Final Report to the Australian Flora Foundation

Epacris impressa Labill.:
Inoculation of cuttings with ericoid mycorrhizal fungus and DNA fingerprinting of floral races

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Abstract

Epacris impressa Labill. is an attractive heathland shrub endemic to the state of Victoria, parts of South Australia and Tasmania and southern New South Wales. The plant has showy red, pink or white flowers for most of the winter and has potential markets in landscaping and revegetation, as well as a cutflower. Flower colours fall into three general flower colour races: red, pink and white (Stace & Fripp 1977a, 1977c, 1977b). Like all members of the Ericaceae, *E. impressa* forms a symbiotic relationship with fungi that colonise its hair roots. It is primarily an outcrossing species with some examples of selfing occuring in each population (Fripp 1982; O'Brien, S. P. & Calder 1989)

Few nurseries propagate *E. impressa* since it has proved difficult to grow from cuttings or seed. Strike rates are often as low as 10% for cuttings and seed germination often fails. Selection of propagation material for revegetation purposes is usually determined by local anecdotal information and provenance delineation is not based on genetic traits.

This study examined the use of ericoid mycorrhizal fungus as an inoculum to stimulate root and shoot production from cuttings. The fungus did not provide any benefits to root and shoot growth or health, but methods for improved propagation success rates were developed during the experiments.

Genetic fingerprinting techniques were also used to examine relationships between geographic sites and flower colour populations and to aid in provenance determination. Results indicated that *E. impressa* has a high level of both inter-site and intra-site genetic diversity. The red and white floral races had a high level of shared genetic traits while the pink-flowered race had a more distinct genetic identity. The results suggested that the pink-flowered populations have developed into a separate floral colour race rather than an F1 hybrid between red and white races.

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1. Introduction

Ericaceous plants are found throughout the world in heathland environments. The types of ecosystems in which they thrive vary greatly but all have low soil nitrogen. The plants have developed a unique 'hair root' system and formed symbiotic relationships with ericoid mycorrhizal fungi.

The flowers of *E. impressa* fall into three general races: red, pink and white. The red race has flowers that range from dark to bright red, while the pink race flowers are a light-pink colour, sometimes with white corolla tips. The white race has flowers that are all-white, with no traces of any colour. The buds of the white flowers are often pink in colour when unopened but are completely white when open.

E. impressa has proved difficult to propagate by seed or cuttings and is currently grown by only a limited number of Victorian nurseries. With higher propagation rates, the plant has a potential market in both revegetation and landscaping and as a cutflower. While not currently under threat, *E. impressa* is not being restored in the wild due to the low numbers of plants in horticultural production.

This report will seek to answer two questions:

1. **Does mycorrhizal inoculum aid in shoot initiation, plant health and growth rates with propagation by field cuttings of *Epacris impressa*?**
2. **What are the intra-race and inter-race genetic relationships of different geographic populations of *Epacris impressa*?**

1.1. Research Sites

The six research sites (figure 1) were selected based on their geographic distances, distinct flower-colour raciation in populations, and varying soil types. The main research sites used for both propagation and genetic studies were Angahook-Lorne State Park, 38°37'27" S, 143°53'97" E (site A), the Royal Botanic Gardens, Cranbourne, 38°07'66" S, 145˚16'48" E (site C) and 38˚08'07" S, 145˚15'65" E (site CA), and

Grampians National Park, Sundial track , 37°10'39" S, 142°30'89" E (site G). The soil types for the four propagation experiment sites are listed below (table 1). Two additional sites were used for genetic studies, Braeside Park Heathland, 37˚59'42" S, 145˚08'00" E (site B) and a privately-owned site in Tulla, Tasmania, 41˚44'35" S, 145˚37'25" E (site T).

Figure 1. Research site locations (Google Earth 2007)

Site	Soil Type
Angahook-Lorne State Park	Brown duplex soils, fine sandy clay loam (Pitt 1981)
RBG Cranbourne, C	Cranbourne sand, dark grey sandy lateric podzols (Sargeant 1975)
RBG Cranbourne, CA	dark grey sandy lateric podzols, sandy loam (Sargeant 1975)
Grampians	Skeletal soils and rocky shallow podzols (Sibley 1967)

Table 1. Site soil types.

1.1.1. Angahook-Lorne State Park

Angahook-Lorne State Park is a coastal heathland south of Melbourne. Since this research was completed, the park has been incorporated into the recently formed Great Otway National Park which combines the former Otway National Park, Carlisle and Melba Gully State Parks, and areas of State forest and Crown land. Site A was located 2.2 kilometres inland along the Wye River Road in Open Forest. The major site vegetation consisted of *Eucalyptus cypellocarpa*, *E. globula*, *E. obliqua*, and *E. sideroxlyon*,

1.1.2. Royal Botanic Gardens, Cranbourne (sites C and CA)

The Royal Botanic Gardens, Cranbourne is a 363 hectare area of Australian native and indigenous vegetation. The park, which opened in 1989, consists of former sand-mining and cattle-grazing land (Aitken & Kershaw 1993). Site C was a sandy soil open heathland dominated by *E. impressa* and bordered by *Eucalyptus viminalis* ssp. *viminalis* and *Banksia marginata*. Site CA was a grassy woodland with clay loam soils dominated by *Acacia dealbata*.

1.1.3. Grampians

The Grampians National Park is located 260km northeast of Melbourne, Victoria. The park is home to unique flora and fauna with over 800 indigenous plant species. The collection site was a heath woodland near the Sundial track containing a polymorphic flower-colour population of *E. impressa* var. *grandiflora*. Other ericoid species present included *Astroloma conostephioides* (Flame Heath), *A. pinifolium* (Pine Heath) and *Styphelia adscendens* (Golden Heath). The dominant species at the site included *Eucalyptus baxteri*, *E. goniocalyx*, and *E. oblique*.

2. Materials and Methods

2.1. Propagation experiments

Two ericoid mycorrhizal fungi that were found to improve plant growth in micropropagation were used as inocula in three separate propagation experiments. For the first experiment, *E. impressa* cuttings were collected from field sites A, C, and CA and from *E. impressa* var. *grandiflora* from site G in spring 2001. The struck cuttings from experiment one had root samples taken for mycorrhizal assessment and were potted into 7cm tubes at 20 weeks. Plants from tubes were potted on to 20cm pots at 52 weeks. Material was collected from *E. impressa* var. *grandiflora* at site G in Spring 2002 for experiment two. *E. impressa* cuttings for experiment three were obtained from sites A, C and from three year-old nursery plants from experiment one in Spring 2004. Mycorrhizal inoculum was prepared six weeks prior to the sourcing of cuttings. A peat moss and pine bark based inoculum was used in experiments one and two and a liquid medium in experiment three.

2.1.1. *Phytophthora cinnamomi* testing

Soil samples were taken from each site and tested for *Phytophthora cinnamomi* prior to any soil being brought into the nursery. The testing method used *Eucalyptus sieberi* cotyledons to bait potential *P. cinnamomi* in the soil samples (Lawrie 2001) First, *Eucalyptus sieberi* seeds purchased from AustraHort Pty. Ltd. Cleveland, Queensland were surface sterilised with 1% chlorine bleach in sterile deionised water. Under sterile conditions the seeds were placed in a TechnoPlas 90mmx14mm Petri dish on Whatman® number one 70mm circle filter paper layed on top of 3cm x 3cm sponge pieces that had been moistened with deionised water. The containers were sealed with parafilm and placed in a growth room at 24°C for one week until germination occurred and cotyledons were present.

The soil to be tested was collected by spade to a depth of 15cm at each experimental site and stored in zip-lock plastic bags until use. In the University of Melbourne, Burnley College plant science laboratory, 1cm of soil was placed in a 200ml LabServ jar and labelled with site information. Two samples were prepared for each site along with a control jar with no soil. Each jar was filled to two-thirds with sterile deionised water and ten cotyledons were removed from the *E. sieberi* seedlings using forceps and sterile razor blades and placed in each jar. Jars were closed, sealed with Parafilm and placed in a growth room at 20°C for one week.

The cotyledons were then transferred from each jar to a Petri dish with 10ml sterile deionised water. Each dish was examined under an Olympus SZ51 dissecting microscope under x100 magnification. Cotyledons were inspected for signs of *P. cinnamomi*.

2.1.2. Propagation experiment one

2.1.2.1. EMF inoculum preparation

Ericoid mycorrhizal fungi Ei1.1 and Ei4.1.2 that had been isolated from *E. impressa* roots (McLean, C. M. 1999) were provided by Dr. Cassandra McLean. Both fungi were found to improve growth of *E. impressa* in micropropagation and warranted further investigation as inocula (table 2) (McLean, C. B. et al. 1998; McLean, C. M. 1999). Fungi Ei1.1 and Ei4.1.2 were plated out onto malt extract agar (MEA) under sterile conditions in a laminar flow cabinet.

Malt extract agar (MEA) was prepared by mixing 50gm powdered Oxoid MEA with one litre deionised water in a two litre Simax Schott bottle. The mixture was dissolved and sterilised in an autoclave at 120°C for 20 minutes, poured into TechnoPlas Petri dishes under sterile conditions and allowed to cool and solidify. A five-millimetre cube of the ericoid mycorrhizal fungus Ei1.1 or Ei4.1.2 was cut from the outer active growth

area of each of the two fungi, placed in the centre of the medium in a TechnoPlas Petri dish and sealed with American National Can Parafilm®. The petri dishes were placed in the dark at 20°C for eight weeks to allow for fungal growth (figure 2).

Fungi	Source	Collection Date	Colour	Benefit provided to micropropagated *E. impressa* plants
Ei1.1	Nowa Nowa	April 1997	dark grey	High infection rate of 65% Increased growth rate of 156%
Ei4.1.2	RBG Cranbourne	December 1995	dark grey	High infection rate of 42% Axenically grown plants were healthy for more than 6 months

Table 2. Fungal inoculum selected for propagation experiments.

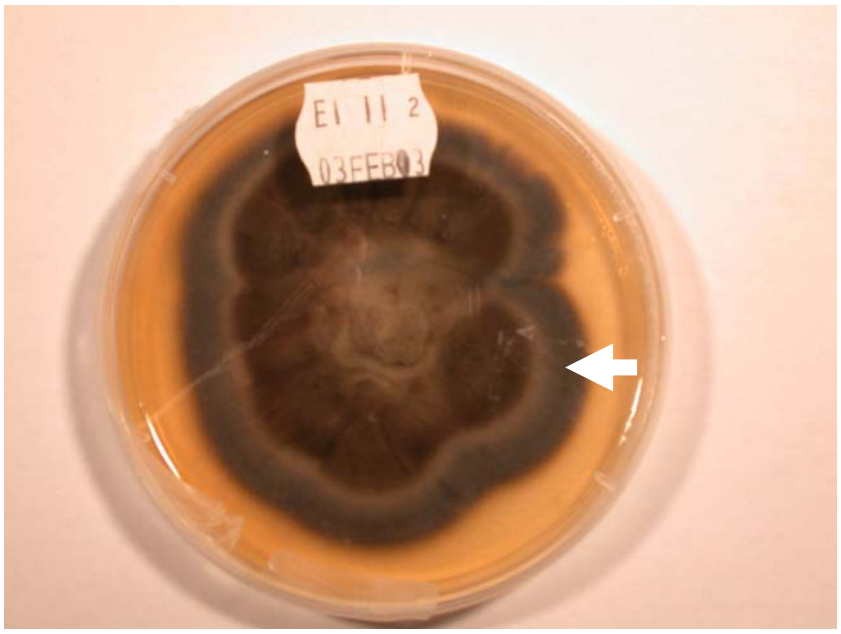

Figure 2. EM Fungi Ei1.1 sub-cultured onto MEA. The white arrow shows the area of new fungal growth from which the inoculum was taken.

The EMF inoculum was prepared in sterilized 100ml jars in the following media: 70% sphagnum peat moss, 20% composted pine bark of 1cm or less in size, and 10% medium grade vermiculite (Starrett, M.C. 2000, pers.comm., 29 August). The medium

was moistened with 1% sucrose solution in deionised water, sterilized for 20 minutes at 120ºC, cooled and then placed into 100ml glass jars under sterile conditions. Next, a 5mm cube of the sub-cultured Ei1.1 or Ei4.1.2 was cut from the outer active growth area and placed in the centre of the medium in each jar. A further 1ml of sterilized 1% sucrose solution was added to rinse in the mycelia and provide nutrients to aid in the growth of the fungus. The jar lids were sealed with parafilm and stored in the dark at 20°C until hyphal growth was visible after 4 weeks. Due to the presence of a fast-growing yellow fungal contaminant in all the jars after two weeks, the Ei4.1.2 inoculum was not used in propagation experiments.

2.1.2.2. Field collection of cuttings

E. impressa cuttings for experiment one were collected from sites A and CA in September 2001 and from site C in November 2001. *E. impressa* var. *grandiflora* cuttings were gathered from site G in November 2001. A research permit was obtained from the Victorian Department of Natural Resources and Environment and meetings were held with the local Rangers-in-Charge prior to any field work.

Records of GPS position using a Garmin GPS 12XL and flower colour using the *RHS Colour Chart* (Royal Horticultural Society c1995) were taken and a herbarium record was created for each sampled plant. Plants at each site were photographed and tagged with the following information: plant identification number, name of researcher, permit number at sites A and G, and date (figure 3). Twenty-eight plants were tagged at site A, fifty at site C, fifty at site CA, and twenty-six at site G. The *E. impressa* var. *grandiflora* plants at Grampians site G were approximately 200-300mm taller than *E. impressa* plants at the other sites.

Figure 3. Tagged plants at Cranbourne Site C. Cutting material was obtained from the tagged plants for propagation and DNA fingerprinting experiments.

Cuttings 30cm in length were collected from each tagged plant and placed in a cooled collection box to retain turgidity until return to the University of Melbourne, Burnley. The collection boxes were brought back to the Burnley College nursery and stored at 4°C for a maximum of 72 hours before the cuttings were potted up. The cutting material retained turgidity and appeared in good condition and each 30cm cutting provided sufficient plant material for several smaller tip cuttings.

Soil was collected by spade to a depth of 20cm from the root area of one plant at each site for use as an inoculum. Approximately one litre of soil was removed and placed in a labelled sealed plastic bag for later use.

2.1.2.3. Potting up cuttings

Prior to potting-up, the cutting material was surface sterilised for two minutes in a 5% sodium hypochlorite (NaOCl) solution and then rinsed in water. Any diseased or insect infested material was discarded. A mixture of soft and woody new season's growth was selected for tip cuttings. Uniform cutting material was not always available due to growth variations at each site.

No.	Treatment
1	Control group with no stem treatment in 100% Burnley cutting mix (BCM)
2	100% BCM
3	90% BCM and 10% site soil
4	75% BCM and 25% Ei.1.1 inoculum

Treatment groups 2, 3 and 4 were dipped for six-seconds in 1000ppm IBA.

Table 3. A summary of the four treatments used in propagation experiments one and two.

Tip cuttings from between 3-5cm in length were taken from each larger field cutting. In previous experiments (McLean, C., Lawrie & Blazé 1994), stem disturbance including stripping of leaves and re-potting of cuttings with no root growth has caused *E. impressa* cuttings to die. In order to minimise stem disturbance, the leaves from the bottom two centimetres of each cutting were removed with small sharp scissors rather than by stripping. Individual cuttings were placed in single cells in treatment groups of forty per tray. There were four separate treatments (Table 3): 1) 100% cutting mix ; 2) 100% cutting mix with 1000ppm Indole Butyric Acid (IBA) stem dip; 3) 10% site soil and 90% cutting mix; 4) 75% cutting mix and 25% Ei1.1 inoculum.

All the cuttings were grown in pasteurised Burnley cutting mix (BCM) with a pH of 5.4 consisting of the following: 2 parts P500 grade perlite; 1 part sieved peat moss; 9 parts 6mm pine bark; additives per m3: 1500g Saturaid, 1000g Dolomite. In treatments 3 and 4 the BCM and soil or fungal inoculum were thoroughly mixed before the cuttings were stuck. The cuttings were placed under fog on bottom-heated beds (24ºC) and hand watered daily for twenty weeks. Dead cutting material was removed periodically.

2.1.2.4. Assessment of cuttings

After 20 weeks the cuttings were removed and scored according to root initiation and shoot growth. Root samples were taken for clearing and staining to determine the

presence of mycorrhizal infection. Struck cuttings were potted on into individual 7cm tubes in Burnley general potting mix (4 parts medium grade pinebark, 1 part coarse mixed sand. Additives per m3: 4000g Debco Green Jacket No. 2 (N:P:K 16.5:4.1:9.6), 1500g Saturaid). The plants were then placed under mist for 5 days to harden off and then transferred outdoors under shade cloth. Healthy unstruck cuttings were re-potted in groups of seven in their original media in 15cm plastic pots.

At 52 weeks plants in tubes were potted on to 20cm pots in Burnley general potting media and mulched with 10-15cm composted graded pinebark. The plants were placed under shade cloth and overhead watered daily. Plants were assessed for health, growth, and flowering at 220 weeks.

2.1.2.5. Mycorrhizal assessment

Root samples were taken from struck plants in experiment one at 20 and 52 weeks for mycorrhizal assessment. Hair root samples were removed with forceps, potting medium was gently shaken loose, and then the samples were stored in 70% ETOH in McCartney vials until clearing and staining. In the laboratory, the roots were cleared and stained using a process modified from McLean, Lawrie and Blazé (1994). Each cleared and stained sample was then placed on a glass slide with a cover slip and examined with a Nikon Eclipse E400 microscope at 400x magnification for presence of typical ericoid mycorrhizal structures (TEMS). Infected samples were photographed using a Nikon Coolpix 995, 3.34 mega pixel digital camera.

2.1.3. Propagation experiment two

2.1.3.1. EMF inoculum preparation

The EMF Ei1.1 that had previously been sub-cultured onto MEA for experiment one was used for inoculum in experiment two. The EMF inoculum was prepared in the same method as in experiment one. Sterile procedures were used at all times for the preparation of the inoculum.

2.1.3.2. Field collection of cuttings

Epacris impressa var. *grandiflora* cutting material was collected from the Grampians National Park site G in November 2002 for experiment two. Adequate cutting material was unavailable at sites A, C and CA in the spring of 2002 due to erratic flowering times and growth patterns caused by drought conditions. Cuttings from soft tip growth had been determined to provide better root and shoot development than hard tip cuttings in propagation experiment one (Conomikes et al. 2002). The new season's growth had already hardened off well before spring collection trips to sites A, C and CA, so a smaller experiment was conducted with soft tip cuttings from *E. impressa* var. *grandiflora* plants from site G.

2.1.3.3. Potting up cuttings

The protocol for potting up cuttings was identical to that used in experiment one. One forty-cell tray of cuttings was prepared for each of the four treatments. The trays were placed under fog on bottom-heated beds (24ºC) and hand watered daily. Any dead cutting material was removed periodically.

2.1.3.4. Assessment of cuttings

The cuttings in experiment two were to be left for 30 weeks to allow additional time for mycorrhizal infection of roots, however, all cuttings had died by week 17. Due to the poor strike rates that occurred with *E. impressa* var. *grandiflora* in experiment one and deaths in experiment two, site G was not included in propagation experiment three.

2.1.4. Propagation experiment three

2.1.4.1. EMF inoculum preparation

For the third propagation experiment, a liquid inoculum was prepared as follows: a potato extract growth medium was made from 500g sliced old potatoes and 500ml sterile deionised water, sterilised for 20 minutes at 120ºC and then filtered. Fifty

millilitres of the cooled solution were placed in 200ml LabServ jars with a 5mm cube of sub-cultured EMF Ei1.1, lids were sealed with parafilm and the jars were stored in the dark at 20°C. After five weeks, a 3mm-thick dark-grey fungal layer had developed on the top of the medium and hyphae were present throughout the jar. One millilitre of the liquid medium with hyphae was removed by plastic syringe and thoroughly mixed into each litre of cutting medium at the time of potting up.

2.1.4.2. Field collection of cuttings

Cuttings for experiment three were obtained from sites A, C and from three year-old plants from experiment one growing in the University of Melbourne, Burnley nursery. Plants at site CA had died due to continuing drought conditions. The same protocols for collection, transport and storage were followed as for experiment one.

2.1.4.3. Potting up cuttings

Tip cuttings of 3-5 cm of soft new season growth material were taken from larger cuttings. Prior to potting-up, the cutting material was surface sterilised for two minutes in a 5% sodium hypochlorite solution and then rinsed in water. Any diseased or insect infested material was discarded. Due to the considerable time involved in manual removal of leaves from the bottom of cutting stems, all leaves were left on the stems.

In propagation experiment three, 40 cuttings per treatment per site were potted up in cell trays (table 4). The cell trays were cut into groups of ten and placed randomly into 56-cell trays. Each tray was made up of three random treatment groups of ten separated by one row of empty cells. Cuttings from nursery plants grown from experiment one (Group N) did not receive the 10% site soil treatment since they were growing in potting media rather than soil. The treatments in experiment three were as follows:

No.	Treatment	No. of cuttings per site		
		A	C	Nursery
1	100% Burnley cutting mix (BCM) as a control	40	40	40
2	90% BCM + 10% Site Soil	40	40	-
3	BCM + Ei1.1 liquid inoculum	40	40	40

All cuttings were dipped for 6 seconds in 1000ppm IBA.

Table 4. A summary of the three treatments used in propagation experiment three.

Treatments were placed under fog on bottom-heated beds (24ºC) and hand watered daily. The trays were raised 1cm above the heated bed to avoid cross-contamination of fungal propagules through runoff water. Dead cutting material was removed periodically. *Stratiolaelaps mile* (Hypoaspis) were scattered on the surface of the media to control larval pests. At 13 weeks, a white powdery residue was visible on the surface of many of the leaves. Under microscopic examination the powder had a crystalline formation and was determined to be a mineral salt. No further testing was done, but the leaf surfaces were thoroughly washed with deionised water using a squirt-bottle to remove the deposit. The cuttings were left for 30 weeks during this experiment to allow a longer period of time for mycorrhizal infection.

2.1.4.4. Assessment of cuttings and hair roots

After 30 weeks the cuttings were removed and scored according to root initiation and shoot growth. Plants were not potted on in experiment three on due to a low strike rate. Hair root samples were taken from struck plants for mycorrhizal assessment using the previously described protocol.

2.2. Genetic fingerprinting

Genetic fingerprinting experiments were conducted using the Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) methods. DNA samples were obtained for RAPD and ISSR experiments from each of the three flower

colour races (white, pink and red) found at each site. After initial primer screening, ISSRs and RAPDs were performed on each sample using primers that produced the clearest reproducible banding patterns.

2.2.1. DNA isolation from plant tissue

Cuttings were collected from tagged *E. impressa* plants at sites A, C and CA in September 2002 and from *E. impressa* var. *grandiflora* in November 2002 from site G. Additional samples were obtained from 10 plants at Braeside Park Heathland (site B) and 12 plants Tullah, Tasmania (site T) in September 2004. A cutting was taken from each sample and pressed for a herbarium record. Plant material collected at each site was then stored at -21 C° until required.

DNA was isolated from the *E. impressa* cuttings from sites A, C and CA in June 2003 and from sites B and T in October 2004 using the Qiagen DNeasy® Plant kit. Each sample was loaded into an agarose gel and an electrophoresis program was run to determine that DNA had been obtained.

2.2.2. Random Amplified Polymorphic DNA (RAPD)

The RAPD OPB set was selected for screening since the primers had been used successfully with *Kunzea ericoides*, *K. pomifera* (Page 2003) and the ericoid *Vaccinium macrocarpon* (Stewart & Nilsen 1995). The entire Operon OPB set of 20 primers obtained from Fisher Biotec was screened with two DNA samples, one a red-flowered plant from site A and the other a white-flowered plant from site C, using the protocol outlined below. Each sample and primer was run twice to test for reproducibility.

Eight primers, OPB1, OPB3, OPB4, OPB5, OPB6, OPB7, OPB15, and OPB19 produced clear reproducible bands in both flower colour samples. Four primers, OPB9, OPB13, OPB14, and OPB18 produced bands in only one of the two samples. Eight

primers, OPB2, OPB8, OPB10, OPB11, OPB12, OPB16, OPB17, and OPB20 failed to produce bands or produced only faint bands. Two of the eight primers that produced clear reproducible bands, OPB6 and OPB19, were selected for the RAPD experiments.

The following protocol was used in a laminar flow cabinet with RAPD OPB primers 6 and 19 (table 5): A master mix was made up with 505µl 1.25mM dNTP (stock solution made from 200µl Fermentas 10mM dNTP Mix and 1.4ml milli-q H_2O), 126µl $MgCl_2$, 458µl sterilised milli-q H_2O, and 315µl BIOTECH International Limited 10x reaction buffer (670 mM Tris-HCl, 166 mM $(NH_4)_2SO_4$, 4.5% Triton X-100, 2mg/mL gelatin). The master mix was pipetted into twelve 2ml aliquot tubes, each containing 117µl, enough for one RAPD/PCR run of 13 samples, and frozen at -21°C.

Experiment two									
Site	**Colour**	**Sample Size**	**RAPD primer**		**ISSR primer**				
			OPB6	**OPB19**	**812**	**814**	**824**	**835**	**836**
A	W	10			●				●
A	P	5	●	●	●				●
A	R	10	●	●	●				●
B	W	10	●	●	●	●	●	●	●
C	W	10	●	●	●	●			●
C	P	8	●	●	●			●	●
C	R	8	●	●	●	●	●	●	●
CA	W	10	●	●	●				●
CA	P	10	●	●	●				●
CA	R	10	●	●	●		●	●	●
G	W	10	●	●	●				●
G	P	10	●	●	●		●	●	●
G	R	10	●	●	●			●	●
T	W	3	●	●	●			●	●
T	P	4	●	●	●	●		●	●
T	R	5	●	●	●			●	●

Table 5. RAPD and ISSR primers used and population sample sizes in experiment two.

A final mix for RAPD/PCR amplification consisted of each of the following per reaction: 9µl master mix, 8µl milli-q H_2O, 1µl Operon OPB series sequence primer obtained from Monash Oligonucleotide Synthesis Facility, .5µl BIOTECH International Limited TAQ DNA Polymerase (*Thermus aquaticus*) were micro-pipetted into in an Axygen 0.2ml domed cap PCR® tube in a laminar flow cabinet. The tubes were mixed by vortexing and were then placed in a Biometra® Personal Cycler top-heating thermocycler. A negative control with no DNA was included in each run to test for contamination. The RAPD/PCR process included a 4-minute strand separation cycle at 94° C, then 45 cycles of one minute at 94° C, two minutes at 36° C, and two minutes at 72° C and a final 72° C extension step for 10 minutes.

Tubes were removed from the thermocycler and mixed by vortexing briefly at 2400rpm. Then, 5µl of the RAPD/PCR product and 2µl of 6X loading dye were pipetted into a 2% agarose gel with 0.05% EthBr and covered with 1% TBE. 1µl of MBI Fermentas GeneRuler™ 100bp Ladder Plus was loaded at one end of the gel. The gel was run through an electrophoresis program at 96v for 60 minutes and then placed on a UV light table to view bands. Bands were photographed with a Kodak Digital Science DC120 digital camera and analysed with the Kodak Digital Science 1D programme. Samples were re-run when contamination was present in the negative control or when no bands were seen. If contamination was still present or no loci were visualised, the results were not included in the analysis of bands.

2.2.3. Inter Simple Sequence Repeat (ISSR)

This research was the first to use Inter Simple Sequence Repeat method with a member of the Southern Hemisphere Ericaceae. A previous Northern Hemisphere study employed RAPD and ISSR to evaluate genetic relationships amongst *Vaccinium* (blueberry) cultivars (Levi & Rowland 1997). ISSRs were selected since they had a high correlation of results with RAPDs in previous experiments (Adams, Schwarzbach & Pandey 2003; Casasoli et al. 2001; Levi & Rowland 1997; Patzak 2001). Both techniques use similar protocols so additional equipment or materials other than primers are not required.

Six standard ISSR primers, 812, 814, 824, 835, 836 and 857 obtained from Monash Oligonucleotide Synthesis Facility were screened based on their ability to amplify loci in a variety of plant genera and species (Casasoli et al. 2001; Ge et al. 2003; Levi & Rowland 1997; Mattioni et al. 2002; Nan et al. 2003; Pharmawati, Yan & McFarlane 2004; Qiu et al. 2004; Wang et al. 2004; Xiao & Berch 1996). The six primers were screened with two DNA samples; one a red-flowered plant from site A and the other a white-flowered plant from site C. Duplicates were run for each sample and primer to test for reproducibility.

Bands were obtained with both samples with the following primers: 812, 824, 835, and 836. Primer 857 produced no bands and primer 814 produced bands in one sample. From these, the two primers that yielded the clearest reproducible bands, 812 and 836, were chosen for ongoing experiments.

The following protocol was used for each ISSR reaction in experiment two: in a laminar flow cabinet, 9µl master mix (as described in RAPDs experiment two), 7.5µl milli-q H2O, 1µl primer, .5µl Fisher Biotech TAQ DNA Polymerase and 1µl DNA were placed in an Axygen 0.2ml domed cap PCR® tube. The tubes were mixed by vortexing and were then placed in a Biometra® Personal Cycler top-heating thermocycler. A negative control with no DNA was included in each run to test for contamination. There was an initial three-minute step at 94° C, then 45 cycles of one minute at 94° C, 45 seconds at 54° C, one minute at 72° C, then a final extension step of 10 minutes at 72° C. The annealing temperature was optimised at 54° C after tests at 56° C and 55° C did not produce bands. Gels were run and bands analysed as discussed with RAPDs. Samples were re-run when contamination was present in the negative control or when no bands were seen. If contamination was still present or no loci were visualised, the results were not included in the analysis of bands.

3. Results

3.1. Propagation experiments

3.1.1. *Phytophthora cinnamomi* testing

The *E. sieberi* cotyledons were inspected microscopically as previously discussed. There was no indication in any of the samples of coenocytic hyphae with terminal, ovoid asexual zoosporangia with exit papilla at each tip that are characteristic of *P. cinnamomi*. Soil samples from all of the sites were therefore determined to be negative for *P. cinnamomi*.

3.1.2. Field collection

The red and pink flowers of *E. impressa* plants were matched to corresponding colour samples in the RHS Colour Chart, Red-Purple group (Royal Horticultural Society c1995). Records of flower colour were tabulated by site (table 6), and RHS colour and race (table 7).

3.1.3. Propagation experiment one - assessment of cuttings

A mixture of soft new season growth and woody second season growth was used for the cutting material at the first two collection sites (Angahook-Lorne site A and Cranbourne site CA). Strike rates were 61% for soft cuttings and 40% for woody cuttings (table 8). Soft tip cuttings were observed to have better overall health and growth than the woody cuttings after the first four weeks of experiment one. More than 50% of the soft tip cuttings from site CA exhibited 10-20mm of new growth after four weeks and 90% had some new growth and minimal leaf mortality. At seven weeks, 68% of the Site A soft tip cuttings showed new tip growth and no leaf necrosis. After these early results, only soft cutting material was used when it was available.

Site	RHS Colours (Red-Purple Group)	Colour Race	Sample size	Percentage
Site A - Angahook-Lorne State Park				
A	White	White	1	3.5%
A	57A	Red	13	46%
A	57B	Red	8	28.5%
A	57C	Pink	3	11%
A	57D	Pink	3	11%
		Site total	**28**	100%
Site CA – Royal Botanic Gardens, Cranbourne				
C	White	White	48	96%
C	57A	Red	1	2%
C	57D	Pink	1	2%
		Site total	**50**	100%
Site CA – Royal Botanic Gardens, Cranbourne				
CA	White	White	12	24%
CA	57A	Red	8	16%
CA	57B	Red	7	14%
CA	57C	Pink	13	26%
CA	57D	Pink	6	12%
CA	61D (corolla) & 62A (tips)	Pink	2	4%
CA	62A	Pink	1	2%
CA	62C	Pink	1	2%
		Site total	**50**	100%
Site G - Grampians National Park				
G	White	White	7	27%
G	57A	Red	7	27%
G	57B	Red	5	19%
G	57C	Pink	6	23%
G	57D (corolla) & white (tips)	Pink	1	4%
		Site total	**26**	100%
Site B – Braeside Park Heathland				
B	White	White	10	100%
		Site total	**10**	100%
Site T – Tullah, Tasmania				
T	White	White	3	25%
T	57A	Red	5	42%
T	57D	Pink	4	33%
		Site total	**12**	100%

Table 6. *E. impressa* plants at each site categorised by RHS colour (Royal Horticultural Society c1995) and flower colour race. The numbers shown are for the tagged plants at each site and the percentage of each colour found at each site.

RHS Colours	Flower Colour Race	Total No.	% of total sample	Sites
57A	Red	34	19%	A, C, CA, G, T
57B	Red	20	12%	A, CA, G
	Red total	**54**	**31%**	
57C	Pink	22	12.5%	A, CA, G
57D	Pink	14	8%	A, C, CA, T
57D (corolla) & white (tips)	Pink	1	0.5%	G
61D (corolla) & 62A (tips)	Pink	2	1%	CA
62A	Pink	1	0.5%	CA
62C	Pink	1	0.5%	CA
	Pink total	**41**	**23%**	
White	White	81	46%	A, C, CA, G, B, T
	White total	**81**	**46%**	
	TOTAL	**176**	**100%**	

Table 7. The distribution of *E. impressa* plants by RHS colour (Royal Horticultural Society c1995). Atypical pink forms were found at sites G (Grampians) and CA (Cranbourne) and site B (Braeside) contained only white-flowered plants.

Site	Soft tip	Woody tip
A		
Total cuttings	57	103
Number that struck	35	47
Percent Struck	61%	46%
CA		
Total cuttings	110	50
Number that struck	67	14
Percent Struck	61%	28%
Total strike rate	**61%**	**40%**

Table 8. Strike rates by type of tip cutting (woody or soft) at the first two sites in experiment one at 20 weeks. Soft tip cuttings had a higher strike rate and were used on later collections.

At 20 weeks the cuttings were assessed with an ordinal scoring system according to shoot health and development (table 9). Living cuttings were given a score from one (lowest) to five (highest) and dead cuttings received a zero (table 10). The plants were also examined for presence or absence of flowers and axillary buds, and the above-

ground shoot height was measured. Statistical analysis was executed with Minitab® Release 14.20. After 30 weeks, 83% of the repotted unstruck cuttings were dead and the remaining 17% had not formed roots.

Score	Shoot Development
0	Dead
1	Extensive leaf loss or browning
2	Leaf loss or browning, little or no new growth
3	Healthy, some leaf loss or browning, some new growth
4	Healthy, no leaf loss or browning, some new growth
5	Healthy, no leaf loss or browning, extensive shoot growth

Table 9. Shoot score definitions. Living cuttings were scored from one to five based on plant health, stem growth and leaf development. Dead cuttings were marked zero.

Site	Shoot Scores						Total
	0	**1**	**2**	**3**	**4**	**5**	
A	55	15	17	23	49	1	160
C	81	11	25	36	7	0	160
CA	42	21	16	37	38	6	160
G	124	18	11	6	1	0	160
Total	302	65	69	102	95	7	640

Table 10. Propagation experiment one: total shoot scores by site. Cranbourne site CA had the highest scores, followed by Angahook-Lorne site A, then Cranbourne site C. The Grampians site G had the lowest scoring cuttings.

A binary logistic regression analysis was carried out for stem development with values of zero (no growth) or one (growth). A table comparing the binary outcome (zero or one) against site is shown below (table 11). A binary logistic regression model was fitted, and only site was shown to be significant to stem growth (P <0.05). A log transformation was used to correct for skewness and satisfy the assumption of normality.

Figure 4 displays an individual value plot for new growth by site. Each point represents a plant with new growth and the mean is illustrated by a diamond, with a 95% confidence interval bar for each mean. The mean new growth results for sites CA, then A, were the highest while the means of sites G and C were the lowest.

Site	Stem height: binary scores		
	0	1	All
A	74	86	160
C	112	48	160
CA	56	104	160
G	130	30	160
Total	372	268	640

Table 11. Propagation experiment one: binary height results by site. Cuttings that had stem growth were scored one, and those with no growth, including dead cuttings, were marked zero. Site CA had the highest number of cuttings with growth, followed by site A. Sites G and C had the lowest number of cuttings that exhibited growth.

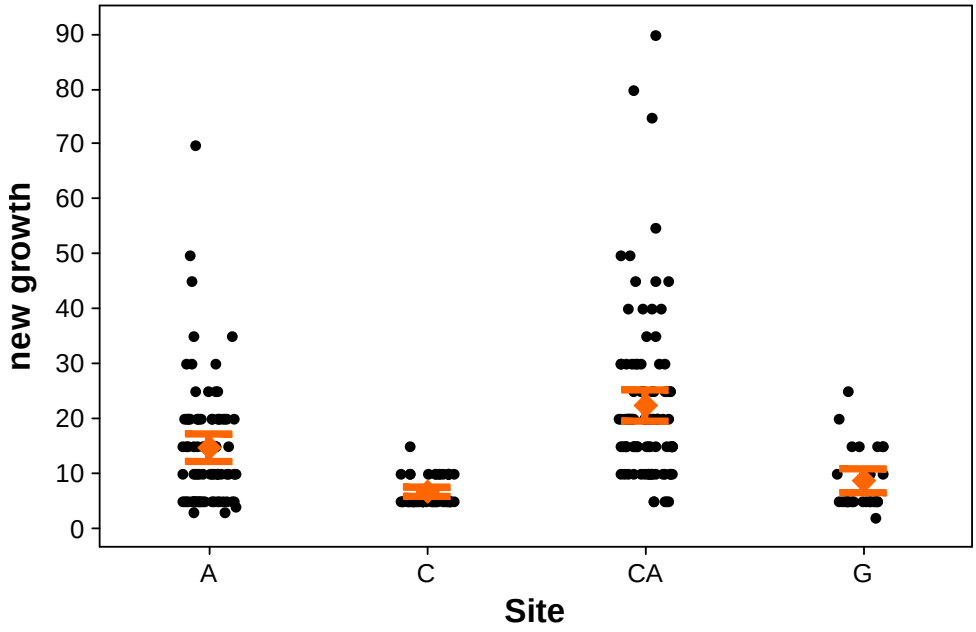

Figure 4. Individual value plot of new growth by site with each point representing a plant with new growth. The mean is illustrated by an orange-coloured diamond, with a 95% confidence interval. Sites CA, then A, have the highest new growth results, followed by sites G and C.

The presence (1) or absence (0) of flowers and axillary buds (table 12) are shown as binary outcomes below. Binary logistic regression analyses were performed and none of the variables were significant for presence or absence of buds. Flower colour and treatment were shown not to be significant variables for the presence or absence of flowers, however, site was a significant variable (P <0.05). Site CA had the most cuttings with flowers (16), followed by sites A (9), C (2) then G (0).

Site	Binary scores					
	Flowers			Axillary buds		
	0	1	All	0	1	All
A	151	9	160	134	26	160
C	158	2	160	114	46	160
CA	144	16	160	142	18	160
G	160	0	160	153	7	160
Total	613	27	640	543	97	640

Table 12. Propagation experiment one: binary flower and axillary bud results by site. An absence of flowers or axillary buds is indicated by 0 and presence of flowers by 1. Cuttings from sites CA and A had the greatest instance of flowering and those from sites C and A had the highest number of cuttings with axillary buds. Site G had the lowest number of cuttings with both flowers and axillary buds.

The cuttings were then removed from their cells and the roots were assessed for strike rate using an ordinal scoring system from zero to five (table 13). Scoring was performed with the potting media still attached in order to minimise root disturbance. Dead cuttings received root scores of zero and living cuttings with no roots were marked with a score of one. Struck cuttings were given a score of two to five depending on root development. Plants that received a score of five had a dense mass of fine roots that filled the cell.

Score	Root Development
0	Shoot dead no roots
1	Shoot alive no roots
2	Less than 5 roots present <1cm
3	1-5 roots present >1cm
4	5-10 roots present >1cm
5	>10 roots present >1cm

Table 13. Root score definitions. Struck cuttings were marked ordinally from two to five based on the number of roots visible while the plant was still in the media. Dead cuttings received a zero and unstruck cuttings a one.

The root scores were first analysed by site (table 14) and treatment (table 15). Site CA had the highest strike rate (51%), followed by sites A (41%), then C (26%). Site G had the lowest strike rate at 4%. The control group (treatment one) and treatment four had the highest strike rates (36%) followed by treatments two (26%) and three (23%).

Site	Root Score						Total	Strike rates (%)
	0	1	2	3	4	5		
A	55	39	27	6	17	16	160	41
C	81	38	13	6	5	17	160	26
CA	43	36	9	15	17	40	160	51
G	123	31	1	0	1	4	160	4
Total	302	144	50	27	40	77	640	30

Table 14. Propagation experiment one: ordinal root scores by site. A score of 0 was given for dead cuttings and 1 for unstruck cuttings. Struck cuttings were scored from lowest (2) to highest (5) based on the number of visible roots. Sites CA then A had the highest strike rates, followed by sites C and G.

Treatment	Root Score						Total	Strike rates (%)
	0	1	2	3	4	5		
1	75	27	27	11	10	10	160	36
2	80	38	4	6	10	22	160	26
3	78	45	9	4	4	20	160	23
4	69	34	10	6	16	25	160	36
Total	302	144	50	27	40	77	640	30

Table 15. Propagation experiment one: total root scores and strike rates by treatment. Treatments 4 (Ei1.1 + IBA) and 1 (control) showed the highest strike rates, followed by treatments 2 (nil + IBA) and 3 (soil + IBA).

The results for roots were analysed by ordinal logistic regression with treatment, site, flower colour and individual plant used as variables. Colour and treatment were shown to be non-significant variables and site and the individual plant were identified as significant variables. Thus, the source plant and site from which the cutting material was collected had the only statistically significant effects on root development. Treatment and flower colour had no effect on root formation.

3.1.3.1. Assessment of plants at 220-weeks

Measurements were taken at 220-weeks of plant heights, number of primary, secondary and tertiary stems, and presence or absence of buds, flowers and seed capsules. The data for plant heights were analysed by ANOVA (analysis of variance) in order to assess the effects of each factor on plant height (treatment, site, source plant, flower

colour and presence or absence of mycorrhizal infection). The outcome of 'plant height' was treated as a continuous response variable. Treatment, flower colour and the presence or absence of mycorrhizal infection (at 52 weeks) were shown to be non-significant variables and were removed from the model. Site and individual plant were revealed to be significant with P=0.01 and P<0.001, respectively. The estimated mean heights from the ANOVA are given in table 16, along with standard errors.

Site	Mean heights (mm)	SE
A	994	39
C	893	81
CA	1147	39
G	1278	135

Table 16. Estimated mean heights with standard errors (SE) of all plants at 220-weeks. Site G plants (*E. impressa* var. *grandiflora*) had the highest mean heights, followed by sites CA, A and C. Sites CA and A also had the highest initial strike rates.

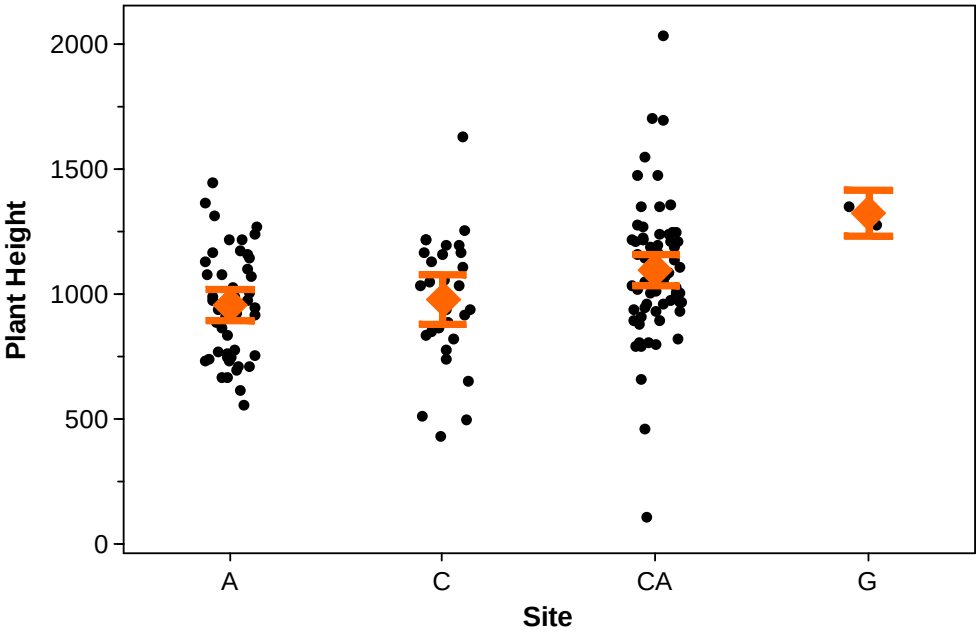

Figure 5. Propagation experiment one: individual value plot of plant height by site at 220 weeks with each point representing a plant. The mean is illustrated by an orange-coloured diamond, with a 95% confidence interval bar in orange. Plants from site G had the highest mean heights followed by sites CA, C then A.

An individual value plot is shown above (figure 5), where each point represents a plant height and the mean is illustrated by a diamond, with a 95% confidence interval bar for each mean. Plants from site G exhibited the highest mean heights followed by sites CA, then C. Site A had the lowest mean plant heights. The larger site G plant sizes correspond to the larger plant sizes from which the initial cuttings were taken. The Grampians site G, *E. impressa* var. *grandiflora*, plants were approximately 200-300mm taller than *E. impressa* plants at the other three sites.

ANOVAs were carried out on the number of primary, secondary and tertiary stems to compare the mean effects of each previously mentioned factor on stem branching structure. A log transformation was used to correct skewed data. Flower colour and treatment did not significantly influence the number of primary, secondary, or tertiary stems. The cutting material source plant and site significantly effected the number of primary stems at $P = .008$ and $P = .01$ respectively. Thus, the individual plant and site from which the cutting was taken had the only significant effects on the number of primary stems at 220-weeks.

None of the factors had a significant effect on the number of secondary stems. The source plant was the only factor to significantly effect on the number of tertiary stems ($P < .001$). This meant that the number of minor stems was primarily determined by the cutting's source plant.

Separate binary logistic analyses were completed for presence (1) or absence (0) of axillary buds, flowers and seed capsules. The site from which the cutting was taken had the only significant effect on the presence or absence of both axillary buds and flowers. Site CA had the most cuttings with seed capsules (14), followed by sites C (9), A (8) and G (2). None of the factors were significant for presence or absence of seed capsules

3.1.4. Propagation experiment three - assessment of cuttings

The cuttings were removed at 30 weeks and scored according to root initiation and shoot growth in the same manner as for experiment one. Shoot scores are shown by treatment (table 17) and site (table 18) and root scores by treatment (table 19) and site

(table 20). The above-ground shoot heights were measured and the plants were examined for the presence or absence of flowers and axillary buds, and whether new shoots were terminal or axillary growth.

Treatment	Shoot Score						Total
	0	1	2	3	4	5	
1	120	19	17	1	4	6	167
2	103	5	3	1	0	0	112
3	110	34	19	5	0	0	168
Total	333	58	39	7	4	6	447

Table 17. Propagation experiment three: total shoot scores by treatment. Treatment 3 (Ei1.1) had the highest shoot scores, followed by treatment 1 (control) then 2 (10% soil).

Site	Shoot Score						Total
	0	1	2	3	4	5	
A	149	16	2	0	0	0	167
C	135	13	14	4	2	0	168
Nursery	49	29	23	3	2	6	112
Total	333	58	39	7	4	6	447

Table 18. Propagation experiment three: total shoot scores by site. Nursery cuttings had the highest shoot scores, followed sites A, then C.

Strike rates were the highest for treatments one (control) (12%) and three (Ei1.1) (11%), followed by treatment two (10% soil) (4%). The nursery cuttings had a considerably higher strike rate (24%) than sites C (7%) or A (3%). Strike rates in general were lower in experiment three than in experiment one, but higher than experiment two.

Treatment	Root Score						Total	Strike rate (%)
	0	1	2	3	4	5		
1	120	27	1	5	1	13	167	12
2	103	5	1	1	0	2	112	4
3	110	39	4	5	2	8	168	11
Total	333	71	6	11	3	23	447	10

Table 19. Propagation experiment three: total root scores and strike rates by treatment. Strike rates were highest for treatments 1 (control) and 3 (Ei1.1), followed by treatment 2 (10% soil).

Site	Root Score						Total	Strike rate (%)
	0	**1**	**2**	**3**	**4**	**5**		
A	149	13	0	3	0	2	167	3
C	135	22	3	0	1	7	168	7
Nursery	49	36	3	8	2	14	112	24
Total	333	71	6	11	3	23	447	10

Table 20. Propagation experiment three: total root scores and strike rates by site. Nursery cuttings had a considerably higher strike rate than sites A and C.

Treatment	Stem height: binary scores		
	0	**1**	**All**
1	120	47	167
2	103	9	112
3	110	58	168
Total	333	114	447
Site	**0**	**1**	**All**
A	149	18	167
C	135	33	168
Nursery	49	63	112
Total	333	114	447

Table 21. Propagation experiment three: binary stem height results by treatment and site.

Variable	Baseline	Level	Odds ratio	95% CI	*P*-value for comparison	Overall *P*-value
Site	A	C	2.06	(1.10, 3.85)	0.02	
	A	Nursery	8.39	(4.44, 15.7)	<0.001	<0.001
	C	Nursery	4.08	(2.33, 7.15)	<0.001	
Treatment	1	2	0.45	(0.20, 1.01)	0.05	
	1	3	1.43	(0.86, 2.37)	0.17	0.01
	2	3	3.18	(1.43, 7.06)	0.004	

Table 22. Propagation experiment three: binary logistic regression analysis for stem growth.

A comparison of the binary outcome for stem height of zero (no growth) or one (growth) by site and treatment is shown above (table 21). A binary logistic regression analysis was carried out for stem heights, and variables of treatment and site were shown to be significant (overall $P < 0.05$) (table 22). The plant height results indicate

that stem growth was achieved most often with treatment 3 (EI 1.1 liquid inoculum), followed by treatment 1 (control) and then 2 (site soil). The nursery cuttings (N) had the greatest number of cuttings with stem growth (63), followed by sites C (33) then A (18).

An ANOVA was carried out on non-zero height results comparing the effects of site and treatment on plant growth. The site from which the original cutting came had the only significant effect on plant growth ($P = 0.002$). The nursery cuttings (N) had the greatest number of cuttings with stem growth (63), followed by sites C (33) then A (18).

Binary logistic analyses determined that none of the factors significantly affected whether new stem growth was terminal or axillary, or presence or absence of flowers. However, the presence or absence of buds was significantly effected by treatment ($P = 0.0$). Plants that received treatment 2 were most likely to have axillary buds, followed by treatments 1, then 3.

3.1.5. Mycorrhizal assessment

Hair root samples were taken from struck plants in trial one at 20 and 52 weeks for microscopic examination. No typical ericoid mycorrhizal structures (TEMS) were present in root samples collected at 20 weeks. However, one non-inoculated sample from site CA displayed vesicles and hyphae that would normally be seen in arbuscular mycorrhizas (figure 7).

Plants with root samples taken at 52-weeks began to show signs of TEMS in cleared and stained hair roots (figure 8). Hyphae are visible coiled inside and penetrating outside thickened epidermal walls. Structures that resembled dark septate endophytic fungi were seen in the hair roots of a struck cutting from site A that had been inoculated with site soil (treatment 3) (figure 9). The stained objects appear to be the sclerotia of fungi that have penetrated and colonised the epidermal cells.

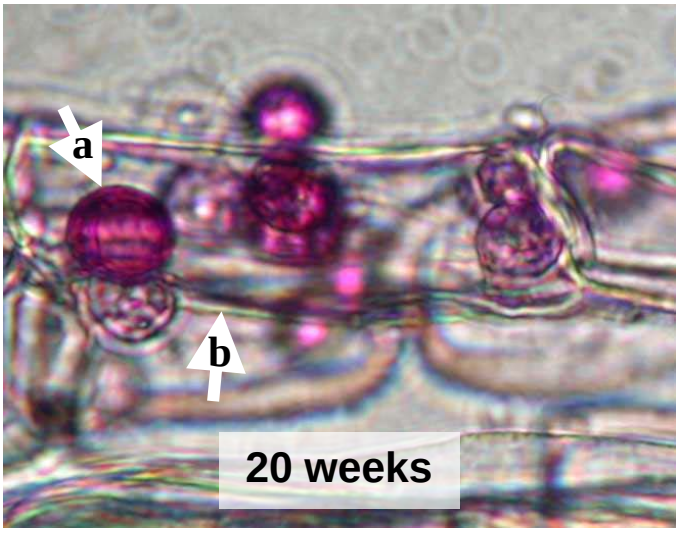

Figure 6. A cleared and lactofushian-stained 20-week root sample from site CA showing vesicles with thickened wall (a) and hyphae (b) typical of arbuscular mycorrhizal fungi.

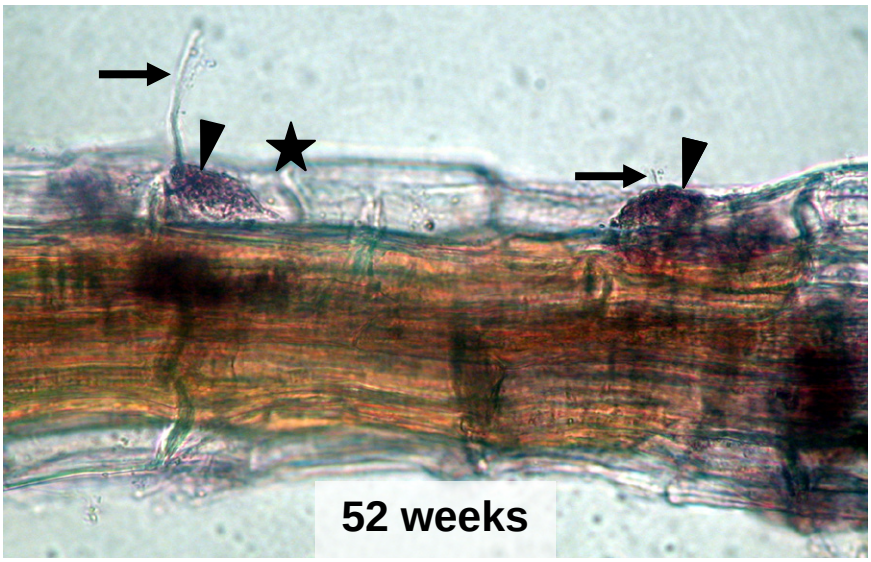

Figure 7. A cleared and lactofushian-stained root sample of a 52-week-old plant grown from a site CA cutting, inoculated with EI 1.1. Typical ericoid mycorrhizal structures (TEMS) include the hyphal coil (arrowheads), hyphae penetrating the epidermis (arrows), and thickened epidermal cell walls (star).

The presence (1) or absence (0) of TEMS in the roots of plants from experiment one at 52-weeks was scored below by site and by treatment (table 23). Site CA had the greatest number of cuttings with TEMS (27) followed by site A (7) and site C (2). TEMS were not visible in any of the root samples from site G plants. Treatment 2 (control + IBA) had the greatest number of cuttings with TEMS (14), followed by treatment 2 (Ei1.1) (13), then treatments 3 (10% site soil) (5) and 1 (control) (4). A

binary logistic analysis revealed that site had the only significant effect on presence or absence of TEMS ($P = .005$). Root samples were not taken in experiment two due to lack of cutting survival or in experiment three due to low strike rates.

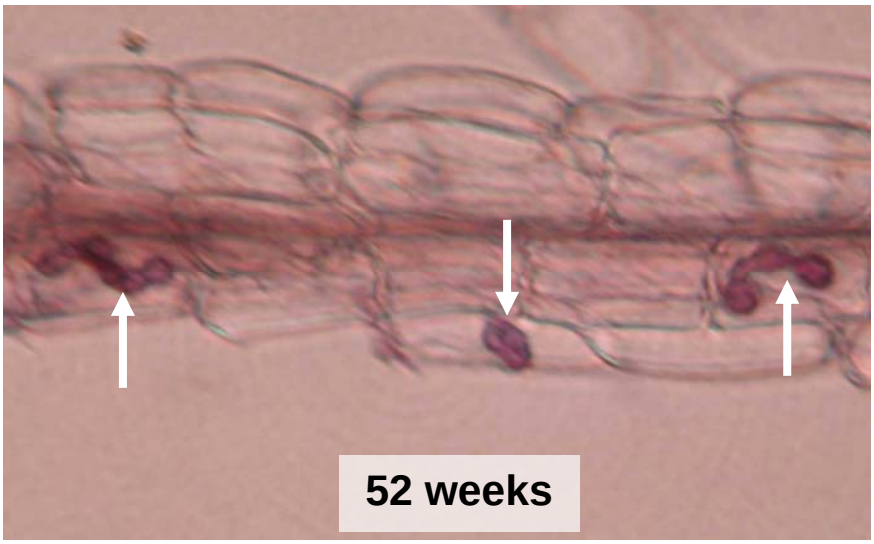

Figure 8. Possible dark septate endophytic fungi (arrows) in a cleared and stained root sample from a 52-week old plant. The original cutting was from site A and was inoculated with site soil.

	TEMS: binary scores at 52 weeks		
Site	**0**	**1**	**All**
A	45	7	52
C	27	2	29
CA	47	27	74
G	3	0	3
Total	122	36	158
Treatment	**0**	**1**	**All**
1	37	4	41
2	25	14	39
3	26	5	31
4	34	13	47
Total	122	36	158

Table 23. Propagation experiment one: number of plants by site and treatment exhibiting the presence (1) or absence (0) of typical ericoid mycorrhizal structures (TEMS) in roots at 52-weeks. Site CA had the greatest number of cuttings with TEMS

3.2. Genetic fingerprinting experiments

ISSR and RAPD gel photos were visually reviewed and faint ambiguous bands were removed from the data set (figure 10) (Williams et al. 1990; Zawko et al. 2001). Each sample was named according to site (A, B, C, CA, G or T), colour (W, R, or P) and the plant of origin. All of the gel photos were reviewed with their corresponding loci information from the Kodak1D analysis program. High (1500, 2000 and 3000 base pairs) and low (100, 200, 300 base pairs) molecular weight bands were also removed from the analysis to minimise the possibility of the inclusion of nested inverted repeats (Stewart & Excoffier 1996; Stewart & Nilsen 1995). Any individuals with no bands were excluded from the data analysis. Monomorphic bands were not present in any of the RAPD or ISSR results. A total of 182 polymorphic bands were scored with four primers (Table 24). The data used in the final analysis discussed below were obtained from RAPD and ISSR experiment two. Experiment one provided an incomplete data set due to previously discussed difficulties and hence was not analysed.

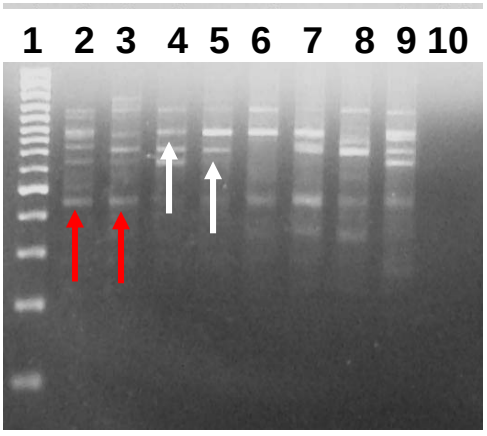

Figure 9. A gel photograph showing examples of scored bright bands (white arrows) and unscored faint bands (red arrows). Lane one is the DNA ladder and lane ten is the control with no DNA. This photograph was from an ISSR run with site C pink-flowered samples and primer 836.

Primer	No. Polymorphic Bands
OPB6	33
OPB19	48
812	43
836	58
Total	**182**

Table 24. Number of polymorphic bands obtained from two RAPD primers and two ISSR primers.

The molecular weight information from Kodak 1D was saved as a text (.txt) file and imported into Microsoft® Office Excel 2003. The weights were then converted into binary data for presence (1) or absence (0) of bands. Each of the data sets from the four primers was combined in a single Excel document with the alleles listed in sequential order by columns and the individual samples by rows. Any samples that were missing data from more than one primer were removed, with the exception of pink-flowered individuals from site CA (CAP). The Exeter software program NTSYSpc 2.20e was used for statistical analyses.

A similarity matrix was created using the Jaccard coefficient, *a/(n-d)* (Jaccard 1908) (table 25). The Jaccard coefficient is applicable for dominant marker analysis since it compares similarities (1) and disregards negative data (0) (Iruela et al. 2002; Mattioni et al. 2002; Meyer et al. 2004; Pharmawati, Yan & McFarlane 2004).

Reduced similarity matrices were created with data sub-sets by site (table 26) and colour race (table 27) to compare the average similarities between and amongst populations. The percentages of similarity ranged from 10% between sites A and G to a 33% average chance of relatedness between individuals at sites C and T. Site A had the lowest percentage of genetic similarity (13%) of plants within its population, followed by sites CA (17%) and B (19%). Site G had the highest amount of intra-site genetic similarity (30%) followed by site C (26%) and site T (24%). There was a 20% inter-site average polymorphic similarity between plants.

Plants of the pink-flowered race had the highest average percentage (31%) of intra-race genetic similarity. White-flowered and red-flowered plants had lower average probabilities of being related to other plants of the same flower-colour race (21% and 19% respectively). Pink-flowered plants had a 21% average genetic similarity to white-flowered plants and a 23% to the red-flowered race. Red-flowered plants had an equal average probability (19%) of being related to either other red-flowered plants or to white-flowered ones. There was a 22% average genetic similarity between plants of any one flower-colour to those of any other race.

	AR18	AR19	AP34	BW11	BW12	CW19	CW20	CR133	CR134	CP101	CP105	CP121	CAW24	CAW27	CAW34	CAR4	CAP15	CAP17	GW118	GW119	GW120	GR137	GP110	GP127	TW1	TW3	TR1	TR2	TP1	TP2	TP4
AR18	1.00																														
AR19	0.75	1.00																													
AP34	0.00	0.00	1.00																												
BW11	0.00	0.00	0.00	1.00																											
BW12	0.00	0.00	0.00	0.57	1.00																										
CW19	0.33	0.38	0.29	0.18	0.08	1.00																									
CW20	0.25	0.29	0.40	0.22	0.20	0.58	1.00																								
CR133	0.25	0.13	0.00	0.33	0.31	0.27	0.21	1.00																							
CR134	0.29	0.14	0.25	0.15	0.14	0.29	0.23	0.75	1.00																						
CP101	0.18	0.20	0.20	0.13	0.29	0.42	0.36	0.20	0.10	1.00																					
CP105	0.13	0.14	0.33	0.17	0.40	0.30	0.38	0.11	0.00	0.56	1.00																				
CP121	0.09	0.10	0.25	0.13	0.29	0.33	0.40	0.20	0.10	0.89	0.63	1.00																			
CAW24	0.00	0.00	0.33	0.00	0.00	0.27	0.20	0.21	0.33	0.14	0.00	0.14	1.00																		
CAW27	0.00	0.00	0.33	0.00	0.09	0.27	0.20	0.21	0.33	0.14	0.00	0.14	0.71	1.00																	
CAW34	0.00	0.00	0.33	0.00	0.10	0.18	0.10	0.23	0.36	0.00	0.00	0.00	0.57	0.83	1.00																
CAR4	0.20	0.25	0.20	0.14	0.29	0.15	0.09	0.08	0.18	0.10	0.00	0.00	0.13	0.29	0.33	1.00															
CAP15	0.20	0.20	0.50	0.25	0.25	0.29	0.33	0.67	0.33	0.50	0.60	0.57	0.00	0.00	0.00	0.00	1.00														
CAP17	0.25	0.25	0.50	0.33	0.33	0.33	0.40	0.33	0.00	0.38	0.75	0.43	0.00	0.00	0.00	0.00	0.75	1.00													
GW118	0.00	0.00	0.25	0.20	0.17	0.20	0.25	0.29	0.14	0.22	0.00	0.25	0.14	0.14	0.00	0.00	0.17	0.00	1.00												
GW119	0.00	0.00	0.25	0.20	0.17	0.20	0.25	0.29	0.14	0.22	0.00	0.25	0.14	0.14	0.00	0.00	0.17	0.00	1.00	1.00											
GW120	0.00	0.00	0.25	0.20	0.17	0.20	0.25	0.29	0.14	0.22	0.00	0.25	0.14	0.14	0.00	0.00	0.17	0.00	1.00	1.00	1.00										
GR137	0.11	0.13	0.20	0.25	0.22	0.29	0.36	0.45	0.36	0.25	0.10	0.27	0.10	0.10	0.11	0.00	0.40	0.20	0.33	0.33	0.33	1.00									
GP110	0.00	0.00	0.33	0.00	0.00	0.27	0.20	0.25	0.50	0.38	0.17	0.43	0.29	0.29	0.33	0.11	0.40	0.20	0.11	0.11	0.11	0.43	1.00								
GP127	0.00	0.00	0.33	0.00	0.00	0.08	0.09	0.25	0.50	0.22	0.00	0.25	0.29	0.29	0.33	0.11	0.17	0.00	0.25	0.25	0.25	0.25	0.50	1.00							
TW1	0.25	0.25	0.25	0.20	0.17	0.33	0.43	0.29	0.14	0.38	0.40	0.43	0.00	0.00	0.00	0.00	0.75	0.50	0.33	0.33	0.33	0.60	0.25	0.11	1.00						
TW3	0.25	0.25	0.20	0.25	0.20	0.33	0.43	0.14	0.00	0.38	0.40	0.43	0.00	0.00	0.00	0.00	0.40	0.50	0.14	0.14	0.14	0.60	0.25	0.11	0.60	1.00					
TR1	0.00	0.00	0.33	0.17	0.17	0.30	0.22	0.43	0.29	0.40	0.43	0.44	0.20	0.20	0.25	0.00	0.60	0.40	0.17	0.17	0.17	0.38	0.40	0.17	0.40	0.17	1.00				
TR2	0.00	0.00	1.00	0.17	0.17	0.18	0.10	0.43	0.29	0.27	0.25	0.30	0.20	0.20	0.25	0.00	0.60	0.40	0.40	0.40	0.40	0.38	0.40	0.40	0.40	0.17	0.67	1.00			
TP1	0.50	0.50	0.17	0.00	0.14	0.56	0.33	0.11	0.13	0.50	0.33	0.38	0.13	0.29	0.14	0.25	0.33	0.40	0.11	0.11	0.11	0.11	0.20	0.00	0.25	0.25	0.14	0.14	1.00		
TP2	0.50	0.50	0.25	0.00	0.10	0.55	0.36	0.18	0.33	0.50	0.33	0.38	0.33	0.50	0.38	0.30	0.33	0.40	0.08	0.08	0.08	0.08	0.36	0.25	0.18	0.18	0.14	0.14	0.67	1.00	
TP4	0.50	0.50	0.20	0.00	0.00	0.63	0.38	0.00	0.00	0.50	0.33	0.38	0.14	0.14	0.00	0.13	0.33	0.40	0.13	0.13	0.13	0.13	0.22	0.00	0.29	0.29	0.14	0.14	0.83	0.56	1.00

Table 25. Similarity matrix for RAPD/ISSR experiment two using the Jaccard coefficient. The data represents the level of similarity between each plant listed on axes x and y. (Hence the top diagonal row of numbers are '1' because they represent a self-self comparison, ie; AR18 to AR18, AR19 to AR19). A phylogenetic tree (dendogram) was then generated to create a visual representation of the numerical data.

SITE	A	B	C	CA	G	T
A	0.13					
B	0.00*	0.19				
C	0.22	0.21	0.26			
CA	0.20	0.15	0.24	0.17		
G	0.10	0.13	0.23	0.14	0.30	
T	0.30	0.12	0.33	0.25	0.23	0.24

* white-flowered plants were not found at site A at the time of collection for DNA isolation.

Table 26. Reduced similarity matrix showing average percentages of similarity by site.

	W	R	P
W	0.21		
R	0.19	0.19	
P	0.21	0.23	0.31

Table 27. Reduced similarity matrix showing average percentages of similarity by colour race.

A dendogram was then created using a clustering algorithm with an unweighted pair-group method, arithmetic average (UPGMA) formula to illustrate phylogenetic relationships within the entire population sample (figure 12) by geographic population (figure 13) and by flower-colour population (figure 14). Individuals from each site are primarily clustered at lower similarity coefficients or are seen grouped with members of other site populations. This indicates a high level of inter-site genetic variability.

The phylogenetic tree in figure 14 shows relationships between plants of different flower-colour populations. Clustering at the highest similarity coefficients are shown primarily by discreet colour race groups. Small but separate clusters of red, pink, and white flowered plants are displayed in the dendogram with coloured bars. The smaller discreet colour groupings, rather than the emergence of three major clusters, represent a relatively high level of intra-race genetic variability.

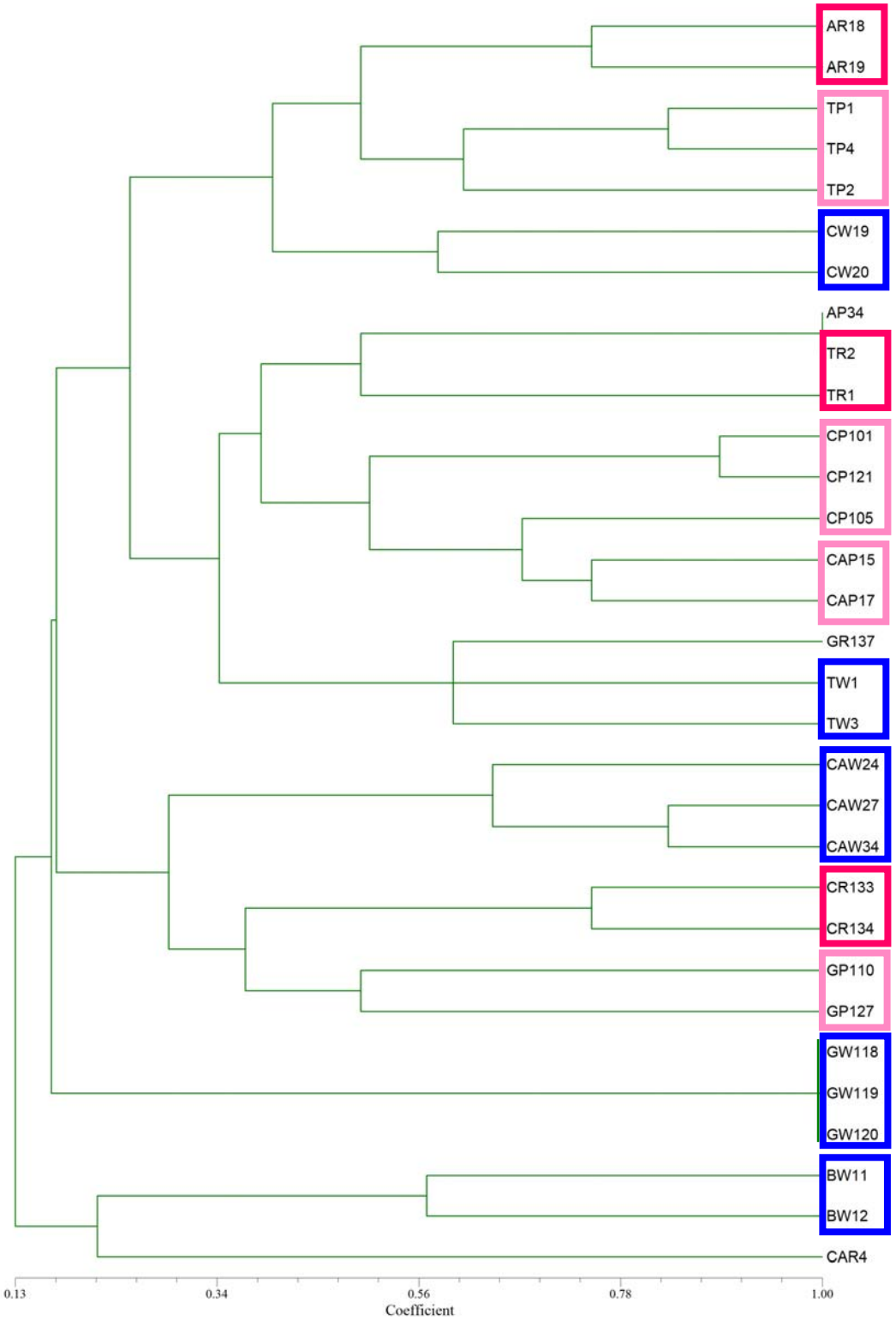

Figure 10. RAPD/ISSR Experiment two: dendogram illustrating phylogenetic relationships between individuals in the entire population sample. The first letter or letters represent the collection site (A, B, C, CA, G, T) and the second letter the flower colour race (R, P, W). The number corresponds to the plant identification number. Clustering can be seen between individuals of the same flower colour race within populations, with the Grampians white-flowered plants (GW118-120) showing the highest similarity coefficient.

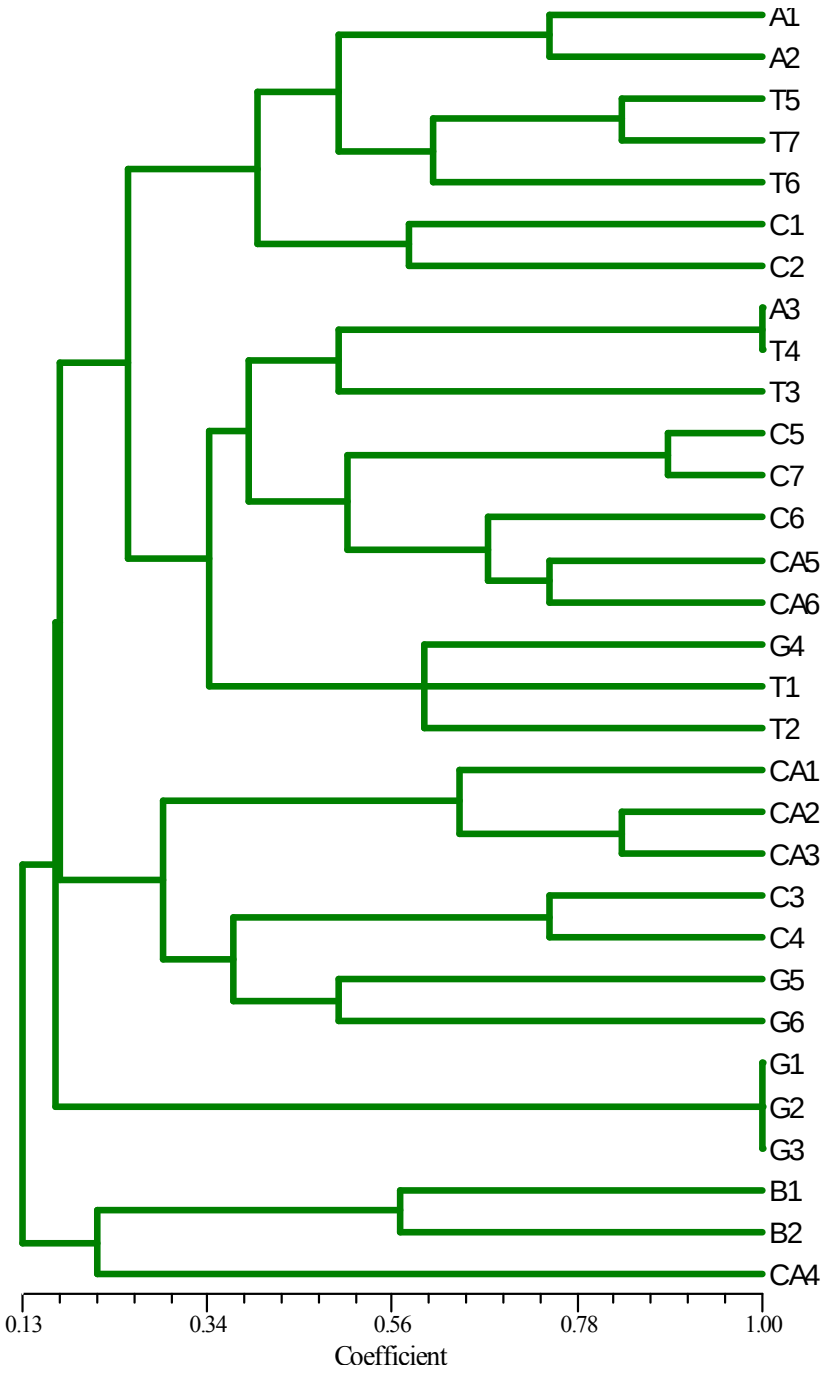

Figure 11. RAPD/ISSR Experiment two: dendrogram demonstrating inter-site phylogenetic relationships (Sites A, B, C, CA, G, and T). Individuals from each site are primarily clustered at lower similarity coefficients or are seen grouped with members of other site populations. This indicates a high level of inter-site genetic variability.

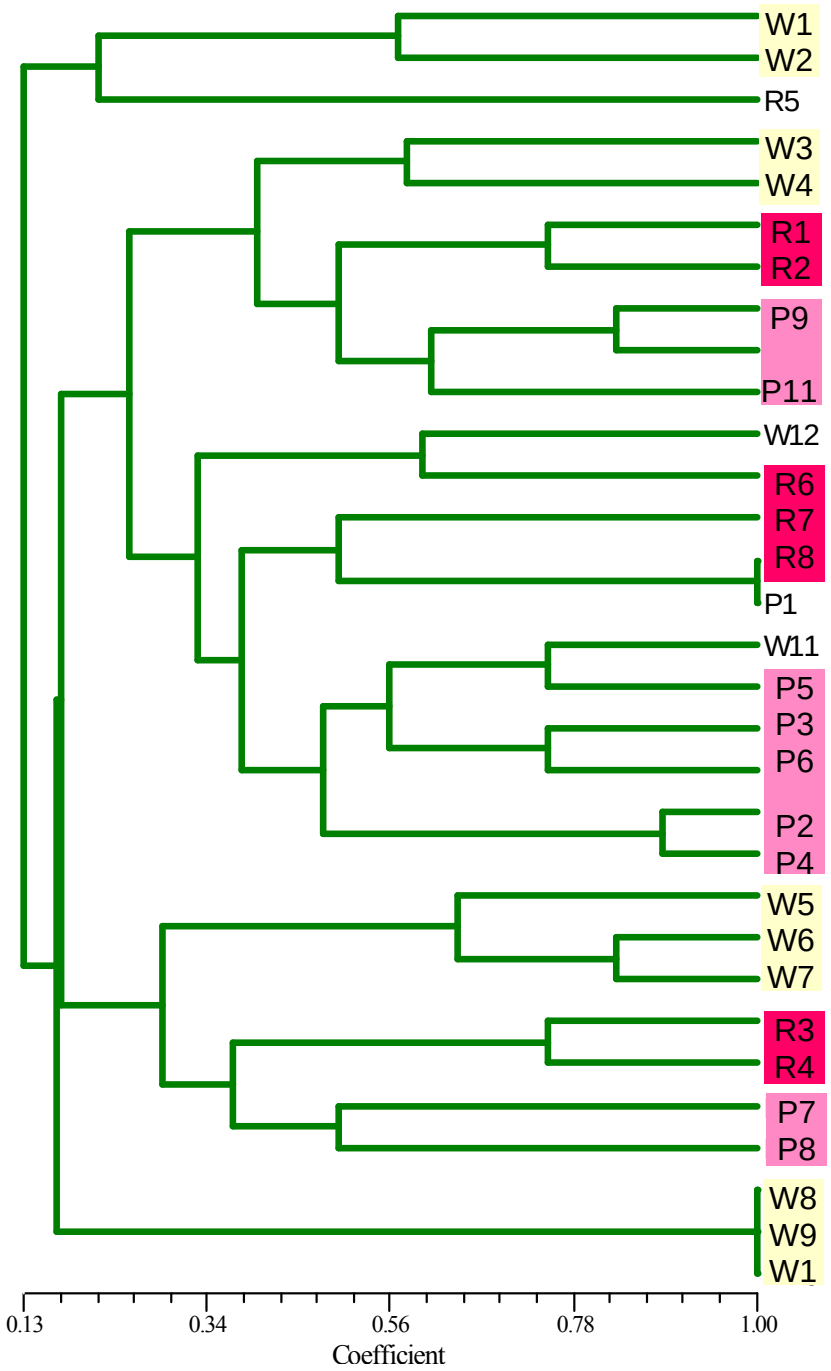

Figure 12. RAPD/ISSR Experiment two: dendogram showing phylogenetic relationships between flower-colour race populations (R=red, P=pink, W=white). Clustering at the highest similarity coefficients (segmented bar) are shown primarily by discreet colour race groups. The colour race groups show a high level of intra-race genetic variability since they are not grouped into three major clusters by flower colour.

4. Discussion

4.1. Propagation experiments

4.1.1. Field collection

The type of cutting material, woody or soft tip, had a great influence on the survival and strike rates of cuttings in the first experiment. Soft new-growth tip cuttings hardened off in the fog house after approximately four weeks had higher strike rates than woody cuttings. Woody tip and mid-stem cuttings provided low strike rates, contrary to findings by Thompson (1986). The soft tip cuttings were taken from plants approximately six to eight weeks after flowering which allowed new meristematic tip growth to reach a length suitable for propagation.

The flowering times at each site were dramatically affected by drought conditions in South-Eastern Australia. However, general flowering patterns by race still appear to follow Stace and Fripp (1977c), with red and pink-flowered plants flowering first followed by white-flowered plants. None of the treatments had any effect on flowering time or number of flowers in the nursery.

4.1.2. Propagation experiments one and two

Site had the only significant effect on above ground stem growth and health and the presence or absence or flowers. Site and the individual plant from which the cutting material was sourced had the only significant effect on root growth. The presence or absence of axillary buds was not affected by any of the variables. Sites CA then A demonstrated the greatest stem growth, overall health of cuttings and strike rates, while sites G and C were the lowest.

Plants from Site CA cuttings exhibited the highest level of growth at 220 weeks, including mean height and greatest number of primary, secondary and tertiary stems. Site G plants also had high growth rates while sites C and A were the lowest. The source material from site G was from *E. impressa* var. *grandiflora* which grow to

approximately one half-metre taller than *E. impressa*. The plants from these cuttings demonstrated growth rates consistent with the *grandiflora* variety.

Since none of the struck cuttings showed signs of EM infection at twenty weeks, it is likely that experiment one was of insufficient length of time to see measurable results from the inoculum. The findings concur with previous findings that the length of experiment time significantly affected EM inoculum results with *Leucothoe* (McLean, C. & Lawrie 1996) and blueberry cuttings (Powell & Bagyaraj 1984).

None of the four treatments had a significant effect on presence or absence of TEMS at 52 weeks. The collection site had a significant effect on infection by TEMS, with Site CA showing the highest infection rate (57%). The other three sites had low infection rates with Site A at 2%, Site C at 1% and Site G at zero.

The differences in colonisation between cuttings from different sites may be a result of host-fungus compatibility related site soil characteristics. Both the Nowa Nowa site from which the inoculum EI1.1 originated and site CA had similar sandy clay soil types. It is possible that the southern hemisphere EM have specialised to have associations only with plants growing in their particular soil types. McLean and Lawrie (1996) found that EM fungal colonisation of soil-inoculated cuttings was dependent on similar edaphic conditions. It would be of interest to determine whether isolated EM also infected other Ericoid plants from similar soil types.

Infection rate differences between struck cuttings may also be a result of the use of only one EM fungus in these experiments. Previous studies have revealed multiple species of EM fungi in a single ericoid hair root (Boyer, Ballington & Mainland 1982; Hutton, Dixon & Sivasithamparam 1994; McLean, C. & Lawrie 1996; McLean, C. B., Cunnington & Lawrie 1999; Midgley, Chambers & Cairney 2004; Perotto, Girlanda & Martino 2002). A single mycorrhizal inoculum may not be sufficient to provide any benefit to the plant. Further research is required to determine whether inoculation with more than one EM is needed.

The seasonal differences between optimal field cutting collection time and the active fungal growth periods may have also been significant to these propagation experiments. Studies have found that mycorrhizal infection in the Southern Hemisphere ericoids is greatest during the cool and wet winter periods (Bell & Pate 1996; Hutton, Dixon & Sivasithamparam 1994; Hutton et al. 1997; Kemp, Adam & Ashford 2003; Reed 1989). This time period is also when *E. impressa* plants are flowering and not producing new stem growth. Inoculation of field-collected cuttings could prove difficult since fungal dormancy starts in warmer weather at approximately the same time that new stem growth begins. The ideal time for collection of field cuttings does not coincide with the optimal fungal growth time. To date, no research has been done to determine whether *in vitro* EMF replicate *in situ* seasonal growth patterns. It would be of interest to determine whether or not this has an effect on fungal infection rates and growth after inoculation. If this is the case, the problem could be surmounted by the use of nursery stock-plants from which winter tip growth could be induced by late-summer pruning.

Treatment, flower colour and presence or absence of mycorrhizal infection were not statistically significant to plant growth, flowering, number of stems, and presence or absence of seed capsules at 220 weeks. The original collection site and the plant from which the cutting material was taken were revealed to be the only significant factors in overall plant growth. Site was the only significant factor for presence or absence of both axillary buds and flowers. None of the variables were significant for presence or absence of seed capsules

E. impressa var. *grandiflora* cuttings from Site G proved difficult to propagate with 11% and 0% strike rates in experiments one and two, respectively. Out of the 18 struck plants that were potted on into tubes, only seven survived for 52 weeks. It is unlikely that the low strike rate was due to the later cutting collection time since flowering times were also later at Site G, necessitating later collection of new tip growth. Plant species in the Grampians National Park display unique biological traits as discussed in section 1.1.3. Further investigation will be needed to determine optimum clonal propagation methods for *E. impressa* var. *grandiflora*.

4.1.3. Propagation experiment three

The cuttings were left for an additional ten weeks to increase the potential for effectiveness of the mycorrhizal inoculum EI1.1. Cuttings were also collected from plants growing in the nursery from experiment one to compare their strike rates with *ex situ* cuttings.

Strike rates by treatment were low, with the highest at 12% for the control and 11% for the liquid inoculum EI1.1. The addition of site soil proved again to be the least effective treatment with a strike rate of 4%. Both external collection sites had low strike rates with 3% for site A and 7% for site C. The nursery-collected cuttings (site N) had a much higher strike rate at 24%.

The cuttings treated with EI1.1 liquid inoculum exhibited the most instances of new stem growth. Both treatment and site had a statistically significant effect on stem growth. However, without a significant effect on root formation, stem growth alone in the cuttings is not a successful propagation outcome. Therefore, the use of a single mycorrhizal inoculum would not be recommended based on these results.

The propagation trays were raised to prevent cross-contamination of inocula, but this appears to have had some negative side-effects. Primarily, the efficacy of the heated beds was greatly reduced by the creation of an air pocket between the tray and bed. Secondly, the media in the individual cells dried out more quickly with the addition of a second source of evaporation which necessitated a variation in watering regime from the original trials. In future experiments, trays should be placed directly on to a container or device that will isolate run-off water and prevent cross-contamination, while still conducting heat to the cutting media.

4.1.4. Mycorrhizal and other root-associated fungi – experiment one

There were no typical ericoid mycorrhizal structures present in the hair-root samples collected at 20 weeks. However, one non-inoculated sample from site CA displayed AM-like structures. McLean and Lawrie (1996) previously found AM 'balloon-like' structures in the roots of *E. impressa* from the Grampians and *Leucopogon ericoides*

from the same area as Cranbourne site C of this study. Arbuscles have also been observed in the cleared roots of *E. microphylla* (Bellgard 1991) and in Hawaiian ericoid roots (Koske, Gemma & Englander 1990). Additionally, researchers have found fungi from the basidiomycete order Sebacinales in association with EM fungi (Allen et al. 2003; Bougoure & Cairney 2005; Selosse et al. 2007) and twenty-five species in the Ericaceae have been described as being colonised by dark septate endophytic (DSE) fungi (Jumpponen & Trappe 1998). Cleared and stained hair-root samples in this experiment appeared to show signs of infection by non-EM fungi. It would be of interest to determine the exact nature of these fungi in future experiments through DNA sequencing.

4.2. Genetic fingerprinting analysis

ISSR and RAPD analyses showed a high level of genetic variability both between and amongst geographic and race populations. The results indicated a 20% average genetic similarity between geographic populations and a 22% average similarity between races. The pink-flowered race had the highest average intra-population polymorphic similarity at 31%. The red and white races had the lowest inter-race and intra-race average genetic similarities (19-23%).

The high percentage of polymorphic similarity between the pink-flowered *E. impressa* individuals suggests that they are a separate race to the white and red-flowered plants. From the results of these experiments, plants of the pink race are more likely to have parent plants from the same race rather than being the result of an F1 hybrid of plants of the white and red races. The pink race has developed a genetic identity that is distinct from the other races based on evidence shown by ISSR and RAPD analysis.

It is possible that this is a result of nectar feeding and pollination patterns of introduced vectors (Castellanos, Wilson & Thomson 2003; Meléndez-Ackerman, Campbell & Waser 1997; Streisfeld & Kohn 2006). Pollinators that select pink-flowered plants only during overlapping periods of flowering of the three floral races would not be cross-pollinating between the races. Further study is needed to determine whether certain

non-native pollinators have developed preferences for pink-flowered plants due to flower colour, temperature or chemical composition. Differences in time of flowering between flower-colours in the same population would also have a great influence on pollination patterns. During this research and previous studies, (Stace & Fripp 1977c) plants of different flower colours in populations that were polymorphic for flower colour were observed to have separate but overlapping periods of flowering. Both of these factors would have an influence on the genetic makeup of the separate floral races.

The geographic isolation of the Grampians has created unique taxa, such as *E. impressa* var. *grandiflora*. It was not surprising that the Grampians population (site G) showed the highest level of intra-site genetic similarity (30%). The separation of many of the Grampians individuals into the *grandiflora* variety is reflected in the high average polymorphic similarities at site G.

A 26% average polymorphic similarity between individuals was recorded at the Royal Botanic Gardens, Cranbourne, Site C. The site primarily contained plants of the white-flowered race, which may account for the high level of intra-site genetic similarity. The Tulla, Tasmania site demonstrated a 24% average intra-site polymorphic similarity. The site consisted of a remnant vegetation site not directly geographically linked to other populations.

Angahook-Lorne site A had red, pink and white-flowered populations, and demonstrated the lowest average genetic similarity within its own population (13%). This concurs with Stace and Fripp's (1977b) findings of high levels of polymorphism for flower-colour race within linked 'mosaics' of populations. The site was also part of a large coastal range of linked *E. impressa* populations within a protected state park region. This provided the opportunity for genetic dispersal to other geographically linked populations. Genetically, the site appears to be part of a larger population or one of a series of linked populations that share genetic traits. The recent creation of the Otways National Park will help to preserve this large polymorphic group.

Braeside site B and Cranbourne site CA also had low average intra-site polymorphic similarities at 13% and 17% respectively. These results were surprising since both sites were geographically removed from other populations. Site B consisted of all white-flower race plants which could certainly explain the polymorphic similarities. Site CA had a robust mix of races but all *E. impressa* plants at the site died during this research. It is possible that nectar-robbing by introduced vectors has significantly altered the pollination regime and genetic makeup of this population (Hingston & McQuillan 1998).

5. Conclusion

5.1. Cutting Collection

Previously suggested late-summer cutting collection times must be re-examined. In current conditions of drought in Eastern Victoria and global climate change, flowering patterns can vary greatly from year to year. It is suggested that cutting collection times are based on observation of plant growth rather than calendar or seasonal dates. Anecdotal collection times such as collecting 'the week after Melbourne Cup Day' are no longer applicable with current climate change conditions.

5.2. Mycorrhizal inoculum

This research found no benefit from the use of mycorrhizal inoculum with clonal propagation by cuttings. In addition, the use of site soil as an inoculum provided no benefit. The site soil treatments had the lowest strike rates in all three experiments. Based on these results, Ericoid mycorrhizal fungus is not recommended as an inoculum for *E. impressa* cuttings at this time.

As discussed in the previous chapter, the members of the Ericaceae have multiple fungal associates in each hair root. Further research is needed using multiple fungi as inocula. From this research, the EMF would also appear to select host roots that have grown in similar edaphic conditions. Thus, in future experiments, fungal inocula should be selected based on similar soil types, or ideally, from the same site.

Ericoid mycorrhizal fungi are slow growing. Previous studies (Powell & Bates 1981) have questioned whether the length of time of their trials was adequate to determine effectiveness of the inoculum . Trials of even greater lengths of time, potentially one to two years, may be needed. It is possible that additional benefits to the plant would follow with greater rates of infection. However, high rates of mycorrhizal infection from inoculum do not always result in improved plant health and growth (Corkidi et al. 2004). Further research is needed to determine whether higher levels of hair-root infection in *E. impressa* result in more robust plants.

It is possible that short-term responses are not adequate indicators of inoculate effectiveness. Previous researchers (Gorman & Starrett 2003) have pointed out the difficulty in selecting which host responses correctly identify mycorrhizal interaction. Because of the slow-growing nature of the fungus, beneficial results may not be seen until the plant is in more advanced stages of growth. A previous AMF study suggested that long-term research, including plant growth and health after planting, may be needed to determine the true efficacy of an inoculum (Corkidi et al. 2004). Measurable results may not be seen in Australian Ericoid plants until years after inoculation. In future studies, cuttings which have struck and show signs of TEMS in their root systems should be planted out and monitored for longer periods of time. It would be of interest to monitor inoculated plants for a period of five years or more to determine longer-term effectiveness of the inoculum.

It has also been suggested in the literature (Cairney & Ashford 2002) that mycorrhizal presence does not guarantee improved plant health. The fungi-host interaction is still not clearly understood in the Australian Ericaceae. It is possible that only a one-way transfer of nutrients, from host to fungi, was taking place in this particular strain of the fungus. Further research is needed to establish what, if any, levels of nutrient transfer are taking place between the EMF and host *E. impressa* plant.

E. impressa plants are also known to suffer from transplant shock. A high number of container grown plants die when transplanted into the field, whether in a landscape or

revegetation project. Further studies are needed to determine if inoculation helps plant survival during transplanting.

Thus, based on these experiments, inoculation of *E. impressa* cuttings with Ericoid mycorrhizal fungus provided no benefit to strike rates or shoot health. Additional research is needed to determine whether inocula from a similar soil type or longer experiment times would be advantageous.

5.3. DNA fingerprinting

E. impressa appears to have high levels of inter-site genetic diversity and relatively low levels of inter-race genetic diversity. These findings concur with Stace and Fripp's earlier work on raciation of the species which found high levels of polymorphism for flower colour race, corolla colour and corolla length (1977a; 1977b; 1977c). Red and white flower race populations had a high level of shared genetic traits while the pink-flowered race had a more distinct genetic identity. The RAPD and ISSR results suggest that the pink-flowered populations comprise a separate floral colour race rather than an F1 hybrid between red and white races.

While geographic populations showed high levels of inter-site genetic diversity, morphological characteristics should still be considered when collecting cutting material for revegetation purposes. Since genetic fingerprinting techniques target unknown regions of the genome, locally adaptive traits may not be represented by the loci in RAPD and ISSR analysis (O'Brien, E., Mazanec & Krauss 2007). A combination of genetic fingerprinting and traditional morphologic observation is recommended to determine provenance (Krauss, Koch & Vlahos 2005). Cutting material should still be collected from populations of similar flower-colour race dispersal (Stace & Fripp 1977b). Hence, when trying to re-establish a population of all white-flowered plants, it is suggested that cutting material is collected from another site of all white-flowered plants. Geographic proximity of populations does not appear to be an issue in provenance determination based on the regional genetic spread of *E. impressa*. This has been found to be the case in other Australian studies where large regional areas of

provenance have been established with DNA fingerprinting techniques (Krauss & Koch 2004; Krauss, Koch & Vlahos 2005).

The intra-site genetic similarities indicate that *E. impressa* populations have probably been geographically inter-connected until fairly recently. The historical geographic isolation of the Grampians *E. impressa* var. *grandiflora* population was reflected in the higher level of intra-site genetic similarity. Further work will need to be done on Tasmanian populations to determine their genetic links to mainland South-eastern Australia plants.

This research highlights the need for continued research into the use of Ericoid mycorrhizal inoculum as a potential propagation inoculum for *E. impressa*. Based on this research, *E. impressa* has a high level of both intra-race and inter-race genetic diversity. As a result of these experiments, significant recommendations to growers are made in the following section to increase strike rates of *E. impressa* when propagating by cuttings.

5.4. Recommendations to growers

Based on these experiments, the following recommendations would be made to growers in relation to propagation of *E. impressa* from cuttings.

- The use of Ericoid mycorrhizal fungus as inoculum for cuttings would not be recommended at this stage. Further research is needed to determine whether fungus from similar soil conditions or site-specific inocula would have greater efficacy.
- Site soil should not be used as inoculum under any circumstances. Cuttings inoculated with site soil performed the worst of all treatment groups in all three experiments.
- Cutting material may be collected from any Victorian population of a similar flower-colour race distribution. With the exception of the Grampians *E. impressa* var. *grandiflora*, Victorian populations of *E. impressa* exhibited high

levels of inter-site polymorphism. More research is needed on Tasmanian populations to determine their genetic relatedness to Victorian plants.

- Soft new tip cuttings should be collected from plants approximately six weeks after flowering ceases and placed under fog for twenty weeks. The lower leaves should be manually removed from the stem with a sharp blade or scissors prior to sticking.

6. References

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