

PROJECT TITLE : Micropropagation of threatened species of *Persoonia*

Abbreviations used in the text : IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; 2,4-D = 2,4-dichloropneoxyacetic acid; 2iP = N⁶-*iso* pentenyl amino purine.

i) Preliminary work

Work on the project commenced in November 1991 with collections of fruits and stem lengths of *P. juniperina* from the Tasman Peninsula for the purpose of conducting germination trials and preliminary micropropagation and cuttings trials on a species which is widespread and not endangered. It was hoped from this to be able to identify problems that might be characteristic of the family as a whole before any work was undertaken on rare members of the family. Of 40 fruits, 31 were found to contain a grub - later identified as a member of the Cecidomyiidae; this left few viable fruits for experimentation but these remaining fruits were surface sterilized, half were treated in 32% HCl at 60° C for 2 min. and they were all then placed on an agar medium and left at 24° C. To date none of the fruits has germinated. For the tissue culture experiments 30 nodal explants and 30 shoot tips were surface sterilized in 2% (av. chlorine) sodium hypochlorite and placed on a high nutrient - MS - (Murashige & Skoog, 1962) and low nutrient - B5 - (Gamborg *et al*) medium. Half of all of these cultures showed fungal contamination within 2 weeks and of the non-contaminated explants, all had turned black and died within 8 weeks. For glasshouse experiments, a total of 30 cuttings were taken and were treated in the standard way used by the Royal Tasmanian Botanical Gardens for cutting material (i.e. no hormone treatment; a "Clonex" dip supplying 3 g/L IBA; a "Clonex" dip supplying 8 g/L IBA) - the cuttings were kept in the mist propagator at the Botanical Gardens. Most of the cuttings turned black and died without developing adventitious roots, within 3 months; cuttings still alive have not produced roots.

This preliminary work thus identified three main problem areas :

- i) germination / viable seed
- ii) phenolic production in tissue culture and cutting material
- iii) contamination in explants taken from field-grown material for tissue culture experiments

In subsequent collections of *P. juniperina* and another species, *P. gunnii* , in January 1992 these problems were confirmed.

At the end of January 1992, we were fortunate enough to be included on a National Parks flight down to Melaleuca in the South-west of the state and used the opportunity to search for the rarest Tasmanian persoonia *P. moscalii* which is thought to occur only in the vicinity of Bathurst Harbour. In habit and habitat, this species is very similar to *P. gunnii* and, in fact, had been included with *P.gunnii* until a recent revision of the Tasmanian Persoonias (Orchard, 1983). The plant proved to be in a fairly inaccessible and remote area and given this, plus the fact that the likelihood of being able to achieve any success with propagation was very low (because of results with *P. juniperina* and *P.gunnii*), we decided that we would focus all efforts on *P. gunnii* until such time as we felt confident about the conditions necessary for successfully handling this species.

ii) Experiments with *P. gunnii*

a) Embryo culture : Because of widespread reports of the difficulty of germinating *Personia* (see e.g. Wrigley & Fagg, 1989) embryo culture offers a possible means of bypassing dormancy problems; the technique involves removal of the intact embryo from the seed and culturing it on a suitable nutrient medium. *P. gunnii* (which occurs at higher sub-alpine altitudes in Tasmania e.g. Mt Field, Hartz Mountains and Lake St Clair) flowers in late March and produces most fruit in mid-April although fruits can still be found on plants almost all year round. The fruit is a fleshy (turning from green to dark purple as it matures) drupe and the seeds are found inside a stony endocarp. Our observations indicated that two seeds are usually produced but that one invariably aborts when the embryos are still very immature (heart-shaped stage). We collected mature fruits throughout the year (January, March, April, May, July, September) from the locations mentioned above and found that the best method of removing embryos was to crack surface-sterilized fruits open with a nut-cracker and aseptically dissect out the embryo; the procedure was not entirely satisfactory because of the difficulty in removing the embryo from the closely adhering testa without damaging it. Embryos not removed from the testa did not respond well; damaged embryos showed marked reddening (phenolics production ?) and death; immature embryos died. The best time of year for taking fruits was April/May - outside this, fruits showed high abortion rates and insect damage.

The embryos were placed on a variety of culture media both liquid and solid testing the effect of growth regulators such as cytokinin, auxin and gibberellic acid (GA_3). Nutrient levels, sucrose concentration and dark/light incubation were also tested. In the end, none of the treatments tried was satisfactory; the embryos in all cases were

extremely slow to grow and tended to be highly distorted, producing a large single root (members of the *Persoonia* genus are not thought to produce proteoid roots [Wrigley & Fagg, 1989] and anyway, such roots require a soil bacterium for initiation and therefore do not develop in sterile conditions, so this finding cannot be explained in these terms) and very little or no shoot development. However, in a few instances (embryos cultured on a medium containing 2 μM 2,4-D + 0.2 μM kinetin for 28 days and then transferred to a medium containing 0.75 (μM IAA + 5 μM 2iP) shoot growth began after 4 months; also, embryos grown in a very small volume of liquid medium containing 0.1 μM 2iP grew quite well although they were vitrified (a physiological condition, essentially brought on by "waterlogging" of tissue) and hence would present problems in planting out to soil. GA₃ was used at various levels (0.1 - 10 μM) and there was some indication that the higher level may be beneficial but unfortunately the results for this experiment were confounded by contamination problems; other workers have suggested that GA₃ treatments may help in germination of intact seeds (Wrigley & Fagg, 1989).

These results suggests that embryo culture may have promise but further experimentation would have to be done at optimal collection time.

b) Micropropagation : The first problem to overcome with this method was disinfestation of field-grown material. Various treatments were tried (e.g. sodium hypochlorite, benzalkonium chloride, mercuric chloride and hydrogen peroxide) in combination with pretreatments in benlate and running water. It was found that a (1h - overnight) treatment in running water was very successful.

The problem of blackening of explants (phenolic production) proved to be insurmountable in the time frame of the project Many treatments were tried including -

- * addition of anti-phenolic agents to culture media - polyvinyl-pyrrolidone, L-cysteine, bovine serum albumin, dithiothreitol, ascorbic acid, benzyladenine, indolebutyric acid, activated charcoal, aminooxyacetic acid

- * pretreatment of explants in anti-phenolics prior to transfer to media

- * low temperature incubation

- * dark incubation

- * various basal media - MS, B5, Woody Plant Medium (Lloyd & McCown, 1980), high calcium medium, addition of growth regulators

- * liquid and solid media

- * orientation of explants in media

- * type of explant taken i.e. shoot tip, nodal
- * time of year at which explants were taken
- * moving explants to fresh medium every day

None of these treatments in any way inhibited blackening - some increased it. This phenolic problem in the Proteaceae is not unique to *Persoonia* with other laboratories reporting severe blackening in *Banksia* (Dixon, pers. com. - Kings Park & Botanic Garden, Perth) and my own laboratory (in a study this year designed to complement the *Persoonia* work) having similar results with *Bellendenia montana* and to a lesser but still significant degree with *Agastachys odorata*, *Cenarrhenes nitida*, *Orites diversifolia* and *Lomatia tinctoria*. In fact, very few members of the family have been successfully tissue cultured because of the phenolic problem. The genus which responds best in tissue culture is *Grevillea* (see e.g. Gorst *et al.*, 1978; Bunn & Dixon, 1992a); there also appears to be some success with *Hakea* and *Telopea speciosissima* (see Barlass, 1991) and *Stirlingia latifolia* (Bunn & Dixon, 1992b).

iii) Experiments with other members of the Proteaceae

This year, as indicated above, an Honours student - Mr Patrick Ball - has surveyed a number of other members of the Tasmanian Proteaceae. There has been varying success with these. *Bellendenia montana* and *Telopea truncata* respond well to embryo culture ; *Cenarrhenes nitida* and *Orites diversifolia* show a marked seasonal response to micropropagation techniques and it is only in recent collections that explants have remained green and showed signs of growth - it remains to be seen whether these explants can now respond to media designed to induce rapid proliferation; *Lomatia tinctoria* explants taken at any time throughout the year initially responded well to culturing but phenolic problems arose as soon as material was subcultured.

iv) Conclusions

Like most other members of the Proteaceae, *Persoonia* seems to present problems in its response to micropropagation techniques. The most significant of these is production of phenolics which leads to rapid blackening and death of explants and which cannot be inhibited by standard anti-phenolic treatments. Until the problem can be overcome, there is no chance of being able to tissue culture *Persoonia* or to achieve any repeatable success with taking cuttings (some amateur botanists have reported successes

with cuttings but the success rates are extremely low and cannot be linked to any particular treatment - Wrigley & Fagg, 1989). The next stage is probably to collaborate with a chemist and attempt to identify the major phenolic(s) produced, in the hope that this might then suggest a means to inhibit synthesis.

Embryo culture of *Persoonia* is much less successful than for other members of the Proteaceae but, with further experimentation, may prove to be a feasible way of overcoming seed germination difficulties. The experiments have, at least, demonstrated that dormancy is probably linked with inhibitors produced within the seed (since the embryos do show precocious germination when removed from the seed) although there are obviously inherent physiological factors acting, as well, to account for the slow development of seedlings.

References

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