings
PDA   4   U1tub1   2 mm   white
PDA   5   N3a1   40mm   brown fluffy
PDA   6   N3b1   50mm   brown fluffy

CULTURES 25/10/2005

CULTURES 28/06/2006 21 deg C dark

	PDA	U1b1	+++	*Tulasnella* match AY634130 N2b4
				+++

Species					Description
Nematoceras macranthus	PDA	tuber 1	Nmt1		contaminated
Nematoceras macranthus	PDA	2	Nmt2	+	42 mm brown wooly
Nematoceras macranthus	PDA	3 tuber	Nmt3	+	32 mm brown wooly
Nematoceras macranthus	PDA	4 tuber	Nmt4	+	40 mm brown wooly
Nematoceras macranthus	PDA	5 old	NmOR1	+	20 mm brown wooly
Nematoceras macranthus	PDA	6 old	NmOR2	+	60 mm brown wooly
Nematoceras macranthus	PDA	7 new	NmNR1	+	15 mm brown young white wooly
Nematoceras macranthus	PDA	8 new	NmNR2 nil		

CULTURES 5/09/2006
Isolate from (04/10/2005) held at 4 degrees C

					Description
Tarata 2 (N. iridescens)	PDA	a 1	T2a1	+	40 mm old d'brown to beige wooly hyphae
Tarata (Aneura spp.) spp A	PDA	2	TAA	+	55 mm white wooly hyphae
Matemateonga (Aneura spp)	PDA	3	MAA	+	white wooly hyphae

CULTURES 12/10/2006
5 samples/plate
Media culture # Code 1°culture cult detail sub-cultured DNA
Isolate from (12/10/2005) held at 12 degrees C

	Media culture #	Code 1°culture		cult detail sub-cultured DNA
Uruti N. iridescens young root	PDA a 29a	U29a	1/5	1 mm white smooth
Uruti N. iridescens old root	PDA b 29b	U29b	0/5	nil
Uruti N. iridescens tuber	PDA tub 29c	U29c	3/5	40 mm buff v. fine mycellium
Uruti Metzgeriales thallus	PDA thallus	29Umetz	1/4	total plate white dense cotton w
Moki N. iridescens young root	PDA a30a	M30a	1/5	20 mm white hyaline v. fine mycellium
Moki N. iridescens old root	PDA b30b	M30b	0/4	nil
Moki N. iridescens tuber	PDA tub30c	M30c	0/5	nil

	Media	culture #	Code	1°culture	cult detail
Moki N. iridescens	-	-	-	-	-
Moki Metzgeriales thallus	PDA	thallus	Mmetz	4/4	1 bacterial 3 white cotton wool Rhizoctinia
Tarata *N. iridescens* young root	PDA	a31a,	T31a	1/4	buff v. fine mycellium
Tarata *N. iridescens* old root	PDA	b31b	T31b	2/4	buff v. fine mycellium

CULTURES 26/10/2006 5 samples/plate
Isolate from (12/10/2005) PDA Calcfl UV held at 12° C

	Media	culture #	Code	1°culture	cult detail
Anzybas rotundifolius old root	PDA	b 1	Anzrot1b	nil	Te Paki
Anzybas rotundifolius young root	PDA	a 2	Anzrot2a	nil	Te Paki
Singularybas oblongus young root	PDA	a 3	Singob1a	+++	Pukeiti
Singularybas oblongus old root	PDA	b 4	Singob1b	+++	Pukeiti

CULTURES 26/10/2006
5 samples/plate Media culture # Code 1°culture cult detail

	Media	culture #	Code	1°culture	cult detail
Uruti Metzgeriales thallus	PDA	thallus 29 I	Umetz	1/4 i	white total plate dense
Uruti Metzgeriales thallus	PDA	thallus 29 ii	Umetz	1/4 ii	white total plate dense
Moki Metzgeriales thallus	PDA	thallus 30	Mmetz	4/4	green-blue fruiting bodies

Uruti *N. iridescens* young root	PDA	a 29a	U29a	1/5	contaminated
Uruti *N. iridescens* old root	PDA	b 29b	U29b	0/5	nil
Uruti *N. iridescens* tuber	PDA	tub 29c	U29c	3/5	nil
Moki *N. iridescens* young root	PDA	a 30a	M30a	1/5	20 mm white total plate
Moki *N. iridescens* old root	PDA	b 30b	M30b	0/4	nil
Moki *N. iridescens* tuber	PDA	tub 30c	M30c	0/5	nil 24/05/2007

Culture comparison library PDA oat and Humate 12C dark PDA + Oatmeal PDA + humate

Uruti 2	U2a3	22 mm white fine colony nil growth
Uruti 3	U3b3	60 mm white fine colony nil growth
Uruti 4	U4a1	20 mm fawn fine colony nil growth
Uruti 4	U4b1	30 mm white cushion nil growth
Uruti 4	U4b2	10 mm fawn cushion 5 mm white fine filament
Moki	M3b2 M2a	80 mm white fine colony nil growth
Moki	M4b1 M3b	15 mm white cushion 5 mm white fine filament
Moki 3	M3b2	85 mm white fine upright filaments nil growth
Moki	M5b2 M4b	15 mm white cushion nil growth
Moki 5	M5b1	nil growth
Moki	M5b2	5 mm white fine filament
Matemateonga	Mate1a1 3	0 mm white fine blue black fruiting contaminated 30 mm white fine blue black fruiting
Tarata	Tara3b2	15 mm white fine colony
Nursery 1	N1tub1	20 mm white fine blue black fruiting contaminated 90mm white fine blue black fruiting
Nursery 1	N1tub2	30mm smooth white (bacterial?) contaminated nil growth
Nursery 1	N1tub3	10 mm white cushion nil growth
Nursery 1	N1tub4	70 mm white fine colony 90 mm fine fawn cushion
Nursery 2	N2b1	90 mm fine fawn cushion
Nursery 2	N2b2	nil growth nil growth
Nursery 2	N2b4	80 mm white fine upright filaments 80 mm white fine upright filaments
Nursery 3	N3a1	15 mm white cushion 15mm brown cushion
Nursery	N3b1	15 mm white cushion 15mm brown cushion

Mollybas red 1 (Matemateonga)	A7	20mm smooth white (bacterial?) contaminated 15mm brown cushion

Mollybas red 2 (Matemateonga)   A8   25 mm white fine colony 20-25mm brown cushion
Mollybas alba (Matemateonga)   A9   30 mm white fine blue black fruiting contaminated 30 mm white fine blue black fruiting

20/07/2006 -
Library marked as 27/07/2006 7/08/2006 9/08/2006

Tarata *N. iridescens*   tuber   1/2 series   Slow, brown, cushion 450mm zoned brown
Tarata *N. iridescens*   a   2/2 "   2T2a nil brown small
Tarata *N. iridescens*   b   3/2 "   v.slow small no colour 2 cultures 30mm zoned fawn – brown
Matemateonga *N. iridescens* b & b 1   4/2 " nil.
Matemateonga *N. iridescens*   a & a   2   5/2 " nil.
Matemateonga Metzgeriales *Riccardia* spp. 1M nil 7/08/2006