Photoautotrophic micropropagation of *Banksia*

Final Report to the Australian Flora Foundation, May 2005

John Godfrey & Rob Cross

Royal Botanic Gardens Melbourne, Birdwood Avenue, South Yarra, 3141

Summary

The objective of this project was to develop a tissue culture propagation method for *Banksia* species. Banksias are an important component of the Australian cut flower export industry, but most plants are derived from seed, with consequent problems of variability. Tissue culture methods offer the prospect of rapid multiplication of selected superior forms. Although there are a few reports of culture of *Banksia* species, no technique giving good *ex vitro* survival has been developed. The initial aim of the project was to look at photoautotropic propagation of these species, but early in the work it became apparent that a major barrier was the surface de-contamination of species to introduce them into culture. Hence the focus was changed into developing a reliable method for introducing *Banksia* species into culture.

The method finally developed involved taking terminal shoots of *Banksia coccinea*, about 150mm long, from greenhouse grown plants, trimming off the leaves, and placing the stems into 0.01M HCl with 3 drops/500ml of Tween 80® with gentle agitation for 3 minutes, then transfer to 1% available chlorine solution for 10 minutes with constant agitation; then to sterile 2.4mM citric acid solution for 5 minutes. The sections were rinsed once and stored in sterile de-ionised water. Seventy-five percent of explants survived, and remained green and viable after surface sterilisation using this treatment, and subsequent placement on tissue culture medium. A higher percentage of explants survived on ½ M & S although bud expansion and growth was observed to be best on WPM.

This procedure offers a fast, simple, safe and effective means of surface de-contamination which does not damage plant cells or destroy surface integrity. This surface sterilisation method has enabled a number of small trials to be undertaken to determine an optimal *in vitro* medium for the initiation and multiplication of *Banksia coccinea*. Although long-term *in vitro* survival and multiplication have been unsuccessful to date, a basis for a full-time research project has been established. The procedure offers promise for the surface de-contamination of other woody species.

Objective
To develop a method of asexual propagation, micropropagation, for *Banksia* species, that will enable the selection of superior cultivars for the nursery and cut flower industries.

Background
The genus *Banksia* offers the nursery and floricultural industries a number of species that are unique in form and texture. They have been an important component of the Australian cut flower export industry, initially through harvesting from natural areas or farmed bush, especially in Western Australia, but increasingly from plantations established from seed propagated material in both the eastern and western states. Large growth rates in the value of cut flower exports from Australia were predicted in the 1990s (Baker, 1994), and by 2002 the value of exports had climbed to $55 million (Australian Flower Export Council, 2003) with *Banksia* being an important component of this. The potential for *Banksia* as a nursery or landscape industry plant is high too.

The development of *Banksia* as a cut flower is impeded by a number of factors. Cultivation of *Banksia* in plantations has become increasingly important, not only to reduce reliance on harvesting from natural vegetation as we become increasingly aware for the need to conserve our natural heritage, but also to lift potential production levels. Plantations have been established using seed raised plants, however further value could be added, by supplying high quality, uniform blooms from plantations established with superior selections of asexually propagated plants, rather than the variability of the current seed produced plantations. A reliable asexual propagation technique would also allow selections to be made that have greater resistance to diseases like *Phytophthora*.

Micropropagation potentially offers a rapid multiplication method and some success has been achieved with the establishment of *B. coccinea, B. ericifolia, B. lemanniana, B. marginata, B. menziesii, B. ornata, B. prionotes, B. serrata* and *B. spinulosa* var. *collina* from nodal segments and shoot tips in culture at the University of Adelaide (Sedgley, 1998, Tynan *et al.*, 2001). The same researchers have also achieved slow growth and multiplication rates for *B. coccinea* and *B. spinulosa* var. *collina*, and *in vitro* root induction has been achieved with *B. coccinea* although with little *ex vitro* survival. Niccol *et al.* (1994) observed shoot multiplication for *B. serrata* and *B. oblongifolia* using explants of shoot apices, or more successfully, hypocotyls, although root initiation in culture was reportedly poor. Other unpublished attempts to initiate *Banksia* into culture have been made by other laboratories with little success.

The purpose of this project was to establish modified micropropagation techniques that will produce plants more suited to the *ex vitro* environment. Originally the project was designed to test whether the survival of explants *ex vitro* could be improved by providing photoautotrophic conditions *in vitro* using methods that have largely been developed by Kozai (1991) at Chiba University in Japan. Kozai's studies have indicated that the leaf, shoot and root tissues produced under the heterotrophic conditions normally experienced in tissue culture, are not well adapted to *ex vitro* conditions. Kozai's photoautotrophic methods have been shown to produce plant tissue more equivalent to that produced by standard nursery propagation methods, and are thus more capable of surviving *ex vitro* conditions. Photoautotrophic metabolism is stimulated by high light and carbon dioxide levels, and low levels of sucrose in the nutrient media. However, the technical difficulties encountered refocused the project into preliminary studies thus setting the groundwork for photoautotrophic micropropagation trials. The first of these was the development of an optimal surface sterilisation technique which eliminated reliance on antibiotics in the *in vitro* medium.

Acknowledgements

We are grateful to the Australian Flora Foundation for the grant that enabled the repair and upgrading of an existing Conviron controlled environment cabinet, and made this work possible.

We also gratefully acknowledge the guidance of Mike Rigby of Vitrotec in planning our trials, the assistance of Dr Marco Duretto of the Royal Botanic Gardens Melbourne with the scanning electron microscopy, and Robin Eichner of the Australian Quarantine and Inspection Service and Dr Ian Pascoe of the Institute for Horticultural Development for the fungal identification.

Participants

Initially, the *Banksia* micropropagation project was to be a part of La Trobe University student Fiona Perry's PhD project, but unfortunately she withdrew shortly after she started the project in 1997.

The Royal Botanic Gardens' Honorary Associate, Dr John Godfrey, has been working on the *Banksia* micropropagation project since 1998, although very much on a part-time basis.

Surface sterilisation of explants

Introduction

Plant surfaces are a habitat for a varied microflora nourished by the plant itself and by surface residues including insect detritus (Cassells, 1991). Most of these microorganisms are exclusively epiphytic and can be removed by surface sterilisation unless dwelling in crevices, under scales or on mucilaginous surfaces. Endophytic microflora may also be present and cannot be removed by surface sterilisation. Gunson and Spencer-Phillips (1994) highlight the danger of epiphytes being transferred to endophytic niches during the excision of explants and subsequent manipulation *in vitro*. Therefore care must be taken in collecting material for explants to avoid transferring epiphytic microflora to intercellular and intravascular sites.

The difficulty of micropropagating *B. coccinea* is compounded by problems in surface sterilisation. *B. coccinea* has proved difficult to disinfest (Tynan, 1994). Figures 1, 2, 3 and 4 show the hairy tomentose structure of the adaxial leaf surfaces and the foreign bodies trapped between the hairs.

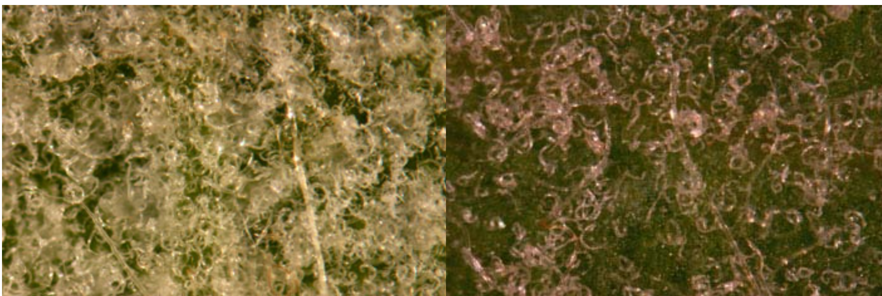

Figure 1: Abaxial (left) and adaxial (right) leaf surfaces of *Banksia coccinea* (x50) showing the mass of hairs that make surface sterilisation difficult.

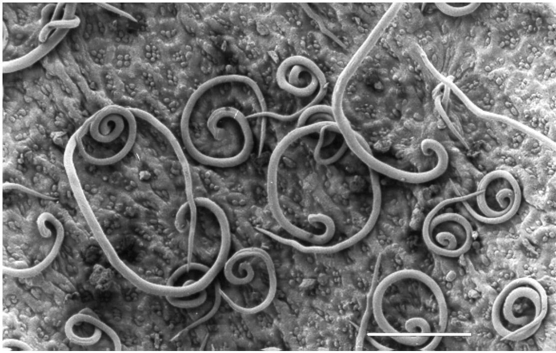

Figure 2: Scanning electron micrograph of the adaxial surface of *Banksia coccinea*, showing the multitude of coiled hairs (x100).

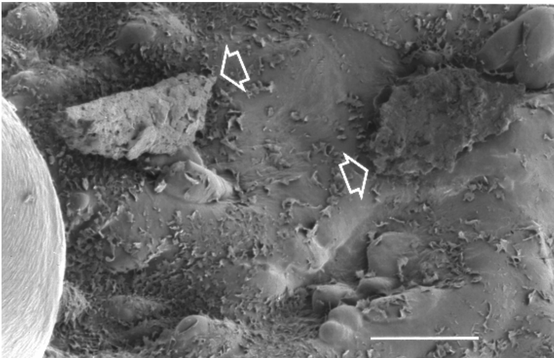

Figure 3: Scanning electron micrograph of the adaxial surface of *Banksia coccinea* (x1000*)*. Arrows indicate some contamination.

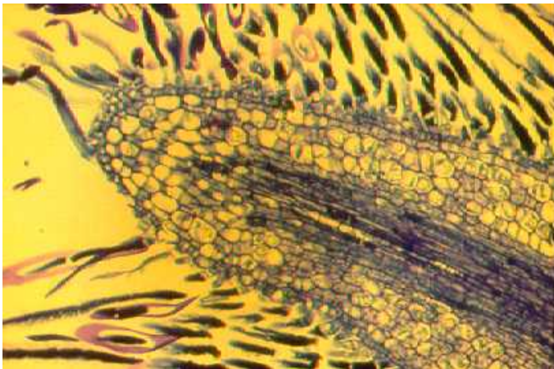

Figure 4: Photomicrograph of a stained section of a developing leaf of *Banksia coccinea* showing the numerous hairs arising from the epidermal layer (x250).

Pre-treatment of the mother plant by growing it in a protected environment and/or using foliar fungicides reduces the incidence of surface contamination, but a protected environment is not always possible and the use of fungicides is best avoided. Fungicides and antibiotics added to culture media have undesirable environmental and health implications. The widespread use of antibiotics in humans and animals increases the potential for antibiotic resistance (Austin *et al.*, 1999) and exposure to fungicides, particularly together with herbicides, results in a significantly increased risk for non-Hodgkin lymphoma (Hardell & Eriksson, 1999). A further danger is that pharmaceutical products and their often more toxic metabolites can be present in the environment at concentrations similar to pesticides (Halling-Sϕrensen *et al.*, 1998) and, like them, are persistent and mobile appearing in ground water, streams and drinking water (Pearce, 1999). Antibiotic resistant epiphytic bacteria have also been detected on plants cultured with antibiotics (Epton, 1993).

PPM (Plant Preservative Mixture), a mixture of two isothiazolones, is an additive which controls contaminants in culture, and an independent assessment (Niedz, 1998) supports the manufacturer's claims of its effectiveness, but its long term safety and effect on the physiology of explants following several subcultures has not been extensively

documented. Reports of other additives with the same purpose e.g. sodium dichloroisocyanurate (Parkinson, Prendergast & Sayegh, 1996) appear from time to time.

Successful disinfestation of *B.coccinea* has been achieved (Tynan, 1994), but the decontamination process, which included soaking explants in Benlate® overnight, was lengthy and may have altered plant cell physiology, resulting in sub-optimal material which subsequently failed to survive. Benlate-induced phytotoxicity has been reported for example in bedding plants (van Iersel & Bugbee, 1997), and DuPont withdrew Benlate® from sale in 2001 due to litigation initiated by many plant growers concerned that the product caused problems with their crops (DuPont, 2003). Physical surface treatment, such as scrubbing, must damage tissue, and the inclusion of antibiotics in the medium can be phytotoxic (Pollock *et al*. 1983; Debergh & Maene, 1984; Duhem *et al*. 1988; Enjalric *et al*., 1988) and can inhibit photosynthesis (Surzycki, 1969).

A fast, simple, safe and effective means of surface de-contamination which does not damage plant cells or destroy surface integrity has obvious advantages.

2. Method

Terminal shoots of *Banksia coccinea*, about 150mm long, were collected from greenhouse grown plants, the leaves trimmed, and the stems placed into 0.01M HCl with 3 drops/500ml of Tween 80® (pH 2.4) with gentle agitation for 3 minutes, transferred directly to 1% available chlorine solution (sodium hypochlorite - Domestos® 1:5; pH 12.4) for 10 minutes with constant agitation; then transferred directly to sterile 2.4mM citric acid (pH2.9) solution for 5 minutes. The sections were rinsed once and stored in sterile de-ionised water.

Ninety-three axillary buds showing obvious signs of growth were selected from 9 different plants and were initiated on Woody Plant Medium (WPM) (Lloyd & McCown, 1980) with 1μM BA and 0.1μM IBA. A further 98 axillary buds from 8 different plants were initiated on ½ Murashige & Skoog medium (M&S) (1962), also with 1μM BA and 0.1μM IBA. The explants were initiated under a light intensity of 20-60$\mu mol.m^{-2}s^{-1}$.

Contamination and explant survival were recorded after five weeks. Microorganisms found on contaminated explants were identified by Robin Eichner and Ian Pascoe at Australian Quarantine Inspection Service and the Institute for Horticultural Development respectively, both at Knoxfield, Victoria.

3. Results:

Contamination and explant survival are recorded in Table 1. Seventy-five percent of explants survived, and remained green and viable after surface sterilisation using the hydrochloric acid/sodium hypochlorite/citric acid treatment, and subsequent placement on either WPM or M & S. A higher percentage of explants survived on ½ M & S although bud expansion and growth was observed to be best on WPM.

Table 1: Explant survival and condition 5 weeks after disinfestation with hydrochloric acid/sodium hypochlorite/citric acid treatment.

Explant condition	Percentage explants		
	WPM	½ M&S	Total
Green viable	60	91	75.5
Contaminated	15	6	10.5
Dead	25	3	14

Scanning electron micrographs of the abaxial surface of treated *B. coccinea* leaves (Figures 5 and 6), show the effect of the treatment on the explant surface. Surface contamination is markedly reduced, most of the surface hairs are abscised, and the surface of the cuticle is smoother than the untreated control (Figures 2 and 3).

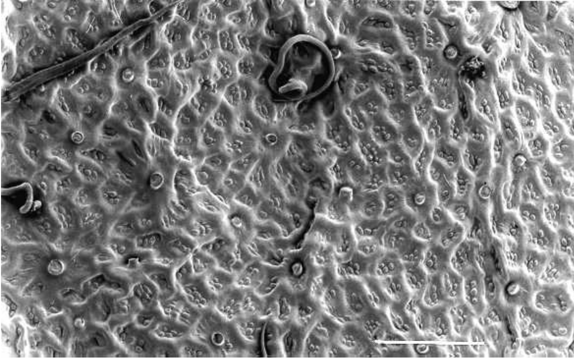

Figure 5: Scanning electron micrograph of the adaxial surface of *Banksia coccinea* after treatment with HCl, sodium hypochlorite and citric acid, showing the reduced contamination and number of hairs (x100).

Figure 6: Scanning electron micrograph of the adaxial surface of *Banksia coccinea* after treatment with HCl, sodium hypochlorite and citric acid, showing the smoother surface of the cuticle, and markedly reduced surface contamination (x1000). The base of an abscised hair can be seen in the top right corner.

The most common fungal contaminant was *Cladosporium* sp. which appeared as a grey fluffy growth, the organism being characterised by lemon-shaped conidia. *Cladosporium* was also the persistent and commonest contaminant of seed. Other contaminants were *Nigrospora* sp. and *Sordaria* sp. which are both saprophytic; *Acremonium* sp., an endophyte in grasses; and *Tilletiopsis* sp. which is a hyperparasite probably parasitic to other fungal parasites. All the fungi identified were not considered to be pathogenic but rather reflected incomplete surface sterilisation.

4. Discussion

The densely hairy surfaces of *B. coccinea* are a haven for microorganisms which are difficult to remove because of inadequate wetting due to the trapped air between and under the hairs. Comparison of Figures 5 and 6 with Figures 2 and 3 shows that the treatment for surface sterilisation of this trial, partially denudes the surface of hairs and epicuticular structures, and with them, surface contaminants are also removed.

During the sterilising process there is a rapid and extreme environmental change in pH from pH 2.36 for 0.01M HCl to pH 12.38 for Domestos® 1:5 solution to pH 2.89 for 2.4mM citric acid solution. It is thought this disrupts the cells of the surface microorganisms causing death (Rigby, 1998, pers. comm.). Although exposure to these extremes of pH is phytotoxic to very young tissue, growing axillary *B. coccinea* buds can withstand over 15 minutes exposure to the Domestos® solution, and still produce some viable explants.

Krebs' Cycle acids, especially citric acid, are growth stimulants, and growth in culture of some plants is enhanced if an organic acid is present in the culture medium (George, 1993). Although omitted from the culture media in this experiment, its use as a final bath was designed to reduce pH after the sodium hypochlorite rinse, and to be absorbed by the explant to stimulate growth.

The advantage of this technique is that it is simple, safe, fast and effective. This method of surface sterilisation has been used successfully on *Leptospermum* spp., *Phalaenopsis* spp. and *Begonia* cultivars.

Further work

This surface sterilisation method has enabled a number of small trials to be undertaken to determine an optimal *in vitro* medium for the initiation and multiplication of *Banksia coccinea*. Although long-term *in vitro* survival and multiplication have been unsuccessful to date, a basis for a full-time research project has been established. With recent advances in the micropropagation of "recalcitrant" woody species, more rapid progress may now be possible.

References

Austin, D.J., Kristinsson, K.G. and Anderson, R.M. (1999) The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. Proceedings of the National Academy of Science USA 96: 1152-1156.

Australian Flower Export Council (2003) A snapshot of Australian floriculture and exports Australian Flower Export Council (http://www.feca.com.au/)

Baker, J (1994) Where is WA's wildflower industry off to? Australian Horticulture 92(9) 44-45

Cassells, A.C. (1991) Problems in tissue culture: culture contamination. In Debergh, P.C. and Zimmerman, R.H. (eds.) 'Micropropagation. Technology and application.' Kluwer, Dordrecht.

Debergh, P. and Maene, L. (1984) Pathological and physiological problems related to the *in vitro* culture of plants. Parasitica 40(2-3): 69-75.

Duhem, K., Le Mercier, N. and Boxus, P. (1988) Difficulties in the establishment of axenic *in vitro* cultures of field collected coffee and cacao germplasm. Acta Horticulturae 225: 67-75.

DuPont (2005) Benlate® Fungicide. E.I. du Pont de Nemours and Company (http://heritage.dupont.com/floater/fl_benlate/floater.shtml) Accessed May 2005.

Enjalric, F., Carron, M.P. and Lardet, L. (1988) Contamination of primary cultures in tropical areas: the case of *Hevea brasiliensis.* Acta Horticulturae 225: 57 –65.

Epton, H.A.S. (1997). Epiphytic bacteria: activities, risks and benefits In 'Pathogen and microbial contamination management in micropropagation'. (Ed. A.C.Cassells) pp.299-308 (Kluwer Academic Publishers: Dordrecht).

George, E.F. (1993). Plant propagation by tissue culture. Part 1. The technology. 2nd edition. Exegetics Ltd., England.

Gunson, H.E. and Spencer-Phillips, P.T.N. (1994) Latent bacterial infections: epiphytes and endophytes as contaminants of micropropagated plants. In Lumsden, P.J., Nicholas, J.R. and Davies, W.J. (eds.) 'Physiology, growth and development of plants in culture' Kluwer, Dordrecht.

Halling-Sϕrensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhϕft, H.C. & Jϕrgensen, S.E. (1998) Occurrence, fate, and effects of pharmaceutical substances in the environment – a review. Chemosphere 36(2): 357-393.

Hardell, L. & Eriksson, M. (1999) A case-control study of non-Hodgkin lymphoma and exposure to pesticides. Cancer. 85: 6: 1353-1380.

Kozai, T. (1991) Micropropagation under photoautotrophic conditions in Deburgh, P C & Zimmerman, R H Micropropagation - technology and application Kluwer Academic Publishers, Dordrecht, The Netherlands.

Lloyd, G. & McCown, B. (1980) Use of microculture for production and improvement of *Rhododendron* spp. HortScience 15: 416(Abst. 321).

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

Niccol, RJ, Regan, PA & De Filippis, LF (1994) Simplified protocol for the micropropagation of selected *Eucalyptus* and *Banksia* species. Australian Forestry 57(4): 143-147.

Niedz.R.P. (1998). Using isothiazolone biocides to control microbial and fungal contaminants in plant tissue cultures. HortTechnology 8: 598-601.

Parkinson.M., Prendergast. M., and Sayegh. A.J.(1996). Sterilisation of explants and cultures with sodium dichloroisocyanurate. Plant Growth Regulation 20: 61-66.

Pearce, F. (1999). Something in the water. New Scientist 161(2176): 18-19.

Pollock, K., Barfield, D.G. and Shields, R. (1983) The toxicity of antibiotics to plant cell cultures. Plant Cell reports 2: 36-39.

Sedgley, M. (1998) *Banksia*: new Proteaceous cut flower crop in Janick, J. (ed.) Horticultural Reviews Volume 22. John Wiley & Sons: New York.

Surzycki, S.J. (1969) Genetic functions of the chloroplast of *Chlamydomonas reinhardi*: effect of Rifampin on chloroplast DNA-dependent RNA polymerase. Proceedings of the National Academy of Science 63: 1327-1334.

Tynan, K.M. (1994). Evaluation of *Banksia* species for response to *Phytophthora* infection. Unpublished manuscript, University of Adelaide.

Tynan, KM; Scott, ES; Sedgley, M, (2001) *Banksia* propagation – *in vitro* multiplication of *Banksia* species. Australian Plants 21(166): 79-82.

van Iersel, M.W. & Bugbee, B. (1997) Increased organic matter in the growing medium decreases Benlate DF phytotoxicity. Plant Disease 81(7): 743-748.