AN INVESTIGATION OF FACTORS INVOLVED IN THE FORMATION OF PROTEOID ROOTS

by

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Summary

Proteoid roots are clusters of dense bottle-brush-like rootlets which form on lateral roots of plants in the family Proteaceae (Figures 1.1-1.3). They have been observed in most genera of the family and also in some legumes (Fabaceae, Mimosaceae and Papilionaceae) and Casuarinaceae. There is evidence to suggest that the formation of proteoid roots in the Proteaceae is initiated by microorganisms. This thesis examines aspects of this hypothesis.

Methods of *in vitro* cultivation of the proteaceaous plants *Macadamia* and *Banksia* were devised to provide cultures free of microorganisms. *Macadamia* was chosen as a test plant because of its importance in the horticultural industry. Methods of initiation and propagation of shoots for *Macadamia* were established. Procedures were also developed for growing *Banksia* seedlings in hydroponic cultures, both aseptically and septically. The septic cultures provided copious amounts of proteoid roots needed for biochemical analysis.

This investigation examines the possibility that proteoid roots are produced as a result of the integration of prokaryotic DNA into the plant genome as typified by the *Agrobacterium*/plant interaction. The morphology of the hairy roots of *Agrobacterium*-transformed plants is superficially similar to proteoid roots. The soil bacteria *A. tumefaciens* and *A. rhizogenes* transfer genetic material from extra chromosomal plasmids to plants. These bacteria are responsible for proliferative diseases known as crown gall and hairy root, respectively.

A characteristic of *Agrobacterium* infection is the presence in transformed plant tissues of a class of biological chemicals known as opines. The opines are used as substrates for the inciting bacteria, but they cannot be catabolised by other soil bacteria or by the plant. One opine-like chemical was found in abundance in proteoid roots. This was subsequently identified as tyramine. However it was also located in the non-proteoid roots of *Banksia grandis* cultured under aseptic conditions. Furthermore, eight bacterial species were isolated from the rhizosphere of proteoid roots that were able to catabolise tyramine. These results were inconsistent with an opine-like role for tyramine.

One approach in this investigation has been the use of molecular probes to search for bacterial DNA sequences in DNA isolated from proteoid roots because such sequences would

constitute clear evidence of bacterial involvement in proteoid root production. Prokaryote-specific genes coding for enzymes for indole-3-acetic acid (IAA) synthesis have been isolated from *Agrobacterium*. These have homologous sequences with other species of soil bacteria. Even under low stringency conditions, no hybridising DNA sequences were found in the DNA of proteoid roots although other genes (α-amylase) with low copy numbers were detected. These data together with that of the opine investigation provides circumstantial evidence that *Agrobacterium* is not involved in the production of proteoid roots. However sequence homology was found between the molecular probes for the genes encoding enzymes for IAA biosynthesis and DNA from bacteria isolated from the rhizosphere of proteoid roots. This indicates that, given an abundance of tryptophan (the precursor of IAA), bacteria within the rhizosphere may, by the production of IAA, be involved in the causation of proteoid roots.

The introduction of *Agrobacterium* to aseptic cultures of *Macadamia* and *Banksia* did not initiate the production of proteoid roots or analogous structures. Attempts to infect *Banksia* roots using bacteria isolated from proteoid roots were unsuccessful in initiating proteoid roots.

The mechanism by which proteoid roots are formed is still not well understood. However this investigation furthers our knowledge of their formation by showing that it is unlikely that *Agrobacterium* or a similar bacterium with oncogenic properties is involved. Further, the compound tyramine isolated from proteoid roots may prove to be of importance in the nutrition of *Banksia* as a chelating agent, especially if it is exuded into the soil. By chelating with iron and phosphorus in the soil, tyramine may increase the flux of iron and phosphorus to the root thereby making it available for uptake by the plant.

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, no material described herein has been previously published or written by another person except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

D.M.Cuthbertson

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Chapter 1

Introduction

The term 'proteoid roots' was first used by Purnell (1960) to describe clusters of highly-modified tertiary roots which can often be found in the upper soil horizons and in the leaf litter. These roots are a characteristic of many species within the family Proteaceae (Figures 1.1-1.3). The complex anatomy of these root structures has been described by Purnell (1960) and Lamont (1972; 1973), and this account of their structure is based on their work. Briefly, proteoid roots are closely spaced roots that form in longitudinal rows along lateral roots which apparently all emerge at the same time. The number of rows produced reflects the number of protoxylem poles in the parent lateral root. Proteoid rootlets arise opposite the xylem poles and cause considerable disruption of the cortical tissue as the rootlets push through it. Proteoid rootlets have normal primary root structure and do not undergo secondary growth. It has been estimated that a 10mm length of lateral root with seven rows of proteoid rootlets can bear over 1000 individual rootlets. These elongate and then form copious, long root hairs which are sometimes forked at the tips. Unlike the normal roots of the plants on which they are growing, the proteoid roots are ephemeral. They form 2 to 6 months after germination and live 12 to 18 months, frequently forming a mat at or near the surface of the soil. The particular section which bears the proteoid root has been termed the axis of the proteoid root and the rootlets which form the proteoid root are referred to as proteoid rootlets. In some species (for example species of the genera, *Adenanthos* Labill. Pl. Nov. Holl., *Conospermum* Sm., *Grevillea* R.Br., *Hakea* Schrad. Sert. Hannov., *Isopogon* R.Br. ex Knight, *Lomatia* R.Br., *Orites* R.Br., and *Telopea* R.Br.) axes of proteoid roots are unbranched and are approximately 0.6 to 3.8cm long, whereas in species of *Banksia* Linn. branching is profuse, particularly in older plants and so the proteoid roots may become a very complex structure. In *Banksia* seedlings proteoid roots may be 2.5 to 5cm long and up to 15cm in mature plants.

Structures morphologically similar to proteoid roots occur in plants other than those in the Proteaceae. They have been reported as being components of the root systems of the legumes *Kennedia* Vent. sp. (Fabaceae) (Trinick, 1977, reported as personnel communication from N. Malajczuk), *Lupinus cosentinii* Guss. (Trinick,1977) and *Lupinus albus* L. (Papilionaceae)

Figure 1.1. Proteoid roots. *Banksia ornata* collected from a leaf litter layer, Kuipo Forest, South Australia. The roots shown in this photograph were adhering to a leaf giving a flattened appearance.

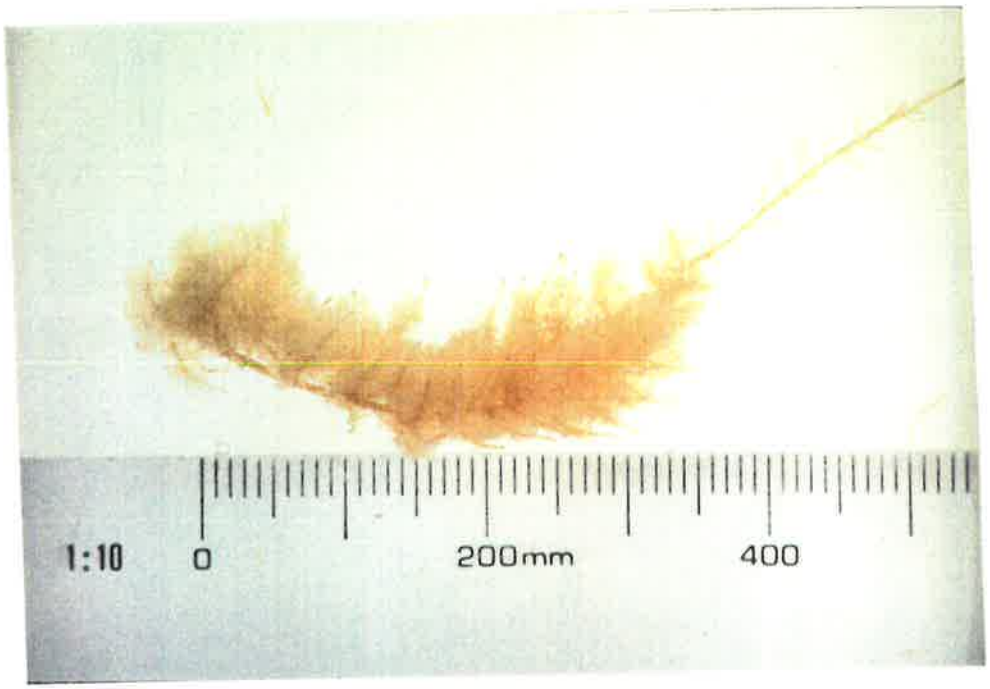


Figure 1.2. Proteoid roots of *Banksia grandis* grown in the liquid culture medium of Dell *et al.*, 1980.

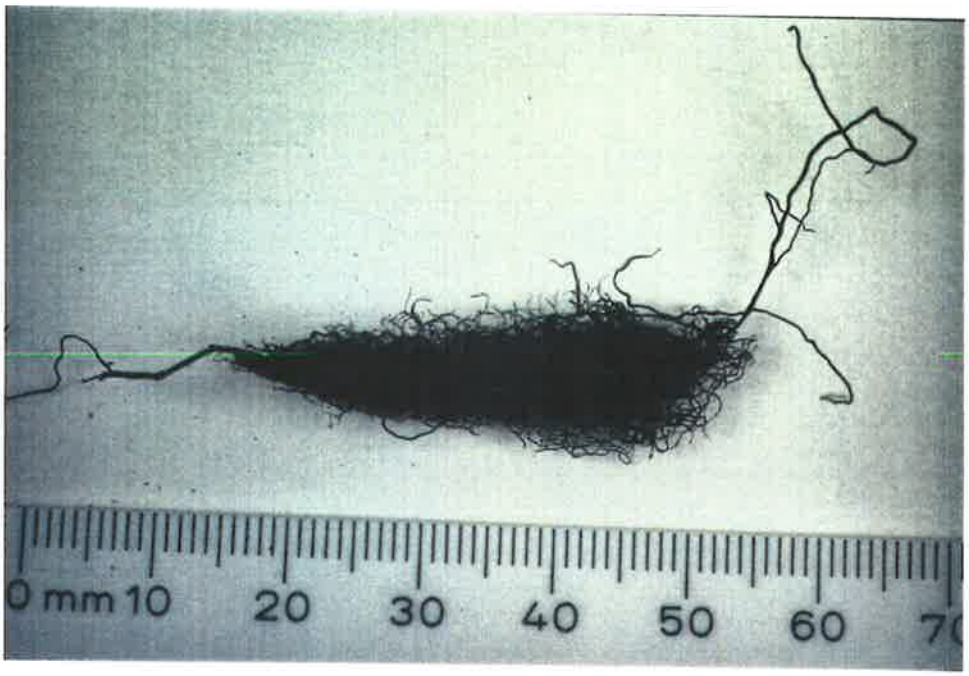


Figure 1.3. Proteoid roots of *Allocasuaina striata* collected from sandy soil, Ashbourne, South Australia.

(Gardner *et al.*, 1981), *Acacia mucronata* Willd. ex. H.L.Wendl. (Mimosaceae) (Sward, 1978), *Viminaria juncea* Schrad. and J.Wendl. (Fabaceae) (Lamont, 1972), and of the root systems of some members of the Casuarinaceae [*Casuarina equisetifolia* L. (Bowen, 1981) *Allocasuarina littoralis* (Salisb.) L. Johnson (cited in Gardner *et al.*, 1982 as personal communication from G.D.Bowen) and *Allocasuarina striata* (Macklin) L. Johnson (personal observation) (see Figure 1.3)]. Dauciform roots (named because of their carrot-like shape) are bunches of specialised rootlets of the sedge family (Cyperaceae), and are said to be analogous to proteoid roots (Davies *et al.*, 1973).

Nutrition

Proteoid roots form in nutrient poor soils and their presence is generally considered to increase the ability of the plant to absorb phosphorus and other nutrients from soil (Jeffrey, 1967; Malajczuk and Bowen, 1974). They form commonly on *Banksia grandis* Willd. when concentrations corresponding to 1 and 3ppm extractable phosphorus are found in lateritic podzolic and sandy soils respectively (Malajczuk and Bowen, 1974). Maximum proteoid root production occurs at low phosphorus and nitrogen concentrations in natural soil or a synthetic rooting medium (Gardner *et al.*, 1982; Groves, 1964; Beadle, 1968; Grundon, 1972; Groves and Keraitis, 1976; Lamont, 1972; Barrow, 1977). Nutrient uptake by proteoid roots is reviewed in Lamont (1981; 1982) (also see Chapter 4 and General Discussion). Malajczuk and Bowen (1974) found that proteoid roots absorbed ^{32}P supplied as KH_2PO_4 about four times faster than non-proteoid roots. Attempts have been made to explain the ability to increase nutrient uptake in terms of the increased surface area provided by the proteoid root structure for nutrient absorption (Jeffery, 1967; Lamont, 1977). Gardner *et al.* (1982), however, questioned this explanation due to the clustered nature of the roots and the resulting overlap of nutrient depletion zones around them. They suggest that the compact nature of these root systems is more likely to be suited to the build up of substances secreted by them in the rhizosphere. This is supported by the findings that proteoid roots of *Lupinus albus* and *B. integrifolia* Linn. produce large amounts of reductants, protons and organic acids compared to those found in the bulk soil (Gardner *et al.*, 1983) and the authors suggest that

citrate, exuded by the roots, chelates and mobilises iron, thereby making phosphorus available for uptake. Grierson and Attiwill (1989) also found an unidentified chelating agent in water extracts of proteoid roots which may have the effect of mobilising iron and phosphorus.

Initiation and development

The initiation of proteoid roots in the Proteaceae has been attributed to an interaction between non-infecting rhizosphere microorganisms and the roots (Lamont and McComb, 1974; Malajczuk and Bowen, 1974). The evidence for this conclusion is that proteoid roots form in non-sterile but not in sterile soil. The experiments have, however, dealt with mixed populations of soil bacteria and there have been no reports of the application of Koch's postulates to the proteoid root phenomenon.

Bacteria, actinomycetes and fungi were not detected in newly formed proteoid roots (Purnell, 1960; Lamont,1972; Lamont and McComb, 1974; Malajczuk and Bowen, 1974; Powell, 1975; and Trinick, 1977). However, direct visual inspection of stained proteoid roots from inoculated pots showed the rhizosphere to be dominated by bacteria (Malajczuk and Bowen, 1974). The same authors counted total bacteria, total aerobic viable bacteria, pseudomonads, Gram-negative bacteria, and *Bacillus* spp. in the rhizoplane of both proteoid and non-proteoid roots of the same plant and found the frequency of these groups were similar per unit length of root. The counts did not allow identification of a causal organism.

Investigations by Gardner *et al* (1982) found that proteoid roots of *L. albus* could develop under aseptic conditions. Van Staden *et al* (1981) observed structures similar to proteoid roots forming on *Protea nerifolia* R. Br. grown in aseptic culture. The sequence of events which leads to the formation of proteoid roots is unclear. The pure culture studies of Van Staden *et al* (1981), using different synthetic auxins, suggested that natural auxins may be involved. Prokaryotic genes coding for enzymes involved in synthesis of the natural auxin indole-3-acteic acid (IAA) synthesis have been isolated in transformed plant tissue (see below). Because proteoid roots look superficially like the transformed roots of carrot, it can be asked whether genetic transformation is a good explanation of the facts so far reported about the initiation of proteoid roots. The biology of

the soil bacterium *Agrobacterium* which is a natural genetic engineer of plants, and its plant relations will be briefly examined.

The biology of Agrobacterium/plant relations

Infection and genetic transformation

Agrobacterium tumefaciens (Smith and Townsend) Conn and *A. rhizogenes* (Riker *et al*) Conn transfer genetic material from their bacterial plasmids to plants. These bacteria are responsible for proliferative diseases known as crown gall and hairy root, respectively (Armitage *et al.*, 1988) in which there is integration of prokaryotic DNA into plant nuclear genomes [for reviews see Kahl and Schell (1982), Caplan *et al* (1983), Gheysen *et al* (1985), Stachel and Zambryski (1986), Walden (1988) and Binns and Thomashow (1988)].

The interaction between *Agrobacterium* and plant hosts is controlled by large plasmids, known as Ti (tumour inducing in the crown gall) or Ri (root inducing in hairy root) plasmids, of which one specific segment (the T-DNA) is transferred into cells of a susceptible plant (Armitage *et al.*, 1988). *A. tumefaciens* and *A. rhizogenes* are considered to have wide host ranges, including many dicotyledonous plants (Keen and Staskawicz, 1988; Anderson and Moore, 1979; De Cleene and De Ley, 1981). Individual strains, however, exhibit relatively narrow host ranges (Anderson and Moore, 1979).

Wounding of the plant is a prerequisite for the development of crown gall or hairy root disease. Although *Agrobacterium* sp. may colonize undamaged plant surfaces, wounding is necessary for the interaction to develop fully and produce disease symptoms (Keen and Staskawicz, 1988). Expression of a specific set of bacterial genes is required for tumor induction. These are the so-called *vir* genes in *Agrobacterium* (Ream, 1989; Bolton *et al.*, 1986; Rogowsky *et al.*, 1987; Stachel *et al.*, 1985[ab], 1986[cd]). The genes are induced by compounds produced from wounded plant tissues (Binns and Thomashow, 1988). The complex series of events triggered by wounding of plant tissues is well reviewed elsewhere (Lippincott and Lippincott, 1975; Kahl, 1982; Binns and Thomashow, 1988; Ream, 1989) and the interest of wounding in the context of

this thesis is mainly related to the methodology. Wounding is now a classical step in the process of plant genetic engineering when *Agrobacterium* is used as the agent for genetic exchange and may well be important in proteoid root initiation. This has been attempted with *Macadamia* F.Muell. cultivars (Chapter 3).

Opine production

Crown gall and hairy roots induced on plants by oncogenic *Agrobacterium* strains, synthesize specific compounds that have been termed opines (Tempé *et al.*, 1979). The discovery that opines serve as specific growth substrates for the pathogenic agent (Lippincott *et al.*, 1973; Kerr and Roberts, 1976; Petit and Tempé, 1978; Ellis and Murphy, 1981) led Tempé and his coworkers to formulate the opine concept (Tempé *et al.*, 1979; Guyon *et al.*, 1980). This proposes that a parasitic agent may incite opine synthesis in its host, creating a chemical environment favourable for growth and propagation of the pathogenic agent and mediating parasitism. Figure 1.4 shows the structures of some of the known opines. In *Agrobacterium* strains, opine synthesis and degradation are encoded by genes located on Ti or Ri plasmids (Tempé *et al.*, 1984). The bacterium can be said to engineer plant cells to produce a substrate which can be utilised by the pathogenic bacterium and not by the plants. Opines can constitute up to 7% of the dry weight of transformed tumour tissue (Firmin and Fenwick, 1978). Generally, each *Agrobacterium* strain catabolises only the opines synthesised by the tumors it induces (Ream, 1989) and, conceivably, the inciting strains can create a niche that offers an environment specifically favorable for their growth.

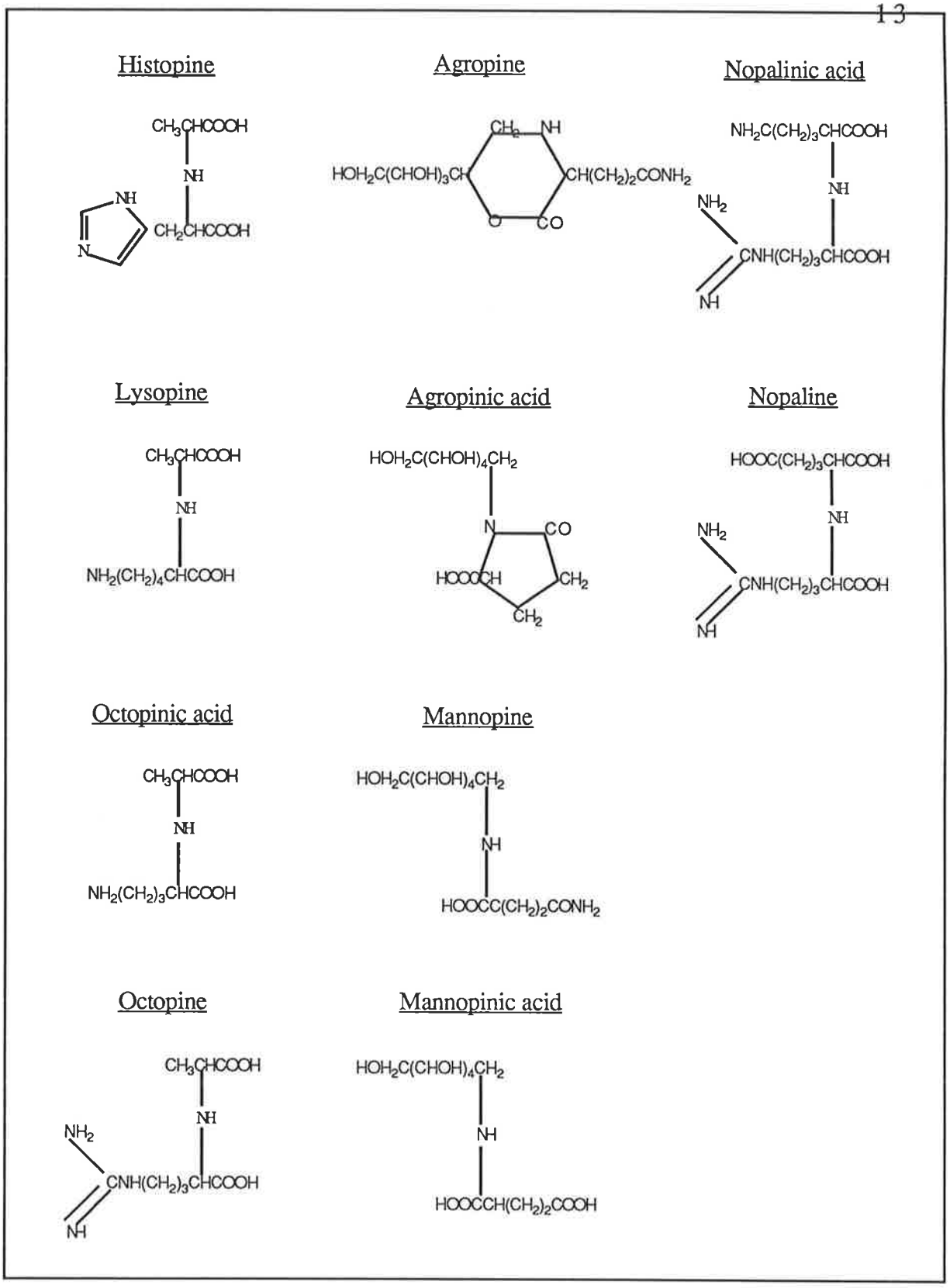


Figure 1.4. Chemical structures of opines (Petit *et al.*, 1983).

Enzymes for IAA synthesis in prokaryotes

IAA is structurally similar to the amino acid tryptophan and is synthesised from it. However, its synthesis in plants is by different enzymes than in bacteria or genetically transformed plants in which the appropriate bacterial genes have been inserted. *A. tumefaciens* genes code for enzymes for the two-step biosynthesis of indole acetic acid (IAA) (Figure 1.5). This is significant because the genes are of prokaryotic origin (see below) and have not been isolated in aseptic, non-transformed cells of eukaryotes (Weiler and Schröeder, 1987). The two enzymes involved are tryptophan mono-oxygenase, and indole-3-acetamide hydrolase and are encoded on the T-DNA. The first enzyme converts tryptophan (a product of the shikimate pathway) to indole-3-acetamide by oxidative decarboxylation. The second enzyme hydrolyses indole-3-acetamide to IAA (Inze *et al.*, 1984; Schröeder *et al.*, 1984; Thomashow *et al.*, 1985; Thomashow *et al.*, 1986; Van Onckelen *et al.*, 1986; Yamada *et al.*, 1985). *A. rhizogenes* also carries genes which are functionally equivalent to those *A. tumefaciens* genes coding for IAA synthesis (Cardarelli *et al.*, 1985; Huffman *et al.*, 1984).

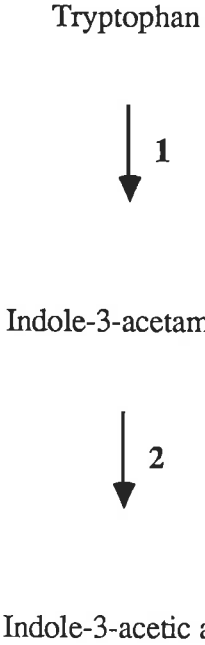


Figure 1.5. The biochemical transformation of tryptophan to IAA, which is catalysed by the enzymes encoded in the T-DNA of *Agrobaterium* (Weiler and Schröder, 1987). 1 = Tryptophan mono-oxygenase, 2 = indole-3-acetamide hydrolase.

Many rhizosphere microorganisms produce auxins (Lynch, 1976). The auxin and cytokinin genes from *Pseudomonas syringae* pv. *savastanoi* (Smith) Stevens may have a common origin with those of *A. tumefaciens* (Yamada *et al.*, 1985; Follin *et al.*, 1985). *P. s* pv. *savastanoi* induces tumorous growths on olive and oleanders, which result from the production of auxins and cytokinins by the pathogen. Unlike crown gall or hairy root disease, the plant tissue is not transformed and continued tumor growth is dependent on the presence of the inciting bacterial pathogen (Surico *et al.*, 1984; Surico *et al.*, 1985).

These observations suggest that the *Agrobacterium* T-DNA sequence encoding for tryptophan mono-oxygenase and indole-3-acetamide hydrolase may be of use as general molecular probes to identify analogous genes in other microbes or in plants, especially in tissues showing 'abnormal' growth. A positive result against DNA from proteoid roots would imply that *Agrobacterium* (or another oncogenic bacterium) is involved (as described above) or that the relationship is an example of a non-integrating, but plant tissue invasive pathogen, as typified by *P.*

s pv. *savastanoi*. This second explanation is unlikely as workers agree that in newly formed proteoid roots there are no internal microbes (Purnell, 1960; Lamont,1972; Lamont and McComb, 1974; Malajczuk and Bowen, 1974; Powell, 1975; and Trinick; 1977). If functionally equivalent genes are found within the population of rhizosphere bacteria, they may indicate an influence of non-invasive bacteria on proteoid root production due to their hormonal effect.

Is Agrobacterium involved proteoid root formation?

Proteoid roots normally form in non-sterile conditions. Central to this thesis is the hypothesis that proteoid roots are produced in response to an invasive microorganism. Previous investigations have suggested that the roots form as a response to non-invasive microorganisms because microscopic examination of newly formed proteoid roots have revealed no microorganisms present in root tissues (see above). The data is, however, contradictory because proteoid roots have been formed in sterile culture on *Lupinus* and *Protea* (see previously). An invasion and subsequent transformation of roots by *Agrobacterium* or another hitherto undiscovered microbe capable of plant transformation, might however go undetected by microscopic examination because the inciting microbe need not be present for the resultant morphological phenomenon to be observed. Infection could conceivably have taken place at an earlier developmental stage. This is an alternative hypothesis which would fit the facts.

If *Agrobacterium* is implicated as the causal agent in the production of proteoid roots, then the question may be resolved by:

a) chemical tests on rhizosphere bacteria associated with proteoid roots to detect the presence of *Agrobacterium* (see Chapter 2),

b) molecular probes using prokaryotic genes coding for enzymes involved in IAA synthesis against DNA from proteoid roots (Chapter 5),

c) a chemical investigation of proteoid root extracts to detect known opines or opine-like compounds and an assessment of the ability of rhizosphere bacteria to catabolise 'putative opines' (Chapter 4).

The approaches outlined above form the main part of this thesis. In addition, Koch's postulates was carried out to examine the question of the aetiology of proteoid root morphogenesis using aseptically grown plants and bacterial isolates from proteoid roots. This approach could either support the *Agrobacterium* hypothesis (*Agrobacterium* is involved in proteoid root formation) or identify another bacterium responsible for proteoid root initiation.

The approaches outlined above have a requirement for aseptic plant tissues, proteoid roots and other non-sterile plant tissues. This has led to pure culture studies with proteaceaous plants of horticultural interest (Chapter 3) and development of other plant culture systems (Chapter 2).

Chapter 2

Plant and bacterial culture

Introduction

Microorganisms and higher plants coexist in nature in constant interaction (Schmidt, 1979). One way to examine if there is a specific interaction which results in the production of proteoid roots and which involves an association with proteaceous plants and a certain species of bacteria is by fulfilling Koch's postulates. These postulates were first established by Henle in 1840 to examine the causal relationship between a microorganism and a disease and first satisfied by Koch in 1876 with his experiments on anthrax. The experimental requirements can be summarised as follows:

1) the same bacterium must be present on all proteoid roots.

2) the bacterium must be isolated from proteoid roots and grown in pure culture.

3) the bacterium from the pure culture must cause proteoid roots to form when inoculated into a healthy, susceptible laboratory plant.

4) the bacterium must again be isolated from the inoculated plant and must be shown to be the same bacterium as the original organism.

It should be noted that although Koch's postulates are useful in determining the causative agent for most diseases, there are some exceptions. For example, it is known that the bacterium *Treponema pallidum* (Schaudinn and Hoffmann) is the causative agent for syphilis, but virulent strains have never been cultured on artificial media (Tortora *et al.,* 1982). Also, vesicular-arbuscular mycorrhizal fungi, which form symbiotic associations in most plant families (Harley and Smith, 1983), can not currently be grown in pure culture (Powell and Bagyaraj, 1984) so that Koch's postulates cannot be satisfied. Another exception is the situation where *Agrobacterium* transforms plant tissue. Often in surface sterilised transformed-tissues *Agrobacterium* cannot be reisolated. This is because, often it is only the T-DNA (Transfered DNA) which remains and is replicated within the plant.

This chapter describes the methods used to isolate and store bacteria from proteoid roots. Given that one of those bacteria might be responsible for proteoid root formation, the bacteria were

characterised by standard microbiological tests. This characterisation of bacteria from proteoid roots also addresses the question, is *Agrobacterium* involved in proteoid root formation?

To fulfil Koch's postulates, bacteria from proteoid roots were used to inoculate aseptic cultures of *B. grandis*. Carrot tissues were inoculated in a similar manner to investigate the possibility of using carrot as a test plant, thereby providing a simplified experimental system.

A fundamental requirement for the study of proteoid roots is their plentiful supply. Methods used to grow proteoid roots and sterile plants are described. Hydroponic culture systems were developed because the roots are easily recoverable from those media and the development of root systems can be observed *in situ*. Plants, whose culture is described in this chapter, have also been used in experiments described elsewhere. However, because there is an overlap in methodologies, all plant and bacterial materials and methods are included in this chapter.

The method used for the isolation of bacteria from proteoid roots (as apposed to the more conventional methods of isolation of bacteria from roots) was chosen because it was reasoned that if the bacteria responsible for initiating the proteoid roots were *Agrobacterium* species or ones with similar opine-like producing properties, then the exudation of opines would give to those bacteria a competitive advantage both within the roots and in the rhizosphere and so they would predominate. This method would then isolate them.

Materials and methods

Isolation and characterisation of bacteria from proteoid roots are described, followed by methods whereby sterile plant material can be generated. The plant materials and methods section also includes information pertinent to, and in common with, other chapters in this thesis: and is included here for convenience and simplicity.

Bacterial culture

Bacteria were isolated from proteoid roots of *B. grandis* growing in hydroponic culture. Roots were first blotted dry and then homogenized by crushing them with a glass rod in a 2ml centrifuge tube. Debris was removed by centrifugation. The supernatant was spread, using a glass spreader, onto LB agar (Appendix A) and incubated at 25°C in the dark. The microbial population was measured by standard plate counts on NB medium (Appendix A). Fifteen pure cultures were isolated from streak plates. Isolated bacteria, together with laboratory strains [*Agrobacterium* strains K565, K568, K598, K301, K303, K750, *Escherichia coli* (Migula) Castellani and Chalmers (HB101) and *Pseudomonas* strains K110, K321 (Appendix C)], were stored either on slants at 4°C or at -80°C in 10% (vol/vol) glycerol. Laboratory strains were supplied by A. Kerr, Department of Plant Pathology, Waite Agricultural Research Institute. Unless otherwise mentioned, cultures were grown at 25°C in the dark on a rotary shaker or in petri dishes also at 25°C in the dark. General procedures for handling bacteria as outlined in Stolp and Starr (1981), were adopted in the course of this study.

Tests to characterise the bacteria were;

1) Gram staining; *Micrococcus lutea* (Schroeter) Cohn and *E. coli* being the positive and negative Gram stain control bacteria, respectively (Bartholomew and Mittwer, 1952; Lamanna and Mallette, 1954; Libenson and McIlroy, 1955; Salton, 1963),

2) 3-ketoglycoside test for *Agrobacterium*; bacteria were streak diluted on KL plates (Appendix A) and flooded with Benedict's reagent. Positive colonies form a yellow ring of Cu_2O around the colonies. Strain K301 was used as a positive control (Bernaerts and DeLey, 1963),

3) fluorescent pigment production on King's B agar; bright green or yellowish green fluorescence (Pyoverdin production) seen under 254nm UV light (King *et al.*, 1954),

4) aerobic/anaerobic growth observed in Hugh and Leifson medium (1953); by covering the surface of one duplicate tube with mineral oil to exclude air,

5) observation of spore forming properties; in wet preparations under a phase contrast microscope,

6) growth on D-1 agar for *Pseudomonas* (Kado and Heskett, 1970),

7) oxidase test (Kovác, 1956),

8) catalase test; where hydrogen peroxide is destroyed by catalases or peroxidases (Cowan, 1974; Vera and Power, 1980),

9) growth in oxidation-fermentation medium (Hugh and Leifson, 1953) and,

10) phase-contrast microscopic examination of motility.

Test strips for the oxidase test and the oxidation-fermentation medium was purchased from a local supplier (Medvet Science Ltd., Adelaide).

Bacteria isolated from proteoid roots were also assayed for their ability to catabolise tyramine. Laboratory strains of *A. tumefaciens* K301 and K303 were included as controls (Appendix B). A crude extract from proteoid roots, containing a compound identified as tyramine, was dried under a gentle stream of nitrogen (Chapter 4), redissolved in Bergersen's salt (1961) medium (Appendix A) and filter sterilized. Bacteria were assayed after incubating a 1/10 vol bacterial suspension (total vol = 0.1ml) in the Bergersen's salt medium plus the resuspended extract from proteoid roots (in a sterile 1.5ml Eppendorf tube) at 25^{o}C on a rotary shaker. The presence of ninhydrin positive components (which include tyramine) was monitored at 0, 24, 48 and 72 hrs by high voltage paper electrophoresis (pH 1.7). Details of high voltage paper electrophoresis and ninhydrin staining are given in Chapter 4.

Dot blot analysis of bacterial isolates from proteoid roots was performed to establish whether or not there were cross-hybridising sequences between their DNA and the DNA of genes for IAA synthesis and/or *vir* region genes of *Agrobacterium*. Bacterial cultures were grown overnight in 5ml of liquid LB broth (Appendix A) at 26^{o}C on a rotary shaker. The resulting populations were sonicated to lyse cells and centrifuged for 2min at 5,000rpm (rotor JA10, Beckman J2-21M). An equal volume of 0.5M NaOH was added to each of the supernatants, which

were left for 30min. These were dotted (using the 'dot blotting' technique of Kafatos *et al* (1979) and a Schleicher and Schuell 'minifold' dot blot apparatus) onto a membrane (Hybond N+) whilst applying suction to the under-side of the membrane and using additional NaOH to wash the wells. The NaOH was neutralised by placing the membrane on a glass plate and gently flooding the membrane with a solution of 0.5M Tris-HCl (pH7.0) and 1.5M NaCl. The membrane was air-dried, UV cross linked for 5min and then sealed in a plastic bag for the prehybridisation reaction. The prehybridisation and subsequent reactions are described in the section entitled 'Southern hybridisation' in Chapter 5. DNA probes for IAA synthesis genes are also described in Chapter 5.

Plant culture

This section covers the requirements for: a) the production of sterile plant material and b) the production of proteoid roots for the various biochemical tests described in Chapters 4 and 5. Chapter 3 deals separately with the sterile culture of *Macadamia.*

Seed germination

Initial work with seed of *B. grandis* gave erratic germination rates, therefore an experiment was conducted to ascertain the optimum temperature for germination. Germination was considered to have taken place if the radicle emerged from the seed coat. *B. grandis* and *B. coccinea* R.Br. seeds (supplied by Nindethana Seed Service, Western Australia) were surface sterilized by swirling for 2min in 70% ethanol. The alcohol was then discarded and replaced with 10% Ca-hypochlorite with a drop of surfactant [Tween 20® (polyoxyethylene sorbitan monolaurate)]. After 45min the sterilant was poured off and the seeds were rinsed 3 times in sterile double distilled water. Seeds that floated were discarded. Seeds were transferred (remnants of the terminal wings pointing upward) to 10ml poly-carbonate centrifuge tubes containing 4ml of a low salt nutrient agar [W.P.M./0.7% agar or half strength M.S.salts/0.7% agar (Appendix A)]. Seeds were pushed into the agar to approximately 3/4 their length. They were incubated at 4, 10, 15, 20, 25 and 30°C in the dark and examined after 8 weeks. For experiments requiring bacteria-free plants, germinated

seedlings were transferred to petri dishes with nutrient agar (NB or LB, Appendix A) for secondary screening. Contaminated cultures were discarded. A number of seedlings, selected at random, were ground with a motor and pestle and the slurry plated onto LB medium to investigate the occurrence of internal bacterial and fungal contaminants in the plant tissues. The incubation conditions for the plates were 25°C and darkness.

The origins of proteoid roots used in this study were diverse. Proteoid roots were collected from *B. ornata* F. Muell. ex Linn. growing in a natural woodland in Kuipo Forest, South Australia and were used to extract DNA (Chapter 5). *Grevillea hookeriana* Meissner and *B. prionotes* Lindley grown in containers were supplied by the Woods and Forests Department of South Australia. Leaves, proteoid and non-proteoid roots were sampled for HPLC analysis (Chapter 4). *B. coccinnea*, cultured in sterile agar (0.7%) or sand (Figure 2.1) (using the nutrient salts of Dell *et al.*, 1980, Appendix A), were grown in a growth cabinet. Plants were confined in 500ml poly-carbonate containers. Incident light measured at plant level (but outside the clear poly-carbonate containment vessels) was 35-50μmol m^{-2} s^{-1}, the day/night time temperatures were 25 and 18°C, respectively and the day length was 14 hr. Leaves, proteoid and non-proteoid roots were sampled for HPLC analysis (Chapter 4).

B. grandis plants were grown with their roots immersed in a non-sterile, aerated hydroponic solution (Dell *et al.*, 1980) which was replaced at monthly intervals. They were grown in 20 litre plastic containers (see Figures 2.2 and 2.3) with the nutrient solution aerated by means of a fish aquarium air pump (Rena Model number 301R). The top was covered by a black plastic lid in which 0.5cm holes had been drilled to accommodate the plant stems. Seedlings often needed to be replaced during their initial establishment because some plants became infected with bacteria which caused their death. This hydroponic culture device was placed in a growth-cabinet with a 14hr day length, light with an intensity of 300μmol m^{-2} s^{-1} (provided by mercury vapour lamps) and 15/25°C day/night temperature regime. Leaves, proteoid roots, non-proteoid roots and the nutrient solution were sampled for HPLC, HVPE, GC/MS and NMR analysis (Chapter 4). Proteoid roots were also used for DNA extraction (Chapter 5).

Figure 2.1. *Banksia coccinea* growing in sterile nutrient sand culture (Dell *et al.*, 1980).

Fig.2.2.

Fig.2.3.

Figure 2.2 and 2.3. *Banksia grandis* growing in hydroponic nutrient culture medium (Dell *et al.*,1980), showing exposed proteoid roots (Fig. 2.2) and the air pump (in top right hand corner of the device) used for the aeration of the nutrient solution (Fig. 2.3).

Figure 2.4. *Banksia grandis* growing in the aerated hydroponic culture medium of Dell *et al* (1980).

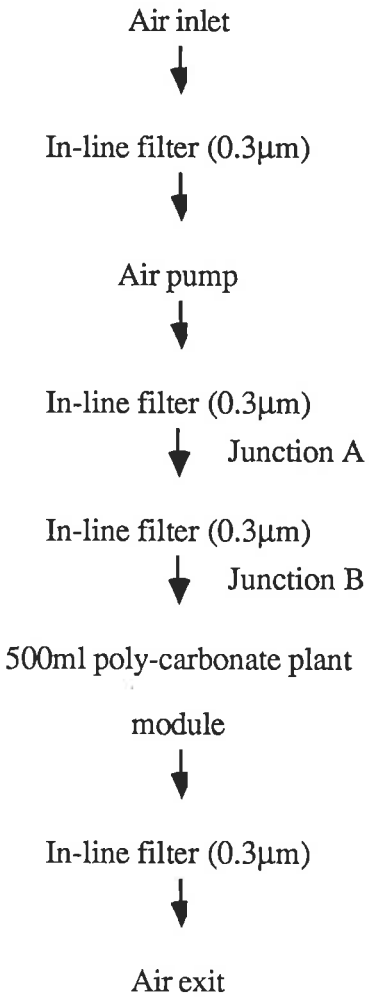


Figure 2.5. Schematic diagram of a device used to grow *Banksia grandis*. Plants were cultured hydroponically by means of filtered air passing through a nutrient solution (Dell *et al.*, 1980) in which the plant roots were immersed. The 500ml poly-carbonate modules isolated plants from unwanted microorganisms. The arrows indicate connecting tubing (see text). At junctions A and B there were 3-way and 4-way connectors which divided the air flow through tubes to each of the 500ml poly-carbonate modules.

B. grandis was also grown in aseptic hydroponic culture. This involved the development of complicated devices which enabled individual plants to grow in totally enclosed conditions in a sterile environment (see Figures 2.4 and 2.5). Two devices were constructed. Each comprised 32 modules; each module was a containment vessel for one plant. The essential features of each module were as follows; a 500ml poly-carbonate container with 200ml of nutrient solution (Dell *et*

al., 1980). The container housed either a paper bridge made of Whatman No 1 filter paper or a 0.3mm thick black plastic disk to support *B. grandis* leaves above the nutrient solution. A 0.3cm hole was made in the centre of the plastic disc to accommodate a plant. The plastic disc was supported by an 'air stone' (used in each module) which was immersed in the solution. The air stone, (obtained from an aquarium shop) was designed for aerating fish tanks. The air was delivered and exhausted by means of tubing (Silastic Brand Medical Grade by Dow Corning No 601-325 and Nalgene No 8000-0030) entering and leaving the vessel by means of holes drilled near the top of the vessels. The holes were made slightly smaller than the outside diameter of the tubes to make an air tight fit. The exhaust tube (~15cm Silastic Brand Medical Grade 2.64mm ID X 3.28mm OD) was either packed with non-absorbant cotton wool and tied into a double loop with the opening pointing down ward or consisted of a 0.3µm in-line filter (see Figure 2.4). The air delivery sequence was constructed as follows. Starting with the air stone the tubing (Silastic Brand Medical Grade 2.64mm ID X 3.28mm OD) left the container and joined a 0.3µm in-line glass micro fibre filter device (Whatman, Hepa-Vent™ No 6723-5000). This was connected to the delivery tubes of 3 other devices with Nalgene tubing (4.76mm ID X 7.93mm OD) and was joined with polypropylene 3-way and 4-way connectors. A further in-line filter device of the type previously mentioned was inserted upstream of the 3-way junction as an extra precaution. The tubing was connected to a central point where air pumps (Rena 301R) were used to delivered the air. The pumps were also protected from microbial contamination with a 0.3µm in-line filter device (Whatman, Vacu-Guard™ No 6722-5000). The device (with the exception of the pumps) was autoclaved at 121°C for 20min and the plants (*B. grandis*) were placed into the containers while in a laminar flow hood. The device was then located in a growth room, the conditions of which are described above for the non-sterile hydroponic cultures of *B. grandis*. Cultures were examined for bacterial contamination by removing individual containers upstream of their filter device (and inserting the exposed tube ends in 10% Ca-hypochlorite until they were reconnected) and swabbing the outside of the containers intermittently for 30min (whilst in a laminar flow hood) with 10% Ca-hypochlorite. The containers were then opened and a sample (1ml) of solution was removed and incubated on LB agar (25°C in the dark) and checked for contamination after 10 days. The tissues

and nutrient solution of sterile liquid cultures of *B. grandis* were sampled for HPLC analysis (Chapter 4).

Inoculation experiments

Aseptic plants were inoculated with bacteria isolated from proteoid roots or with various strains of *Agrobacterium*. There were 2 replicates per treatment. Inoculation was made by two methods: 1) the inclusion of 10ml of individual bacterial cultures into the nutrient medium. Cells were grown to late log phase (2 days) at 28°C until there were ca. 10^9 cells/ml, or 2) plants were individually inoculated 5 times with 0.05ml bacterial suspension (ca. 10^8 cells made up in sterile double distiled water) through wounding with a hypodermic needle. Inoculations were made in the laminar flow hood. Plants were examined at weekly intervals for 10 months for the presence of proteoid roots or abnormal growth.

Carrots were inoculated by the method of Ryder *et al* (1985) on the apical surfaces of discs. Three *A. rhizogenes* strains (K598, K301 and K303) (Appendix B) and 15 bacterial isolates from proteoid roots were used. Inoculations were made with bacterial suspensions of approximately $2x10^9$ cells/ml. The discs were placed on water agar (0.6%), sealed in petri dishes with waxed tape (Parafilm™) and incubated at 25°C in the dark. Hairy roots produced as a result of infection by *A. rhizogenes* strain K598 were maintained on Monnier's (1976) salts with Morel and Whetmore's (1951) vitamins (Appendix A) without hormones.

Results

Bacterial characterisation

The abundance of bacteria varied between 10^9 to 10^{12} cells per gram dry root weight of hydroponically grown proteoid roots of *B. grandis* (5 replicates). Fifteen distinct bacterial isolates were obtained. All bacteria were rod shaped and motile. Eleven were Gram negative and 4 Gram positive.

All 4 Gram positive bacteria were catatase positive, oxidase positive and spore forming. Using the diagnostic keys of Skerman (1967) and Kado (1986) these data suggests that the 4 Gram positive isolates are *Bacillus* Chon species .

The Gram negative bacteria did not produce 3-ketolactose, indicating that they are not *Agrobacterium* species. They were Kovac's oxidase positive, not fermentative in Hugh and Leifson's medium (glucose) and grew on D4 medium. After incubation on King's B medium the bacteria produced a yellowish-green fluorescence when observed under UV light at 254nm. These data are consistent with the characteristics of *Pseudomonas* species (Skerman, 1969; Kado, 1986). The ability of the isolates to catabolise tryamine is reported in Chapter 4.

DNA sequences containing genes encoding enzymes of IAA synthesis from *Agrobacterium* cross hybridised to DNA of 7 proteoid root bacteria (all exhibiting the characteristics of *Pseudomonas*) and the *Agrobacterium* (K301, K565, K598, K303, K750) and *Pseudomonas* (K110, K321) controls (Figures 2.6). *E. coli* (HB101) was used as a negative control and did not produce a positive blot. The DNA from the *vir* region of *Agrobacterium* did not hybridise with DNA from the proteoid root bacteria (Figure 2.7).

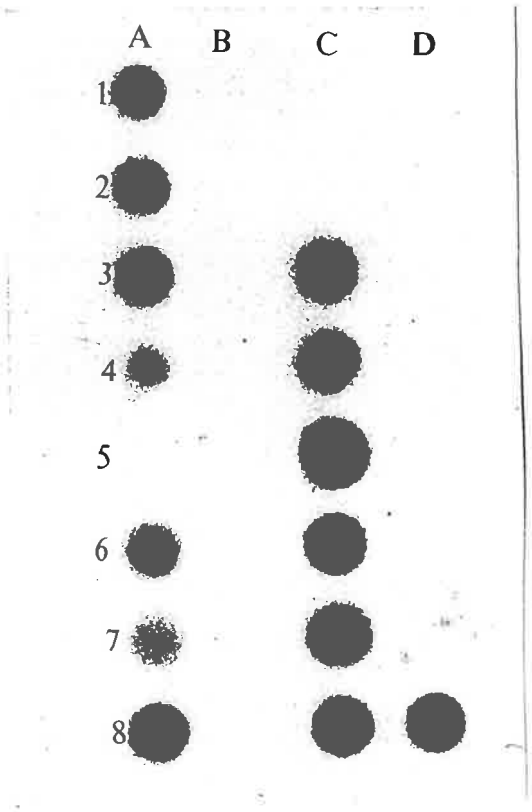


Figure 2.6. Dot blots of soil bacteria. The probe (132-10) contained a 7.6kb BamH1 fragment of TiC58 (from *A. tumefaciens*) cloned into pBR322 which encompassed the loci encoding most of the genes tryptophan mono-oxygenase and indole-3-acetamide hydrolase. Wells A1-A8 and B1-B7 contain DNA from bacteria isolated from the rhizosphere of proteoid roots (positive dots correspond to bacteria with characteristics of *Pseudomonas*); B8, C1 and C2 were blanks with no added DNA; C3, *Agrobacterium* (K565); C4, *Agrobacterium* (K598); C5, *Agrobacterium* (K301); C6, *Agrobacterium* (K303); C7, *Agrobacterium* (K750); C8, *Pseudomonas* (K321); D1-D7, blanks with no added DNA; D8, *Pseudomonas* (K110).

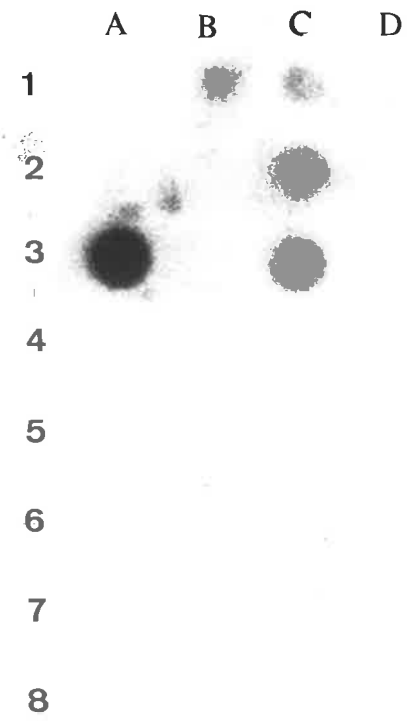


Figure 2.7. Dot blots of soil bacteria. The probe (PGV0361) was from the *vir* region of *Agrobacterium* TiC58 plasmid and is a Hind111 fragment cloned into pBR322. Positive spots correspond to *Agrobacterium* strains K301 (A3); K565 (B1); K303 (C1); K598 (C2) and K750 (C4). DNA from *Pseudomonas* controls (K110 and K321) (A1 and A2) and 15 bacteria isolated from the rhizosphere of proteoid roots (A4-A8, B2-B8 and C4-C6) did not hybridise with DNA of the probe. C7-C8 and D1-D8 were blanks with no added DNA.

Seed germination

Table 2.1 shows the optimum temperature for the germination of *B. grandis* to be in the vicinity of 15°C. The experiment used seed that had been surface sterilised and an effect of the sterilisation treatment was observed. Table 2.1 also shows that the sterilisation treatment was effective with 16.7% (or less) of seeds infected with bacteria. The percentages of seed infected with bacteria at different temperatures reflect the interaction between temperature and growth of contaminating bacteria. In two other experiments, 'sterile' cultures were subjected to a secondary screening for bacterial contamination on LB or NB agar. Two percent were found to harbour bacteria. Seeds selected at random (n=20), ground in a mortar and plated on LB or NB media showed no contamination.

Table 2.1. The relationship between temperature and seed germination of *B. grandis* and the effect of surface sterilisation of seed on bacterial contamination (unsterilised control seeds showed 100% contamination at every temperature). Means and standard errors of means of 4 replicate batches of 60 seeds except for treatment temperatures of 4°C and 30°C where there was only 1 batch of 60 seeds each.

Temperature °C	Percentage germination		Percentage infected with bacteria
4	0		1.7
10	23	±2.4	5.0
15	85	±3.6	10.0
20	51	±5.2	16.7
25	27	±3.6	13.3
30	11		15.0

Root inoculation of *Banksia*

Bacteria isolated from proteoid roots were used to inoculate the roots of *B. grandis* in an attempt to initiate proteoid root formation and thereby fulfil a part of Koch's postulates with respect to the question: is a bacterium responsible for proteoid root development? Strains of *Agrobacterium* were also inoculated into the cultures of *B. grandis* as control organisms to ascertain if they induced proteoid roots or other abnormalities on the roots.

Growth rates of *B. grandis* plants growing in sterile enclosed plant-modules were slower than those growing in non-sterile culture and not contained within a plant-module (see Materials and Methods this Chapter). Proteoid roots did not form in sterile culture.

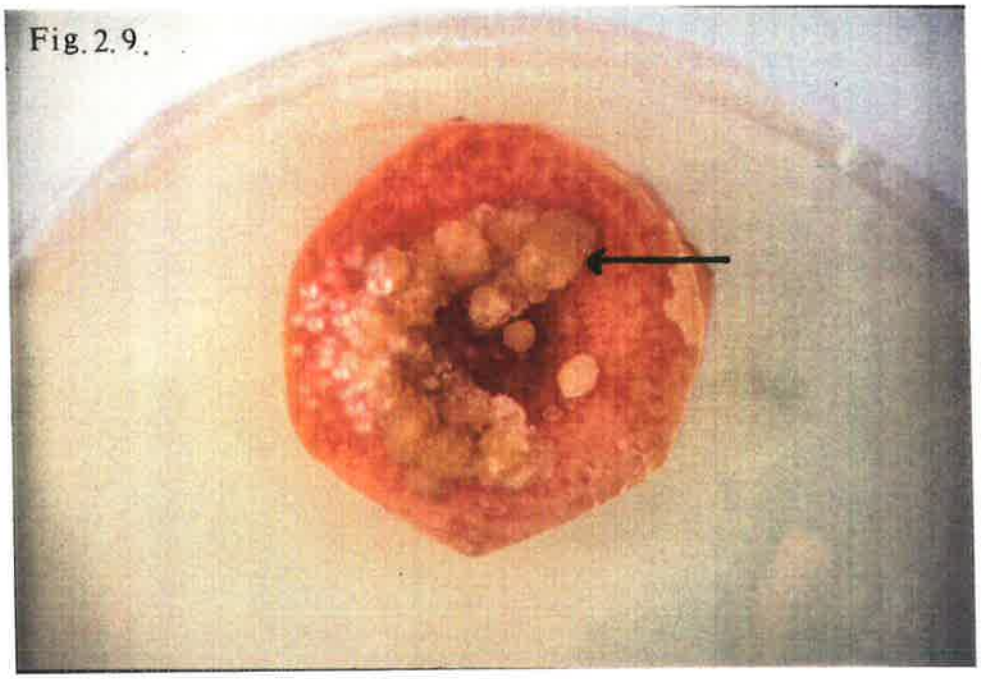
The inoculation of aseptic cultures of *B. grandis* with bacteria isolated from proteoid roots did not result in proteoid root formation. The bacteria were inoculated as a mixed population or as individual isolates. Observations of plants were made for 10 months (or until plants died). Inclusion of individual bacteria or mixed populations of bacteria often resulted in the death of the plants.

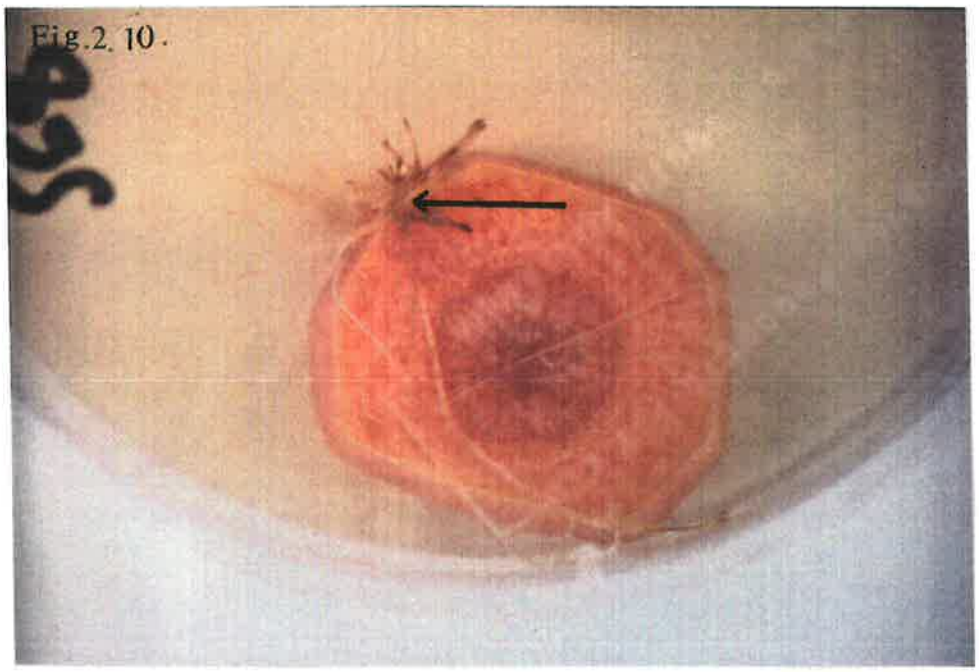
Inoculation of *B. grandis* with *Agrobacterium* strain K301 resulted in a gall forming on one plant (see Figure 2.8). The gall was assayed for opines using the methods described in Chapter 4. However, there was insufficient material for a thorough analysis.

Figure 2.8. Gall formed on the roots of *Banksia grandis* (arrowed) after inoculation with *Agrobacterium* strain K301. The plant on the left is an uninoculated control plant.

Carrot disc inoculation

Carrot discs were inoculated with the bacteria isolated from proteoid roots to see if abnormal growth would be induced in a simple test system. The reaction of the tissues was similar to those of *B. grandis*, that is, the plants died as a result of bacterial invasion of their tissues. The carrot discs decomposed completely. Inoculation of the carrot discs with *Agrobacterium* (strains K598, K301 and K303) did, however, produce galls and hairy roots (Figures 2.9 and 2.10). Integration of bacterial DNA into the carrot tissues was confirmed by Southern Blot analysis (see Chapter 5) with a probe covering the genes encoding tryptophan mono-oxygenase, and indole-3-acetamide hydrolase.





Figures 2.9 and 2.10. Crown gall and hairy roots (arrowed) forming on carrot disks after inoculation with *A. tumefaciens* (Crown gall strain K301) (Fig. 2.9) and *A. rhizogenes* (Hairy root strain K598) (Fig. 2.10).

Discussion

This chapter has covered many aspects which are purely methodological and of those only the germination of *Banksia* and aspects of the Koch's postulates experiment will be included in the discussion. The findings of the dot blot analysis of rhizosphere bacteria will also be discussed.

The experiment to examine the effects of temperature on seed germination was useful in establishing methodology for growing *B. grandis*. Optimum germination temperatures around 15°C are not surprising because these temperatures are normal in southern Western Australia during spring, when there is suitable soil moisture for *Banksia* to germinate in the natural environment. Indeed with most seed, the temperatures most effective for their germination are similar to those in their natural environments (Hartman and Kester, 1983). The viability of the batch of seed was 85%. This figure could be expected to vary from one seed batch to the next depending on, for example, environmental conditions at seed formation and the length of time the seed has been stored.

The same experiment examined bacterial contamination after surface sterilisation. The contamination rates of seed after surface sterilisation was found to be lower at temperatures of 4 and 10°C. This is probably a reflection of the incubation temperatures required for bacterial growth and it highlights a problem in determining whether a given plant can ever be said to be free of microbial contamination. It is probable for example, that those bacteria growing at 4°C do not grow well at higher temperatures which makes the problem of isolation of bacteria very difficult. This means that without destructive tests of seedlings and testing a large range of environmental conditions (for example, temperature and media), a plant or plant culture cannot be said to be sterile with absolute certainty. This, however has not been a problem in this study because the 'sterile' *Banksia* cultures did not produce proteoid roots and there is no indication that those cultures were anything other that sterile.

The isolation of bacteria from proteoid roots was not exhaustive, nor was it intended to be. As the size of the experiment needed to fulfil Koch's postulates was already large with 15 bacterial isolates (treatments duplicated), certain assumptions needed to be made. The qualitative composition of bacterial populations could be expected to differ in the rhizosphere due to the

selective stimulation or inhibition of specific organisms and changed conditions of competition for both carbon substrate and mineral nutrients. However, it is a fundamental assumption that the bacteria/bacterium responsible for causing proteoid root formation would comprise a large proportion of the bacteria in the rhizosphere of proteoid roots, and it/they can be isolated on a wide spectrum bacterial culture medium. Therefore the fact that none of the bacteria isolated from proteoid roots induced changes in root morphology can be interpreted either as an indication that those microbes responsible for proteoid root production were not isolated from proteoid roots in this investigation or as a failure of the experimental system to test adequately the proteoid root phenomenon. The culture system could be improved by the addition of inlet and exit ports in each culture vessel designed to enable easy aseptic transfer of nutrient solutions. This would be expensive in terms of cost of the equiptment. It should be stressed that by using the approach just outlined, the task of fulfilling Koch's postulates is very large in terms of time, space, equipment and labour.

Carrot discs were inoculated with bacteria isolated from proteoid roots to discover whether they could be substituted for *Banksia* as an experimental plants. No abnormal growth was observed. The dot blot analysis did not reveal the presence of *vir* genes of *Agrobacterium* in DNA of bacteria isolated from proteoid roots nor were *Agrobacterium* strains isolated from the rhizosphere. This suggests that *Agrobacterium* is not involved in the formation of proteoid roots.

Genes responsible for IAA synthesis were detected in just over 46% of those rhizosphere bacteria assayed. A peculiarity of the hormone IAA is it can cause different effects in different plants, or even different effects in the same species at different times. Auxin is characteristically synthesised in plants at the stem apex and in young tissues (Bidwell, 1979) and moves mainly down the stem. It thus tends to form a concentration gradient from the shoot tip to the root. Its activities include both stimulation and inhibition of growth and the same cell or structure may exhibit opposite responses, depending on the concentration of IAA. Furthermore, different tissues respond to very large differences in concentration - roots are stimulated by concentrations several orders of magnitude below those that stimulate shoots (Bidwell, 1979). The fact that IAA may only be required in low concentrations to affect root development allows speculation about whether bacteria in the rhizosphere of proteaceous plants can by the action of IAA stimulate proteoid root

formation. If IAA in the rhizosphere induces proteoid root formation, the problem then becomes one of tryptophan availability in the soil around the roots and whether sufficient tryptophan can be converted to cause an effect on the growth of the plant. Whilst IAA has significant effects on root growth in laboratory situations, the substrate/product pathways in the soil are difficult to test. IAA synthesis genes are found within the population of rhizosphere bacteria and this may indicate an influence of non-invasive bacteria on proteoid root production due to their hormonal effect.

Chapter 3

Tissue culture of *Macadamia*

Introduction

The study of proteoid roots on an intact plant *in vivo* does not readily allow the identification of physiological factors which affect their development. Proteoid roots cultured *in vitro*, on the other hand, provide a simplified system, unaffected by many factors which could otherwise affect the outcome of experiments. For this reason procedures in the *in vitro* culture of *Macadamia* (*Macadamia integrifolia* Maiden and Betche, *M. tetraphylla* L.Johnson and hybrids) and *Banksia* species have been developed. This chapter outlines the development of procedures for *in vitro* propagation of *Macadamia,* a plant which under normal circumstances has proteoid roots.

Rationale for selecting *Macadamia* as an experimental plant for the study of proteoid roots

Macadamia cultivars were chosen as experimental plants because the development of plant cloning techniques could be useful for the Australian *Macadamia* nut industry. The industry claims that the macadamia nut is one of the best edible nuts in the world which yields high amounts of oil (Trochoulias *et al.*, 1980).

Macadamia is indigenous to coastal rainforests of subtropical regions of Australia east of the Great Dividing Range. Since the European settlement of Australia, coastal rainforests have been steadily cleared for their timber and for farming purposes. Potentially valuable genetic material for development of *Macadamia* as a crop species, has been lost or is at risk. This germplasm needs to be preserved either by extensive collecting from wild populations and/or by preservation of surviving rainforest communities (Sedgley, 1983). Tissue culture provides one method of germplasm rescue from wild communities not previously achieved. The germplasm could be stored or outplanted into orchards.

Very little is known about the genetics of *M. integrifolia* and *M. tetraphylla* since they are both outcrossing species which have a long generation time (at least six years). This makes asexual

reproduction a useful horticultural alternative to seedling plants for propagation of *Macadamia*. Tissue culture can potentially produce plants genetically identical to the parent material. The most significant advantage offered to industry by aseptic methods of clonal propagation (popularly called micropropagation) over conventional vegetative methods, is that in a relatively short space of time large numbers of plants can be produced from a single individual. In this investigation the aims were to develop methodologies for the production of sterile plantlets which would not only fulfill a requirement in the *Macadamia* nut industry for clonal material but also produce a sterile test plant for the study of the proteoid root phenonenon.

Plant Micropagation

The various procedures employed to produce plants by *in vitro* techniques have been frequently reviewed (de Fossard, 1981; Hussey, 1983; Bonga and Durzan, 1982; Krikorian, 1982; Bhojwan and Razdan, 1983; Hu and Wang, 1983; George and Sherrington, 1984; Tisserat, 1985; Styer and Chin, 1983). The method of choice for woody plants which maintains clonal integrity is 'axillary shoot proliferation', where shoots develop from pre-existing vegetative meristems.

Procedures used for plant propagation by tissue culture involve three major stages: the establishment of an aseptic culture, the multiplication of propagules (e.g. shoots) and the induction of roots. The objectives of each stage are different. However, there are several factors in common that need to be investigated at each stage. These include: the chemical formulation of the nutrient media (inorganic salts, organic substances and growth regulators), physical properties of the media (i.e. agar concentration) and culture requirements such as temperature and light.

There are no reported instances where *Macadamia* has been successfully cultured *in vitro* for purposes of micropropagation, although Schroeder (1961) was able to induce callus growth from *Macadamia* fruit tissue, cultured on Nitsch (1951) medium. Relatively little research work has been done on micropropagation of other proteaceous species. Complete methods for *in vitro* propagation have been devised for some *Grevillea* R.Br. sp. (Gorst *et al.*, 1978; Ben-Jaacov and Dax, 1981). These are the only proteaceous plants reported to have been commercially propagated (George and Sherrington, 1984). Other research work in tissue culture of proteaceaous plants

includes *Protea* R.Br. sp. (Van Staden *et al.*, 1981; Ben-Jaacov, 1986), *Leucospermum* R.Br. sp. (Van Staden and Bornman, 1976; Ben-Jaacov, 1986), *Serruria florida* R.Br. (Ben-Jaacov, 1986) and *Telopea speciosissima* R.Br. (Seelye *et al.*, 1986). *T. speciosissima* was the only plant of the above mentioned where rooting and successful outplanting was achieved.

Two of the principal considerations involved in the handling of stock plants are first reducing the potential for contamination by microorganisms and second the physiological condition of the stock plant when tissue is being harvested (Hartman and Kester, 1983). The time the plant material is collected from the field has an effect on the number of explants established *in vitro* and the number of cultures which are contaminated with bacteria after sterilisation procedures (Hutchinson, 1984). Explants taken from the apices of a stem are at a younger stage of development than explants taken from the base. A younger stage has often been found to give higher percentages of shoot regeneration than older plant material (Hu and Wang, 1983). This has been demonstrated in *Dianthus* Linn. (Roest and Bokelmann, 1981) where the percentages of shoot regeneration from nodal segment culture were 88.6 and 69.8% for the top and the base of the plant respectively. With *Chrysanthemum* [Tourn.] Linn. Hollings and Stone (1968) ascertained that the success rate (measured as shoot proliferation) of explants obtained from terminal buds was 32%, whereas from lateral buds it was 18%. Hasegawa (1979) observed that a higher percentage of shoot tip explants of *Rosa* L. developed multiple shoots from lateral buds.

White's medium (1943) was the most widely used medium during the early days of tissue culture. Many improvements have been made since then, the most noticeable of which are increased N, P and K levels, reduction of the Ca level and prevention of iron precipitation at high pH (Hu and Wang, 1983). There is no general purpose medium yet available for plant tissue cultures. The salt concentration and other growth factor requirements of the medium vary between species and within species from one developmental stage to the next (Hu and Wang, 1983). The question of how to select a medium to optimise the growth of *Macadamia in vitro* is, given normal practical restraints (industry resources such as space, explants, laboratory equipment and time), difficult to answer. There are several media (for example, Morel and Whetmore, 1951; Murashige and Skoog, 1962; Blades, 1966; Nitsch and Nitsch, 1967; Gamborg *et al.*, 1968 and Hildebrandt, 1962) which have become well known for tissue culture of plants. Numerous modifications of these

media have also been reported. One approach to select a medium for *Macadamia* would be to try media reported in the literature that have proved successful for other proteaceous or woody plant species. This 'hit or miss' approach may allow a successful medium to be selected. However, a more systematic system of media selection is desirable. The concentration of salts and organic components were the first factors investigated in this study. The salt mixture of Murashige and Skoog's (1962) medium (Appendix A) has been satisfactory for many crop plants (Bhojawani and Razdan, 1983). It is often possible to use the same medium (MS medium) for culture initiation and multiplication of shoots. A basal medium containing inorganic salts of MS medium plus 170mg/l $NaH_2PO_4.H_2O$, 80mg/l adenine sulphate dihydrate, 0.4mg/l thiamine HCl, 100mg/l inositol and 30g/l sucrose has been successfully used by Murashige and his co-workers (Murashige, 1977) for the micropropagation of a large number of plant species. For some plants, however, the level of salts in the MS medium is either toxic or unnecessarily high (Anderson, 1975). Blueberry shoots, for example, grow extremely well in a medium with MS salts reduced to quarter strength (Bhojwani and Razdan, 1983). Higher levels were toxic. Leaf explants died even on half strength salts. It is, therefore, important to test media with reduced levels of salts for species which do not survive well on high salt formulations.

The concentration of agar, used as a physical support for *Macadamia* explants was assessed in this study. It is recognised that with a plantlet growing on solidified media, nutrient concentration gradients will be generated; plantlets growing in liquid medium permit better definition of the nutrient environment (Teasdale, 1986).

The general concept propounded by Skoog and Miller (1957) that organ differentiation in plants is regulated by an interplay of two groups of hormones, auxins and cytokinins, is universally applicable. A higher cytokinin-to-auxin ratio promotes shoot formation and a higher auxin-to-cytokinin ratio favours root formation. The exogenous requirements for hormones depend on their endogenous levels in the plant system and this varies with the tissues, plant type and phase of growth (Bhojwani and Razdan, 1983). Therefore, to initiate organ differentiation the inclusion in the culture medium of only one or other hormone may be necessary. The use of the cytokinin 6-benzylaminoadenine (BAP) to stimulate bud emergence *in vitro* was suggested by the work of Boswell *et al.* (1981) where it was applied to intact *Macadamia* trees and shown to stimulate the

outgrowth of auxiliary buds. Furthermore, BAP is known to be effective in promoting axillary shoot proliferation *in vitro* in, for example *T. speciosissima* (Seelye *et al.*, 1986) and *G.* cv. 'Crosbie Morrison' (Gorst *et al.*, 1979).

The ability to initiate roots on explants, especially in the context of this investigation, is important. Tree, shrub, and vegetable species are commonly planted as bare rooted plants. Initial establishment of the transplant is dependent on its ability to produced roots and ultimately acquire and meet the water needs of the plant. Inoculation of bare almond (*Prunus amydagalus* Butsch) and olive (*Olea europea* L.) roots with *A. rhizogenes* led to significant increases in leaf number, stem diameter, and shoot elongation during the first growing season after treatment (Strobel and Nachmias, 1985). Both number and weight of new roots were increased. Presumably, this could be attributed to the genetic transformation of the roots via the Ri plasmid of *A. rhizogenes* (Chilton *et al.*, 1982). Since inoculation with *A. rhizogenes* may have some potential benefit to the horticultural industry by virtue of its ability to promote root formation on plants, it was desirable to test the infectivity of *Agrobacterium* on cultivars of *Macadamia*. This could open the way for genetic engineering of *Macadamia* through gene transfer with the T-DNA of *Agrobacterium* plasmids as well as stimulating root production of tissue-cultured shoots.

Experimental approach

This chapter relates to practical considerations involved with the collection of stock plant tissues and their eventual establishment *in vitro*. The requirement was to establish methodology whereby plant cultures can be induced to proliferate and to grow roots *in vitro*. To meet this requirement a number of aspects related to tissue culture methodology have been investigated, which include:

1) the decontamination of explants by surface sterilisation.

2) the effect of the time of the year on explant establishment *in vitro* and bacterial contamination.

3) the optimum developmental stage of explants for establishment *in vitro*.

4) the explant type (apical or nodal buds) and their orientation on the medium.

5) the concentration of salt and organic components in the tissue culture medium.

6) the concentration of agar that will allow prolific growth.

7) the cytokinin concentration required for shoot proliferation.

8) root initiation by conventional means (by use of auxins, different light regimes and by out planting into glasshouse).

9) root initiation by inoculation with *Agrobacterium.*

Materials and methods

Plant material

There were two sources of stock plants; a) glasshouse-grown seedlings of *M. tetraphylla* (the seeds were kindly supplied by Dr R.A.Stephenson at the Maroochydore Research Station, Department of Primary Industries, Queensland) and b) field grown plants from the Claremont orchard, Waite Agricultural Research Institute. Plants from the Claremont orchard included the Australian *Macadamia* cultivars 'Schimke', 'Renown', 'Eggshell', 'Hinde', an Hawaiian cultivar '508' (Kakea) and three *M. tetraphylla* seedlings. There are important advantages in using glasshouse grown material because the plants are less contaminated with microorganisms and therefore easier to disinfect. However, as previously mentioned, it was important to use the selected material because of the potential nut yield advantage to the horticultural industry. There was the possibility of using glasshouse-grown grafted seedlings but not only does this method take considerable time to produce large quantities of explant material but also requires large amounts of glasshouse space. As this study progressed limited explant material was made available from young field grown plants from the Claremont orchard and so the use of glasshouse grown seedlings was discontinued.

Explants

Explants consisted of either apical buds or shoots or axillary buds arising from nodes.The leaves were cut off approximately 5mm from the bud or shoot and trimmed with 10mm of stem

attached on either side (Figure 3.1). Explants from field grown *Macadamia* were taken from the tops of plants or the slower growing side branches. The plants were approximately 2 metres tall and 4 years old. Explants from glasshouse grown seedlings of *M. tetraphylla* were taken from the tops of the plants. The plants were approximately 20cm tall.

Explant collection

Explants were harvested over a whole year in order to identify the optimum season for collection of stock material for micropropagation and also to enable enough propagated material to be gathered and established in culture for other ongoing experiments.

Culture conditions

Polycarbonate culture tubes were used exclusively. These were 105x42mm and contained 40ml of medium except when liquid medium was used. For liquid medium, the lower third of explants was covered. Cultures were incubated at 25°C with a 16h photoperiod or in the dark. The light source was cool-white fluorescent tubes providing an incident light of 35-50μ mol m^{-2} s^{-1} measured at a level corresponding with the top of the culture vessels. Plants were subcultured at 4 week intervals.

A B C

Figure 3.1. Shoots from a field grown*Macadamia* cv. 'Shimke'. A, shoot from field; B, with leaves trimmed off; and C, cut into segments prior to sterilisation.

Surface sterilisation

Explants were immersed in 70% ethanol containing the emulsifier Tween 20® (0.01%) for 1min. Tween 20 was added to improve the surface coverage. This was followed by immersion in freshly prepared and filtered 10% calcium hypochlorite (w/v) (also containing Tween 20). The solution (plus explants) was stirred for periods of 10, 20, 30 or 40min under partial vacuum to determine the optimum time for sterilisation. The explants were then rinsed 3 times with sterile distilled water to remove any residual disinfectant. All subsequent manipulations were carried out in a laminar flow cabinet. Fungal and bacterial contamination in plant cultures was usually detected within 14 days of culture initiation and contaminated cultures were discarded.

Tissue culture media

Various dilutions of the basal salt medium and organic components of Murashige and Skoog (1962) (MS) and Woody Plant Medium (WPM) (Lloyd and McCown, 1980) were used (see Appendix A). *Macadamia* cv. 'Shimke' explants were used to determine a ratio of various dilutions of the salt and 'organic nutrient' components of the MS medium suitable for micropropagation of *Macadamia*. Full strength organic nutrients were made up with 100mg/l inositol, 2mg/l glycine, 0.5mg/l nicotinic acid, 1mg/l thiamine.HCl. 30g/l sucrose, 10μM 6-benzylaminoadenine (BAP) and 6g/l agar were used with the pH adjusted to 5.8 proir to autoclaving at 121^0C for 20min. A similar experiment compared various proportions of MS salts and WPM medium salts. Other components (30g/l sucrose, 6g/l agar, 100mg/l inositol, 2mg/l glycine, 0.5mg/l nicotinic acid, 1mg/l thiamine HCl and 10μM BAP. pH 5.8) remained constant.

Separate experiments were carried out to evaluate the effects of hormone additions of the cytokinins BAP and isopentenylaminopurine (2iP) and the auxins α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). Explants were preincubated on WPM salts with 100mg/l inositol, 2mg/l glycine, 0.5mg/l nicotinic acid, 1mg/l thiamine HCl, 30g/l sucrose and no hormones for 1 week to screen for bacterial contamination.

Unless otherwise stated the medium formulation adopted as a result of earlier experiments was 30% strength MS salts with the following organic nutrients; 100mg/l inositol, 2mg/l glycine, 0.5mg/l nicotinic acid, 1mg/l thiamine HCl, 30g/l sucrose and 40μM BAP. Media were solidified with Difco Bacto agar (unless otherwise indicated) at 0.6%. The pH of all media was adjusted to 5.8 prior to autoclaving at 121^0C for 20min.

Outplanting

Shoots were outplanted into coarse sand in a mist propagator unit situated in a glasshouse. The mist propagator unit maintained high humidity under a plastic tent. The device was produced by a local manufacturer. Shoots were preconditioned for 2 weeks on either basal medium with no hormones, basal medium plus 5μM IAA or on basal medium with no hormones but dipped for 30sec in a 3,000ppm IAA solution prior to outplanting.

Agrobacterium transformation

Proliferating shoot cultures of the *Macadamia* cultivars 'Shimke', 'Hinde' and 'Renown' were inoculated with *A. rhizogenes*. There were six replicates per treatment. Culture conditions were the same as those for *in vitro Macadamia* culture except that hormones were omitted from the medium. Controls to assess the infectivity of *Agrobacterium* strains consisted of infected carrot discs which were inoculated by the method of Ryder *et al.* (1985) on the apical surfaces of the discs (see Chapter 2 for details). *A. rhizogenes* strains used in this study were K565, K568, and K598 (Appendix B). The bacteria were grown on YM medium (Appendix A) containing 0.027M mannitol and 0.011M $NaNO_3$, as the major constituents (White and Nester, 1980). They were grown to stationary phase (2 days) at 28°C when the density was ca. 10^9 cells/ml. Each plant was inoculated five times (total ca. 10^8 cells made up in sterile double distilled water) and transfer was made using the tip of a hypodermic needle which simultaneously wounded and inoculated plant tissues. One week after introduction of bacteria, plants were transfered onto medium (see above) containing 500mg/l Claforan® (cefotaxime) to inhibit growth of *Agrobacterium.*

Results

Surface sterilisation

Table 3.1 shows the level of microorganism contamination on explants after emersion in a 10% calcuim hypochlorite solution (w/v) for various times. Explants in the 40min treatment suffered considerable tissue damage. The 30min treatment gave acceptable results and was adopted as the standard sterilising time for all field grown material.

Table 3.1. Sterilisation of field grown *Macadamia* cv. 'Shimke' explants in 10% calcium hypochlorite (*n*=30).

Minutes in sterilant	Contaminated cultures (%) after 2 weeks
10	93.3
20	60.0
30	16.7
40	3.3

Effect of time of year on establishment of cultures and on bacterial contamination

Table 3.2 shows that the optimum time for collecting field grown material is during the summer months. Plants were undergoing a strong growth flush during this dry period of the year and this period corresponds with the maximum availability of explant material from the orchard and good rates of explant establishment onto sterile nutrient medium.

Table 3.2. Effect of time of year on: 1) bacterial contamination of explants and 2) survival of explants (n=100 for spring and summer; n=20 for autumn and winter).

Time of year	Survival (%)	Contamination (%)
Spring	80	15
Summer	90	10
Autumn	45	30
Winter	20	60

Concentrations of salts and organic components in the medium

Macadamia cv. 'Shimke' explants were used to determine a ratio of various dilutions of the salt and 'organic nutrient' components of the MS medium suitable for micropropagation of *Macadamia*. Full strength organic nutrients were made up with 100mg/l inositol, 2mg/l glycine, 0.5mg/l nicotinic acid, 1mg/l thiamine.HCl. It was found that of salt concentrations tested, the 30% MS formulation of inorganic salts with full strength organic nutrients supported the highest rate of survival (see Table 3.3). A similar experiment compared various proportions of MS salts and WPM medium salts (all with full strength organic nutrients). WPM is a low salt medium compared to full strength MS salts (Appendix A).

Table 3.3. Survival of field grown *Macadamia* cv. 'Shimke' explants (apical buds) on basal medium containing various proportions of the inorganic salts of MS medium and 'organic nutrients'. Other components (30g/l sucrose, 6g/l agar, and 10μM BAP, pH 5.8) remained constant. Means and standard errors of means of 6 replicates for each nutrient formulation.

MS salts	Organic nutrients	% survival of explants after 12 weeks	
Full strength 100%	100%	0	
100%	half strength 50%	0	
60%	100%	33.3	± 9.6
60%	50%	22.2	± 5.5
30%	100%	77.7	± 5.3
30%	50%	72.2	± 11.0

Table 3.4 shows that of those formulations tested, a 30% MS salt formulation gave the highest survival rate for *Macadamia*. In succeeding formulations 30% MS salts were used as standard protocol.

Table 3.4. Survival of field grown *Macadamia* cv. Shimke explants (apical buds) on basal media containing various proportions of the inorganic salts of MS and WPM medium. Other components (30g/l sucrose, 6g/l agar, 100mg/l inositol, 2mg/l glycine, 0.5mg/l nicotinic acid, 1mg/l thiamine HCl and 10μM BAP. pH 5.8) remained constant. (*n*=20).

Basal media	% survival of explants after	
	4 weeks	12 weeks
MS salts	0	0
60% MS salts	28	20
30% MS salts	95	95
WPM salts	85	75

Hormone concentrations

Five concentrations (5, 10, 20, 30 and 40μM) of each of two cytokinins (BAP and 2iP) were evaluated for their ability to induce proliferating shoot cultures. The results shown in Table 3.5 indicate that there was no difference in the response of the explants to BAP and 2iP. With both BAP and 2iP at concentrations of 5μM, there was little or no shoot multiplication. The treatments did, however, result in shoot elongation and the production of normal leaves (Figure 3.2). The higher concentrations of either cytokinin resulted in tighter clumps of shoots whereas at lower cytokinin concentrations, shoots were elongated (Figures 3.3 and 3.4). BAP was adopted for general use because it is cheaper. A concentration of 40μM was used to initiate and maintain proliferating shoot cultures of field grown material of *Macadamia* cultivars 'Shimke', 'Renown', 'Eggshell', 'Hinde' and '508'. The number of shoots produced after 12 weeks was 9.0 ± 0.71, 8.6 ±.0.67, 7.3 ± 0.56, 9.6 ± 0.76 and 6.4 ± 0.48 for each cultivar respectively.

Table 3.5. Effect of five concentrations of the cytokinins (2iP and BAP) on shoot proliferation of apical bud explants of *Macadamia* cv. 'Shimke'. Means and standard errors of means of 5 replicates of each cytokinin treatment.

Cytokinin	Number of shoots per explant		
(μM)	4 weeks	8 weeks	12 weeks
BAP			
5	3.4 ± 0.4	3.8 ± 0.49	3.8 ± 0.49
20	5.2 ± 0.37	6.8 ± 0.37	8.6 ± 0.24
30	5.4 ± 0.24	7.2 ± 0.2	9.4 ± 0.24
40	5.6 ± 0.24	7.6 ± 0.24	10.4 ± 0.51
2iP			
5	2.2 ± 0.49	2.2 ± 0.49	2.6 ± 0.75
20	4.0 ± 0.45	6.2 ± 0.37	7.2 ± 0.66
30	4.4 ± 0.4	6.0 ± 0.71	7.8 ± 0.66
40	4.8 ± 0.37	7.0 ± 0.55	9.0 ± 0.71

Figure 3.2. *Macadamia* cv. 'Shimke' growiing on sterile nutrient medium [30% MS salts and full strength organic nutrients (MS)] with 5μM BAP. 'Normal' leaves and elongated internodes formed at this cytokinin concentration.

Figures 3.3 and 3.4. *Macadamia* cv. 'Shimke' *in vitro* proliferating shoot cultures [30% MS salts and full strength organic nutrients (MS)] with 40µM BAP (Fig. 3.3) and 15µM BAP (Fig. 3.4). Note the short internodes and poor leaf development shown in Figure 3.3 and the elongating shoots shown in Figure 3.4.

Concentration of agar support

The agar concentration best suited for proliferating shoot cultures of *Macadamia* was determined using 30% MS salts with full strength organic nutrients. Treatments consisted of 0.6% and 1% agar and nutrient solution only. For the latter, care was taken that the liquid medium covered only the distal third of the explants to ensure good aeration of cultures. Table 3.6 shows that liquid medium gave the highest number of shoots followed by the soft agar (0.6%). However, solidified medium proved more convenient because manipulations were faster.

Table 3.6. Comparison of number of shoots produced by explants of*Macadamia* cv. Hinde cultured on 0.6%, 1% agar or liquid culture. Means and standard errors of means of 20 replicates of each treatment.

Comparison	Number of shoots per explant		
	4 weeks	8 weeks	12 weeks
1% agar	6 ± 1	9 ± 1	11 ± 2
0.6% agar	8 ± 1	12 ± 1	17 ± 3
liquid	9 ± 1	16 ± 3	19 ± 4

Timing of explant harvest from field

The number of explants available for use from the Waite Agricultural Research Institute was limited due to the number and size of stock plants. Therefore the type of tissue best suited for *in vitro* culture was examined. *Macadamia* forms an apical bud which then undergoes a stationary phase followed by growth and rapid elongation. An experiment was carried out to compare the difference in growth stage in terms of their potential as explant material. A difference was observed

in numbers of explants surviving the introduction to *in vitro* environments. Results shown in Table 3.7 demonstrate clearly the benefit of using stationary apical buds for initiating *in vitro* culture of *Macadamia.*

Table 3.7. Survival of stationary apical buds and elongating shoots of *Macadamia* cv. Shimke after 4 weeks in culture (*n*=20).

Treatment	Survival %
Stationary apical bud	80
Burgeoning shoots	10

Effect of explant type and orientation in the culture vessel on shoot proliferation

Since there is only one apical bud per shoot, it was desirable to ascertain the potential for using axillary buds of *Macadamia* for shoot regeneration. A comparison of the survival rates of axillary and apical buds was made. In the same experiment the effect of the orientation of the explant on the medium was determined. Treatments consisted of axillary buds or apical buds placed vertically or horizontally into media. Table 3.8 shows the percentage survival.

Table 3.8. Comparison of survival rates of *Macadamia* cv. Shimke explants. The treatments were: axillary buds or apical buds placed vertically or horizontally into media (n=20).

Treatment	% Survival
Axillary buds (vertical)	45
Axillary buds (horizontal)	100
Apical buds (vertical)	75
Apical buds (horizontal)	85

Nodal sections placed horizontally gave the highest survival rate (100%). However, they produced copious amounts of callus growth, as did the horizontally placed apical buds. Horizontally placed apical buds produced less callus partly because callus growth originated from cut ends of stems and apical buds had only one end cut. This data indicates that vertical placement of apical buds is the preferred method, combining good survival rates with lack of callus growth.

Root initiation (Auxin type and light/dark regimes)

Five concentrations (1, 5, 10, 20 and 30μM) of each of three auxins (IAA, IBA, NAA) were tested in factorial combination with dark and light regimes for the ability to initiate roots. The cytokinin concentration was reduced from 40μM BAP to 5μM BAP for this experiment (this is often a standard procedure for rooting of plants). There were 5 replicates per treatment. No treatments produced roots. All dark treatments produced callus growth. After one subculture (6 week interval) all cultures showed signs of stress, callus production and necrosis. There was no obvious correlation between auxin type or concentration and the extent of necrosis.

Root initiation (acclimisation of explants *in vivo*)

The transfer of explants from the tissue culture to the shade house environment is a very important transition stage for the plants. One rooting experiment involved the transfer of *in vitro* cultured *Macadamia* cv. Shimke shoots to a 'mist propagator' unit in non-sterile conditions (n=6). Treatments included shoots preconditioned for 2 weeks on basal medium with no hormones, basal medium plus 5µM IAA or on basal medium with no hormones but dipped for 30sec in a 3,000ppm IAA solution [a traditional method of rooting woody plant cuttings (Hartman and Kester, 1983)] prior to outplanting. Although the plants were protected from desiccation by the high humidity that was maintained within the plastic tent, no shoots survived longer than six weeks. There was no root formation.

Root initiation (*Agrobacterium* transformation)

Macadamia cultivars inoculated with *Agrobacterium* did not show visible signs of infection. The same *Agrobacterium* strains (K565, K568, and K598) produced hairy roots on carrot disks (see Chapter 2). In the case of infection of *Macadamia* susceptibility was judged on the basis of hairy root formation, not DNA transfer. The lack of hairy roots indicates that DNA transfer did not take place.

Discussion

The results described in this chapter clearly show that a number of factors are important for the establishment and proliferation of shoot cultures of *Macadamia* cultivars.

There was a high survival rate of explants from material taken during spring and summer because of low contamination and the fact that explants were in a suitable 'biological state' during these months. The low contamination rates experienced during spring and summer months are probably due to the hot dry conditions which are not conducive to microbial growth and the short time the material had been exposed to airborne contaminants.

The low salt medium required to establish and grow *Macadamia in vitro* is not unexpected. The proliferation rates obtained with shoot apices were good and compare well with other woody plants. For example, *Malus* L. cultivars (Hutchinson, 1984) have similar shoot multiplication rates. However the high concentration of cytokinin needed to obtain dividing shoot cultures was surprising. Lane (1978) published a dose-response curve for BAP using *Malus* 'McIntosh' showing that 5μM was optimal, 0.5μM was slightly less effective, and 50μM killed the explants. Similar results were obtained with M.4 (*Malus*) root stocks (Dunstan *et al.*, 1985) and other woody plants (Hu and Wang, 1983). Jones (1967) however, also found a high concentration (ca. 44μM) of BAP was required to inhibit stem elongation with the *Malus* rootstock M.26, this compares well with the results obtained for *Macadamia* (the inhibition of stem elongation increased shoot formation in *Macadamia*).

Roots did not form on any *Macadamia* explants produced in this investigation. This stage is frequently the most difficult in production of plantlets. For example difficulty is still commonly encountered with the induction of roots derived from woody explants of *Eucalyptus* (Bennet and McComb, 1982; Cresswell and Nitsch, 1975) and *Santalum* (Barlass *et al.*, 1980). Agar at a concentration of 0.6-0.8% is the most commonly used support for explants. Werner and Boe (1980) found that by reducing the agar to a concentration of 0.2% (and MS salts to one-third), they were able to obtain root initiation in M.7 (*Malus*) with 5-15μM IBA. Treatments ranging from liquid to 1% agar had no effect on rooting with *Macadamia*, although more shoots were obtained with liquid media and lower agar concentrations. This is probably due to a steeper nutrient

concentration gradient effect in the more solidified media which would result in partial nutrient starvation.

Survival rates obtained with types of explant other than newly formed apical buds, are interesting. It was expected that the nodes closer to the base would have given the highest survival rates (see Introduction). However they gave the lowest rates. Horizontally placed explants produced copious amounts of callus growth making this orientation undesirable from a micropropagation view point. The data indicate that vertical placement of apical buds is the preferred treatment for micropropagation. However, these data are interesting as the callus (which was also produced if auxin was added to the medium) was produced from the cut ends of horizontally placed explants. When the auxin concentration is high, callus will form at the shoot base and this inhibits root development (Lane, 1979). The callus may be produced as a result of endogenous auxin. The copious callus production of horizontal explants is probably due to the phenomenon that auxin, which is produced in young leaves and meristemic regions and which ordinarily moves in a basipetal direction, has a bipolar movement in horizontal explants. Root formation was not achieved. The callus produced in rooting experiments suggests that the concentration of auxin was either too high or the cytokinin concentration needed to be increased. This is consistent with the concept of Skoog and Miller (1957) which states that organ differentiation in plants is regulated by the interplay of the two hormones, auxins and cytokinins. Although with many species of plants a low salt medium has proved satisfactory for the rooting of shoots (Bhojwani and Razdan, 1983), even transfer of explants to a sand medium with no added salts failed to produce roots on *Macadamia*. This also indicates that a successful method for producing roots on *Macadamia* explants will be achieved after the appropriate concentrations of hormones have been ascertained.

It was considered that infection by *A. rhizogenes* might provide an alternative way of inducing roots to form on *Macadamia* explants. Roots were not produced. The *Agrobacterium* system appears to be limited to the transformation of susceptible plants and, while the list of susceptible plants is extensive (De Cleene and De Ley, 1981), there are many plants which have been reported to be non-susceptable to infection. At present, *Macadamia* can be classified as one of those non-susceptable.

Other factors should be investigated to induce rooting of *Macadamia* shoots, for example Jones (1976) and his co-workers found phloroglucinol promoted rooting in a number of rosaceous fruit trees. There are numerous other treatments that could be investigated to establish roots, for example, different temperatures (Lane, 1978) and sucrose concentrations (Sriskandarajah and Mullins, 1981). More work will need to be carried out to achieve the objective of producing roots on *Macadamia* explants.

The investigation of procedures suitable for the micropropagation of *Macadamia* provides a starting point for application of this technology to horticultural practice. The important aspects of the protocol can be summarised as follows: The choice of plant material to initiate *in vitro* cultures is important and apical buds collected during spring and summer are the best. Plant tissue can be adequately surface sterilised by immersion in 70% ethanol containing Tween 20 (0.01%) for 1min followed by immersion in freshly prepared and filtered 10% calcium hypochlorite (w/v) (also containing Tween 20) for 30 min whilst stirring under partial vacuum. A formulation of thirty percent MS salts with 100mg/l inositol, 2mg/l glycine, 0.5mg/l nicotinic acid, 1mg/l thiamine.HCl, 30g/l sucrose, 6g/l agar at pH 5.8 with BAP concentration of 40μM are effective in establishing proliferating shoot cultures of *Macadamia* cultivars. The dividing shoot cultures can be subcultured onto the same medium.

Although procedures for the initiation and proliferation of axillary shoot cultures have been developed, there needs to be more research into factors involved in root induction and the acclimisation of plants to shade house environments. The use of proliferating shoot cultures for grafting material could be of benefit to the industry. This also requires further investigation.

Chapter 4

An abundant carbon-nitrogen compound in proteoid roots

Introduction

One way of investigating whether *Agrobacterium* is involved in the production of proteoid roots is to look for opines in proteoid root tissues. Opines serve as specific growth substrates (carbon and nitrogen sources) for their inciting pathogenic agent. Opines are not catabolised by the plant or other bacteria. The *Agrobacterium* species incites opine synthesis in its host which creates a chemical environment favourable for growth and propagation of itself, thus mediating parasitism. Opines can constitute up to 7% of the dry weight of transformed tumour tissue (see Chapter 1).

If *Agrobacterium*, or another bacterium which incites opine production, is involved in the production of proteoid roots then opines would be expected to be produced in proteoid roots but not in other tissues.

An investigation of abundant nitrogenous compounds in proteoid roots with similar chemical properties to known opines, formed the starting point in this investigation.

If an opine-like compound is present it would: 1) be present in high concentrations in proteoid roots but not in other plant tissues, 2) be catabolised by the inciting bacteria, and 3) have its synthesis encoded on the DNA of the inciting organism which may be transferred to the plant (not investigated in this thesis). Further, it may be possible to detect the exuded compound in the soil medium.

The opine hypothesis was examined by identifying potential opines using high voltage paper electrophoresis, describing their chemical structure, measuring their concentrations, determining in which tissues they are to be found and also ascertaining if they are exuded by the roots into the medium. It is also possible to identify which of the bacteria might be responsible for proteoid root production by providing bacteria isolated from the rhizosphere of proteoid roots with the putative-opines as their sole carbon and nitrogen source.

Materials and methods

Plant material and bacterial isolates

The plant material and bacterial isolates used in this investigation and methods for their culture are described in Chapter 2. The plant material consisted of: 1) non-sterile (proteoid) and sterile (non-proteoid) roots of *B. grandis,* 2) leaves of *B. grandis*, 3) proteoid roots and leaves of *B. prionotes, B. coccinea* and *G. hookeriana*, and 4) shoots of *Macadamia* cv. 'Eggshell'. Bacteria used were *Agrobacterium* strains K750 and K301, *E. coli* strain HB101 (Appendix B) and 15 bacterial isolates from proteoid roots also described in Chapter 2.

Extraction of 70% EtOH soluble fraction from plant material

Plant material (50g fresh weight) was powdered in liquid N_2 using a mortar and pestle and extracted with 70% EtOH (2ml/g fresh weight) for 1 hr. The homomogenate was then filtered through Whatman No. 1 filter paper and the volume reduced to approximately one tenth in a rotary evaporator at 30^{o}C. The extract was examined for known opines or opine-like compounds.

High voltage paper electophoresis (HVPE)

HVPE was performed as described by Tate (1968), using the following solutions: 0.75M formic acid/1M acetic acid, pH 1.7; 0.05M dihydrogen citrate pH 5.0; 0.05M potassium dihydrogen phosphate pH 6.5; 0.2M triethylamine/carbonate pH 7.2; 0.2M ammonium/0.1M carbonate pH 9.2; 0.05M oxalate/carbonate pH 11.25, and 0.05M Tris orthophosphate pH 12.75.

The method of Tate (1981) was used to measure electrophoretic mobility between pH 1.7 and 12.75. Reference markers were Orange G (pH 1.7-7.2) and fructose (pH 9.2-12.75). Other markers used as reference points were the opines deoxymannityl glutamic acid, deoxymannityl glutamine, histopine and cucumopine. Also histidine and an amino acid mixture consisting of asparagine, glycine, alanine, serine, valine, isoleucine, glutamine and cystine were used.

Detection reagents

There are no specific chemical reactions that enable an opine to be identified, because opines are a biological class of chemicals, that is, it is their relationship to *Agrobacterium* and the host plants that classifies them as opines. The detection reagents listed below provided the starting point of the investigation because they stain other known opines. The following detection reagents reacted with a putative opine isolated from the roots of *Banksia* to give positive spots after HVPE:

(a) alkaline silver nitrate dip (Smith, 1969) was used first because it locates all known opines as well as many other reducing compounds (Smith, 1969),

(b) the Pauly reagent (sulphanilic acid) locates imadazoles (Ames and Mitchell, 1952),

(c) ninhydrin (Smith, 1969) locates all α-aminoacids and a variety of other compounds,

(d) ferric chloride-ferricyanide reagent (Barton *et al.*, 1952; Kirby *et al.*, 1953) which detects many phenols and tannins.

Purification of putative opine

The extract was applied as a band (approximately 50µl/cm) to Whatman No 1 filter paper and electrophoresed for 15 to 20min at 4,000V in 0.75M formic acid/1M acetic acid buffer. The position of the putative-opine was detected by using ninhydrin reagent on reference strips cut from the sides of the electrophoretogram. The compound was then eluted with sterile distilled H_2O using the method described by Lethbridge (1990). The eluate was concentrated (to dryness for NMR studies) in a stream of gaseous N_2 or by lyophilisation.

Nuclear magnetic resonance (NMR)

NMR (Fourier transform) spectra were recorded at 89.55 MHz for ^{1}H and 22.49 MHz for ^{13}C on a JEOL FX90Q spectrometer. Samples (approximately 50mg) were dissolved in D_2O. The internal reference was t-butanol which was used according to standard techniques (Williams and Fleming, 1987). The spectra were prepared by Dr G. Jones of the Department of Plant Physiology at the Waite Agricultural Research Institute.

Gas chromatography/mass spectrometry (GC/MS)

A Hewlett Packard GC/MS (Model 5992B) was used to obtain mass spectral data, and similarty indicies were calculated on a Hewlett Packard desk top computer (Model 9825A) using the method as described by Tate *et al.* (1982).

High-performance liquid chromatography (HPLC)

HPLC analysis was carried out on various proteaceous plant tissue extracts and on the root media in which some plants were growing (see Table 4.1). Crude plant extracts were prepared for HPLC as previously described for HVPE analysis. The hydroponic nutrients (Chapter 2) assayed were concentrated 1000 fold by rotary evaporation at 30°C. The nutrient agar media assayed were prepared by lyophilisation and powdering (to disrupt the agar structure) and extraction with sterile distilled water (volume equal to that of the original nutrient agar) followed by concentration (1000 fold) of the solution by rotary evaporation at 30°C. The samples were derivatized using an unpublished modification by H. Gockowiak of the methods of Mayer and Pause (1984) and Jones and Gilligan (1983). See Appendix C for details. HPLC experiments were performed at the Australian Wine Research Institute.

Results

Characterisation of a putative opine in proteaceous plants

HVPE offers a rapid and reproducible method for the separation and identification of small molecules such as aminoacids, sugars, indoles, phenols, imidazoles, purines, pyrimidines, nucleotides and peptides. The technique was adopted to separate, and purify a putative-opine. After separation by HVPE one of the compounds in the extract of proteoid roots (see Materials and Methods) (Figure 4.1), showed a strong silver nitrate staining reaction and also proved positive to Pauly reagent and ninhydrin (Figure 4.2). There were no other compounds in the extract which could be considered as clear contenders for a role as an opine based on staining reactions. These findings encouraged further investigation because the staining reactions mentioned above showed a similar intensity to reference markers (see Figure 4.3) indicating the compound was present in proteoid roots in high concentrations. Also the compound was not detected in the above-ground parts of *B. grandis* when analysed by HVPE and ninhydrin staining (see Figure 4.3).

The Pauly reaction for imidazoles suggested that the putative opine might have histopine or histidine residues, however, electrophoretic pH-relative mobility profiles shown in Figure 4.4 demonstrate a charge difference between histopine or histidine and the compound found in the proteoid root extract. The ninhydrin reaction coupled with the electrophoretic data indicated that an amino-phenolic compound had been isolated, as the Pauly reagent will also detect phenolics (Hatherway, 1969).

Figure 4.1. A compound (arrowed) in the extract of proteoid roots separated by HVPE (0.75M formic acid/1M acetic acid buffer, pH 1.7 at 3500V, for 20min) showed a strong silver nitrate staining reaction. Lanes C, D and E contain 2, 4, and 6µl of extract, respectively. Reference markers were Orange G (Lanes A and G), deoxymannityl glutamine (Lane B), and deoxymannityl glutamic acid (Lane F).

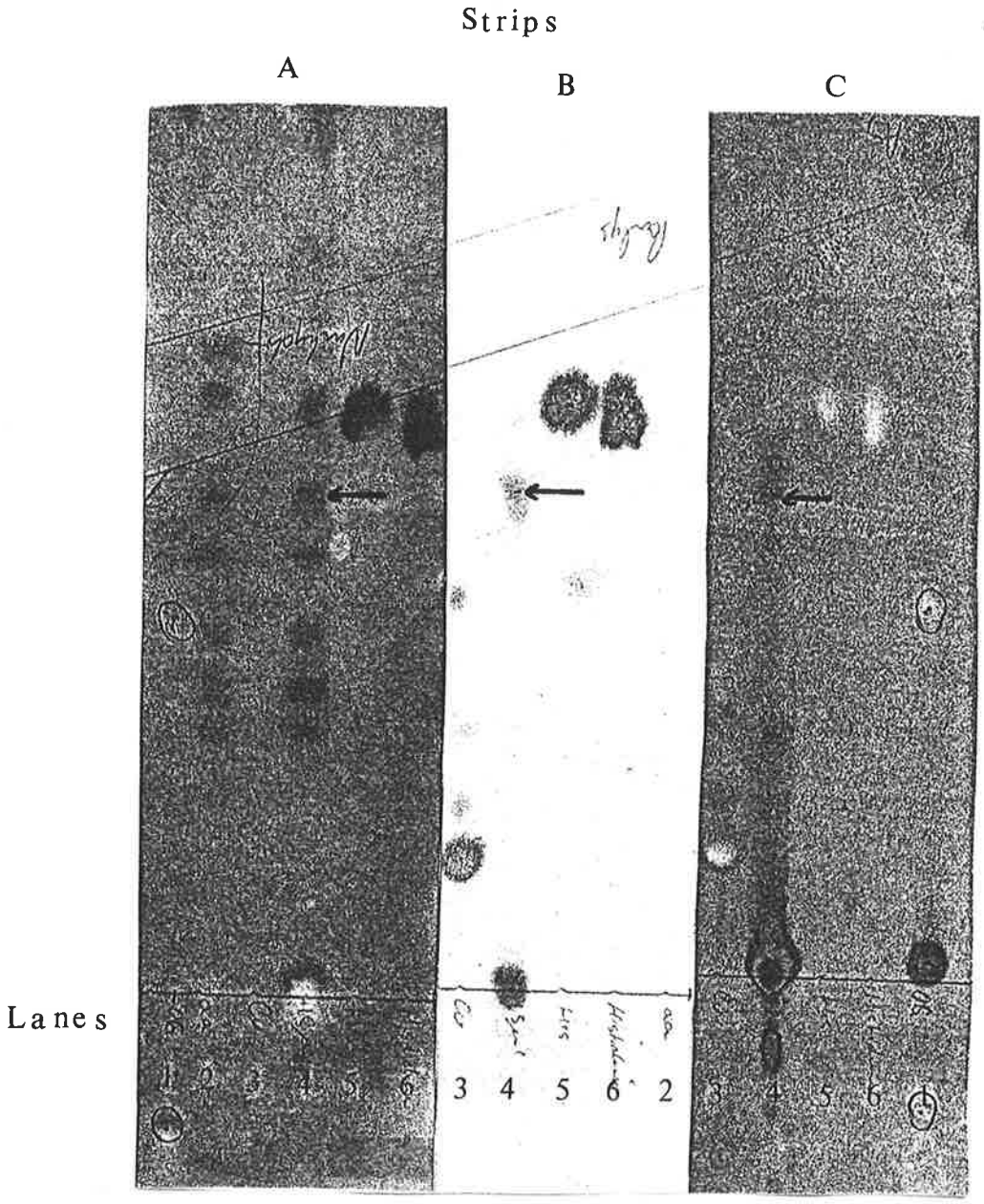


Figure 4.2. Extract from proteoid roots, separated by HVPE (0.75M formic acid/1M acetic acid buffer, pH 1.7 at 3500V for 20min), showed positive reactions to ninhydrin (Strip A), Pauly reagent (Strip B) and silver nitrate (Strip C). Lanes 1, Orange G marker; Lanes 2, amino acid markers (from origin are: asparagine, glutamine, serine, valine, isoleucine, alanine and glycine); Lanes 3, cucumopine; Lanes 4, proteoid root extract; Lanes 5, histopine; Lanes 6, histidine. The 'putative-opine' is arrowed.

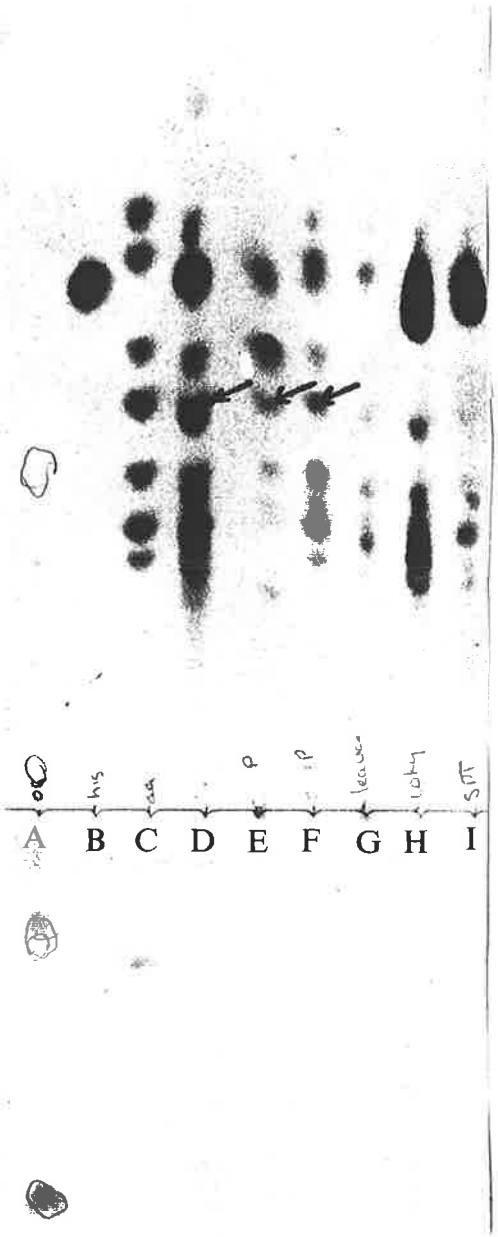


Figure 4.3. Ninhydrin reaction after HVPE (0.75M formic acid/1M acetic acid buffer, pH 1.7 at 3500V for 20min) of 70% ethanol extracts from *B. grandis* tissues. Lane A=, Orange G marker; Lane B, histidine; Lane C, amino acid markers (from origin are: asparagine, glutamine, serine, valine, isoleucine, alanine and glycine); Lane D, E and F, three extracts (all from the same plant) from proteoid roots (the opine-like compound is arrowed as before); Lane G, leaves; Lane H, cotyledons; and Lane I, leaves grown in aseptic conditions.

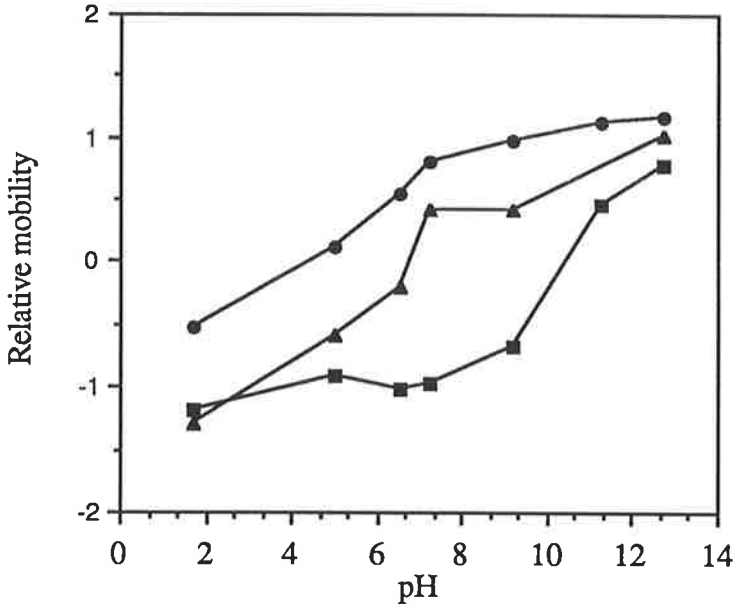


Figure 4.4. Electrophoretic mobility profiles for histopine (●), histidine (▲) and the compound isolated from proteoid roots (■) (subsequently demonstrated to be tyramine).

A ^{1}H NMR spectrum of the semi-purified plant extract (Figure 4.5) also indicates a phenolic compound with characteristic peaks at 7 parts per million (ppm) (Williams and Fleming, 1987). The higher frequency peak at 8-9 ppm is interpreted as an unrelated compound in the plant extract. Upfield peaks at 5 ppm can be identified as contaminant H_2O while the two triplets at approximately 3 ppm indicate a CH_2-N< structure (Williams and Fleming, 1987). The ^{13}C spectrum (Figure 4.6) also indicates an aromatic ring with the addition of two other carbon atoms with 4 peaks clearly visible.

These data are consistent with the identification of the compound as tyramine. This was confirmed by HVPE co-migration studies at pH 1.7 and 11.25 using authentic tyramine. GC/MS data on the acetylated derivative showed a similiarity index of 0.995 compared with a derivatised reference sample of tyramine. The structure is shown in Figure 4.7.

Fig. 4.5.

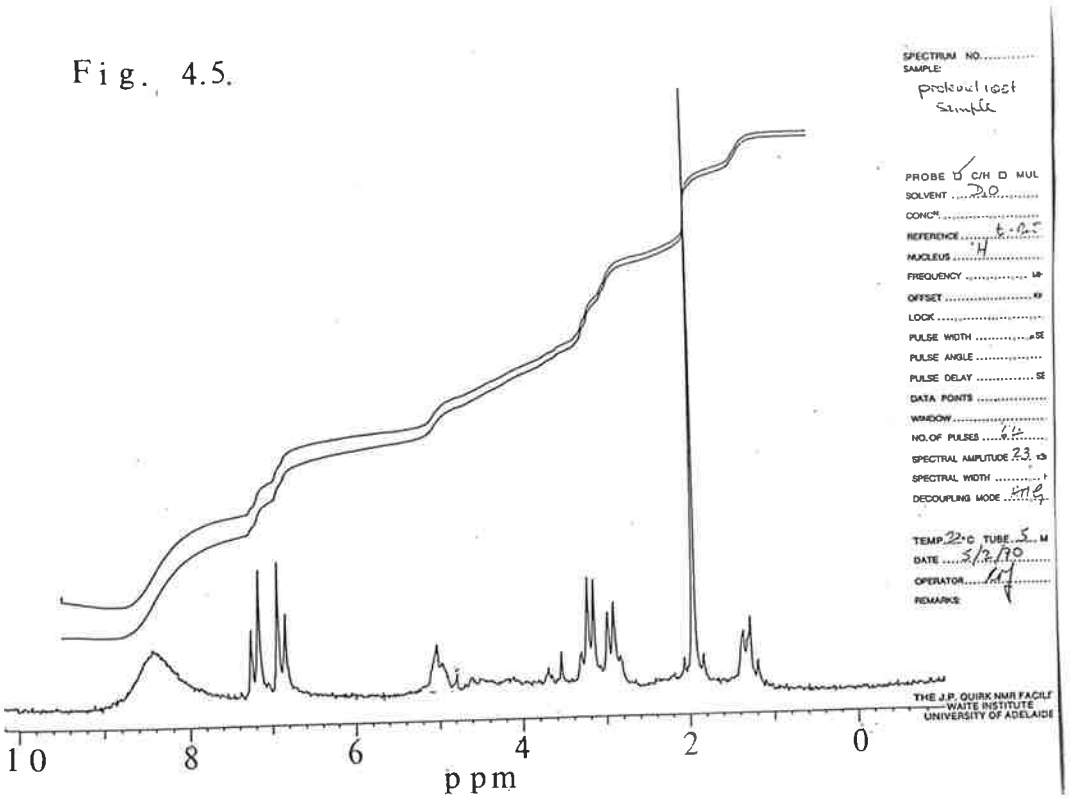
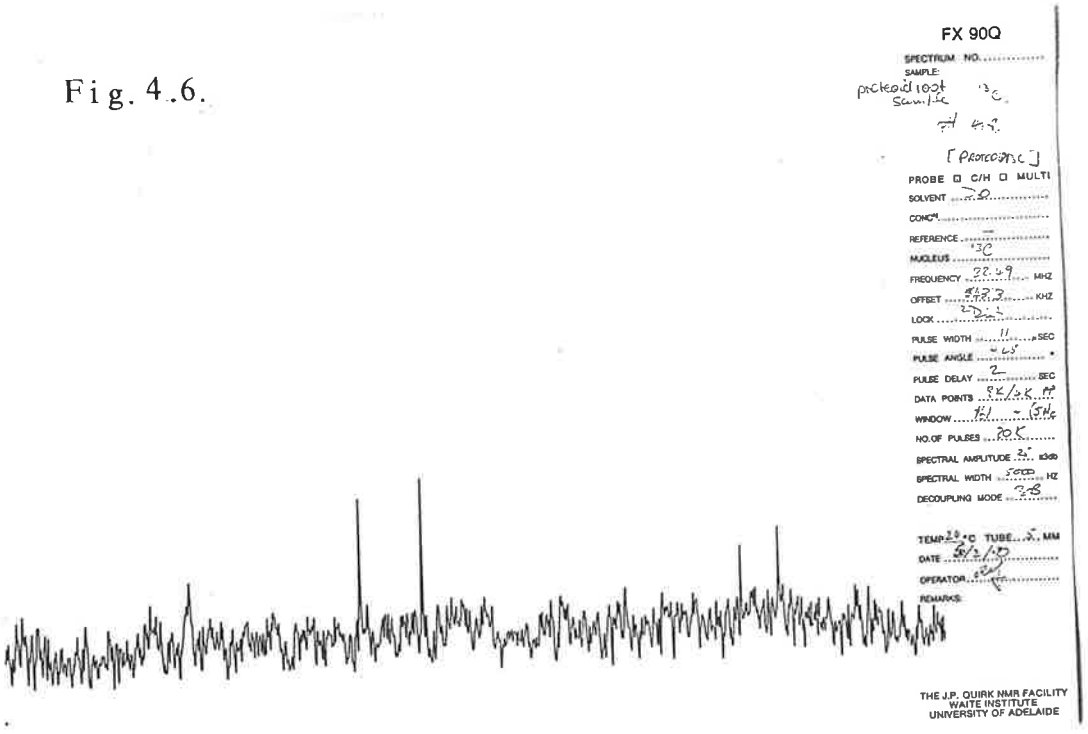
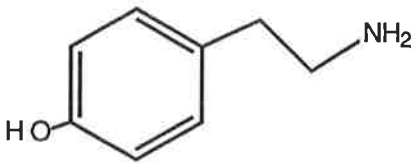


Fig. 4.6.



Figures 4.5 and 4.6. ^{1}H NMR (Fig. 4.5) and ^{13}C spectra (Fig. 4.6) of the semi-purified plant extract. NMR (Fourier transform) spectra were recorded at 89.55 MHz for ^{1}H and 22.49 MHz for ^{13}C on a JEOL FX90Q spectrometer. Samples (approximately 50mg) were dissolved in D_2O and t-butanol served as an internal reference.

Figure 4.7. Structure of tyramine, molecular weight 137.18.



Localisation of tyramine in plant tissues

Extracts from a variety of proteaceaous plant tissues (Table 4.1) were analysed by HPLC to determine both the localisation of tyramine and the concentrations accumulating in the tissues. It was possible to detect concentrations as low as 0.027mg/g fresh weight. The plant tissues consisted of above-ground vegetative parts (leaves and shoots) and both proteoid and 'non-proteoid' roots. The hydroponic and nutrient agar media in which they had been growing were assayed to determine if tyramine could be detected as a root exudate. Tyramine was not detected in any of the above ground organs sampled which included the leaves and shoots of *B. grandis, B. coccinea, B. prionotes, G. hookeriana* and *Macadamia* nor was it detected in the proteoid and non-proteoid roots of *B. coccinea* and *G. hookeriana.* Sterile and non-sterile nutrient media of *B. coccinea* and *B. grandis* were also assayed for tyramine, however none was detected. The positive results of the tyramine assays are shown in Table 4.1.

Table 4.1. HPLC analysis of tyramine in various proteaceous plant tissues. H hydroponic, A aseptic, NS non-sterile, P pot.

Species	Type and age of plant culture	Material sampled	Tyramine mg/g fresh weight
B. grandis	H, NS,12 month	proteoid root	6.93
		non-proteoid root	5.5
B. grandis	H, A, 8 month	non-proteoid root	5.7
B. prionotes	P, NS, 8 month	proteoid root	2.24

Tyramine was found in the proteoid and non-proteoid roots of the hydroponically grown *B. grandis* cultures [as had already been demonstrated by HVPE and the appropriate staining reactions (see above)] at concentrations corresponding to 6.1% (6.93 mg/g fresh weight) and 5.5% (5.5 mg/g fresh weight) dry weight respectively and in the proteoid roots of *B. prionotes* [2.3% dry weight (2.24 mg/g fresh weight)]. The concentration of tyramine is similar to the concentrations of opines found in tumor tissue of *Agrobacterium* transformed plant tissue (Firmin and Fenwick, 1978) and so, in this respect, it fulfils one of the criteria for the role of an opine. Tyramine also conforms to chemical characteristics of opines because of its carbon/nitrogen structure (Figure 1.4). However, because tyramine was detected in non-proteiod roots of an aseptic *B. grandis* culture [5.5% dry weight (5.7 mg/g fresh weight)], this rules out tyramine as an opine candidate produced by a proteoid root causing organism.

Bacterial catabolism of tyramine

The opine hypothesis proposes that bacteria responsible for transforming roots are able to catabolise the opines. Therefore the ability of selected bacterial isolates from proteoid roots to catabolise tyramine was investigated. Fifteen species of bacteria were isolated from proteoid roots of *B. grandis* growing in hydroponic conditions (see Chapter 2). The assay system consisted of

incubating the bacteria in a crude proteoid root extract which had been dried and resuspended in a low salt solution (see Chapter 2, Materials and Methods). Bacterial controls consisted of *Agrobacterium* strains K750 (which had been cured of its ability to catabolise opines, see Appendix B), K301 (anabolic and catabolic genes intact for octopine and agropine, see Appendix B) and the *E. coli* strain HB101. It was expected that if tyramine was an opine *Agrobacterium* strain K750 and *E. coli* strain HB101 would not catabolise it whereas *Agrobacterium* strain K301 would. The continuing persistence of ninhydrin positive chemical species, including the compound subsequently identified as tyramine, was monitored in the plant extracts by HVPE over a period of 72 hours. Examples shown in Figures 4.8 and 4.9 show the disappearance of ninhydrin positive compounds. Of the 15 bacterial isolates, 8 gram-negative isolates catabolised tyramine within 72 hours, whilst 3 gram-negative and all 4 gram-positive species did not. Control strains *E.coli* (HB101) and *Agrobacterium* (K301) were able to catabolise tyramine whereas *Agrobacterium* (K750) did not.

Fig.4.8.

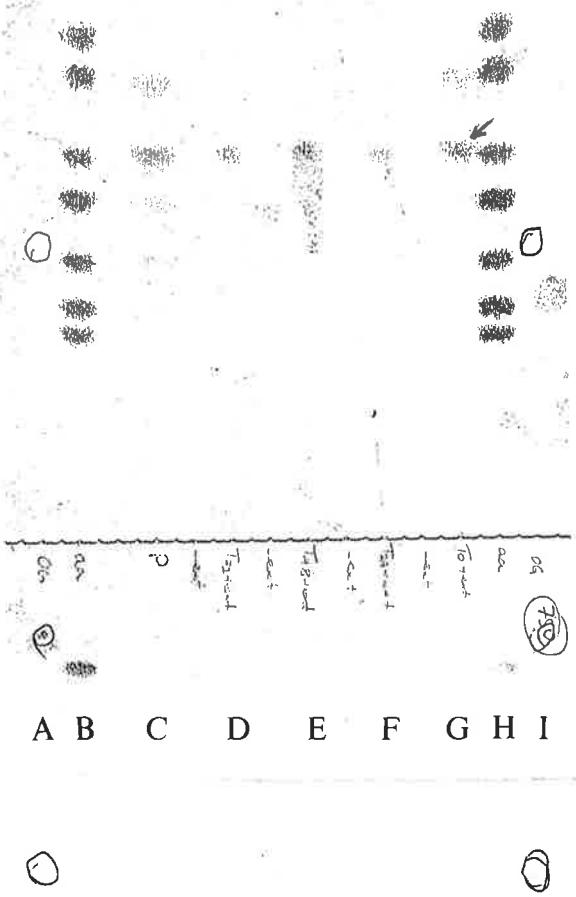
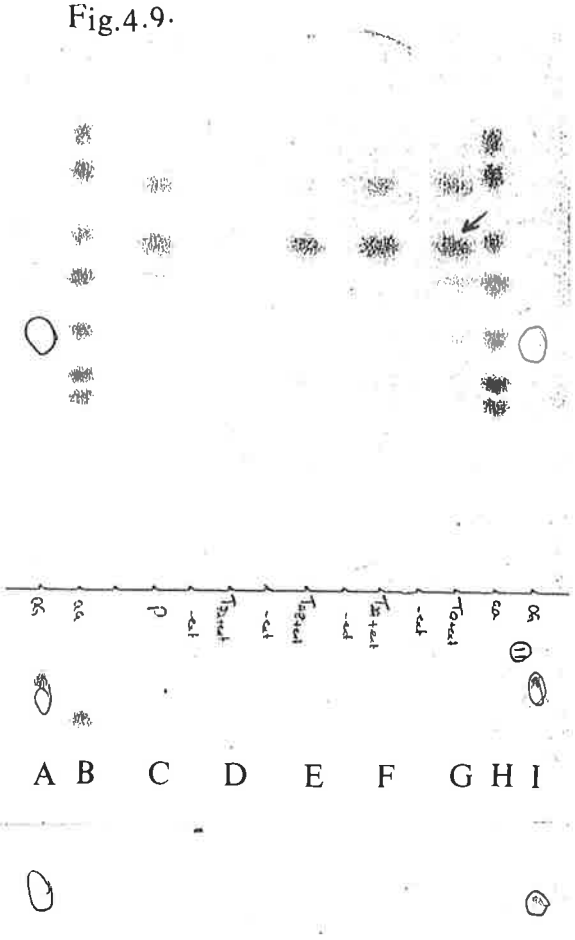


Fig.4.9.



Figures 4.8 and 4.9. Examples of ninhydrin reactions following HVPE (0.75M formic acid/1M acetic acid buffer, pH 1.7 at 3500V for 20min) separation. Reactions demonstrate catabolism by a gram-negative bacterium isolated from proteoid roots of tyramine (Fig. 4.8), and the persistence tyramine when incubated with *Agrobacterium* (K750) (Fig. 4.9). Bacterial isolates were incubated with the proteoid root extract for periods ranging from 0hr to 72hr. Lanes A and I, Orange G reference markers; Lanes B and H, amino acid reference markers (from origin are: asparagine, glutamine, serine, valine, isoleucine, alanine and glycine); Lane C, 0hr; Lane D, 24hr; Lane E, 48hr; Lane F, 72hr; Lane G, proteoid root extract control. The position of tyramine is arrowed.

Discussion

The data provides circumstantial evidence that *Agrobacterium* sp. are not involved in the formation of proteoid roots because neither opines nor related compounds were detected in proteoid roots of the test plant, *B. grandis*. The data also suggests that no other bacterium with a similar biology to *Agrobacterium* is associated with proteoid roots because the compound isolated as a putative opine is a non-specific bacterial metabolite, whereas the opine concept calls for a compound which can only catabolised by the inciting bacterium, this was not the case. The compound identified as a putative opine in proteoid roots in early experiments was found to be tyramine [4-(2-aminoethyl) phenol]. Tyramine is the decarboxylation product of tyrosine derived from the shikimic acid pathway and is found in putrefied animal tissue, ripe cheese, ergot (Merck Index) and in various plant tissues. Tyramine is widespread in the plant kingdom (Harbourne, 1973). Tyramine was found in both proteoid and non-proteoid roots grown in aseptic culture and therefore cannot be regarded as an opine. Tyramine was not detected in leaf or shoot tissues. Rapidly-metabolising leaf and shoot tissues would be expected to synthesize a large proportion of phenolic metabolites which occur in the various organs of the intact plant. Translocation from the leaves may be physiologically possible. However, it is probable that aromatic biosynthesis of tyramine takes place in roots, as was evidenced by the HPLC data, and that this involves precursors with probably originate in the leaves in normal circumstances.

Specific roles have been attributed to tyramine in other plants, for example tyramine is a major cell wall compound in the suberized tissues of potato periderm (Borg-Oliver and Monties, 1989), exhibits cytokinin-antagonistic activity in plant cell cultures (Christou and Barton, 1989) and is a deterent to some leaf sucking insects (Kurata and Sogawa, 1976). Tobacco mosaic virus has been shown to stimulate tyramine production (Negrel and Jeandet, 1987) and indeed phenolics generally have a protective function to virus infection in intact plants (Hatherway, 1969). Phenolic compounds often act in unison rather than individually as shown by their allelopathic effect on seed germination (Friend, 1979; Lynn, 1985).

Tyramine was expected to be found as part of the root exudate because of its abundance in the proteoid roots and because its concentration was consistent with the opine concentrations found

in other plant tissues. Tyramine was not detected in the nutrient media assayed. This is perhaps not surprising because it was demonstrated that rhizospere bacteria were able to catabolise tyramine. Tyramine may act as a chelation agent and thereby affect the nutrition of *Banksia* plants. Plants with proteoid roots inhabit low nutrient soils (particularly those low in phosphorus). In such situations iron and phosphorus nutrition is important. The precipitation of Fe phosphates may not only take place in the soil but also in the conductive tissues of the plant (Sideris and Young, 1956; Rediske and Biddulph, 1953). The importance of Fe in both soils and in plants is the way it readily forms organic complexes or chelates (Mengel and Kirkby, 1978). The chelating effect of tyramine could be important in *Banksia* within the plant and possibly in the soil. Roots of some species from low-phosphorus habitats increase the flux of phosphate to the root surface by secreting chelating agents that increase the solubility and mobility of phosphate in the labile phosphate pool (Marschner, 1986). For example, proteoid roots of *Lupinus albus* secrete large quantities of citrate, which increases the solubility of ferric hydroxy phosphate polycations (Gardner *et al.* 1982; 1983). Marschner *et al.* (1986) found in a mixed culture of white lupin (*L. albus*), phosphorus uptake per unit root length of wheat (*Triticum aestivum* Linn.) plants from a soil low in available P is increased, indicating that wheat can take up phosphorus mobilised in the rhizosphere of proteoid roots. The chelation increases the rate of diffusion of these polycations to the root surface, where the iron is reduced releasing the iron and phosphate which can be adsorbed by the root. Grierson and Attiwill (1989) found an unidentified chelation agent in the proteoid root/soil leachate of *B. integrifolia* which may well be tyramine. This still needs to be investigated.

Other possibilities need to be considered when examining the roles of tyramine because it was demonstrated (see Chapter 2) that bacteria isolated from within the proteoid root rhizosphere have the capacity to catabolise tyramine (8 of 15 isolates). Soil bacteria play important roles in plant development. For example, the presence of soil microorganisms strongly affects the fertility of the soil (Bidwell, 1979). Microorganisms may exert their effect by competing with plants for ions that are present in low concentrations (because many of these ions become unavailable in organic form) or alternatively microbial populations may greatly increase the availability of iron, boron or molybdenum (Bidwell, 1979) while root-induced changes in the rhizosphere, may affect mineral nutrition in various ways (Marschner *et al.*, 1986) by for example, the exudation of chelates.

Furthermore, the activity of both plants and microbes in the soil may effect the exchange of ions by altering the pH so that certain elements become more or less available to plants. There are obviously many microbial, chemical and physical changes that may, in a direct or indirect way be involved in the production of proteoid roots. These aspects of microbial/plant interaction have not been looked at during the course of this study.

Chapter 5

Prokaryotic genes encoding IAA biosynthesis in DNA of proteoid roots?

Introduction

The mechanism of action of the phytohormones, auxin and cytokinin, are poorly understood. An intriguing aspect of phytohormones and one which might provide insight into the aetiology of proteoid roots, is their synthesis by numerous microorganisms (Lynch, 1983). Although IAA occurs naturally in plants, the enzymes tryptophan mono-oxygenase and indole-3-acetamide hydrolase, the enzymes responsible for IAA synthesis in prokaryotes, do not occur in plants with the exception of *Agrobacterium* transformed plant tissues. Since these genes are not present in the plant genome they may be of use as molecular probes to identify bacterial genes in plant tissues. If such genes can be detected in DNA from proteoid roots then this would support the postulate that bacteria are involved in proteoid root production.

The methods used in this investigation are those of Southern hybridisation (Southern, 1975). This technique has a requirement for clean, undegraded DNA. Therefore, extraction of DNA from proteaceaous tissues was a prerequisite for the study. A problem in extraction of nucleic acids and proteins from plant tissues is the presence of interfering substances, such as phenols and quinones. These are released from the vacuoles, oxidise rapidly (causing darkening of tissue homogenates) and complex with plant proteins and organelles (Loomis, 1974). For this reason nucleic acid extraction from woody plants has been found difficult (Newbury and Possingham, 1979; Flores *et al.*, 1985). A number of protocols for DNA extraction were evaluated for their suitability for use with proteoid roots. These included the cetyltrimethylammonium bromide (CTAB) method of Murray and Thompson (1980) in which plant material is initially lyophilized. This allows the DNA to be extracted in the presence of detergent and EDTA immediately on hydration, thereby minimizing nuclease degradation. The authors could see no evidence, while using their technique, of polyphenol formation and they inferred polyphenoloxidase activity was inhibited. Techniques developed by Rezaian and Krake (1987) (Medium 2) and Randles and Palukaitis (1979) were also examined. The medium of Rezaian and Krake was designed to counteract the effects of phenolic compounds and oxidants. It contained sodium azide, 2-

mercaptoethanol and polyvinylpyrrolidone to inhibit poly-phenol oxidation. The technique of Randles and Palukaitis incorporates a protease treatment by which it was hoped to increase DNA yield. It also included sodium citrate in the medium to inhibit poly-phenol oxidation.

Materials and methods

Plant material

DNA was extracted from: 1) hairy roots growing on Monnier's medium (Appendix A), initiated on carrot disks with *A. rhizogenes* strain K598 (Chapter 2), 2) hairy roots originating from tobacco leaf disks transformed with *A. rhizogenes* strain K598 (supplied by M. Francki, Waite Agricultural Research Institute), 3) untransformed tobacco leaves (also supplied by M. Francki), 4) proteoid roots from *B. grandis* and *B. ornata* (Chapter 2), and 5) non-proteoid roots of *B. grandis*.

Preparation of plant DNA

Due to the locally abundant supply of healthy proteoid roots of *B. ornata* from a natural woodland near Ashbourne, South Australia (Figure 1.1), attempts were made to extract DNA directly from a freshly collected mat of densely growing roots, sand and other organic matter. The phenol/chloroform technique of Guidet *et al* (1991) for DNA extraction from cereals was used. The extraction procedure used the sand in which the roots had been growing as an aid to grinding and disruption of plant cell walls. This was removed together with other extraneous material during the first phenol/chloroform step. In a separate experiment, but utilising the same root supply, a technique involving the careful separation with tweezers and a dissecting microscope, of roots from the sand and other organic material, was examined. Otherwise the same phenol/chloroform extraction technique was used.

When a plentiful, fresh and clean clean supply of hydroponically grown roots of *B. grandis* (Figure 1.2) became available, they were utilised to evaluate various DNA extraction protocols. These included those previously mentioned in this chapter [phenol/chloroform technique used in

extraction of cereal DNA (Guidet, *et al*), CTAB method of Murray and Thompson (1980), Rezaian and Krake (1987) (Medium 2) and Randles and Palukaitis (1979)]. A successful technique most closely resembling the phenol/chloroform method used in extraction of cereal DNA (Guidet, *et al.*, 1991) was developed. The major difference between this and previous methods was the inclusion of an antioxidant in the extraction buffer and the re-extraction of the chloroform/phenol interphase. The method is outlined below. Fresh plant tissues were ground in liquid nitrogen using a pre-cooled mortar and pestle. The powdered tissues were homogenised in 4 volumes of extraction buffer (0.1M Na_2SO_3, 4% Sarcosyl, 0.1M Tris-HCl, 10mM EDTA, pH 8.0) and immediately extracted (with a volume equal to that of the buffer) with phenol:chloroform:isoamyl alcohol (25:24:1). This was then mixed end over end in an orbital mixer (20rpm) for 1 hour at 4°C. The phases were separated by centrifugation for 5min at 7,000rpm and 4°C (rotor JA10, Beckman J2-21M). The organic phase was discarded and the aqueous phase, together with the interface, were re-extracted as before. The aqueous phase was then transferred to a fresh container using the wide bore of a 10ml pipette and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) for 1 hour at 4°C. The phases were again separated by centrifugation (5min at 7,000rpm, 4°C) and the aqueous phase transferred to a siliconised beaker. One tenth volume of 3M Na-acetate (pH 4.8) was added and mixed. This was followed by 2 volumes of pre-cooled (-20°C) ethanol (99%) carefully layered on top. The mixture was then placed in a -20°C freezer compartment overnight prior to recovery of the DNA by centrifugation at 9,000rpm at 4°C for 10 min. The recovered DNA was washed 4 times with ethanol, partially dried in a gentle stream of air, and resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0) by allowing it to stand overnight at 4°C, followed by gentle inversion also at 4°C.

Southern hybridisation

Approximately 5µg of total plant DNA were completely digested with restriction endonucleases (as recommended by the manufacturer, Boehringer Mannhein), separated on 0.8% agarose gels (20mA overnight) in TAE buffer (0.04M Tris-acetate, 0.001M EDTA, pH 8.3). The restriction endonucleases used in this study were EcoR1, Pst1 and Hind111 and were supplied by

Boehringer Mannhein. The success of the restriction digestion was tested by staining gels with ethidium bromide (1μg/ml) and visualising DNA bands over a UV transilluminator. Successful digestion was indicated by an even distribution of the ethidium bromide stain over the entire length of the lane. The DNA was then transferred to Hybond N^+ membrane (Amersham) using the following procedure (Figure 5.1):

1) The gel were transferred to plastic sandwich box and soaked in 2-3 gel volumes of depurinating solution (0.25M HCl) for 7 min on a rocker platform.

2) After decanting the solution, the gel was rinsed with distilled water and soaked in 2-3 gel volumes of denaturing solution (0.5M NaOH, 1.5M NaCl) for 30 min, agitating as in step 1.

3) After decanting the solution, the gel was rinsed with distilled water and soaked in 2-3 gel volumes of neutralising solution (3M NaCl, 0.5M Tris-HCl pH 7.4) for 30 min, agitating as in step 1 and 2. The gel was rinsed with distilled water.

4) A tray was filled with 20x SSC (3M NaCl, 0.3M Na citrate pH 7.0) in which sponges had been placed. Four layers of Whatman 3MM paper (cut to the size of the sponges) were placed on the sponges and allowed to soak up the 20x SSC.

5) Using the gel base plate as template a gel size hole was cut in Parafilm™ laboratary film and placed on the sponge and filter paper stack in such a way that the film formed a screen bordering the gel.

6) After ensuring there were no air bubbles trapped in the sponge/filter paper stack the gel was placed on the stack within the parafilm hole taking care not to trap air bubbles.

7) A Hybond N^+ membrane was carefully placed on the gel, again excluding air bubbles.

8) Two layers of Whatman 3MM filter paper was the soaked in 20x SSC and placed on the Hybond N^+ membrane.

9) A stack paper tissues (10cm thick) was placed on the top of the stack followed by a glass plate and a 300g weight. The apparatus was left overnight.

10) After dismantling the apparatus the membrane were briefly rinsed with 3x SSC, blotted dry (using Whatman 3MM filter paper) and dried at 65^0C for 20 min.

11) When dry the membrane was exposed to UV light (Phillips TUV 15W tube at a distance of 12cm) for 5 min.

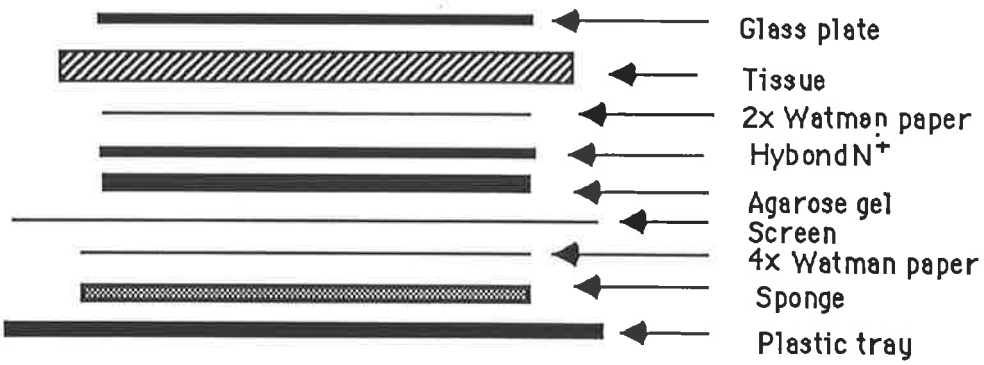


Figure 5.1. Diagram of apparatus used for Southern (1975) transfer.

The membrane (15 x 17cm) was prehybridised in a sealed plastic bag for 4 hours at 65°C with 30ml of a solution containing 3ml 10x Denhardt's 111 (2% gelatin, 2% Ficoll, 2% polyvinylpyrollidone, 10% sodium dodecyl sulfate, 5% tetrasodium pyrophosphate), 6ml 5x HSB (3M NaCl, 0.1M PIPES, 0.02M Na_2EDTA, pH 6.8) and 1.5ml of sonicated and denatured salmon sperm DNA (10mg/ml). Hybridisation was carried out overnight at 65°C with 10ml of a solution containing 2ml 5x HSB, 1ml 10x Denhardt's 111, 100µl freshly boiled salmon sperm DNA (10mg/ml), 4ml dextran sulfate (25% w/v) and the radioactive probe. Probes were labeled with ^{32}P using the 'oligo priming' labelling kit from Amersham (Rapid hybridization system - Multiprime RPN1517). Following the hybridisation, membranes were washed three times with 20min washes at 27°C. The washes were carried out successively as follows; 1) 2xSSC, 0.1% SDS; 2) 1xSSC, 0.1% SDS; and 3) 0.2xSSC, 0.1% SDS. The membranes were exposed to X-ray films (Fuji, NIF) for 2 days at -80°C using intensifying screens.

Molecular probes were supplied by D. Sciaky, Department of Biological Sciences, University of Cincinnati, Ohio, USA. Plasmid 132-10 consisted of a 7.6kb BamH1 fragment of TiC58 (from *A. tumefaciens*) cloned into pBR322. The BamH1 fragment encompassed the loci encoding most of the tryptophan mono-oxygenase and indole-3-acetamide hydrolase. Plasmid 180-1 consisted of an EcoR1 fragment cloned into pBR328 and contained most of the indoleacetamide hydrolase locus. The *vir* region probe (PGV0361) from the*Agrobacterium* TiC58 plasmid used in

dot blots (Chapter 2) is a Hind111 fragment cloned into pBR322 (Depicker *et al.*,1980) was not used in Southern blots and is included here for convenience of reading. The plasmids were grown in *E. coli* strain DH1 and were amplified using 200μg/ml chloramphenicol prior to plasmid isolation. The cDNA clone for barley α-amylase was used as a low copy number control probe and was provided by Dr.P. Chandler, CSIRO, Division of Plant Industry, Canberra. The insert was a 800bp Pst1 fragment in the plasmid pUC18.

Results

Extraction of DNA and Southern blot analysis

The problem of obtaining a plentiful supply of proteoid roots was solved by growing *B. grandis* in hydroponic culture (see Chapter 2). The attempts to extract DNA from *B. ornata* roots together with the sand or by carefully separating roots from sand were unsuccessful. The resulting extracts contained large amounts of black substances attributed to polyphenol oxidase activity. The CTAB method of Murray and Thompson (1980) produced extremely low yields of DNA (ca. 10µg from 10g fresh weight of proteoid roots) which would not cut with restriction endonucleases. A technique developed by Rezaian and Krake (1987) (Medium 2) also gave low yields and poor quality. The Randles and Palukaitis (1979) extraction technique yielded DNA heavily contaminated with black compounds. A satisfactory method described in Materials and Methods gave good quantities (ca. 20µg/10g fresh weight of proteoid roots) of DNA which cut with a range of restriction endonucleases (Figure 5.2).

The nucleotide sequences of tryptophan mono-oxygenase and indole-3-acetamide hydrolase, the prokaryotic enzymes that catalyse the conversion of tryptophan to IAA, were not detected in DNA from proteoid roots using hybridisation at low stringency (membranes were washed three times with 20min washes at 27°C successively in the following solutions; 1) 2xSSC, 0.1% SDS; 2) 1xSSC, 0.1% SDS; and 3) 0.2xSSC, 0.1% SDS). Figure 5.3 shows strong homology between the probe and DNA from transformed tissue of carrot and tobacco. It was important to demonstrate that other genes with low copy numbers could be detected. As a control an α-amylase cDNA clone from barley was used to probe the proteoid root DNA. This should detect the low copies of the α-amylase genes present in the *Banksia* genome. Figure 5.4 shows that the *Banksia* α-amylase genes were easily detectable and that the techniques described are capable of locating low copy gene sequences in the DNA from proteoid roots.

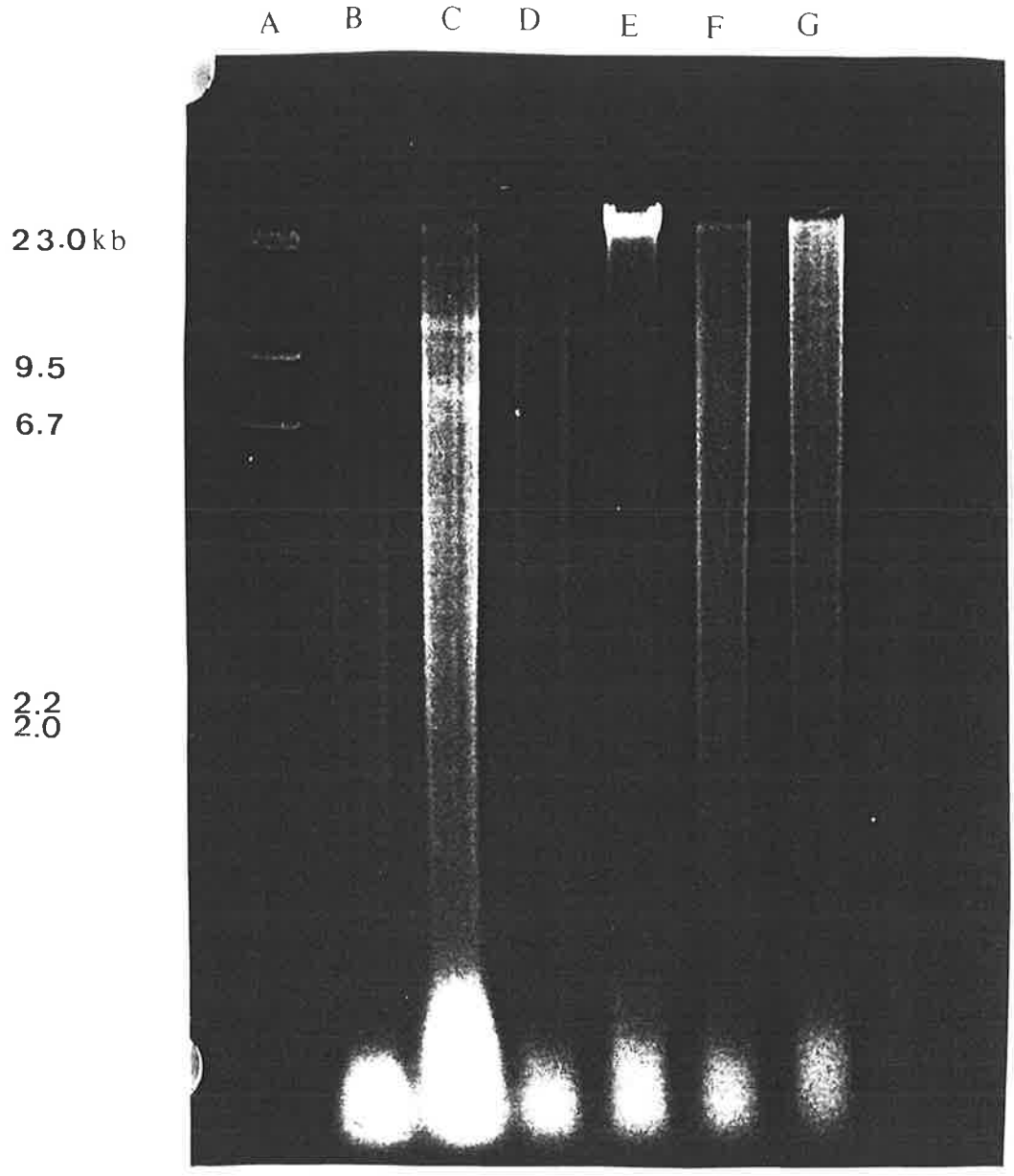


Figure 5.2. Quality of DNA preparations. Ethidium bromide stained agarose gel (0.9%) showing the effect of digestion of DNA with restriction endonucleases. Lanes: A, Lambda DNA digested Hind111 (size marker); B, tobacco leaf DNA digested with EcoR1; C, tobacco leaf DNA digested with Pst1; D, hairy root DNA digested with EcoR1; E, hairy root DNA digested with Pst1; F, proteoid root DNA digested with EcoR1; G, proteoid root DNA digested with Pst1.

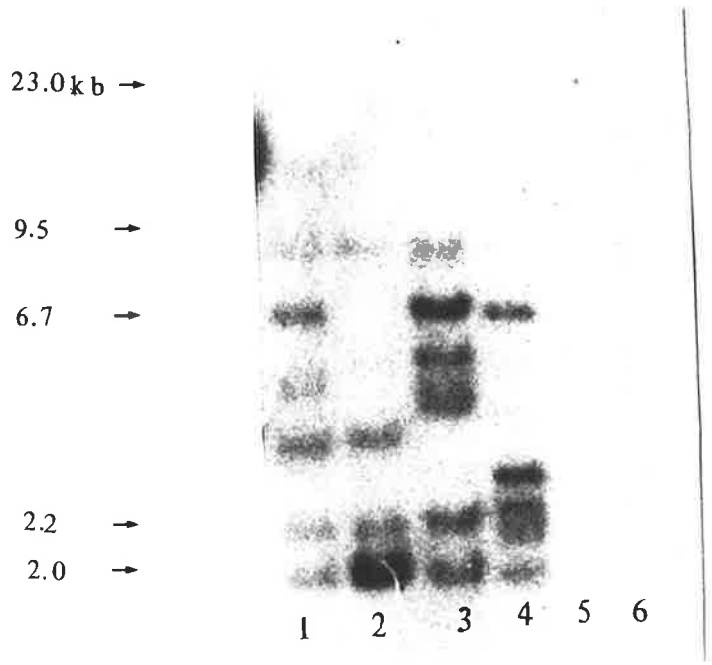


Figure 5.3. Detection of indoleacetamide hydrolase genes. DNA from *B. grandis* proteoid roots (Lanes 5 and 6), carrot (Lanes 1 and 2) and tobacco (Lanes 3 and 4). Lambda DNA digested Hind111 was the size marker. The carrot and tobacco were both transformed with *A. rhizogenes* strain K598. The probe (180-1) is a EcoR1 fragment cloned into pBR328 which contains most of the indoleacetamide hydrolase locus. The restriction endonucleases were EcoR1 (Lanes 1, 3 and 5) and Hind111 (Lanes 2, 4 and 6).

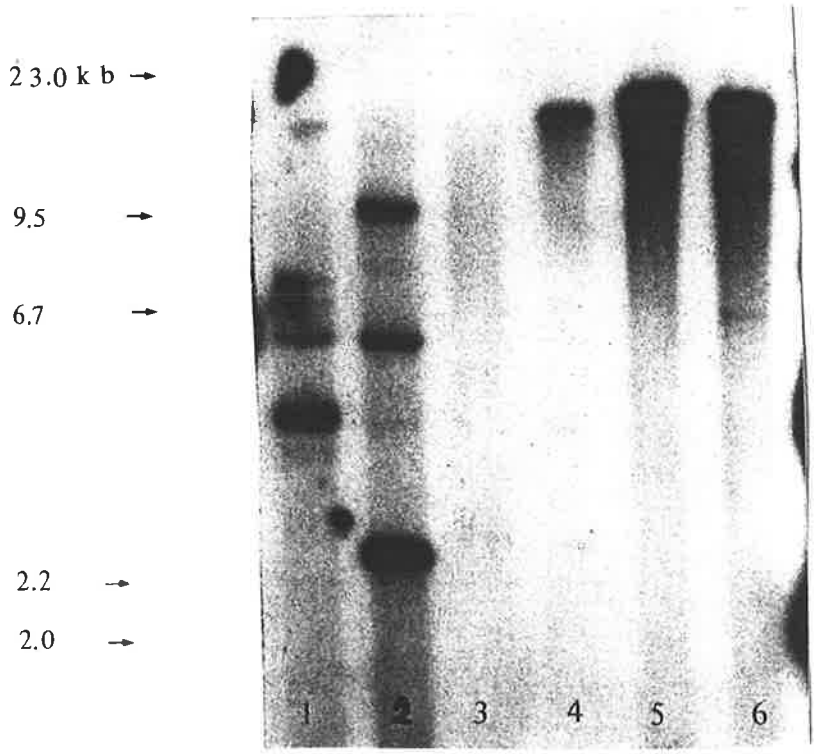


Figure 5.4. Detection of α-amylase genes. Southern blot analysis of DNA from proteoid roots of *B. grandis* (Lanes 5 and 6), tobacco leaves (Lanes 1 and 2) and tobacco leaves transformed with *Agrobacterium* strain K598 (Lanes 3 and 4). Lambda DNA digested Hind111 was the size marker. The probe, α-amylase is a 800bp Pst1 fragment in the plasmid pUC18. The restriction endonucleases were EcoR1 (Lanes 1, 3 and 5) and Hind111 (Lanes 2, 4 and 6).

Discussion

The attempts to use roots that had grown naturally in the field for DNA extraction, were in hindsight, destined to failure. In the case where roots were separated from the soil by hand, there was considerable mechanical damage to the roots during collection and this may have initiated polyphenol oxidase activity. The other technique, in which the root/sand complex was extracted together, was not discriminating with respect of the age of the proteoid roots harvested. The sample probably contained a high proportion of old roots which are not suitable for extraction of clean DNA. Other relevant aspects of the protocol finally adopted were the inclusion of 0.1M Na_2SO_3 to act as an antioxidant and the re-extraction of the interface after the initial extraction with phenol, chloroform and isoamyl alcohol. To the plant biochemist, plant phenols can be a considerable nuisance, because of their ability to complex with protein by hydrogen bonding. When plant cell constituents come together and the membranes are destroyed during isolation procedures, the phenols complex protein and as a result, there is often inhibition of enzyme activity in the crude plant extracts (Harbourne, 1973). Tyramine, due to the high concentrations found in proteoid roots, may have interfered (perhaps in conjunction with other compounds) with DNA extractions. This was suggested because in many DNA extractions a black or reddish pellet formed which was attributed to the interference by phenolic compounds.

This thesis has examined whether proteoid roots are produced in response to an invasive microorganism. Previously published data has found no evidence to support this, indeed investigations have suggested that the roots form as a response to non-invasive microorganisms because microscopic examination of proteoid roots have revealed no microorganisms present in root tissues. However, one of the hypotheses put forward in this thesis allows for the data to be reinterpreted. If an *Agrobacterium* species infected and transformed root tissues then its presence might go undetected within the roots because the bacteria need not be present for the 'disease' symptoms to be observed. Infection could conceivably have taken place at an early developmental stage. Because the DNA sequences used in this study as probes (IAA synthesis genes) are not found in untransformed plants, and are conserved within the prokaryotic kingdom, their use has been a powerful tool in addressing this hypothesis. No foreign sequences coding for IAA synthesis

were found in proteoid roots, this provides strong circumstantial evidence that neither *Agrobacterium* nor another bacterium with similar genes are involved in proteoid root formation.

It should be noted that other prokaryotic DNA sequences could have been used as molecular probes, for example the *rol* genes from *A. rhizogenes* which have been implicated in hairy root disease (Ream, 1989). However, the advantage of using the IAA synthesis genes from *Agrobacterium*, was that these genes are found in many species of bacteria and most importantly do not comprise part of the plant genome.

This study has been important in establishing protocols for working with *Banksia*. The difficulty of extracting clean DNA was overcome by developing a plant culture system which produced healthy clean roots for extraction and the use of an antioxidant in the extraction procedure. This now means that good quality Southern blots can now be produced which has previously not been reported. The production of Southern blots using DNA from *Banksia* can now be used for plant variety identification, plant breeding and studies of the plant genome.

Chapter 6

General Discussion

Proteoid roots are mainly found mainly on proteaceaous plants and consist of clusters of dense bottle-brush-like rootlets which form on lateral roots of plants. In field conditions proteoid roots tend to be concentrated in the top 10 cm of soil and often form a dense continuous mat at the soil/litter interface (Lamont, 1982). Plants with proteoid roots are notable for their ability to grow in soils low in available phosphorus and other nutrients. The conditions at the root/soil interface differ in many respects from those in the bulk soil (Marschner, 1986). Preferential uptake of either ions or water leads to the depletion or accumulation profiles of ions. Proteoid roots may enhance nutrient acquisition by the plant in different ways. The presence of proteoid roots might increase nutrient uptake simply by their increased surface area for nutrient absorption and the larger soil volume which they explore as compared with non-proteoid roots. Alternatively, root exudation of low-molecular-weight organic compounds may mobilise mineral nutrients directly or indirectly by providing the energy substrate for microbial activity in the rhizosphere. The effect of proteoid roots on plant nutrition is important and understanding how proteoid roots form is likely to influence practices for the cultivation of proteaceaous plants.

What triggers the formation of proteoid roots? It is important to note that these root structures do not always form and it is, consequently, presumed that some signal must induce the plant to initiate a radical restructuring of its roots. Since proteoid roots characteristically form in nutrient poor soils and are said to enhance nutrients uptake from the soil, it has been suggested that the nutrient status of the soil acts as the signal to induce proteiod root formation (Groves, 1964; Moore and Keraitis, 1966; Jeffery, 1967). The development of various culture systems for *Banksia* has allowed an assessment of the role of plant nutrition in the induction of proteoid roots. Although the culture media differed considerably (agar, sand and hydroponic culture conditions) the nutrients concentration remained constant. However, hydroponic culture experiments showed that proteoid roots formed well in non-sterile culture. It is significant that the use of a broad range of solid support systems for plant growth failed to influence the formation of proteoid roots. Indeed no correlation can be drawn between the root environment and the type of root system produced.

Nevertheless, the environment in which the plants were growing differed in that the sterile grown plants were grown in poly-carbonate containers which reduced the light and increased the humidity around the leaves. However, it is difficult to conceive of a link between leaf humidity or light intensity and the production of proteoid roots. The major correlation in this series of experiments is the link between non-sterile conditions and proteoid root formation. This strongly supports the contention that microorganisms are involved in the formation of these roots. If there is an environmental effect on proteoid root formation then it may be through its influence on the microorganism population in soil which in turn affects root production.

An involvement of soil microorganisms in the formation of proteoid roots has been postulated by various researchers (Lamont and McComb, 1974; Malajczuk and Bowen, 1974). There are two possible ways in which a microbial interaction with the plant might occur; via infection or invasion of the plant, or through production of plant growth regulators by an organism located in the vicinity of the plant roots. The first type of interaction appears unlikely since no bacteria, actinomycetes and fungi have been detected in newly formed proteoid roots (Purnell, 1960; Lamont,1972; Lamont and McComb, 1974; Malajczuk and Bowen, 1974; Powell, 1975; and Trinick, 1977) although direct visual inspection of stained proteoid roots from inoculated pots showed the rhizosphere to be dominated by bacteria. However, an invasion and subsequent transformation of roots by *Agrobacterium* or another microbe capable of plant transformation, might go undetected by microscopic examination since after the plant has been genetically transformed the microbe need not be present for the resulting morphological phenomenon to be observed. *Banksia grandis* has been used to examine the hypothesis that proteoid roots are formed in response to the genetic integration of bacterial genes into its genome. Hairy root disease and grown gall are examples of such events. However, the prokaryotic gene sequence coding for IAA biosynthesis showed no homology with the DNA of proteoid roots, no opines could be found in the roots and no evidence was found for *Agrobacterium* infection of *Banksia* tissues. These are all characteristics of *Agrobacterium* transformed plants. Although none of these results are conclusive in themselves, taken together they strongly refute the contention of bacterial transformation of the plant. This leaves the proposition that proteoid roots are formed by soil microorganisms which do not invade the root tissues.

If proteoid roots are formed in response to non-invasive microorganisms then it is probably due to the release into the plant rhizosphere of plant growth substances. The finding that *Pseudomonas* species isolated from the rhizosphere of *Banksia* have the genes for IAA synthesis suggest one possible way that microorganisms might influence the production of proteoid roots. A peculiarity of the limited number of plant hormones is that a hormone such as IAA can cause different effects in different plants, or even different effects in the same plant species at different times. Examples of the varied responses of plants to IAA include: stimulation of cell division, stimulation of shoot growth, control of vascular system differentiation, control of apical dominance, delay of senescence, promotion of flowering and fruit setting and ripening (Bandurski and Nonhebel, 1984). Given such varied responses to IAA, it is conceivable that IAA produced by rhizosphere microorganisms may, in certain circumstances, be involved in promoting proteoid root formation. This hypothesis is supported by the observations of Van Staden *et al* (1981) who observed structures similar to proteoid roots on *Protea nerifolia* growing *in vitro* and supplied with synthetic auxins. The involvement of IAA in the production of proteoid roots, however, is likely to be difficult to demonstrate because of the complexity of rhizosphere ecology. Two main plans of experimentation can be adopted to investigate whether or not IAA regulates a process: 1) apply the IAA and observe its effects, and 2) adjust the environmental conditions and relate the concentration of IAA present to the response of the plant. Both of these are unsatisfactory because they only provide correlative data. Furthermore the applied compound may be inappropriate for the tissue at the time chosen, it may not penetrate into the correct cellular compartment, it may be applied in super- or sub-optimal amounts or the response may not be proportional to concentration. Experimentation with growth inhibitors is particularly hazardous because almost any compound will cause inhibition of growth, toxicity and death if applied at a sufficiently high concentration (Milborrow, 1984). If bacteria with genes for IAA biosynthesis are responsible for proteoid root formation by the production of IAA, then the question could be answered using mutants of those bacterial species where the genes encoding the enzymes involved in IAA biosynthesis were inactivated. These could be tested in pot trials of sterile grown and susceptible plants.

Many aspects of the microbial interaction with proteaceaous plants remain open to question. The problems future researchers will need to solve relate initially to aspects of plant culture. The

time required to grow a plant from seed until it produces proteoid roots is approximately four to six months for *B. grandis*. This means there is a considerable lag time for experiments. Further, to grow individual plants in sterile conditions requires a large input of resources. Development of *in vitro* procedures for fast growing plants might be the best approach. *Lupinus* is a faster growing plant that *Banksia* or *Macadamia* but also has proteoid-like roots. However, *Lupinus* produces proteoid roots in sterile conditions and this may make it difficult to relate the results of experiments to the biology of proteaceaous proteoid roots. The culture of *Protea nerifolia in vitro* by Van Staden *et al* (1981) perhaps provides the best clue as to how to approach the problem because they were able to produce proteoid roots in sterile conditions. If adjacent cultures of suitable proteaceous plant tissues (for example *P. nerifolia*) and 'nurse' cultures of bacteria isolated from proteoid root rhizospheres were separated by a filter membrane, it may be possible to investigate the role of diffusable compounds synthesised by certain bacteria in proteoid root production. This method may permit the identification of bacterial species responsible for proteoid root induction and may allow isolation of the compound responsible.

Another factor which might relate to proteoid root formation is the high concentration of tyramine found in the roots of *Banksia*. Growing roots release considerable amounts of organic carbon into the rhizosphere (Marschner *et al.*, 1986). Three major components are involved: low-molecular-weight organic compounds, high-molecular-weight compounds (mucilage) and sloughed-off cells and tissues. This organic carbon may affect the solubility and uptake of mineral elements from the rhizosphere either directly by chelation or indirectly by stimulating microbial activity. The importance of low-molecular-weight organic compounds has been demonstrated by the reduction/chelation of Fe^{111} by phenolics released from the roots of Fe deficient dicots (Römheld and Marchner, 1983). Tyramine isolated from the roots of *Banksia* may function in similar ways. This low-molecular-weight phenolic nitrogen compound was observed from root tissues at concentrations which suggest it comprises a major fraction of the root exudate. Furthermore, it was demonstrated that tyramine can be catabolised by bacteria associated with proteoid roots and may act as a chelating agent and/or have a role of stimulating growth of specific microbes. The unidentified chelating agent found in the soil leachate of *Banksia* by Grierson and Attiwill (1989) may turn out to be tyramine; this needs further investigation.

These findings have suggested new research paths for the study of the biology of proteoid roots. However, there have also been several significant technical advances made through this study. Sterile culture of proteaceaous plants was considered a prerequisite for examining the aetology of proteoid roots. This has lead to advances in the sterile culture of *Macadamia* although there are still problems of initiating roots on these plants. The are no reports of commercial production of *Macadamia in vitro* but the clonal production of selected plants may have important ramifications in the nut industry. Although the sterile culture of *Banksia* was carried out during this study, there are further improvements that can be made in the hydroponic culture systems. Most problems relate to space and the technical difficulty of the sterile culture of individual plants. One of the main reasons for using tissue culture techniques on *Macadamia* was the savings that could be made in these aspects. The non-sterile culture of *Banksia* produced large amounts of root material essential for DNA extraction and biochemical analysis. This is the first report of a procedure for DNA extraction from *Banksia* and may be useful in DNA extractions of other Australian woody plants.

The increasing significance of proteaceaous plants for the floriculture and nut industries has encouraged an expansion of research that will improve our understanding of the biology of this family of plants. Furthermore, the mechanisms evolved by the proteaceous plants to optimise nutrient uptake, may have ramifications for plant nutrition generally.

Appendix A

Culture media

1.Hydroponic solution (Dell *et al.*,1980).

A	$Ca(NO_3).4H_2O$	118g
	$MgCl_2.6H_2O$	51g
	$CaCl_2$	37g
B	K_2SO_4	87g
C	KH_2PO_4	6.8g
D	H_3BO_3	0.93g
	$CuSO_4$	0.14g
	$MnSO_4$	0.21g
	$NaMoO_4.2H_2O$	0.02g
	$ZnSO_4$	0.07g
	$CaSO_4$	0.05g
E	EDTA NaFe	9.3g
	dist. water	to 1 litre

2. LB medium (Maniatis *et al.*, 1982).

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
dist. water	to 1 litre

Adjust to pH 7.5 with NaOH.

3. Bergersen's salts (Bergersen, 1961).

$Na_2HPO_4.12H_2O$	0.45g
$MgSO_4.7H_2O$	0.1g
$CaCl_2$	0.04g
$FeCl_3$	0.02g
thiamine	1.0mg
biotin	0.25mg
dist. water	to 1 litre

Bergersen's medium may be supplemented with 2 g/l mannitol and 1 g/l $(NH_4)_2SO_4$. The salt solutions were stored as 5x concentrates.

4. Monnier's salts with Morel's vitamins (Monnier, 1976; Morel and Whetmore, 1952).

KNO_3	1.9g	
$CaCl_2.2H_2O$	0.88g	
NH_4NH_3	0.825g	Use 100ml/l of
$MgSO_4.7H_2O$	0.37g	a 10x stock
KCl	0.35g	solution.
KH_2PO_4	0.17g	
Na_2EDTA	14.9mg	Use 2ml/l of a
$FeSO_4.7H_2O$	11.1mg	500x stock solution.
H_3BO_3	14.4mg	
$MnSO_4.H_2O$	33.6mg	
$ZnSO_4.7H_2O$	21.0mg	Use 2ml/l of a
KI	1.66mg	500x stock
$Na_2MoO_4.2H_2O$	0.5mg	solution.
$CuSO_4.5H_2O$	0.05mg	
$CoCl_2.6H_2O$	0.05mg	
Ca pantothenate	1.0mg	
myo-inositol	100.0mg	Use 2ml/l of a
biotin	0.01mg	500x stock
nicotinic acid	1.0mg	solution
pyridoxine	1.0mg	(filter-sterilise
thiamine	1.0mg	stock separately).
sucrose	30g	
agar	7g	
dist. water	to 1 litre	

Adjust to pH 6 with NaOH.

5. D1 agar (Kado and Heskett, 1970).

mannitol	15.0g
$NaNO_3$	5.0g
LiCl	6.0g
$Ca(NO_3)_2.4H_2O$	2.0mg
K_2HPO_4	2.0g
$MgSO_4.7H_2O$	0.2g
bromthymol blue	0.1g
agar	15.0g
dist. water	to 1 litre

The medium has a pH of 7.2 after autoclaving and appears dark blue.

6. King's B medium (King, *et al.*, 1954).

Proteose peptone #3 (Difco)	20.0g
K_2PO_4	1.5g
$MgSO_4.7H_2O$	1.5g
agar	15.0g
glycerol	15.0ml
dist. water	to 1 litre

7. MS salts and vitamins (Murashige and Skoog, 1962).

NH_4NO_3	165.0g/l
KNO_3	190.0g/l
$MgSO_4.7H_2O$	37.0g/l
$MnSO_4.H_2O$	1.69g/l
$ZnSO_4.7H_2O$	0.86g/l
$CuSO_4.5H_2O$	0.0025g/l
$CaCl_2.2H_2O$	44.0g/l
KI	0.083/lg
$CoCl_2.6H_2O$	0.0025/lg
KH_2PO_4	17.0g/l
H_3BO_3	0.62g/l
$NaMoO_4.2H_2O$	0.025g/l
$FeSO_4.7H_2O$	2.784g/l
$Na_2.EDTA$	3.724g/l
thiamin.HCl	0.1g/l
nicotinic acid	0.05g/l
pyridoxine.HCl	0.05g/l
glycine	0.20g/l
myo-inositol	10.0g/l

These are 100x the final concentration. Use 10ml of each of the above stock solutions for preparing 1 litre of the culture medium.
$NaH_2PO_4.7H_2O$ is 150mg/l added directly to the medium as supplement (Hartman and Kester, 1983).
30% MS salts (3ml of each salt stock in 1 litre of the culture medium) was used for proliferating shoot cultures of *Macadamia* with full strength organic nutrients (10ml of each stock for preparing 1 litre of medium).

8. Woody plant medium (Anderson, 1975).

NH_4NO_3	40.0g/l
$Ca(NO_3.4H_2O)$	56.6g/l
K_2SO_4	99.0g/l
$MgSO_4.7H_2O$	37.0g/l
$MnSO_4.H_2O$	2.23g/l
$ZnSO_4.7H_2O$	0.86g/l
$CuSO_4.5H_2O$	0.0025g/l
$CaCl_2.2H_2O$	9.6g/l
KH_2PO_4	17.0g/l
H_3BO_3	0.62g/l
$NaMoO_4.2H_2O$	0.025g/l
$FeSO_4.7H_2O$	2.784g/l
$Na_2.EDTA$	3.724g/l
thiamin.HCl	0.1g/l
nicotinic acid	0.05g/l
pyridoxine.HCl	0.05g/l
glycine	0.20g/l
myo-inositol	10.0g/l

These are 100x the final concentration. Use 10ml of each of the above stock solutions for preparing one litre of the culture medium.

9. NB medium.

nutrient broth (Difco)	8.0g
dist. water	to 1 litre

10. KL medium (Bernaerts and DeLey, 1963).

lactose	10.0g
yeast extract	1.0g
agar	12.0g
dist. water	to 1 litre

Appendix B Bacteria

Bacteria	Strain	Opines	Plasmid
A.rhizogenes (Biovar 2)	K565	Catabolic and anabolic for agropine, mannopine, mannopinic acid, and agrocinopinic acid	pArA4(pAr A4a), pRiA4(pAr A4b)
A.rhizogenes (Biovar 2)	K568	Catabolic and anabolic for manopine and mannopinic acid	data not available
A.rhizogenes (Biovar 1)	K596	Catabolic and anabolic for agropine	data not available
A.rhizogenes (Biovar 1)	K597	Catabolic and anabolic for cucumopine	pRi2655
A.rhizogenes (Biovar 1)	K598	Catabolic and anabolic for cucumopine	pRi2657
Agrobacterium tumefaciens (Biovar 1)	K301	Catabolic and anabolic for octopine and agropine	pTiAch5
A.radiobacter (Biovar 1)	K303	No catabolic and anabolic activity	none
A.tumefaceins (Biovar 1)	K750	No catabolic and anabolic activity	none
Pseudomonas fluorescens	K110		
P.syringae	K321		
Escherichia coli	HB101		none
Micrococcus lutea			

*Bacteria and the above mentioned data came from the collection of Professor A.Kerr, Department of Plant Pathology, Waite Agricultural Research Institute.

Sample preparation: delivered as membrane filtered (0.45µm) samples then diluted 1:1 with 0.4M potassium borate buffer.

Standard preparation: Stock of standard tyramine (760µg/ml) diluted 1/16 with deionised H_2O, then diluted 1:1 with potassium borate buffer as above.

Buffer	A	25mM Na acetate pH 6.8; 9% Methanol; 1% Tetrahydrofuran.					
	B	95% MeOH.					
Gradient	min.	0	10	13	16	20	30.
	%B	50	62	100	100	50	50.

Run time: 25 min.

Flow rate: 1µl/min.

Injection parameters: sample volume 5µl.
reagent volume 5µl.
injection volume 5µl.

Derivatizing reagent: 25mg o-phthaldehyde (Sigma).
2ml Methanol (Ajax).
0.5ml potassium borate 0.4M, pH10.4.
25µl Brij 35 (Pierce Chemical Company) 30% solution.
13µl 2-mercaptoethanol (Sigma).

Derivatizing reagent is diluted 1:3 with potassium borate buffer ready for use.

Detector gain: 8.

Column Temp. 29^0C.

Column: Brownlee RP 18 precolumn HPLC Technology 3µm Techsphere ODS 2.

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