Final report on the Australian Flora Foundation funded project:

Lomatia tasmanica and *Persoonia muelleri* propagation and commercial horticulture

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Abbreviations: BAP: Benzylaminopurine, DKW: Driver and Kuniyuki, IAA: indolacetic acid. IBA: indolbutyric acid, MS: Murashige and Skoog, NAA: Naphtalenacetic acid, NaOCl: Sodium hypochlorite, PVP: Polyvinylpyrrolidone, WPM: Woody Plant Medium,

1- Micropropagation of *Lomatia tasmanica* and *Lomatia tinctoria*

Abstract

A micropropagation method for the rare and endangered Proteacaea *Lomatia tasmanica* was developed using, micropropagation of the related *Lomatia tinctoria* as a model. Actively growing shoots of *L. tinctoria* were collected from the field or from potted plants, disinfected and introduced in tissue culture. A modified woody plant medium plus 1 µM benzylaminopurine and 0.1 µM indolacetic acid appeared to be the most efficient medium for shoot proliferation. Best rooting was obtained by dipping the basal end of shoots in a 1 mM solution of indolbutyric acid. Rooted plants were established in soil. Using the same protocol, a micropropagation method for *L. tasmanica* was set up from only 8 buds.

Introduction

Lomatia tasmanica is a small Tasmanian endemic tree occuring in mixed forest of the south west Tasmania. Only two populations are known, each one including approximately 100 plants (Lynch and Balmer, in prep). No seed has ever been observed from these trees, and propagation appears to be vegetative by rhizome (Brown and Gray, 1985). Tissue culture propagation would be of great importance for the

conservation of this species, which is thought to be on the brink of extinction (Lynch et al., in prep).

The difficulty of collecting explants, due to the location of the sites and the rarity of the plants, and the high rate of contamination obtained with the first field-collected material (96%) led us to use the related *Lomatia tinctoria*, widespread in Tasmania, as a model for micropropagation.

Material and methods

According to the previous work conducted by P. Ball (1992) on *L. tinctoria* and *L. polymorpha*, two main problem areas have been identified:

* High percentage of contaminations whatever disinfection treatment applied;
* Rapid blackening of most of the explants due to phenolic production (this phenomenon is common in most other members of the Proteaceae)

The standard procedure for tissue culture establishment was as below:

The explants were rinsed overnight in running tapwater, treated in 70 % alcohol for a few seconds and in 2% NaOCl for 10 min (explants from field-grown plants) or in 1 % NaOCl for 8 min (explants from potted plants).

After three rinsings in distilled water, the explants were dissected into single node microcuttings, treated for 1 h in PVP 0.7%, and then for 24 h in a liquid medium with rifampycin 100 mg/1 (both on an orbital shaker at 4º C). After rinsing in distilled water, the microcuttings were placed on a WPM basal mineral medium (McCown and Lloyd,1981) modified by the elimination of K_2S0_4, supplemented with MS vitamins (Murashige and Skoog, 1962), 30 g/1 sucrose, 1 µM BAP, 0.1 µM IAA, and solidified with 0.7% agar (Difco). The pH was adjusted to 5.7 before autoclaving at 120ºC for 20min. For tissue culture initiation, medium was dispensed in 150 * 25 mm test tubes (10 ml each) and for shoot multiplication and rooting, in 250 ml conical flasks (50 ml each). Cultures were incubated in the dark for 2 weeks and then transferred into light at 23±2ºC on a 16 h photoperiod. For each *in vitro* experiment, at least 30 explants were treated in each way and the experiment repeated on two separate occasions.

For rooting experiments, shoots 10 to 15 min long were cut at the time of subculturing. Preliminary experiments showed that culture on auxin-containing rooting media induced a high rate of callusing. So a two step procedure was used:

- quick dip of the basal end of isolated shoots in a concentrated auxin solution
- transfer to an auxin-free rooting medium consisting in a half strength macrosalt WPM.

After 4 weeks of culture on the rooting medium, microcuttings were assessed for root development.

For acclimatisation, rooted plants were dipped in 4g/1 'Mancozeb' fungicide solution and transplanted into a mixture of pine bark and peat moss 1:1 in a mini greenhouse where humidity was maintained close to 100%. Every week, plants were sprayed with fungicide and Pyrethrum insecticide.

Results

Lomatia tinctoria

3 different types of explants were collected:

1- Growing shoots from two year-old plants in the field; (Tasman Peninsula near Cape Pillar)

2- Growing shoots sprouting up actively after a bush fire;(Snug plains)

3- Etiolated shoots from potted plants grown in the dark for at least 2 weeks.

The following results were obtained from experiments with different explants and treatments:

- A 24 hr treatment in a solution of rifampycin 100 mg/1 after disinfection with NaOCl significantly improved the percentage of non contaminated explants (62 instead of 32 without rifampycin treatment)
- The percentage of contaminated buds was dramatically reduced using shoots sprouting up after a bush fire and etiolated shoots (respectively 11% and 7.5%).
- Addition of activated charcoal 5 g/l known to adsorb phenolic compounds, decreased significantly the percentage of explants producing shoots (72 % without activated charcoal, 44 % with).
- Addition of Polyvinypyrrolidone (PVP) did not improve that percentage, compared with the medium with activated charcoal

- A one week treatment in the cold room at the beginning of the *in vitro* culture had no influence on bud survival.

In order to reduce the blackening of the explants, they were inoculated onto four different media: Knop (1865), Knudson (1946), modified WPM and DKW (Driver and Kuniyuki, 1984).
The percentages of bud flushing, blackening and vitrification were recorded after two subcultures on the same medium and are reported Table 1.

Table 1. Effects of four different mineral media on the establishment of *L. tinctoria* buds in tissue culture.

Culture medium	Knop	Knudson	DKW	modified WPM
% of bud flushing	63.2 a	72.1 ab	61.5 b	76.5 a
% of explants turning black	10.5	23.6	59	19
% of vitrification	67	46.7	50	36.7

Results scored after two times 4 weeks of culture. Within a line means followed by the same letter are not significantly different based on X2 test at $p< 0.05$.

No significant differences were recorded in the percentage of bud break on Knop, Knudson and modified WPM. The percentage on DKW is significantly lower than on the modified WPM. A low mineral medium (Knop, 1865) proved to be the best to avoid blackening of explants but the high rate of vitrification, so that the plants became translucent and hyperhydric, prevented us from using this medium. The high mineral content medium DKW gave the highest rate of browning (59%). The modified WPM induced the highest rate of bud flushing (76.5%) and lowest rate of vitrification (36.7%) with a moderate rate of browning of the explants (19%). After the third subculture, vitrification was no longer observed. Therefore modified WPM medium was used in further experiments..

Shoots were propagated by axillary branching on medium based on the modified WPM macrosalts and containing 1 µM BAP and 0. 1 µM IAA, and subcultured every 6 weeks with a propagation rate varying from 4 to 10 fold. Each shoot was 10 to 30 mm long.

The shoots might also be propagated using single-node culture on the same medium, each bud giving rise to a new shoot. BAP 44 µM dramatically inhibited shoot elongation and induced some adventitious bud regenerations on leaves in contact with the culture medium.

A range of IBA concentrations from 200 µM to 5 mM was tested to induce rooting. A 1 mM IBA concentration was suitable to induce a high rate of root formation with a suitable dipping time (Table 2). Higher concentrations (up to 5 mM) promoted a high degree of callusing. Dipping plants for 10 min in auxin mixtures including IBA l.0 mM, IAA 500 µM and/or NAA 200 µM did not improve rooting.

Table 2. Influence of the duration of treatment in a 1.0 mM IBA solution on root initiation on *L. tinctoria*. Fifteen replicated explants per treatment. Results were scored after 4 weeks.

duration of IBA treatment	rooting percentage	average number of roots per plant
3 s	20	0.2 (⇧ 0.3)
1 min	40	1.9(⇧1.8
10 min	67	2.8 (⇧ 1.6)
30 min	100	4.1 (⇧ 1.8)

For some plant species, rooting has been preferentially induced by auxin mixtures. Hence some mixtures were tested using a 10 min treatment. Results are shown in Table 3.

Table 3. Effects of dipping treatments in auxin mixtures on root initiation on *L. tinctoria* shoots. 15 replicated shoots per treatment. Results scored after 4 weeks.

Auxin mixtures	rooting percentage	average number of roots per plant
IBA 1.0 mM	67	2.1 (⇧ 1.2)
IBA 1.0 mM + IAA 500 µM	67	2.3 (⇧ 1.2)
IBA 1.0 mM + IAA 500 µM + NAA 200 µM	46.7	1.5 (⇧ 1.2)
IBA 1.0 mM + NAA 200 µM	60	2.3 (⇧ 1.3)

Treatments in auxin mixture did not improve either the rooting percentage or the average number of roots per plant.

Twenty eight rooted plants were transferred into the glasshouse for acclimatisation. After 4 weeks 20 of them were healthy and started to grow. However, in the following weeks some shoots died. This death was attributed to a severe fungus attack.

The acclimatisation experiments should be repeated by specialists with a larger number of plants.

Lomatia tasmanica

Explants were collected either from field grown plants or, following the results obtained with *L. tinctoria*, from etiolated potted plants.

-Field collected material:

In December 1994 shoot cuttings of *L. tasmanica* were taken from the Goring Creek population near Cox Bight in the Southwest National Park. The shoots were disinfected and placed into culture according to the standard procedure. Of 60 explants, only 4 were free of fungal and/or bacterial contaminations. Bud flushing occurred within 4 weeks and the explants were subcultured every 4 weeks. Growth was very slow, with very little elongation of the stems. After being isolated from the original explants (2 months after inoculation), shoots began to turn black.

Another collection was made from the same site in January, and the explants were surface sterilized using the same procedure, or with a fungicide and/or different NaClO treatments. All the explants (160) were contaminated, probably due to the long storage period before use (10 days in the cold room).

-Potted plants:

Two one-year old cuttings were placed in the dark (under a carton, in the glasshouse) to promote etiolation and fast growth of the shoots. Within 15 days these two. plants turned black, probably because of the high moisture percentage and lack of aeration under the carton. However, one of them produced a 20 cm long etiolated shoot. From this shoot, 8 buds were introduced into tissue culture. None of these 8 buds were contaminated, whereas 100% of the explants taken from other potted plants were contaminated. Seven of the 8 buds showed expansion within two weeks and had a much faster growth rate compared with the bud explants collected from the rainforest. No

blackening was observed and the shoots propagated slowly with a multiplication rate of 1 to 3 every 6 weeks. Some explants produced a cluster of adventitious buds at their base. An internal bacteria appeared then which induced some death of explants. The addition of 10 mg/1 rifampycin in the culture medium limited the proliferation of the bacteria.

Due to the lack of material, only one rooting treatment was tried with IBA 1.0 mM and IAA 500 µM but no roots were visable after 4 weeks. After 6 weeks, some roots initials were observed.

Discussion

These studies have resulted in mass propagation of two *Lomatia* species including the rare and endangered *L. tasmanica,* for which propagation by cuttings has met little success (Fountain, pers. corn.). Selecting a suitable source of material is essential to obtaining contamination free explants. A dark treatment of the stock plants for 2 weeks followed by the collection of etiolated shoots resulted in a low rate of contamination. A rifampycin treatment for 24 hours at 100 mg/1 dramatically reduced the contamination rate of explants when collected in the field. Addition of activated charcoal (5 g/1), known to adsorb phenolic compounds, significantly decreased the percentage of explants producing shoots.

For micropropagation, we used a modified WPM without K_2S0_4, as potassium ions have been proved to favour blackening of cultured explants (Anderson 1975). The WPM medium is suitable for micropropagation of many woody species (Mc Cown and Lloyd 1981) including other members of the Proteaceae family, *Grevillea robusta* (Rajasekaran 1994) and *Grevillea scapigera* (Bunn and Dixon 1992). Gorst et al (1978) also used a low salt medium for propagation of two *Grevillea* hybrids.

In vitro root initiation on *L. tinctoria* microcuttings was successfully achieved. However, for a commercial application, the method should be adapted for a larger number of plants. On *Grevillea scapigera* and *Stirlingia latifolia*, two other members of the Proteaceae family, Bunn and Dixon (1992 a and b) induced root formation by dipping the bases of the shoots in a commercial softwood cutting powder before direct

transfer to the greenhouse. It would be interesting to try this method with *Lomatia tinctoria* shoots.

Concerning *L. tasmanica*, further experiments must be done to obtain roots. The plants used in the only experiment carried out lacked of vigour because of a previous treatment under reduced light and the proliferation of internal bacteria. However, the sporadic formation of roots from *L tasmanica* shoots on the propagation medium showed the ability of this species to produce roots. This experiment should be repeated with more vigorous plants, propagated under normal light and subcultured on a medium containing rifampycin to restrict the proliferation of the bacteria.

2- Micropropagation of *Persoonia muelleri*

Abstract

Apical and axillary buds were used to initiate micropropagation but all the explants turned black and died within 6 weeks. On the other hand, with *Persoonia muelleri* embryo cultures an in *vitro* plant stock was established from embryos aseptically removed from seeds, and is currently being propagated. Furthermore, plantlets from tissue culture have proved their ability to root.

Materials and methods

Stem lengths and immature fruits were collected in Cox bight in December 1994 and January 1995, and disinfected with the procedure described previously for *Lomatia* species

Fruits of *Persoonia* are drupes with a woody endocarp containing one or two seeds. For embryo cultures, the fleshy mesocarp was removed with a scalpel and the rest was soaked in 2% NaOCl for 15 min and then rinsed in sterile water. To break the stony endocarp without damaging the embryo a small vice was used, and then the embryo was aseptically dissected. The embryos were placed on a variety of culture media in order to induce germination, adventitious bud regeneration or somatic embryogenesis.

The rooting ability was investigated with 10 to 15 mm long plants and using the same procedure as previously described for *Lomatia tinctoria*, i.e., a quick dip in a concentrated auxin solution followed by subculture on auxin free media.

Results

Somatic explants

In agreement with the previous studies by J. Gorst and P. Ball on micropropagation of *Persoonia* (especially *P. gunnii),* blackening of the explants was a major problem. Apical and axillary buds were used to initiate micropropagation but all the explants turned black and died within 6 weeks.

The following treatments were tried without success:

* Pretreatment of explants in PVP
* Dark incubation
* Low temperature incubation
* Low and high mineral nutrient media (Knop, Knudson, MS, MS/4, WPM, DKW)
* Addition of activated charcoal and/or PVP to the culture medium.

Floral buds were tested too, but all turned black whatever treatment or culture medium was used.

Embryos

All the embryos were free of contaminations and less than 10% were damaged. In the first set of experiments, testing the effect of benzylaminopurine (BAP) as cytokinin, chlorophyllian shoots were regenerated. It was difficult to determinate if these shoots originated from the development of epicotyl or were adventitious buds formed at the bottom of the cotyledons.

Different results were obtained with the fruits of the second collection made at the same site one month later. Most of them exhibited an abnormal germination and very few produced adventitious buds.

All the shoots obtained were subcultured every 6 weeks on a modified WPM containing l.0 µM BAP and 0.1 µM IAA. They propagated quite well with a propagation rate of 2 to 4, and blackening of shoots was never observed.

To induce rooting, four different treatments were tried for 10 min, and only 5 plants were treated in each. Results are given in Table 4.

Table 4: Effects of dipping treatments in auxin mixtures on P *muelleri* shoots. Five replicated shoots per treatment. Results scored after 4 weeks.

Auxin treatment	number of rooted plants
IBA 1.0 mM	5
IBA l.0 mM + IAA 500 µM	5
IBA l.0 mM + IAA 500 µM + NAA 200 µM	5
IBA l.0 mM + NAA 200 µM	2

The roots obtained were very thick and with no laterals. Twelve plants were transferred in the greenhouse, after 4 weeks 10 were healthy with normal development.

Discussion

Propagation of *Persoonia* cuttings appears to be particularly difficult (Fountain, pers. com.; Ketelhohn et al. 1994) and rooting trials have led to very little rooting and a high rate of dead cuttings. Attempts to germinate *Persoonia* seeds by gibberellic acid treatments have shown promise but need further investigation (Wriggley and Fagg, 1989). The effects of chemical and mechanical scarification on the germination percentage of *P. sericea* seeds were investigated by Ketelhohn et al. (1994). The seeds were cultured aseptically and a maximum of 50% germination was obtained when the endocarp was half removed. Unfortunately, no information was given on further growth. *Persoonia gunii* embryos were aseptically cultured by Ball (.1992) and exhibited very poor development.

The results we obtained with *Persoonia muelleri* embryo cultures are very satisfactory as an in *vitro* plant stock was established from embryos aseptically removed from seeds, and is currently being propagated. Furthermore, plantlets from tissue culture have

proved their ability to root. Further studies on acclimatisation are necessary as only 10 plants were successfully transferred to the greenhouse.

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References

Anderson (1975) Propagation of rhododendrons by tissue culture. Part I. Development of a culture medium for multiplication of shoots. Proc Int Plant Prop Soc, 25: 129-135.

Ball P (1992) Micropropagation of Proteaceae, Honours Thesis, Hobart.

Brown MJ and Gray AM (1985) *Lomatia tasmanica*: A rare endemic plant from Tasmania's south-west. The Tasmanian Naturalist 83: 1-3.

Bunn E and Dixon KW (1992a) In vitro propagation of the rare and endangered *Grevillea scapigera* (Proteaceae). HortSci. 27 (3) 261-262.

Bunn E and Dixon KW (1992b) Micropropagation of *Stirlingia latifolia* (Proteaceae), an important cut flower from Western Australia. HortSci 27 (4) 368.

Driver JA and Kuniyuki AH (1984) In vitro propagation of paradox walnut rootstock. Hort. Sc. 19 (4) 507-509.

Gorst JR, Bourne RA, Hardaker SE, Ricchards AE, Dirks S and de Fossard RA (1978) Tissue culture propagation of two *Grevillea* hybrids. Int. Pl. Prop. Soc. Comb. Proc. for 1978. 28: 435-446.

Ketelhohn LM, Johnston ME and Gage J (1994) Propagation of *Persoonia* species by seeds and cuttings. Comb. Proc. Intl. Plant Prop. Soc. 44: 72-76.

Knop W (1865) Quantitative Untersuchungen über die Ernahrungsprocesse der Pflanzen. Landwirtsch Vers Stn. 7: 93.

Knudson L (1946) A new method for the germination of orchid seeds. Am Orchid Soc Bull 15: 214-217.

Lloyd GB and McCown BH (1981) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc. Intl. Plant. Prop. Soc. 30: 421-437.

Mc Cown BH and Lloyd C. (1981) Woody Plant Medium (WPM) A revised mineral nutrient formulation for microculture of woody plant species. HortSc 16: 453.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Wriggley LW and Fagg M (1989) Banksias, waratahs and Grevillea and all other plants in the Australian Proteaceae family. Collins, Sydney.