In vitro propagation of *Banksia brownii*, *B. coccinea* and *B. grandis* and possibilities for its use in Dieback (Phytophthora spp.) research

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Preface

Working in Western Australia for 5 months was a wonderful opportunity, not only because I learned much about phytopathology but also about a new country which is indeed a different continent. Therefore, I would like to thank the people who made it possible for me to work at CALM, Como as a part of my thesis Phytopathology. I would like to thank my supervisors for their help and support and also all the people at Como Research who made life in Australia very pleasant.

Jacco Duindam

Abstract

The Dieback disease in Western Australia, caused by the introduction of the pathogenic fungus *Phytophthora cinnamomi* can cause enormous damage. Economical damage occurs as the Jarrah tree (*Eucalyptus marginata*), an important forestry product, is highly susceptible to Dieback. Environmental damage occurs because of the irreversible changes in the composition of plant communities in an infected area.

Many plant species are susceptible to *P. cinnamomi*. Some are even threatened with extinction because in nature their last locations are infected. One of the threatened species is *Banksia brownii*, a very rare plant in south-west Australia. To save the genotypes of the present populations for the future, *in vitro* storage and cryopreservation are being explored. *In vitro* techniques can also assist Dieback research. Both in vitro propagation of *B. brownii*, *B. coccinea* and *B. grandis* and *in vitro* techniques were a part of this research.

In vitro germination of *B. brownii*, *B. coccinea* and *B. grandis* seed at 15°C overcame the sterilisation problems and provided material for further research. *B. coccinea* cotyledons gave callus formation on both 1/2 and 1/4 MS (Murashige & Skoog, 1962) media with different concentrations of 2,4-D (0.1 and 0.5 mg/l) and BA (0, 0.1 and 0.5 mg/l). *B. brownii* cotyledons gave some (10-30%) callus formation on 1/4 MS with different concentrations of 2,4-D and BA. *B. grandis* cotyledons had the ability to form callus-like structures on 1/2 and 1/4 MS media with different concentrations of 2,4-D and BA but callus of *B. grandis* could not be isolated. Calli from *B. coccinea* were isolated and grown separately. Most calli stayed alive but they did not grow vigorously.

Shoots and leaves of *B. brownii* could be sterilised and grown *in vitro*. Axillary buds of *B. brownii* were forced to form new shoots in 1/4 MS media supplemented with different BA concentrations (0.1, 1.0, 5.0 and 10.0 mg/l). The new shoots did not grow vigorously although some formed acceptable shoots. Callus was also formed on about 10% of these shoots, especially at the higher BA levels. This callus could be isolated and grown separately in 20% of the cases. The leaves formed callus on 1/3 and 1/6 MS media in some instances. *B. coccinea* leaves and shoots could not be sterilised because of contamination problems relating to the abundant hair growth on leaf and shoot surface.

Callus from these experiments was eventually to be used in Dieback research but better media for both callus formation and callus isolation and growth are necessary to provide a good working system.

In vitro grown seedlings of *B. grandis* were inoculated *in vitro* with zoospores and mycelium (solution) of different isolates of *P. cinnamomi* and *P. citricola*. Two isolates of *P. citricola* (HSA 1211 and 1450) do not affect *B. grandis* where as the *P. citricola* isolate JW 20 and *P. cinnamomi* isolate SC 72 do affect *B. grandis*. However, the seedlings were affected by all isolates. Penetration of the epidermis and colonisation of the root surface was abundant by all isolates but no example was found where penetration of the epidermis was followed by penetration of the root tissue. A brown layer of cells was formed underneath the epidermis and dark cells appeared after some days. Dissolving of the tissue between vascular system and endodermis was

observed in some cases by the isolates JW 20 and SC 72. Hyphae, growing inter- and intracellular, were found in small numbers by all isolates but a direct connection between hyphae and symptoms could not be found.
The symptoms seemed to be caused by a physiological and/or biochemical reaction of the plant to the penetrating fungus. However, the roots were colonised to a great extent and in the end the roots died. The symptoms could therefore be influenced by this dying of the roots. The system of inoculating *in vitro* grown seedlings was probably too artificial to get a good comparison between the different isolates.

Oospore formation was found in the presence of host (*B. grandis* and *B. attenuata* seedlings) and fungus (*P. megasperma*) in the soil under laboratory conditions. *In vitro* methods can therefore be used to predict the suitable conditions for oospore formation (of *P. megasperma*) in the field.

1 Introduction

1.1 *Phytophthora cinnamomi*

Since the discovery of Australia by Europeans, a great number of exotic live forms have been introduced to this continent. Australia, being separated from other main continents for millions of years, has developed its own characteristic nature with many endemic species. Too often, an introduced specie becomes a pest for native species.

The introduced fungus *Phytophthora cinnamomi* Rands is the cause of the Dieback disease in Western Australia (Podger, 1972). This Dieback disease threatens great parts of the native bushland and forests as (economical) important species, as the Jarrah tree (*Eucalyptus marginata* Donn. ex Smith) and *Banksia* species, are highly susceptible to *Phytophthora cinnamomi*. Irreversible changes in the composition of plant communities appear in an infected area. A changing plant community has a great effect on the ecosystem of the whole area and in this way not only plants are affected. About 14% of the Jarrah forest is affected by *P. cinnamomi* (Campbell, pers.comm.).

Phytophthora cinnamomi is a soil-borne fungus belonging to the Oomycetes. It is a pathogenic fungus which requires plant tissue as food. The life cycle of *P. cinnamomi* depends on moist conditions which favour survival, sporulation and dispersal. When conditions are warm and moist, sporangia and chlamydospores are produced from mycelium. The sporangia release motile zoospores in free water to infect host roots. Mycelium of different mating types (A1 and A2) can grow together and form long lasting oospores (Shearer & Tippet, 1989). The suitable warm and moist conditions occur in Western Australia normally in autumn and spring. Although *P. cinnamomi* is favoured by moist conditions, it is found throughout (Western) Australia, even in parts that have a low annual rainfall.
After being released, zoospores that find their way, active or passive, to roots of susceptible plants can encyst and penetrate. Penetrated roots will rot preventing water and nutrient uptake and in that way the plant, eventually, dies (Shearer & Tippet, 1989). Because of the presence of the fungus in the soil, *Banksia* species and other susceptible plants will not be able to grow on the same spot again. By 1980, Australian research had provided nearly half of the 1000 species known worldwide as being susceptible to *P. cinnamomi* (Zentmeyer, 1980).

Other fungi of the Phytophthora family also proved to be a (introduced) pest. *P. citricola, P. cryptogea, P. drechsleri, P. megasperma* var. *megasperma, P. megasperma* var. *sojea* and *P. nicotianae* have been recovered from the Jarrah forest (Shearer *et al*, 1991). They all have a wide host range and a dependency on moisture to complete their life cycles (Shearer & Tippet, 1989). Research to examine their impact on Australian nature is going on (Shearer, 1992).

1.2 *Banksia*'s

Banksia species are members of the diverse Proteaceae family. The Proteaceae family is spread over all (sub)tropical parts of the word, but mainly in the southern hemisphere. Australia has the greatest diversity with about 860 species of Proteaceae (George, 1986).

In Australia, 75 *Banksia* species are found. Their distribution is widespread along the coastal areas but eastern and western Australia have only one specie (*B. dentata*) in common which is also the only specie found outside Australia.
The genus *Banksia* varies from prostate plants to trees of about 25 meter. They have beautiful flowers which are important for honey eaters. These flowers produce a cone in which normally only a small number of seeds are produced. These seeds remain in the cone until a fire releases them, or until they are released after maturation or because the plant is dying. After a fire, which plays an important role in Australian nature, some species (like *B. brownii* and *B. coccinea*) die and relieve on the released seed for further propagation. Other species can survive fire or regenerate from their roots.

Banksia species are often regionally restricted and adapted to live in a special ecological niche which makes them very vulnerable. *Banksia* species are susceptible to *Phytophthora cinnamomi* although resistance is found in some of the eastern species. After being decimated by land clearing for agricultural purposes, some are now being threatened with extinction from nature by Dieback.
Some *Banksia* species are suspected of spreading *P. cinnamomi* through their root system. One of the control measurements to protect the Jarrah forest against a rapid spreading of Dieback is to burn the highly susceptible *B. grandis*, an important understorey specie in the Jarrah forest, very frequent (Shearer *et al*, 1988). *B. grandis* is killed by fire and needs a couple of years to regenerate from seed, form a new plant, flower and have seed ready for the next fire. *B. grandis* populations are destroyed by frequent burning and even a low degree of resistance against *P. cinnamomi* would be welcome. Resistance within *B. grandis* populations could be tested *in vitro*.

Banksia brownii is found at only 18 different locations. Most sites have a small number of plants so there are few left in their original habitat (Taylor & Hopper, 1988). All the sites are already infected with *P. cinnamomi* so conservation must be done quickly in order to avoid extinction from nature (Shearer, 1992). As, at present, there is no protection from *P. cinnamomi*, the genotypes from the 18 *B. brownii* populations should be stored to preserve their genetic variability. Cryopreservation techniques to store seeds and *in vitro* cultures of Australian native plants are presently being investigated. For success the technique requires homogenous tissues, for example callus, or young shoots which are frozen and stored in liquid nitrogen until required. In Kew Gardens the cryopreservation of seed is being examined while *in vitro* cryopreservation is being investigated at Kings Park, Perth. So far, cryopreservation has succeeded for a very rare *Grevillea* (Proteaceae family), which shoots can be stored and later successfully used to grow a new plant (Dixon, pers.comm.). Cryopreservation has a number of advantages above alternatives as grafting (McCredie *et al*, 1985[c]) or constant multiplication *in vitro*. Cryopreservation is much cheaper and there is almost no chance that the genotypes of the stored plants change during the process.

Through tissue culture, *B. brownii* can be conserved and additionally multiplied. This has already been done for other endangered Australian species (Williams et al., 1985; Williams & Taji, 1987, Bunn et al., 1989; Taji & Williams, 1991, Dixon, pers.comm.). For conservation through cryopreservation, callus tissue and shoots are the best options. The search for resistance against *P. cinnamomi* in the *B. brownii* population (and other species) is also possible with the use of tissue culture techniques, using callus or clones to search for resistance. The use of callus as a model for resistance in the mother plant has been described for a number of plants. Correlation between

resistance in callus cultures and resistance in the mother plant was found in several cases but not everywhere.
Successful attempts to grow other Proteaceous plants in vitro have been reported (Ben-Jaacov & Jacobs, 1986; Seelye et al., 1986; Kunisaki, 1989 & 1990). So far, attempts to grow *B. brownii, B. coccinea* and *B. verticillata in vitro* have failed (Barbour, pers.comm.). Bunn (1991) claimed some success with the tissue culturing of other *Banksia* species (*B. dentata, B. menziesii* and *B. hookeriana*) but he couldn't produce longliving plants (Bunn, pers.comm.). Shoots from *B. brownii* are difficult to sterilise as the hairs on the stem surface of *B. brownii* prevent the contamination under them from being reached by the sterilisation solution. The seeds of *B. brownii* (and other species) are difficult to grow *in vitro*. Although sterilisation is successful on the seeds, the germination of the seeds and the growth of the young plants are the major difficulties.

The aims of this research are to find a way to grow and multiply *B. brownii in vitro* and to find a way of using *in vitro* techniques to contribute to the research of the Dieback disease.

2 Materials and methods

Media preparation

The basal medium used for the tissue culturing of Banksias consisted of half-strength (1/2) Murashige & Skoog (1962) (MS) salts. The different components (appendix 1[a]) were mixed prior to the addition of hormones (if required), sucrose (25 g/l) and agar (7 g/l; Sigma Cell Culture agar: A-1296, Lot 121H00925, 9002-18-0). The pH was set at 5.6 using HCl or NaOH to adjust the pH if necessary. Twelve ml of medium was added to 2.6 x 8 cm polycarbonate tubes with a polypropylene cap and autoclaved for 20 minutes.
Polycarbonate jars (6.2 x 7.8 cm, with polypropylene cap) were filled with 20 ml vermiculite and 15 ml deionised water. Two filter papers were placed on top of the vermiculite. The jars were autoclaved for 30 minutes. Jars with sand (60 ml) and water (15 ml) were autoclaved twice, 30 minutes each, with 24 hours in between. Deionised water was autoclaved for 60 minutes if this water was needed in the sterilisation process. Autoclaving was done at 121°C and 103.5 kPa.

Seeds

Seeds of *Banksia* spp. were sterilised in a 1:1 (v/v) alcohol (95%) and hydroxenperoxide (H_2O_2, 30%) for 20 (*B. grandis*) or 15 (*B. attenuata, B. coccinea* and *B. brownii*) minutes. Afterwards they were put directly on the medium.

Seedlings

Cotyledons were taken from seedlings who had fully developed, green cotyledons without true leaves. The cotyledons were cut from the seedlings close to the bud and cultured with their cutting edge in the media. The seedcoat was removed if it still covered some of the cotyledon, in this case the covered part had to be green. Tubes with cotyledons, which were placed in the dark, were covered with aluminium foil and placed in the same culture room as the light treatment. Cotyledons, which were cut in two, were cultured either with their long edge or their short edge in the media (Appendix 4).

Banksia brownii plants

Banksia brownii plants were grown in an greenhouse and a shade house. The greenhouse was shaded and held at an maximum of 25°C with the help of evaporative cooling equipment. Six plants were purchased from a local nursery. They were 2 years old and had an average hight of 50 cm. Five plants were grown in the CALM nursery at Dwellingup. They were 3 years old and had an average hight of 80 cm. The 12 plants in the shade house were 3 years old and had an average hight of 70 cm.

Plant material

Leaves and young shoots from *B. brownii* plants were successfully sterilised by rinsing them for 4 hours in tap water and sterilising them in 2% NaOCl and 0.5% Tween 70 for 10 minutes. Finally they were rinsed 6 times in autoclaved deionised water for 5, 5, 10, 10, 10 and 15 minutes.

Liquid media

Liquid medium was made according to Bunn (1991). This medium contained 1/2 MS, 20 g sucrose and 2.25 mg/l BA and had a pH of 6.0 or a pH of 5.6. Another 1/2 MS medium with 0.5 mg/l 2,4-D (pH 5.6) was also used. The media were placed on an orbital shaker (80 revolutions per minute) which received no light. Four cotyledons were cultured in a 100 ml glass containing 50 ml medium.

Callus

Callus was isolated and grown on the medium on which it was formed. Calli from both *B. coccinea* (from 1/2 MS media) and *B. brownii* were also isolated and cultured on 1/4 MS medium supplemented with 0.1 mg/l 2,4-D and 0.1 mg/l BA.

Inoculations

Sterile zoospore solutions (Appendix 1^d) and sterile mycelium solutions were inoculated in a hole 1 cm under the agar surface, made with a pipette. The sterile pipette was used to remove the agar without damaging the roots. The zoospores and mycelium solution were inoculated with a sterile syringe. The average number of zoospores per water drop was found by counting the zoospores in 10 drops of zoospore solution. About 150 zoospores per plant were inoculated. The mycelium solution was made by adding 5 x 10 mm mycelium, grown on uncleared V8-agar (appendix 1^b), to 10 ml of sterile water. The mycelium was cut from the border of a growing fungus. The number of mycelium parts was counted in 10 water drops after 2 minutes of vigorous shaking. About 40 pieces of mycelium per plant were inoculated. Some plants were inoculated with a piece of mycelium (1 x 5 mm) which was put 1 cm under the agar surface, close to the root. This mycelium was cut from the edge of a growing fungus.

Root sections

Roots of seedlings of *B. grandis*, growing in inoculated agar, were washed and stored in water prior to their use. Transverse sections of the roots were cut with a razorblade. The sections were immediately stained with a red stain (3% erythrosine in 10% NH_4OH). This stain was used to colour the fungus. The sections were washed with water after 20 minutes to remove the stain. They were transferred to a glycerine solution on a object glass and covered with a cover glass.

Oospore formation

Three seeds per jar of *B. attenuata* and *B. grandis* were sown and germinated at 15°C. After 48 days, the number of seedlings per jar for *B. grandis* was reduced till 2, the number of seedlings per jar for *B. attenuata* was reduced till 1. This reduction was necessary because not all seeds had germinated. They were placed at 25°C afterwards. They were inoculated by placing an (clear) V8 agar disk (diameter 5 mm; Appendix 1^c) with *P. megasperma* 5 mm from the seedling on the sand after 20 days at 25°C.
The sand was sieved using a tier of sieves (1000 µm, 250 µm, 106 µm, 63 µm and 38 µm). The roots were stained using 2 methods (Appendix 5). Both sand and roots were microscopically examined.

Growth room

The 25°C (±1°C) growth room, in which most explants were placed after culturing, had one fluorescent (Thorn power saver, 36 W white, 1 K, made in Australia) which was placed 33 cm above the surface (50 cm width) on which the tubes and jars were placed. The light level varied between 18.7 and 25.4 μmol $s^{-1}m^{-1}$ on both borders and 33.4 μmol $s^{-1}m^{-1}$ right under the fluorescent. The explants received 14 hours light per day. The 15°C (±1°C) growth room had 2 fluorescents which gave 12 hours light per day. The light level varied between 15 and 5 μmol $s^{-1}m^{-1}$.
The results were analysed with the help of the computer program Microsoft Excel version 4.0.

3 Results

3.1 Seed germination

3.1.1 *Banksia grandis*

To investigate the germination of *B. grandis in vitro*, tubes and jars were used. The tubes contained one seed each, the jars contained 5 seeds each equally divided over the surface of the filter paper.
The germination at three different temperatures (15°C, 20°C and 25°C) was examined and the treatments light/dark and tubes/jars were compared (Table 1). The trial started on 22 September.

Table 1: Treatments used to examine the germination of *Banksia grandis* (numbers 1-10 are the different treatments)

temperature	tubes		jars	
°C	light	dark	light	dark
15	1	2	6	7
20		3		8
25	4	5	9	10

The treatments 1 to 5 contained 22 tubes each, the treatments 6 to 10 contained 6 jars each. The treatments 1, 2, 6 and 7, placed at 15°C, had the best germination (Figure 3) after 21 days. All treatments were put at 15°C/light after 21 days. The germination was also counted 7 and 14 days after this transfer. The results are shown in Figure 1.

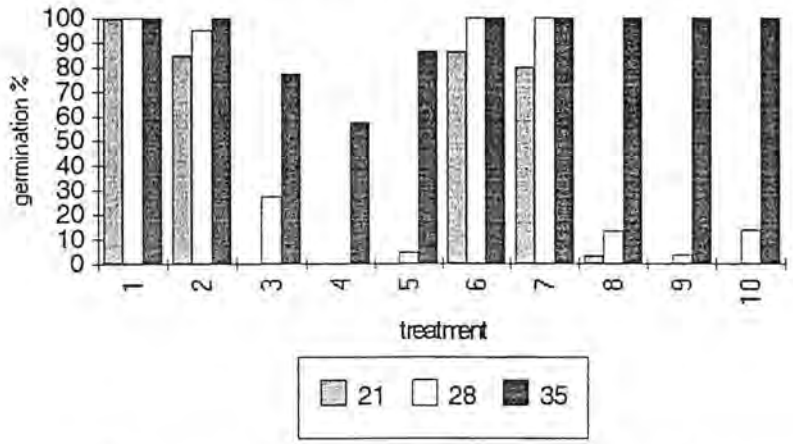

Figure 1: The germination of *Banksia grandis* for the 10 treatments counted after 21, 28 and 35 days.

As displayed in Figure 1, the germination of the treatments 1, 2, 6 and 7 was very good after 21 days. The other treatments gave almost no germination after 21 days so there was a clear temperature effect. Fourteen days after the transfer to 15°C/light, the germination of most treatments was comparable. Treatment 4 had a germination which was lower than the others. In general there was no difference between the germination in tubes (treatments 1 to 5) and in jars (treatments 6 to 10). Light or dark did not seem to influence the germination either.

3.1.2 *Banksia coccinea*

In order to see if the results of the trial with *B. grandis* could be repeated with another *Banksia* specie, *B. coccinea* seeds were used in a second germination trial. Because there was no difference between tubes and jars in the first germination trial, only tubes were used in the second germination trial. The second germination trial (Table 2) was comparable with the first germination trial.

Table 2: Treatments (1 to 5) used to examine the germination of *Banksia coccinea*

temperature	tubes	
°C	light	dark
15	1	2
20		3
25	4	5

Twenty-two *B. coccinea* seeds per jar were sown on 1/2 MS medium on 13 October. All the treatments were put at 15°C/light after 21 days. The germination was counted before and after the transfer of all treatments to 15°C/light. The results are shown in Figure 2.

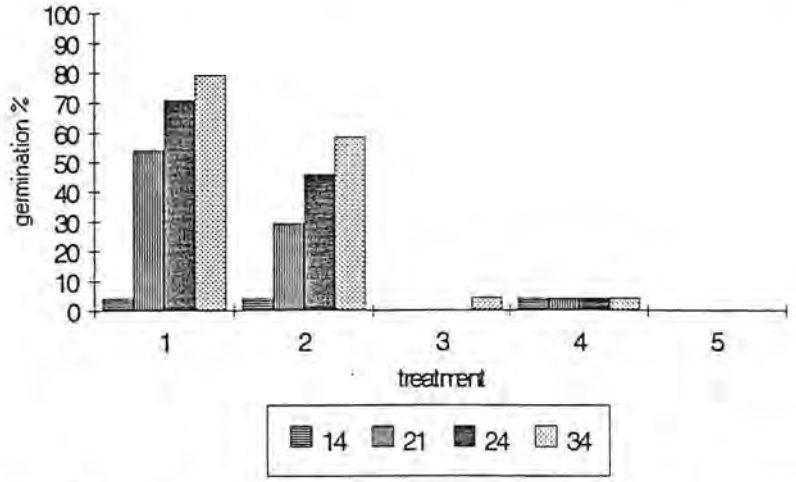

Figure 2: The germination of *Banksia coccinea* for the 5 treatments counted after 14, 21, 24 and 34 days.

As displayed in Figure 2, germination after 21 days occurred almost only by the treatments 1 and 2. Both treatments were put at 15°C, this result is therefore comparable with the results found by the germination of *B. grandis* (Figure 1). There was no difference between dark and light in this trial which was also comparable with the results found by *B. grandis* (Figure 1). The germination of the treatments 3, 4 and 5 did not improve after the transfer to 15°C/light. *B. grandis* seed of treatments comparable with the treatments 3, 4 and 5 of *B. coccinea* gave normal germination after their transfer to 15°C/light (Figure 1). The treatments 1 and 2 still germinated after 21 days, so germination was still possible. The germination results of the treatments 1 (80%) and 2 (60%) after 35 days were not as high as the results of *B. grandis* (Figure 1). *B. coccinea* seed can have a germination of more than 90% (Bathgate, pers.comm.). The seeds used in this trial came from cones who were repeatedly heated in an oven (50°C) for various time periods on and after 18 August 1992 (Collins, pers.comm.), 8 weeks before sowing. The cones released their seeds

after soaking in water for 3 days and drying in the sun. The repeated heating of the cones probably damaged the seeds which led to relative low germination rates.

3.1.3 *Banksia brownii*

The best germination of *B. grandis* and *B. coccinea* seed occurred at 15°C. Forty seeds of *B. brownii* were sown in tubes on 1/2 MS medium and put at 15°C/light. The germination of this seed was counted after 14, 21 and 34 days. They were sown on 13 October. The results are shown in Table 3.

Table 3: The results of the germination of 40 *Banksia brownii* seeds at 15°C/light counted after 14, 21 and 34 days.

time (days)	germinated
14	8
21	35
34	35

As displayed in Table 3, seed of *B. brownii* gave a good germination after 21 days (87.5%) at 15°C/light. Seed germination of *B. grandis*, *B. coccinea* and *B. brownii* (*in vitro*) can easily be done at 15°C/light.

3.2 Seedling growth

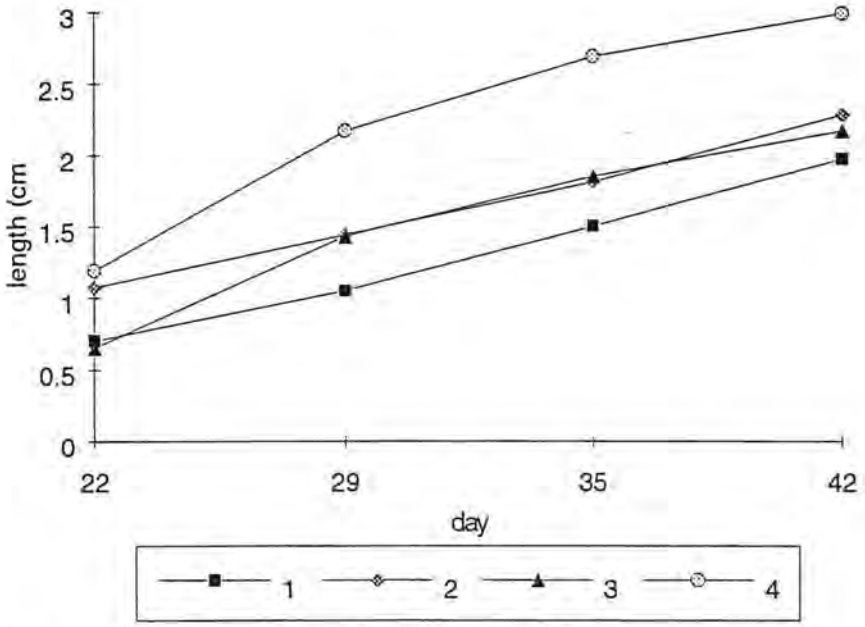

Figure 3: The root length of *Banksia grandis* seedlings between 22 and 42 days after sowing. The different treatments are:
1: root length under 1.0 cm, left at 15°C
2: root length between 1.0 and 1.6 cm, left at 15°C
3: root length under 1.0 cm, placed at 25°C (14-10)
4: root length between 1.0 and 1.6 cm, placed at 25°C (14-10)

Seedlings of the seed germination trial with *B. grandis* (paragraph 3.1.1) were used to examine growth and development conditions after germination. The seedlings were taken from the treatments 1 and 2, which were sowed on 22 September. The seedlings were individually followed over a period of 21 days in which the length of the roots was measured (Appendix 2). This period started on 14 October. The length of the roots was measured because germinating *B. grandis* seeds start by forming a root prior to the unfolding of the cotyledons. This unfolding can take some time while the growth of the roots will continue. The length of the roots is therefore a measure for the development of the seedling in the period before the unfolding of the cotyledons and, later, the formation of the first true leaves.
Twenty-one germinated seeds from treatment 1 and 16 seedlings from treatment 2 were followed. Eleven germinated seeds of treatment 1 and 8 germinated seeds of treatment 2 were left at 15°C/light while the rest was placed at 25°C/light. There was no difference between the two treatments so the results, as displayed in Figure 3, are the averages of the treatments 1 and 2.

The results of the individual plants (Appendix 2) showed a big variation so the plants in Figure 3 are divided in two groups: the plants with roots up to 1.0 cm and the plants with roots of 1.0 cm or longer. The two groups contained each about half the plants. The best development of the average root length of *B. grandis* seedlings is at 25°C for both groups. Treatment 4 gives the best average root length after 42 days. The difference between both groups (3 and 4) placed at 25°C is bigger after 42 days so plants with roots over 1.0 cm and placed at 25°C give the best average root growth. Because they give the best average root growth they are also likely to give the best plant development. The seeds were sown on the same day but there was difference in germination. It is possible that seeds, who germinated late and therefore had a short root after 3 weeks, had a low vigour. This low vigour could explain (some of) the differences after 42 days between both groups. The individual seedlings of both groups show a big variation so a low vigour by seedlings with a root length under 1.0 cm is not likely. Growth conditions in the 15°C and the 25°C room were not the same so this could explain (some of) the differences between both temperatures. Still, the best growth and development conditions seems to be at 25°C for plants with roots of 1.0 cm or longer.

3.3 Cotyledons

3.3.1 Cotyledons of *Banksia grandis*

Young parts of seedlings, such as root, hypocotyl and cotyledon, often have a high capacity to form callus. If the seeds are germinated and grown *in vitro* they are also free of contamination. In this way plants, who don't form callus easily and/or are difficult to sterilise, can form callus. This callus can be used for multiplication or for other research purposes.

Cotyledons, from seed used in the germination trial, were used to investigate the capacity of *B. grandis* to form callus. The cotyledons were cut from the seedlings and cultured in 6 different media with their cutting edge in the media. Different concentrations of the hormones 2,4-D (a synthetical auxin) and BA (a cytokinin) were added to the media. The concentrations were based on different papers dealing with the formation of callus in woody species (Pratap-Narayan & Jaiswal, 1985; Sasamoto *et al*, 1989; Stoehr *et al*, 1989; Tewary *et al*, 1989). 2,4-D is known to induce callus

formation but it can also induce mutations. BA is known to induce growth and development but it can also lead to callus formation (Pierik, 1985). The media, which were used, are given in Table 4.

Table 4: The different media (0 to 6) used to induce callus formation on the cotyledons of *Banksia grandis*

		BA (mg/l)		
		0	0.1	0.5
2,4-D (mg/l)	0	0		
	0.1	1	2	3
	0.5	4	5	6

The first trail started on 15 October when 4 cotyledons per medium were used. On 20 October, a bigger trial with 8 cotyledons per medium was started. When the cotyledons were examined for callus growth, structures who looked like callus were found. The number of structures per cotyledon were counted on 3 different dates. The results of the trial, started on 20 October, are shown in Figure 4 and 5.
Most media in Figure 4 give an improvement in the percentages of structures after the first week. Medium 4 seems to be the best medium with the other media having percentages of cotyledons with a structure of approximately 40%. Some cotyledons died (partly) during the trial so the number of structures can decrease since only structures who looked viable were counted. The average number of structures for the cotyledons who had structures was more than 2.5 after 27 days for the media 1, 3, 4 and 6 (Figure 5). The control medium 0 (Appendix 3^a) gave 2 structures after 7 days and 3 structures after 27 days. These structures appeared to be of a different kind compared to those who were formed by the media 1 to 6. For that reason, the results of medium 0 are not shown in Figure 4 and 5.

The structures formed by the media 1 to 6 looked like callus but they had a brown colour (instead of the normal green colour) and they did not continue to grow after approximately 3 weeks. Some structures formed (root)hairs, something which happened by the media 1 to 6. The control medium 0 gave structures who looked like roots and not like callus. These roots were never found with the other media. The structures, formed by the media 1 to 6, are therefore a result of the added hormones. The trial started on 15 October (Appendix 3^b) gave results who are comparable to the results shown in Figure 4 and 5.

The same media (Table 4) were used to investigate if cotyledons who are cut in two and placed in different positions gave better results. Ten cotyledons were used for each medium. If they were cultured with the small edge in the media they had their 'normal' upright position. If they were cultured with their long edge in the media they were 'lying' down (Appendix 4). This trial was started on 21 October. The formed structures were counted after 6, 13 and 26 days. There was not much structure formation (Table 5) and no difference was found between both culturing methods (not shown). Most of the cotyledons had died after 26 days.

From the trials with *B. grandis* started on 15, 20 and 21 October can be concluded that *B. grandis* cotyledons have the capacity to form callus-like structures. It seemed that the media with 2,4-D and without BA gave the best results. To examine if higher 2,4-D concentrations led to better results, the media shown in Table 6 were used. Medium 5 is added because Bunn (1991) claimed some success with 2.25 mg/l BA by other

Banksia species (the media are further comparable). The trial was started on 5 November. A part of the cotyledons (6 cultured cotyledons for the media 1, 2 and 7; 7 cultured cotyledons for the media 3 and 4) was put in the dark to examine what effect it had on the callus formation. The results of the cotyledons who were put in the dark are given in Figure 6 and 7. The results of the cotyledons (10 per medium), who were put in the light, are given in Figure 8 and 9.

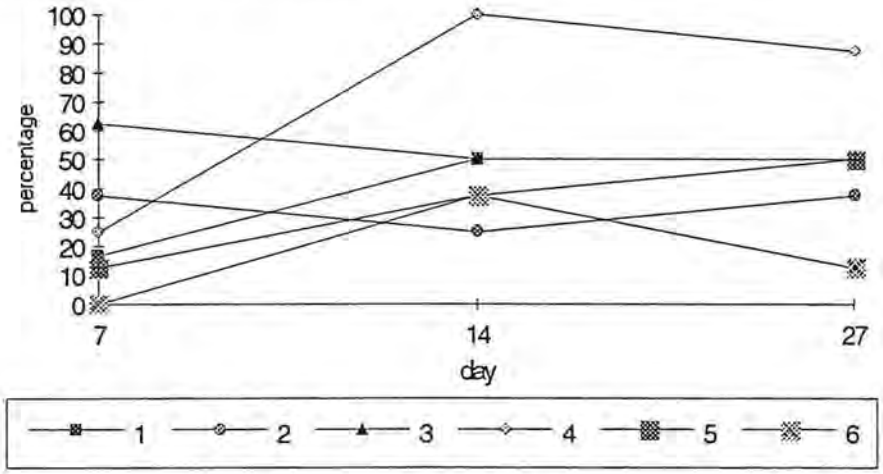

Figure 4: The percentage of cotyledons with a structure counted after 7, 14 and 27 days by 6 different media.

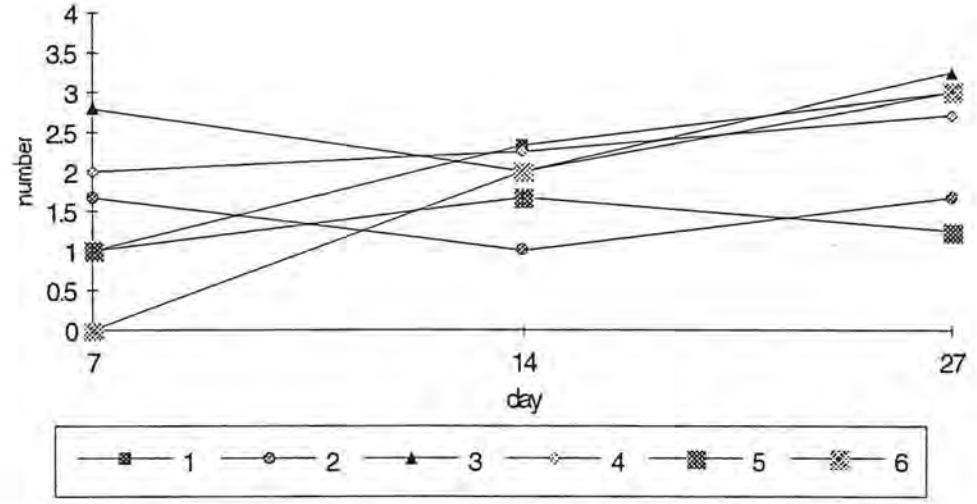

Figure 5: The average number of structures, for the cotyledons who had structures, counted after 7, 14 and 27 days by the 6 different media.

Medium 1 gives the best results (100%) in Figure 6. The other media, except medium 4, give also good results. The highest 2,4-D concentration, medium 4, gives the lowest percentage of structures. Media 2 and 3 differ not much. It seems that a higher 2,4-D concentration does not give a higher percentage of structures. The number of used cotyledons, though, is very small. In Figure 4 is also a comparison between the media 1 and 2 (0.1 and 0.5 mg/l 2,4-D) possible. Media 1 and 4 in Figure 4 are the same as media 1 and 2 in Figure 6. Medium 4 gives better results then medium 1 in Figure 4. The concentrations 0.1 and 0.5 mg/l 2,4-D seem therefore better than the concentrations 1.0 and 1.5 mg/l 2,4-D with not much difference between the

concentrations 0.1 and 0.5 mg/l 2,4-D. Medium 5 gives a high (84%) and constant percentage of cotyledons with structures.

Table 5: The results of the trial started on 21 October with *Banksia grandis* cotyledons. The percentage (%) of the plants who formed a structure, the average number of the structures (#) for the cotyledons who formed structures and the total number of viable cotyledons (sum) are given.

	6 days			13 days			26 days		
media	%	#	sum	%	#	sum	%	#	sum
0	0		9	0		8	40	1.3	4
1	0		8	10	1	8	40	2.5	5
2	0		8	10	1	8	0		3
3	0		8	20	1	8	20	1	3
4	20	1	10	40	1.8	8	0		0
5	0		8	10	1	8	0		1
6	14	1	7	0		7	25	1	4

Table 6: Media (1 to 5) used to examine the capacity of *Banksia grandis* cotyledons to form callus

	2,4-D	BA
0.1 mg/l	1	
0.5 mg/l	2	
1.0 mg/l	3	
1.5 mg/l	4	
2.25 mg/l		5

Figure 7 shows the average number of structures for the cotyledons who had structures. The media differ not very much with 2, 3 and 4 higher than 1 and 5. Medium 5 has an average of 1 by all 3 dates. The cotyledons cultured in medium 5 gave a typical kind of structure. It looked more like a stem than like a callus-structure. Every cotyledon gave only one of those stem-like structures, on a place close to where the bud had been. Medium 1, who had a high percentage of cotyledons with structures, had a low average number of structures.

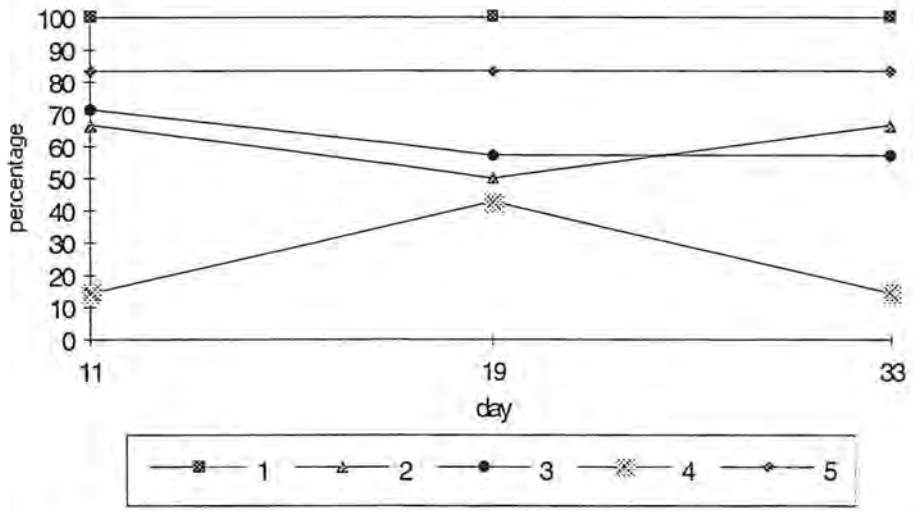

Figure 6: The percentage of cotyledons with a structure counted after 11, 19 and 33 days by 5 different media. The cotyledons were placed in the dark.

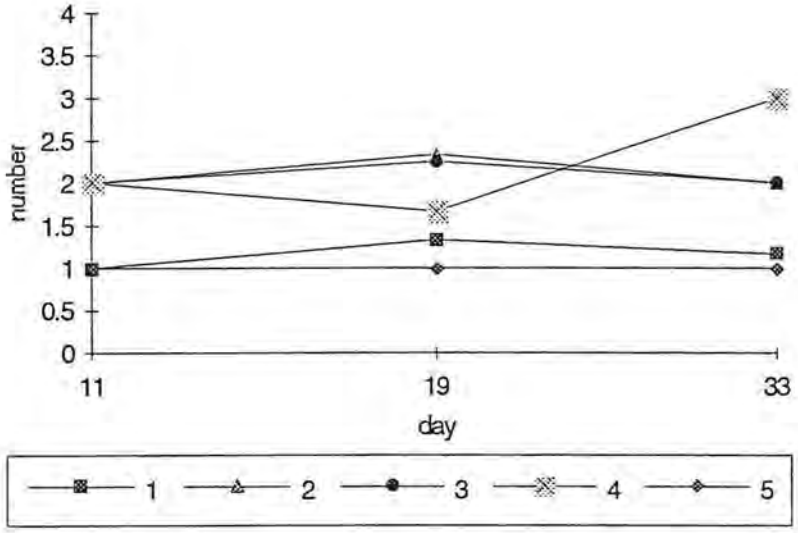

Figure 7: The average number of structures for the cotyledons who had structures counted after 11, 19 and 33 days by the 5 different media. The cotyledons were placed in the dark.

All media in Figure 8 give comparable results. The media 2, 3, 4 and 5 give percentages of cotyledons with structures ranging generally between 50% and 80%. Medium 1 starts low (40%) but increases and ends on 100%. The average number of structures, for the cotyledons who had structures, is high (Figure 9) for medium 4 (ranging from 2.3 to 3.3), lower for media 2 and 3 (ranging between 1.3 and 2.6) and the lowest for media 1 and 5 (ranging between 1 and 2). Medium 5 gave two cotyledons with respectively 2 and 4 stem-like structures. The media 1 to 4 gave callus-like structures comparable with the ones formed in the dark.

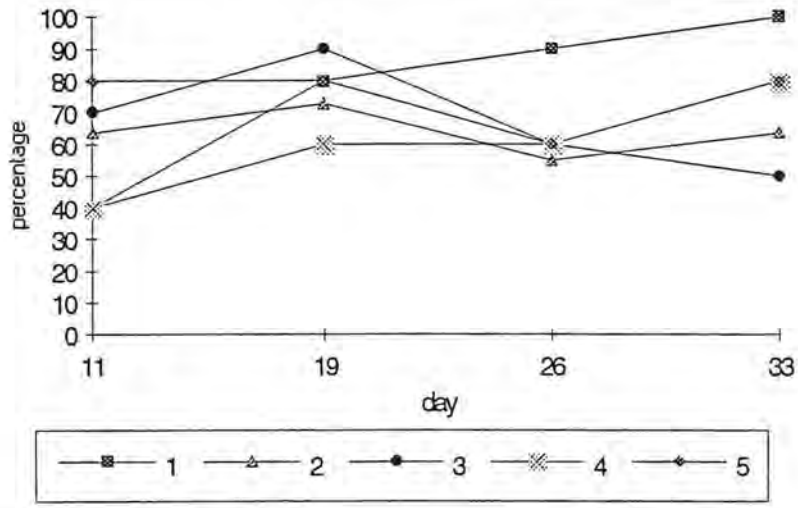

Figure 8: The percentage of cotyledons with a structure counted after 11, 19, 26 and 33 days by 5 different media. The cotyledons were placed in the light.

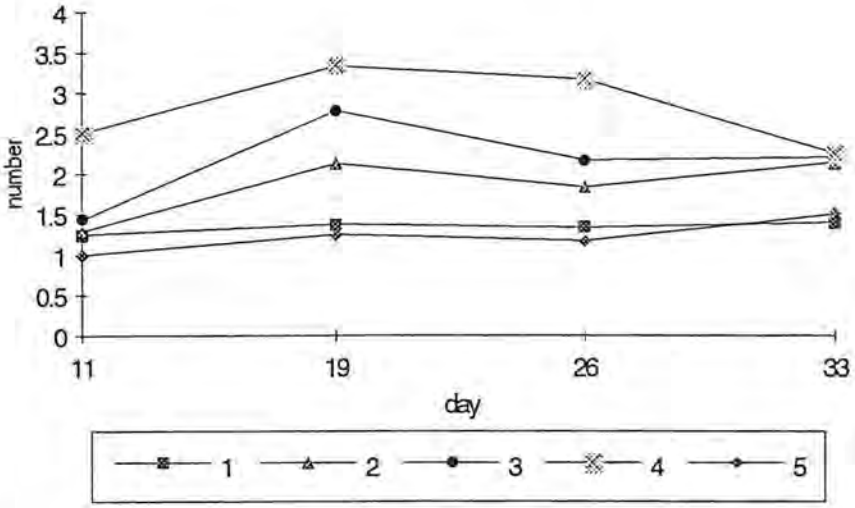

Figure 9: The average number of structures for the cotyledons who had structures counted after 11, 19, 26 and 33 days by the 5 different media. The cotyledons were placed in the light.

A comparison between Figure 6 and 8 shows that the percentage of cotyledons who formed structures is comparable in both figures. Medium 4 in Figure 6 gives very low results compared with the other results in the Figures 6 and 8. This, and other differences, could be a result of the relative small number of cotyledons used. A comparison between Figure 7 and 9 shows that the average number of structures, on cotyledons who formed structures, is also comparable in both figures. Medium 4 in Figure 7 gives low results compared with medium 4 in Figure 9. The other media differ not much.

Seedlings of *B. grandis* were cultured after their cotyledons had been cut off in the same medium in which they had been grown. They could form a complete plant without cotyledons.

An increase in 2,4-D concentration does not lead to a higher percentage of cotyledons with structures (Figure 6 and 8). The number of structures, for the cotyledons who had structures, seemed to be higher by the increased 2,4-D concentrations in light (Figure 9). The callus-like structures who were formed in this trail generally stopped growing after approximately 3 weeks. The media which led to the higher percentages of cotyledons with structures seem therefore more important than the media which led to a better structure growth. The 2,4-D concentrations (0.1 and 0.5 mg/l), who were used in earlier trials, were therefore better than the increased 2,4-D concentrations (1.0 and 1.5 mg/l). Cotyledons, who were cultured in the dark, did not give better results than the cotyledons cultured in light.

Cotyledons of *B. grandis* were cultured in 1/4 MS media. The concentrations 2,4-D and BA of the media 0 to 3 from Table 4 were used to compare 1/4 and 1/2 MS media. The trial was started on 7 January when 15 cotyledons per medium were cultured. The results are shown in the Figures 10 and 11.

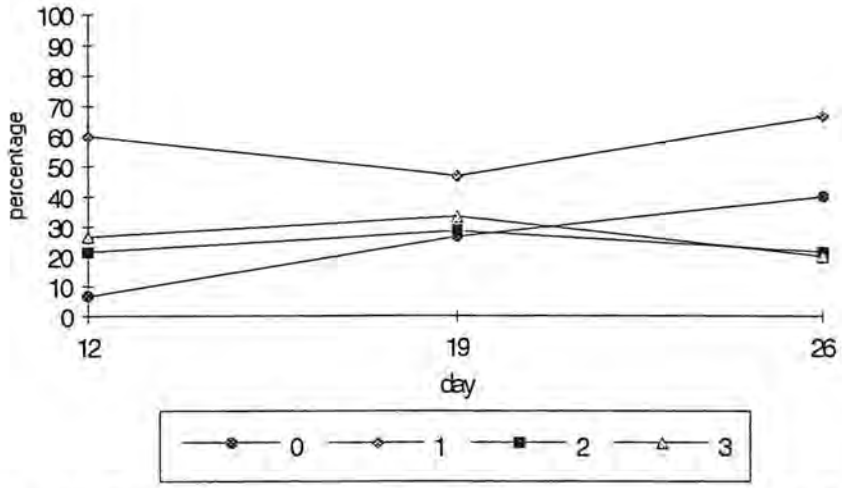

Figure 10: The percentage of cotyledons with a structure counted after 12, 19 and 26 days by 4 different media.

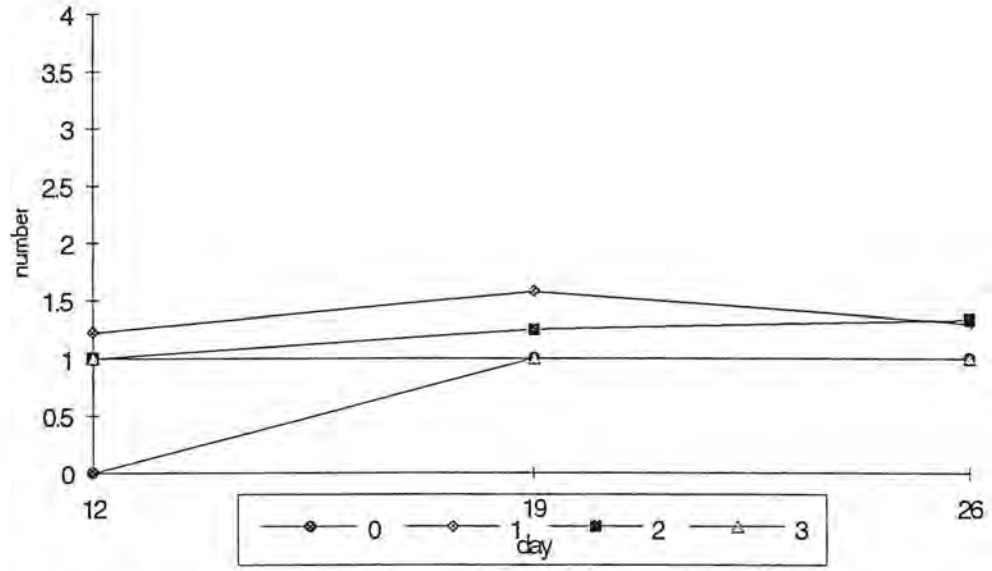

Figure 11: The average number of structures for the cotyledons who had structures counted after 12, 19 and 26 days by 4 different media.

Figure 10 gives lower results for the media 2 and 3 than Figure 4. Medium 1 gives comparable results in Figure 4 and 10. The average number of structures (Figure 11) is low compared to Figure 5. Medium 0 gave some roots during these trials but not the callus-like structures which were formed by the media 1 to 3.
Half MS media (Figure 4 and 5) gave better results than 1/4 MS (Figure 10 and 11) media.

3.3.2 Cotyledons of *Banksia coccinea*

Cotyledons of *B. coccinea* were used to examine their ability to form callus. The same media (1 to 6, Table 4) were used as in the trials with *B. grandis*. The trial was started on 2 December when 6 cotyledons per medium were cultured. The results are shown in the Figures 12 and 13.

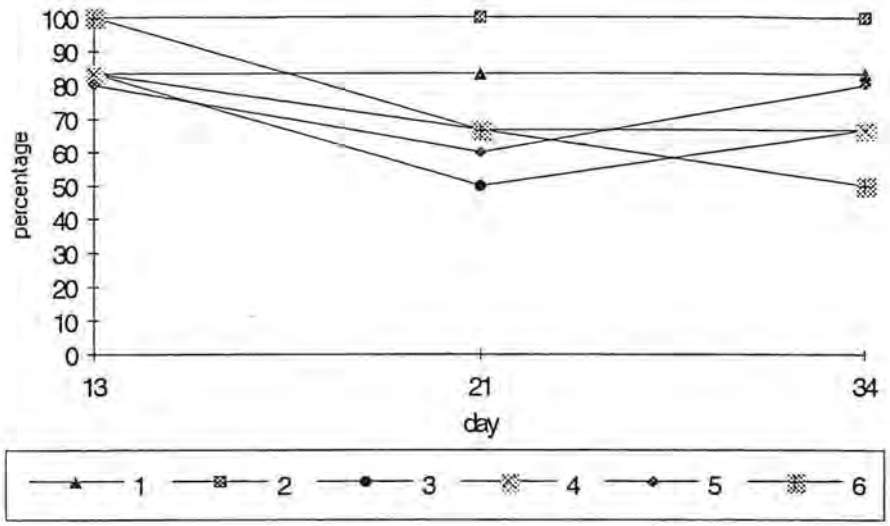

Figure 12: The percentage of cotyledons with a structure counted after 13, 21 and 34 days by 6 different media.

Figure 12 gives percentages of cotyledons with a structure between 50% and 100%. These results are comparable with the results found with cotyledons of *B. grandis* in Figure 8. Medium 2 is the best medium with a constant 100% score. The rest of the media are comparable although medium 6 decreases from 100% to 50%. The average number of structures, for cotyledons who had structures, is comparable for the media 2 to 5 with most of the average values between 1.5 and 2.5 (Figure 13). Medium 1 increases from 2.2 to 3.6. *B. grandis* gave in comparable circumstances (Figure 9) lower values with most points between 1.0 and 2.3.

Seedlings of *B. coccinea* were cultured after their cotyledons had been cut off in the same medium in which they had been grown. They could form a complete plant without cotyledons.

The structures who were formed during this trial looked like callus. They had a brown green colour and some of them continued growing. They had an irregular shape and were formed on the cutting edge. Some hypocotyls, who were also cultured, formed also callus-like structures both on the top and the rear end of the hypocotyls (results not shown).

Cotyledons of *B. coccinea* were cultured in 1/4 MS media. The concentrations 2,4-D and BA of the media 0 to 3 (Table 4) were used to compare 1/4 MS and 1/2 MS media. The trial started on 7 January and the results are shown in Figure 14 and 15.

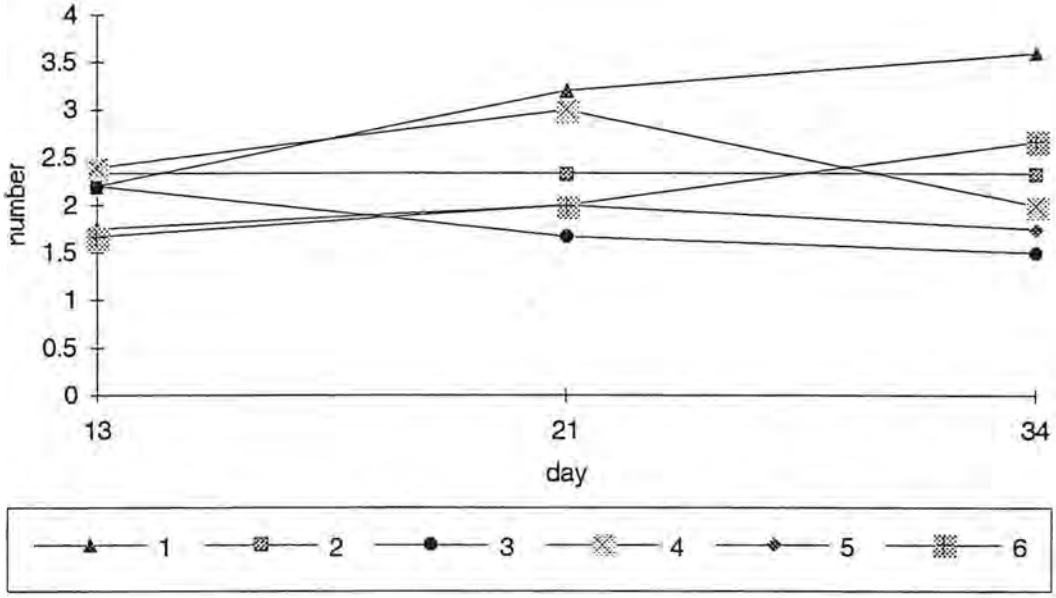

Figure 13: The average number of structures for the cotyledons who had structures counted after 13, 21 and 34 days by 6 different media.

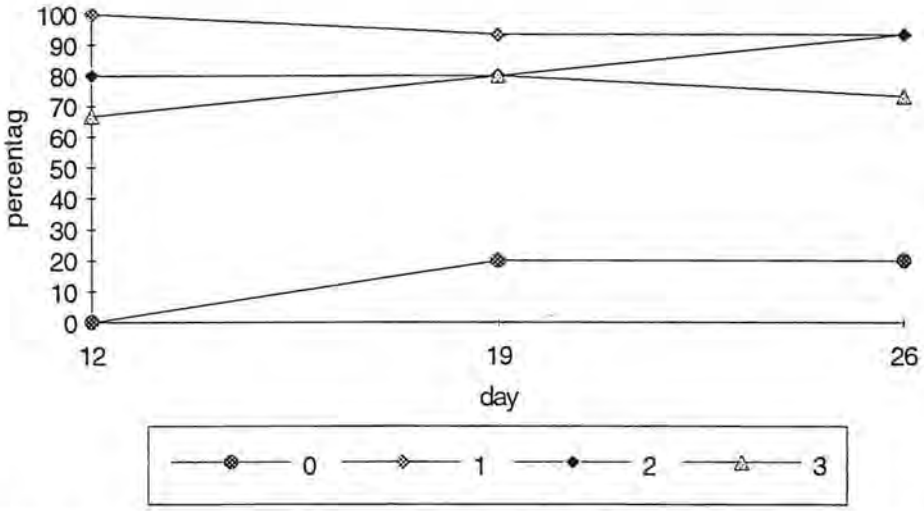

Figure 14: The percentage of cotyledons with a structure counted after 12, 19 and 26 days by 4 different media.

Figure 14 gives the same results for the media 1 to 2 as Figure 12. Medium 3 gives lower results in Figure 12 but the difference is small. The results in Figure 15 are lower than in Figure 13. Medium 0 (Figure 15) gave some roots but not the callus which was formed on the cutting edge of the cotyledons by the media 1 to 3.
From Figure 12, 13, 14 and 15 can be concluded that *B. coccinea* has the ability to form callus. The structures who grew from the cutting edge of cotyledons resembled the expected callus-like structures. The percentage of cotyledons with a structure and the average number of structures for cotyledons who formed a structure are both quite high for both 1/2 MS and 1/4 MS.

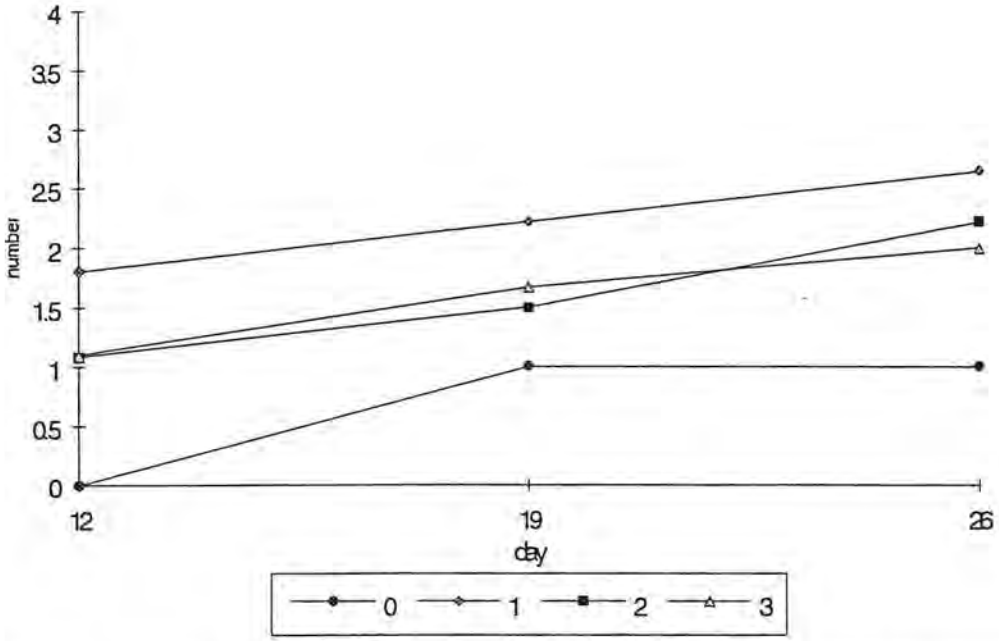

Figure 15: The average number of structures for the cotyledons who had structures counted after 12, 19 and 26 days by different media.

3.3.3 Cotyledons of *Banksia brownii*

Cotyledons of *B. brownii* were used to examine their ability to form callus. The same media (1 to 6, Table 4) were used as in the trials with *B. grandis*. The trial was started on 2 December when 6 cotyledons per medium were cultured.
All the cotyledons were dead when they were checked after 13 days. The cotyledons had turned black. They did not form anything before they died.

A 1/4 MS media and different concentrations of 2,4-D and BA were used in a second trial. The concentrations 2,4-D and BA of the media 0 to 3 (Table 4) were used. Sixteen cotyledons were cultured in medium 0 and 1. Fifteen cotyledons were cultured in medium 2 and 3. The trial was started on 10 December. The results are shown in Figure 16 and 17.

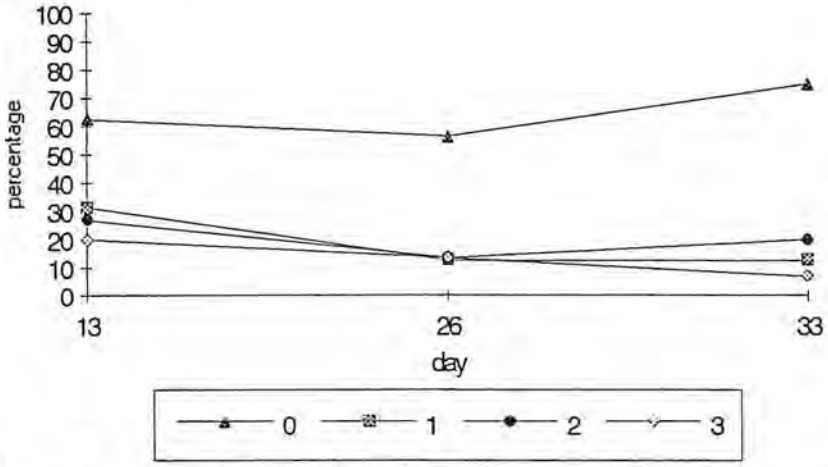

Figure 16: The percentage of cotyledons with a structure counted after 13, 26 and 33 days by 4 different media.

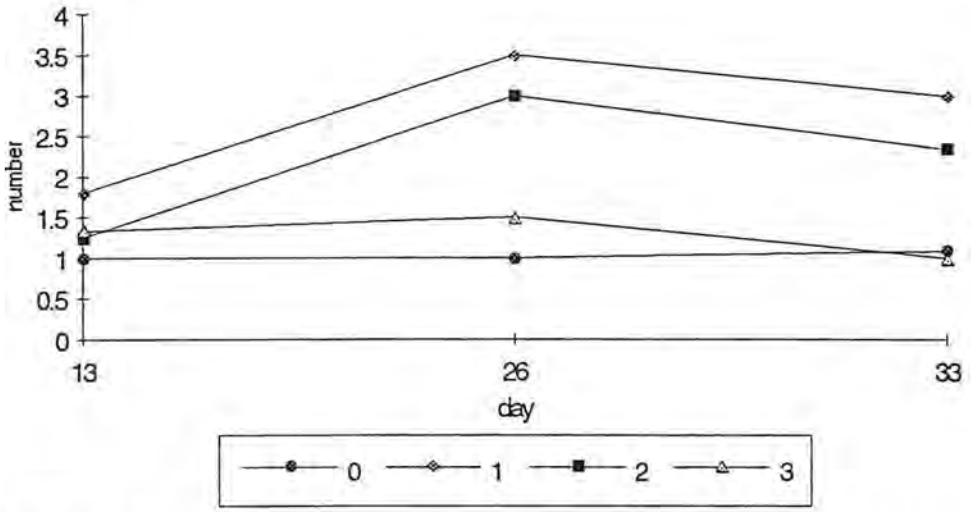

Figure 17: The average number of structures for the cotyledons who had structures counted after 13, 26 and 33 days by 4 different media.

The percentage of cotyledons of *B. brownii* with a structure was high for medium 0 (Figure 16). Medium 0 gave percentages between 55% and 75% and was therefore comparable with the percentages found by *B. grandis* (Figure 12) and *B. coccinea* (Figure 14). The structures formed by medium 0 were roots, except for 2 cases after 33 days when callus was formed. The media 1 to 3 gave low percentages (between 10% and 30%) callus-like structures who were formed on the cutting edge. These structures had a brownish colour and an irregular shape and they continued to grow in some cases. The average number of structures for the cotyledons who had structures was high for the media 1 and 2, compared to Figure 12 and 14.

Seedlings of *B. brownii* were cultured after their cotyledons had been cut off in the same medium in which they had been grown. They could form a complete plant without cotyledons.

From Figure 16 and 17 can be concluded that *B. brownii* has the ability to form callus. The structures, who grew from the cutting edge by the media 1 to 3, resembled the expected callus-like structures. The percentage of cotyledons who formed structures was low compared to *B. grandis* and *B. coccinea*.

3.4 Liquid media

Bunn (1991) claimed some success with *B. hookeriana, B. attenuata* and *B. menziesii* explants (leaves, buds, flower parts and seedlings) cultured in liquid medium on an orbital shaker (40 revolutions per minute) which received light (40 $\mu m\ s^{-1}m^{-1}$). Bunn used pH 6.0 while in this research pH 5.6 was used. To compare both pH-levels, 4 glasses (with each 4 cotyledons) of each pH-level were placed on a shaker. This trial was started on 28 October. Five glasses containing a comparable medium, with 0.5 mg/l 2,4-D instead of 2.25 mg/l BA and pH 5.6, were placed on the shaker on 5 November. The percentage of individual cotyledons with a structure was counted after 11 days (Table 8).

The cotyledons did not look viable after 18 days, they were turning yellow instead of green. The trial was stopped after 26 days.

The cotyledons cultured in BA gave a high percentage (81.2%, Table 8) of structures after 11 days. But these structures were the same stem-like structures which also appeared in earlier trials (paragraph 3.3.1). Cotyledons with a structure gave only one of these stem-like structures each. The cotyledons cultured in 2,4-D gave the same callus-like structures as in earlier trials (paragraph 3.3.1) but the percentage of cotyledons who gave structures was very low (15%, Table 8).

Table 8: The percentage of *Banksia grandis* individual cotyledons with a structure cultured on liquid media after 11 days.

media	%
pH 5.6	81.2
pH 6.0	81.2
0.5 mg/l 2,4-D	15.0

The results of Table 8 and Bunn (1991) are difficult to compare. Bunn used different *Banksia* species and cultured his explants in light. The high percentage of cotyledons with a structure by the media with BA indicates that light is (initially) not very important for these media. The cotyledons in medium with 2,4-D gave a low percentage (15%), compared to solid media with 2,4-D in light. In an earlier trial (paragraph 3.3.1), a solid medium with 0.5 mg/l 2,4-D, which was placed in the dark, gave higher percentages than the 15% found after 11 days (Table 8). A difference in pH-level led to comparable results (Table 8). It seems that cotyledons in liquid media have a comparable ability to form structures as cotyledons cultured on solid media. Bunn reported that *B. hookeriana* seedling apices gave shoots from the cut ends in liquid medium supplemented 2.25mg/l BA. *B. grandis* formed stem-like structures on a medium supplemented with 2.25 mg/l BA which are probably comparable with the shoots reported by *B. hookeriana*.
Liquid media did not seem to improve the results found in earlier trials with solid media.

3.5 Leaves and shoots

Leaf and shoot explants were taken from *B. brownii* plants who were placed in a greenhouse. A new sterilising method was designed since none of the earlier trials gave good sterilising results (Collins, pers.comm.). This sterilising method was based on some general accepted methods (Pierik, 1985). Eleven *B. brownii* leaves were made wet by dipping them in 70% ethanol for a few seconds. They were sterilised in 1% NaClO and 0.5% Tween 70 solution for 15 minutes. Finally, they were rinsed 3 times in sterilised water for 10 minutes each. The eleven leaves were cut in 3 cm pieces and cultured both polair and apolair with their cutting edge in the media. The media, used in this trial, were the same media as in Table 4. There was 100% contamination within 14 days. The contamination was caused by both fungi and bacteria.

Leaves of other *B. brownii* plants, cultured in a shade house, were used in a second trial. They were wet by putting them in 70% ethanol for 1 minute. They were sterilised in 1.5% NaClO and 0.5% Tween 70% for 15 minutes. Finally, they were rinsed 3 times in sterilised water for 10 minutes each. The leaves were cut in 3 cm pieces and cultured both polair and apolair with their cutting edge in the media. The media, used in this trial, were the same media as in Table 4. There was 100% contamination within 14 days. The contamination was caused by both fungi and bacteria.

The sterilising method given by Bunn (1991) was used in a third trial. Eight leaves and 2 shoots of *B. brownii* were washed for 4 hours in running tap-water. They were sterilised in 2% NaClO and 0.5% Tween 70 for 10 minutes. Finally, they were rinsed 6 times in sterilised water for 5, 5, 10, 10, 10 and 15 minutes each. The leaf parts were cultured polair. The lower leaves from the shoots were removed. This trial had less than 5% contamination but still all leaves and shoots died within 14 days. The contamination was caused by fungi.

Different media, with reduced MS concentrations, were used in a fourth trial (Table 9). The media contained 25 g/l sucrose, 7 g/l agar and had a pH of 5.6. Shoots and leaves of *B. brownii* were sterilised following the Bunn (1991) method. Five shoot parts and 15 leaf parts per medium were cultured. The leaves were cultured polair. The shoot parts had at least two axillary buds and a length of 1 to 3 cm. Their leaves were removed.

Table 9: Media (1 to 4) used for the culturing of *Banksia brownii* leaves and shoots.

	2,4-D (mg/l)	
	0	0.5
1/3 MS	1	2
1/6 MS	3	4

The leaves cultured in media 1 and 3 were still green after 13 days while the leaves cultured in media 2 and 4 turned brown and died. Two of the 5 shoots of both media 2 and 4 had turned brown and died. One shoot, cultured in medium 4, had callus growth on top. There was no contamination after 13 days. All leaves and shoots, cultured in media 2 and 4, had died after 20 days. The results of the media 1 and 3, after 20 and 28 days, are given in Table 10.

Table 10: The results of the remaining *Banksia brownii* leaves and shoots counted after 20 and 28 days

		20		28	
	media	callus	nothing	callus	nothing
leaves	1	5	8	3	2
	3	5	5	1	2
shoots	1	2	3	3	
	3	1	4		1

The callus on the leaves was formed close to the vein just above the agar surface. The callus on the shoots was formed on both ends of the explant. The callus had a white-greenish colour and it continued to grow in most cases. Although there were not much remaining explants after 28 days, it is possible to grow callus on both *B. brownii* leaves and shoots.

B. brownii shoots were sterilised following the Bunn (1991) method. They were cut in pieces who had at least 2 axillary buds and a length between 1 and 3 cm. They were cultured in media which contained different concentrations of BA (Table 11). BA has the ability to break the dormancy of axillary buds (Pierik, 1985). Nineteen shoots were cultured in each medium.

Table 11: The media (1 to 4) used to try to break the dormancy of *Banksia brownii* axillary buds.

	BA (mg/l)			
	0.1	1.0	5.0	10
media	1	2	3	4

Table 12: The results (numbers) of *Banksia brownii* shoots counted after 21, 28 and 35 days by 4 different media.

	21			28			35		
media	nothing	shoot	callus	nothing	shoot	callus	nothing	shoot	callus
1	7	2	1	6	3	1	3	1	1
2	4	5	1	1	9	1	1	7	1
3	8	6	2	5	7	1	0	7	2
4	9	5	2	4	5	4	3	4	5

The *B. brownii* shoots had developed both callus and new shoots after 21 days. The number of shoots with callus or new shoots increased between 21 and 28 days but only the number of shoots with callus had increased after 35 days. The number of shoots with new shoots decreased after 28 days because some shoots had died. The total number of shoots (= #nothing + #shoot + #callus) decreased especially between 28 and 35 days. For the media 2, 3 and 4 there was one shoot each with both callus and a new shoot after 28 and 35 days. The newly formed shoots had grown only a few millimetres after 21 days. The newly formed shoots had a maximal length of 5 mm after 28 days and only in some cases they had a length of over 1 cm after 35 days. Differentiation of the newly formed shoot was visible after 28 and 35 days. The callus was generally formed on the (old) shoot itself, just below an axillary bud. The callus had the same white-greenish colour as found previously and it continued to grow. The callus was formed on all media with the highest numbers by the higher BA-levels.

Axillary buds of B. brownii could be forced to form new shoots in 1/4 MS media supplemented with different BA-levels. The number of new shoots was not very high and the new shoots did not grow very vigorously so an adjustment of the media is necessary to achieve a high level of new, vigorous growing, shoots.

Young leaves and shoots of *B. coccinea* were collected in an abandoned nursery. They were sterilised following the Bunn (1991) method. They were cultured on the 1/4 MS media given in Table 7 and the 1/2 MS media given in Table 13. There was 99% contamination within 8 days. These *B. coccinea* shoots and leaves had much more hairs than *B. brownii* shoots and leaves. The hairs on the shoots and leaves of *B. coccinea* prevented probably the sterilising solution from reaching the contamination between the hairs.

Table 13: Media (x) used to culture *Banksia coccinea* shoots

		BA (mg/l)		
		0	0.1	0.5
2,4-D (mg/l)	1.0	x	x	x

3.6 Callus

Calli from *B. coccinea* cotyledons were isolated. They were cultured on 17 December on the same media as on which they were formed. Calli formed on the media 1, 2, 3 and 4 (Table 4) were used. Three calli were available for each of the media 1, 2 and 4. Four calli were available for medium 3. The calli were cultured with half the callus in the medium. The results are shown in Table 14. Calli which were green and looked viable were rated as 'successful'.

Table 14: The successful results of the isolation of *Banksia coccinea* callus, cultured on 4 different media, counted after 7, 26 and 33 days.

media	7	26	33
1	3	1	1
2	3	1	1
3	4	4	3
4	3	0	0

Green and viable *B. coccinea* calli survived (at least) 33 days (Table 14). Sparse growth occurred by all viable calli in this period.

Calli from both *B. coccinea* (from 1/2 MS media) and *B. brownii* was isolated and cultured on 1/4 MS medium. Five Calli from shoots and leaves were cultured by *B. brownii* while the 6 calli from *B. coccinea* came from cotyledons. The trial started on 14 January. All calli looked green and viable after 5 days but after 12 days only 1 (shoot) callus from *B. brownii* still looked viable. *B. coccinea* calli were all viable after 12 days. Surviving calli showed sparse growth in this period.

Calli from *B. coccinea* were relative easy to isolate and to grow on both 1/2 and 1/4 MS media. Calli from *B. brownii* survived only in 1 (out of 5) case. However, it was observed that small calli had a less change to survive than bigger calli. The calli from *B. brownii* were small calli while calli from *B. coccinea* were bigger.

3.7 Inoculations of seedlings of *Banksia grandis*

B. grandis is known to be very susceptible to *Phytophthora cinnamomi*. *P. citricola* seems (generally) not to affect *B. grandis* at all. However, one isolate (JW 20) of *P. citricola* has the ability to affect *B. grandis* (Bunny, pers.comm.). This is of interest because it can provide information about the way *B. grandis* roots deal with pathogenic fungi, especially of the *Phytophthora* family.

In vitro grown seedlings, sown on 22 September, with 2 true leaves were inoculated with zoospores on 26 November. The zoospores were hard to produce and only zoospores of *P. cinnamomi* isolate SC 72 (A2 strain, IMI 264384) and *P. citricola* isolate JW 20 were available. Zoospores were inoculated in the medium near the root. Five plants per fungus were used. The zoospores had germinated and formed mycelium near and on the roots after 4 days. The surface of the roots started to get colonised with mycelium which was penetrating the epidermis. It was clear that both isolates penetrated epidermis cells but apparently they did not penetrate the cells under the epidermis (as in Figure 18). The fungi colonised the roots by penetrating epidermis cells, establishing themselves and producing more mycelium that also penetrated

epidermis cells. A brown layer was formed by both fungi in the cell layer underneath the epidermis after 9 days (as in Figure 19). It was difficult to find hyphae growing inside the root although black cells started to appear in the tissue and vascular system. These black cells were a result of the presence of the fungus because there were no black cells in healthy roots. The vascular system started to dissolve by both fungi after 11 days (Figure 20). The cells between the vascular system and the endodermis were loosing shape and collapsed. Some hyphae could be found growing inside the root especially on the places were the cells had collapsed. It seemed that the collapsing was caused by the presence of the fungi (on the root surface) rather than by the cutting technique used. The vascular system was stained red, just like the fungi, but the red stain of the cells was (probably) not a result of the presence of the fungi.

A mycelium solution was used to inoculate *B. grandis* seedlings in a second trial on 16 December. Two non-affecting isolates of *P. citricola* (HSA 1211 and 1450), 1 affecting isolate of *P. citricola* (JW 20) and an isolate of *P. cinnamomi* (SC 72) were applied unto 4 seedlings each. Two other seedlings per isolate were inoculated with a small piece of mycelium. The plants, who were inoculated with the mycelium solution, showed mycelium growth in the medium and near the roots after 4 days but the isolates HSA 1211 and 1450 had established themselves only in half the inoculated plants. The plants, who were inoculated with the mycelium pieces, gave a good mycelium growth after 4 days. The isolates JW 20 and SC 72 gave the same symptoms as in the first trial except for the dissolving of the cells between vascular system and endodermis which was not found in the second trial. Some examples of hyphae growing inside the root were found. The isolates HSA 1211 and 1450 gave comparable symptoms. Mycelium established itself by penetrating an epidermis cell in the same way found by SC 72 and JW 20 (Figure 19). They colonised the root surface without visible penetration of the layers underneath the epidermis. However, examples of mycelium growing inside the root could be found by HSA 1211 and 1450 (Figure 21 and 22). The growth of the mycelium was intercellular and intracellular (by isolate 1450, Figure 21 and 22). The layer underneath the epidermis had a light brown colour and not the dark brown colour as found by SC 72 and JW 20.

No major differences were found between different isolates of *P. citricola* and *P. cinnamomi*. Non-affecting isolates gave the same results as affecting isolates. All the isolates seemed to penetrate the epidermis but no example was found where the penetration of the epidermis was followed by penetration of the root tissue. Intercellular mycelium of all isolates was found in limited numbers growing inside the root. Intracellular mycelium was found in isolate 1450 (*P. citricola*; Figure 21 and 22). The layer underneath the epidermis showed a dark brown colour by the isolates SC 72 (*P. cinnamomi*) and JW 20 (*P. citricola*) and a light brown colour by the isolates HSA 1211 and 1450 of *P. citricola*. Dissolving of the tissue between the vascular system and the endodermis was found in the first trial by SC 72 (*P. cinnamomi*) and JW 20 (*P. citricola*). It seemed that this dissolving was caused by the fungi rather than the used cutting technique.
The fungi found in this trial were not tested on identity according to the postulates of Koch because there was not enough time to do this properly. The fungi were isolated on a selective Phytophthora medium but not tested on identity.

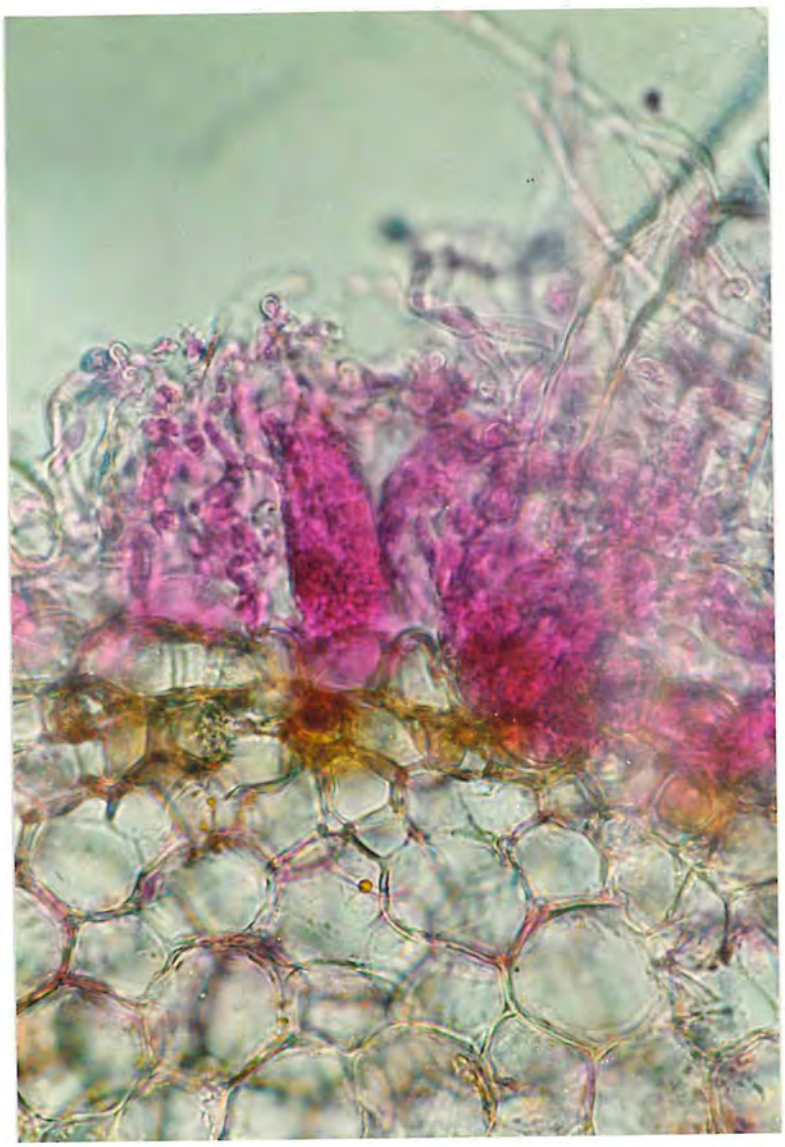

Figure 18: An example of the penetration and colonisation by *Phytophthora citricola* isolate 1450 on a *Banksia grandis* root grown. Mycelium, which has penetrated the epidermis, has a purple stain. (transverse section, 400x; stain: 3% erythrosine in 10% NH_4OH)

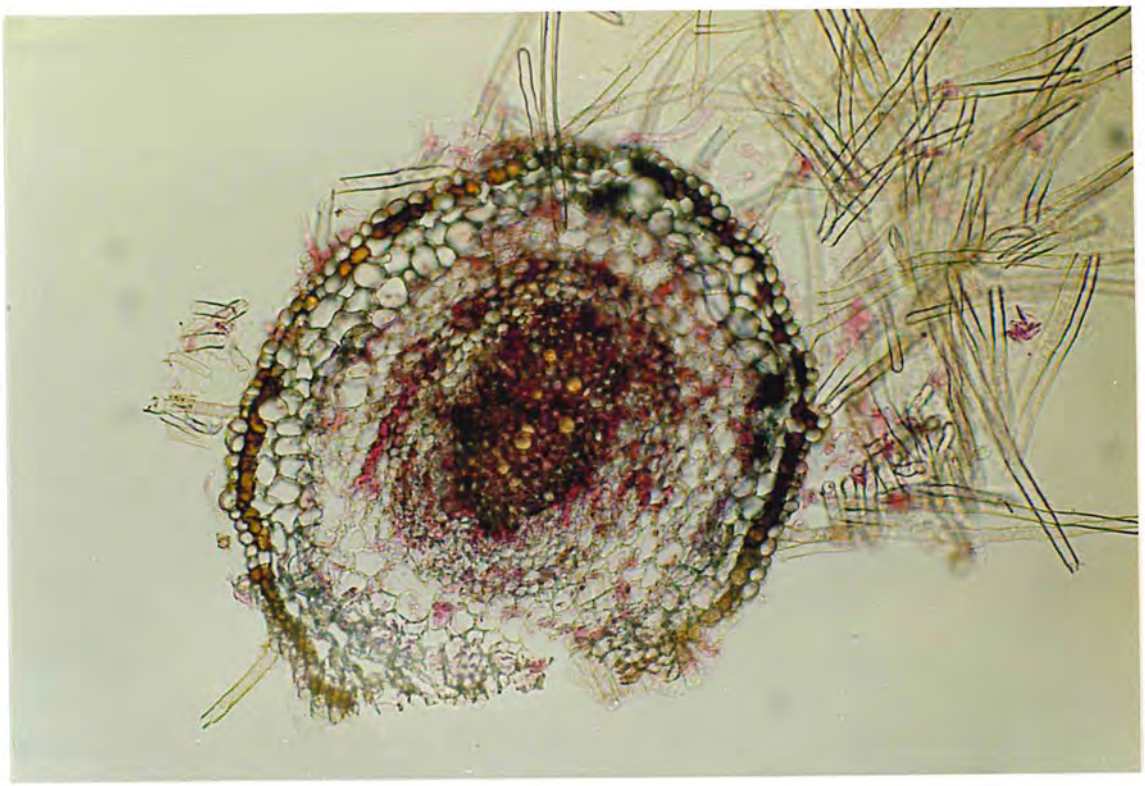

Figure 19: An example of the brown layer underneath the epidermis of a *Banksia grandis* root grown *in vitro* after colonisation by the fungus *Phytophthora cinnamomi* (purple stained: 3% erythrosine in 10% NH_4OH) on the outside. In the vascular system are at least 3 black cells. (transverse section, 100x)

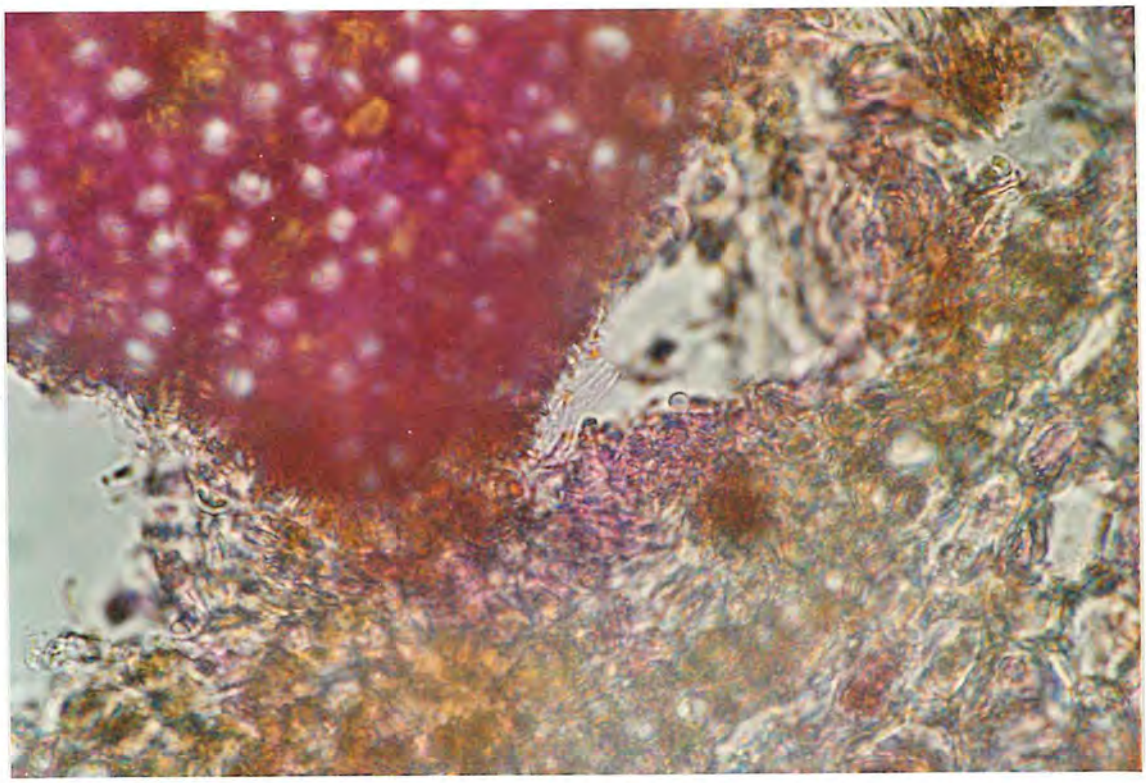

Figure 20: Detail of the dissolving of the tissue between vascular system and endodermis of a *Banksia grandis* root grown *in vitro* by *Phytophthora citricola* isolate JW 20. (transverse section, 400x)

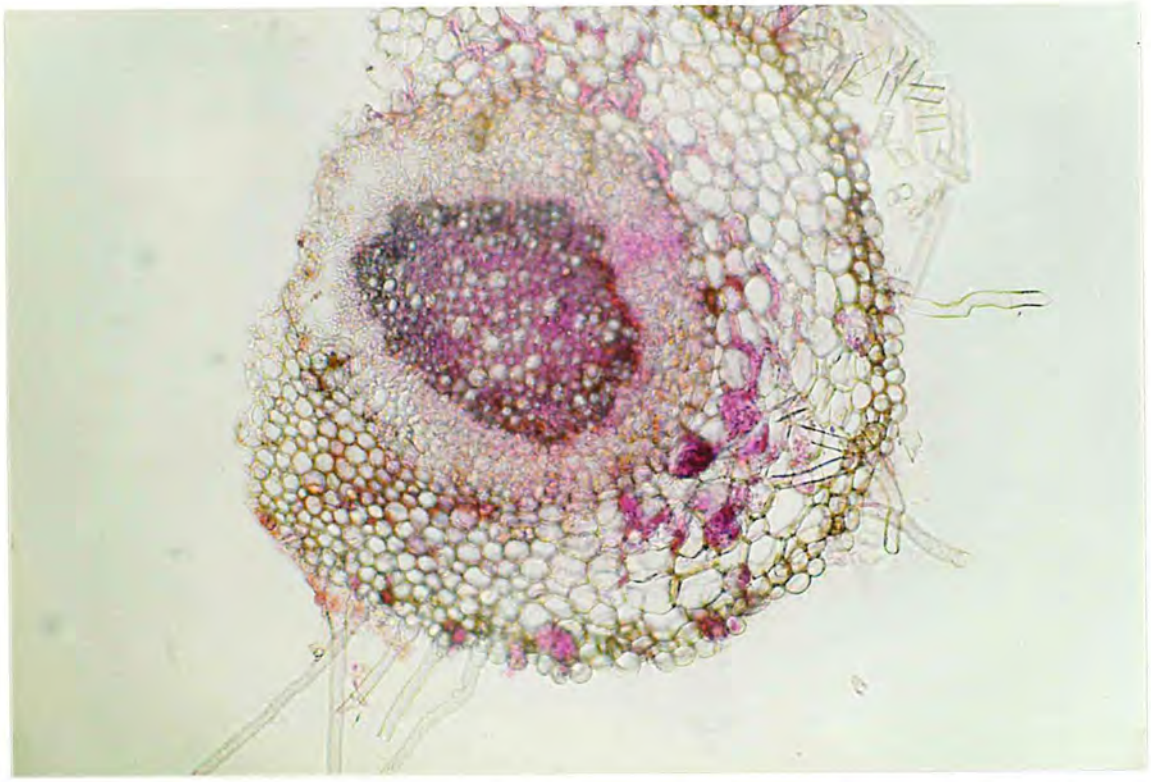

Figure 21: An example of fungal growth (*Phytophthora citricola* isolate 1450) inside the tissue of a *Banksia grandis* root grown *in vitro* (purple stained: 3% erythrosine in 10% NH_4OH). A light brown layer is visible just underneath the epidermis. (transverse section, 100x)

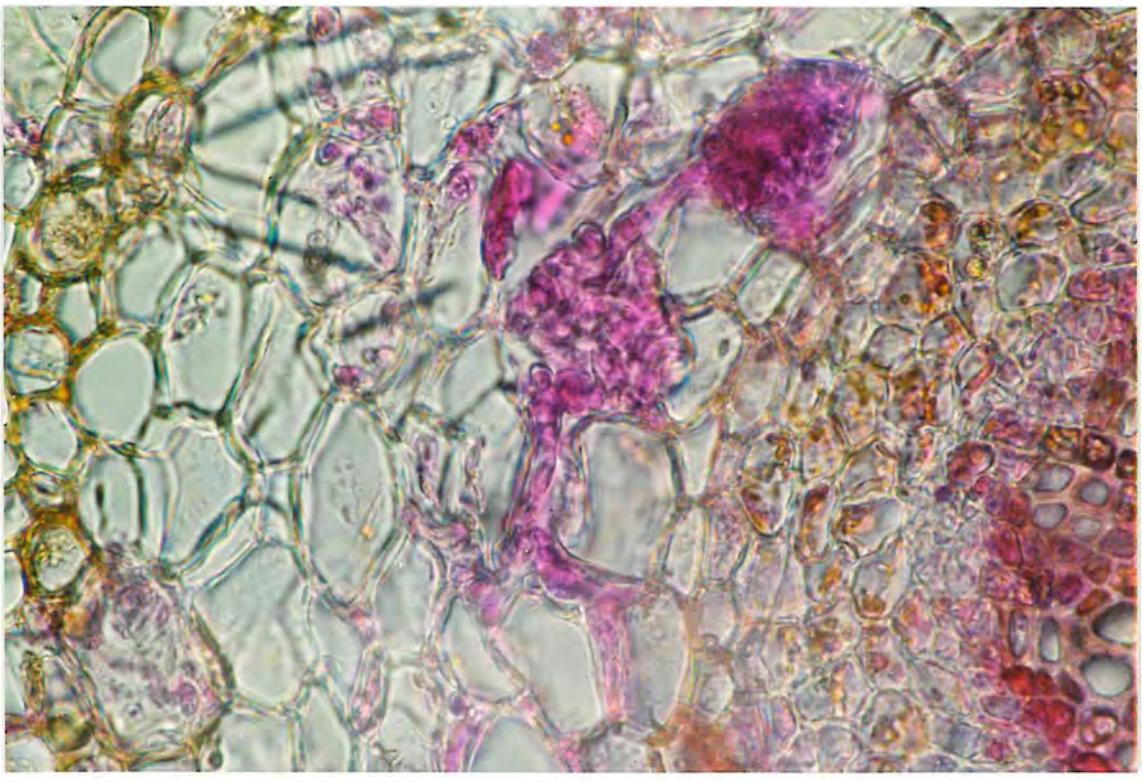

Figure 22: Detail of Figure 21, inter- and intracellular hyphal growth (purple stained) in the root tissue. (transverse section, 400x)

3.8 Production of oospores

Production of oospores (by *P. megasperma*) can be important for survival in the ground during (long) periods of unfavourable circumstances for the fungus. *P. megasperma* forms these oospores on agar plates but it is not clear where and when these oospores are formed in nature since there are no records of oospores found in nature. Wet circumstances may lead to the production of zoospores and mycelium growth while dry circumstances may lead to oospore production as a reaction to unfavourable circumstances (Bellgard, pers.comm.).

The trial was started on 6 November when the seeds of *B. grandis* and *B. attenuata* were sown. *P. megasperma* isolate TH 2 was used. The different treatments (5 jars per treatment per specie) are given in Table 15. Oospore formation was expected in presence of both host (*Banksia* specie) and fungus (A and B), the other treatments are controls. After 97 days, the sand was sieved per jar and the roots were stained.

Table 15: The 6 treatments (A to F) used by the oospore trial. The treatments varied in the presence (+) of host and fungus and whether the plants were dried (+) or not.

treatment	host	fungus	dried
A	+	+	+
B	+	+	-
C	+	-	+
D	+	-	-
E	-	+	+
F	-	+	-

Almost no oospores were found after sieving although the smallest sieve (38 µm) was small enough to collect the oospores. Only one of the oospores (*B. attenuata* treatment B) was a clear example of *P. megasperma* while the rest (*B. grandis* treatments B, E and F) looked like a *P. megasperma*-oospores but had a different appearance (not completely round, darker colour).

The roots were stained using 2 methods (Appendix 5). The KOH method gave a good result and the roots were clear so microscopical examination was possible. Mycelium growing on the root surface was stained but no oospores were found.

Almost no oospores were found in this trial. This seemed to be a result of bad oospores formation rather than of the used techniques. Since mycelium was found on the root surface, it looked like the inoculation with *P. megasperma* had worked. But other spores of different fungi were also found, probably as a result of the opening of the lids in the last 8 days. The mycelium could have belonged to these fungi. A control on specific (*Phytophthora*) medium to test the identity of the fungi according to the postulates of Kochwas not possible because of a lack of time.
A small success were the oospores which were found for they prove that oospore formation by *P. megasperma* is a real possibility in nature. The presence of oospores without host (treatment E and F) seems to indicate that oospore formation does not necessarily need the presence of both host and *P. megasperma*, but these oospores had a different appearance and could have been from other fungi.

4 Discussion

Banksia grandis and *B. coccinea* were used to find ways to grow *B. brownii in vitro*. Results of both species were not always comparable with *B. brownii* but they provided a general model.

Seeds of *B. grandis*, *B. coccinea* and *B. brownii* were easy to germinate *in vitro* at 15°C. Light or dark conditions did not influence the germination but higher (20°C or 25°C) temperatures inhibited germination (Figure 1 and 2, Table 3). The growth of the seedlings of *B. grandis* was better at 25°C then at 15°C (Figure 3). The three *Banksia* species are all native to the south-west of Western Australia. Germination of their seed occurs in early winter when conditions are optimal to provide a successful establishment. The optimum temperature found in this research, 15°C, is a normal temperature in this period and was also found by Cowling & Lamont (1985) in a research with comparable *Banksia* species. Germination in summer, with temperatures above 20°C, would not be very successful because of a lack of rain. The germination is clearly temperature dependent, the germination of more tropical *Banksia* species occurs already at 20°C (Cowling & Lamont, 1985, Bunn, pers.comm.).

Cotyledons proved to be a potential source of callus. Callus formation by both *B. coccinea* and *B. brownii* was achieved by culturing whole cotyledons. Callus of *B. coccinea* was produced in a fairly high percentage (between 50% and 100%) of the cotyledons (Figure 10). Callus of *B. brownii* was produced in a low percentage (between 10% and 30%) of the cotyledons (Figure 16). Callus of *B. coccinea* and *B. brownii* was isolated and was grown successfully by *B. coccinea* (Table 14). The contents of the media used to induce callus formation were very important. The auxin 2,4-D led, alone or in combination with BA, to callus induction. *Banksia grandis* had the capacity to form callus-like structures (Figure 4) but the cotyledons were never active enough to produce so much callus that it could be isolated. There was, however, a clear difference between reactions in different media for *B. grandis*.

Liquid media were not superior to solid media. Cotyledons of *B. grandis* formed the same callus-like structures as on solid media (Table 8) but died shortly afterwards.

The problem of sterilising *B. brownii*-leaves and -shoots was overcome but *B. coccinea*-leaves and -shoots were still very difficult to sterilise. The leaves and shoots of *B. brownii* died on 1/2 MS media within days. Most of them stayed viable on plain 1/3 and 1/6 MS and even produced callus (Table 10). One-third and 1/6 MS media supplemented with 2,4-D (0.1 and 0.5 mg/l) led to the dead of leaves and shoots within 10 days. BA in different concentrations (0.1, 1.0, 5.0 and 10.0 mg/l) was able to break the dormancy of axillary buds in about half the shoots (Table 12). The new shoots, formed from the axillary buds, did not grow very vigorously. About 10% of the shoots also produced callus, especially by the higher BA concentrations, which could be isolated and grown separately. Callus and shoots are both generally accepted ways to grow and multiply a specie *in vitro* (Pierik, 1985). The newly formed shoots can be cut in pieces and cultured again. If each piece is able to form a new shoot, multiplying can be done. Only a part of the shoots formed new shoots so the media or the growth conditions need to be adjusted in order to get a good working system. Callus can be used to multiply a specie if the growth of the callus is fast enough. Calli of *B. brownii* were difficult to grow but this could be a result of the small calli which were isolated. The media can also be a problem since only 10-30% of cotyledons and about 10% of the shoots formed callus. If these media give low percentage of callus growth it could

be that the media cause the problems. Surviving calli from *B. brownii* and *B. coccinea* were slow growers but an adjustment of the media or growth conditions can lead to good results both in callus formation and growth.

Clones of the same genetic material can be developed with both shoots and callus and they can be used for research purposes. The leaves and shoots of *B. brownii* were from plants which were grown outside and later placed in a green- or shade house. It maybe possible to sterilise material from wild populations as well since the disinfection is successful on green- and shade house grown plants. Thus, the original genotypes of the wild populations can be stored by using leaves and shoots.

The concentration of MS (Murashige & Skoog, 1962) macro and micro salts proved to be important. Cotyledons of *B. coccinea* produced callus on 1/2 MS as well as on 1/4 MS. Cotyledons of *B. brownii* died on 1/2 MS but produced some callus on 1/4 MS. Cotyledons of *B. grandis* formed callus-like structures on both 1/2 MS and 1/4 MS but not the callus *B. coccinea* and *B. brownii* formed on these media. Leaves and shoots of *B. brownii* died on 1/2 MS but were able to grow on 1/3, 1/6 and 1/4 MS (shoots). The soils in Western Australia are generally poor in nutrients compared to soils elsewhere. Species, who are adapted to these poor soils, can have problems if they are grown on richer nutrient soils. This could explain why a strong reduction in MS salts is necessary *in vitro*. Native species in South-Africa, from comparable climate, soil type and families, are also often grown on 1/3 MS-media (Barbour, pers.comm.).

An important goal in this research was to produce callus from *Banksia* species. A callus tissue culture system can be used to screen plants for resistance and susceptibility to fungal pathogens. Jang & Tainter (1990[a]) and Miller *et al* (1984) give some advantages and disadvantages of this system:

advantages:
- simplified experimental system
- better control of environmental and nutritional factors
- opportunity to test large numbers in a small area
- use of abundant replicates from a given genotype
- capability to work with haploids

disadvantages:
- cultured plants may be physiologically and genetically different from intact plants
- differences between response at the whole plant versus single cell (callus) level

Callus tissue culture systems have been used to demonstrate the expression of differential resistance in pine callus tissues inoculated with *Phytophthora cinnamomi*. The calli were inoculated by placing a small piece of mycelium on top of them. The number of intracellular hyphae in the callus after 3 days was used as an index of resistance. Major differences between susceptible and resistant reactions were found at cellular level (Jang & Tainter, 1990[a&b]). McComb *et al* (1987) examined the expression of field resistance against *P. cinnamomi* in callus tissue of several Australian native and horticultural species. The extent of hyphal growth (after 24 hours) on callus correlated with the susceptibility of the plant from which the callus was derived. Resistance was detected by limited, sparse fungal growth on callus, in contrast to the prolific, aerial growth seen on media or on callus derived from susceptible species. Callus from Jarrah trees, growing on dieback infected sites, showed resistance where callus from Jarrah trees dying on dieback infected sites showed prolific growth of *P.*

cinnamomi mycelium. These and other studies (for example: Deaton *et al*, 1982; Helgeson *et al*, 1972; Maronek & Hendrix, 1978; Miller *et al*, 1984) show that resistance in the field is expressed *in vitro* but the opposite was also found (for example: Branchard, 1982; Helgeson, 1983). Jang & Tainter (1990[a]) note however that a number of factors in the *in vitro* screening may greatly influence results. Each specific system needs probably a specific *in vitro* screening method. Callus reactions with the pathogen or its metabolites are influenced by growth conditions but it should be possible by systematically modifying a culture/inoculation system to produce the most useful expression of disease resistance.
A callus tissue culture system can be used to screen *Banksia* species for resistance against *P. cinnamomi*. Differences in susceptibility to *P. cinnamomi* are found between and in *Banksia* species. Several *Banksia* species and *Banksia* plants were found to be tolerant, especially species from eastern Australia (Cho, 1981 & 1983, McCredie *et al*, 1985[a&b]). Less susceptible or tolerant plants can be used to save threatened species from extinction in nature. The remaining *B. brownii* population can be screened by using leaves and (young) shoots to produce callus. Callus from cotyledons can be used to screen large numbers of seedlings for resistance especially since it was possible to grow a plant from a seedling from which the cotyledons were taken. This is also possible with other *Banksia* species and other pathogens. An improvement of the methods used to produce and grow callus is necessary to achieve this.

Inoculations of *B. grandis* seedlings, grown *in vitro*, with *P. cinnamomi* and *P. citricola* emphasised the difficult character of both fungi. Two *P. citricola* isolates (HSA 1211 and 1450) do normally not affect *B. grandis* while *P. citricola* isolate JW 20 and *P. cinnamomi* isolate SC 72 affect *B. grandis*. Inoculations, with both zoospores and mycelium (solution), in the medium led to the similar results, roots of *B. grandis* seedlings were affected by all isolates. The first group led to a less coloured cell layer underneath the epidermis and the vascular system of the *B. grandis* roots seemed to dissolve in some cases by the second group but major difference were not found. The fungi penetrated the epidermis easily but no example was found in which penetration of the epidermis was followed by penetration of the root tissue. Intercellular hyphae inside the root were found in small numbers by all isolates and some intracellular hyphae were found by isolate 1450. However, black cells in the tissue and the brown layer underneath the epidermis indicated a much stronger presence of the fungi. Inter- and intracellular hyphae were found by several other plant species (Tippett *et al*, 1977 & 1983; Cahill *et al*, 1989). Black cells (cell necrosis) in the tissue and cell wall digestion or disorganisation was found well in advance of the hyphal front in several susceptible species by Tippett *et al* (1977) and Cahill *et al* (1989). Jang & Tainter (1990[a&b]) suggest that the basis for resistance to *P. cinnamomi* is not anatomical but that it is regulated by physiological and biochemical mechanisms. They base this hypothesis on their work with pine callus tissues inoculated with *P. cinnamomi*. The lack of examples of mycelium penetrating the layer underneath the epidermis seems to be a result of anatomical resistance. The colouring of the layer underneath the epidermis could be a result of activating defence mechanisms. These defence mechanisms could well be fysiological or biochemical since the layer underneath the epidermis exists of normal tissue cells who do not have special (defence) functions as the epidermis and endodermis. A fysiological or biochemical reaction is also supported by the black cells which appeared in the tissue. The black cells did not seem to be connected with mycelium growing in the tissue but they inevitable appeared.
Whatever mechanism was able to restrict the penetration to the epidermis, it was not able to stop the colonisation of the surface of the roots. It appeared that the reactions

in the roots were induced by the presence of the fungus on the outside. The rapid colonisation of the roots could have caused the root to die which could also explain the symptoms. Symptoms of cell death (other than the black cells) were not observed.

The *B. grandis* seedlings were grown and inoculated *in vitro*, an artificial system. The lack of differences between affecting and non-affecting isolates could be a result of these artificial circumstances. A control treatment with seedlings grown and inoculated in the ground was not possible in this research and the inoculations with mycelium can also give different results compared to inoculations with zoospores. The fungi were inoculated in a medium which was rich in sucrose. The fungi did not have to depend on plant tissue for survival and the observed reactions are maybe different on another medium. Seedlings can react different than older, mature, plants and this also could explain the results.

A system of using *in vitro* techniques to examine the formation of oospores by *P. megasperma* on roots of *B. grandis* and *B. attenuata* seedlings (*in vitro* grown) was partly successful. Some oospores were found in the sieved ground solution but only in a small part of the trial and also in low numbers. The KOH staining of the roots was successful and mycelium growing on the root surface as well as the root itself were very clear but no oospores were found. This indicates that oospores form outside the root. However, the low numbers of oospores found in the sieved ground and the pilot character of this trial make a conclusion difficult.
It seemed that the inoculation was successful since both mycelium on the root surface and disease symptoms were found. The spores of other fungi, which were found, must be a result of infection in the last 8 days because the germination, growth and inoculation were all conducted under sterile circumstances. Oospores were especially expected by the dry conditions but were only found in 1 jar which was left to dry. The jars, which were left to dry, dried very quickly and this time period might have been too short to form oospores.
The oospores which were found, however, are the first record of oospores of *P. megasperma* formed in the soil under field conditions. The presence of oospores without host indicates that *P. megasperma* does not need a host to form oospores. *P. megasperma* may have much more opporunities to survive in the field then (for example) *P. cinnamomi* which has no record of oospore formation under field conditions.
In vitro techniques can be of use to predict the formation of oospores under (artificial) field conditions

5 Conclusions

Light or dark germination conditions did not influence the germination of *Banksia grandis*, *B. coccinea* and *B. brownii* seed *in vitro*. The germination was temperature dependent with good germination at 15°C but almost no germination at 20°C and 25°C. The best growth of the seedlings occurred when they were transferred to 25°C instead of 15°C after 21 days.

Callus of *B. coccinea* and *B. brownii* was produced on cotyledons which were cultured in media supplemented with different levels of the auxin 2,4-D (0.1 and 0.5 mg/l) and the cytokinin BA (0, 0.1 and 0.5 mg/l). *B. coccinea* produced this callus on 1/2 MS (Murashige & Skoog (1962) macro and micro salts) and 1/4 MS where *B. brownii* only produced callus on 10-30% of the cotyledons on 1/4 MS. *B. grandis* formed callus-like structures on both 1/2 and 1/4 MS but no callus could be isolated and grown separately as with *B. coccinea*.
Liquid media did not improve the results achieved on solid media.

Shoots and leaves of *B. brownii*, grown in a greenhouse, could be sterilised and grown *in vitro* on 1/3, 1/4 and 1/6 MS media. It was not possible to sterilise *B. coccinea* shoots and leaves because of abundant hairs on their shoot and leaf surface. Axillary buds of *B. brownii* could be forced to form (small) new shoots in 1/4 MS media supplemented with different BA levels (0.1, 1.0, 5.0 and 10.0 mg/l). Callus was also formed on about 10% of the shoots in these media, especially by the higher BA levels. This callus was isolated and grown separately in some cases. Both the cotyledon and the shoot media need adjustment since only a small part of the cotyledons and the shoots formed enough callus to isolate and to grow separately and although about half the shoots formed new shoots only some grew vigorously.

Callus of *B. coccinea* and *B. brownii* was isolated and grown separately but not all isolated calli survived and the ones who survived did not grow fast enough to allow multiplication. An adjusted medium might induce enough callus growth and better multiplication. If so, callus could be used for screening plant species for resistance against pathogens as *Phytophthora cinnamomi* by inoculating them *in vitro* with mycelium.

Seedlings of *B. grandis* were inoculated *in vitro* with zoospores and mycelium (solution) of different isolates of *P. citricola* and *P. cinnamomi*. Two isolates of *P. citricola* (HSA 1211 and 1450) do normally not affect *B. grandis* where isolates JW 20 (*P. citricola*) and SC 72 (*P. cinnamomi*) do affect *B. grandis*. All the isolates affected *B. grandis* seedlings grown and inoculated *in vitro*. The epidermis of the roots was easily penetrated by all isolates but no example was found where penetration of the epidermis was followed by penetration of the root tissue. The layer underneath the epidermis turned brown after some days and black cells in the root and even, in some cases (by isolates JW 20 and SC 72), dissolving of the tissue between vascular tissue and endodermis was observed.
Only a small number of (inter- and intracellular) fungal hyphae growing in the root could be found and they did not seem to be connected directly to the observed symptoms. Therefore, the symptoms could be caused by a physiological and/or biochemical reaction of the plant to the penetrating fungus but they could also be caused by the colonisation of the fungi on the root surface which causes, in the end, dying of the root. The system of inoculating *in vitro* grown seedlings is probably too artificial to get a good comparison between the different isolates.

Oospore formation was found in the presence of host (*B. grandis* and *B. attenuata* seedlings) and fungus (*P. megasperma*) under (artificial) field conditions. *In vitro* techniques can, therefore, be of use to predict the circumstances under which this oospore formation occurs.

Growth of *Banksia brownii in vitro* was achieved in this research but multiplication was not possible (yet). In vitro techniques could be of use by some aspects of the Dieback research but adjustments in both media and techniques are necessary to achieve this.

6 Recommendations for further research

Cotyledons proved to be a source for callus. This callus can be valuable for other research and modifications (other hormones, different levels of hormones) in both media and treatment (light level, temperature) might lead to a better system of callus formation and callus growth.
Shoots and leaves can also produce callus. This callus can be used for the storage of the genotypes of the remaining populations of rare *Banksia* species if the disinfection of shoots and leaves and the formation of callus is more successful. The disinfection of *B. coccinea* might improve if smaller parts (buds, leaves) are used. The use of higher concentrations of disinfective solution might also improve success.
Newly formed buds from shoots can provide a simple and quick way to multiply a specie. The media used to produce these shoots by *B. brownii* need adaption since only a small percentage was successful. Char coal can adsorb excretion from the shoot in the media. Although no excretion was visible in the media, it is a common problem in the *Proteaceae* family and it might reduce survival chances even in low, invisible concentrations.
Callus isolation was successful with *B. coccinea* but it did not grow vigouresly. Adaptations in media and treatment (might grow better in dark conditions) can lead to a better growth. These adaptations might also be valuable for callus of other species, like *B. brownii*, who have problems surviving isolation.

In vitro techniques can be of use (in Dieback research). Callus systems might be useful to screen a population for resistance against a pathogen. Improvement of the used techniques and development of specific systems are necessary. Field trials can give information about *in vitro* trials and make better interpretation of the results possible. Small, fast *in vitro* trials are possible on a small area and application in this way can contribute to field trials.

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Appendix 1[a]: Macro and micro salts according to Murashige & Skoog (1962).

macro salts	(mg/l)
KNO_3	1900
$CNHO_3N_3$	1650
KH_2PO_4	170
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370

micro salts	(mg/l)
$FeSO_4.7H_2O$	27.8
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.3

Appendix 1[b]: The components of uncleared V8-$CaCO_3$-medium (Ribiero, 1978).

900 ml	distilled water
100 ml	V8-juice
2.0 g	$CaCO_3$
15.0 g	agar

After these components are mixed, the medium is autoclaved for 20 minutes at 121°C, 103.5 kPa.

Appendix 1[c]:Cleared V_8-$CaCO_3$ medium + tryptophan + β-sitosterol (Ribiero, 1978)

900 ml	distilled water
354 ml	V_8-juice
5.0 g	$CaCO_3$
15 g	agar
30 mg	β-sitosterol
20 mg	tryptophan
100 mg	$CaCl_2.2H\ O_2$
1 ml	thiamine hydrochloride solution

The procedure for medium preparation:
Add 5 g $CaCO_3$ to 354 ml V_8-juice and mix well. Centrifuge for 20 minutes at 4000 rpm. Decant the clear solution carefully into a flask. Add 15 g agar and 900 ml distilled water to 100 ml supernatant. Measure 30 mg β-sitosterol, dissolve in 2 - 5 ml absolute ethanol with heating. Add β-sitosterol to the rest in a hot water bath. Add 20 mg tryptophan and 100 mg $CaCl_2.2HO_2$. Autoclave media and millepore filter (pore

diameter 0.22 µm). Cool media to 50-60 °C. Add 1 ml thiamine hydrochloride solution per litre agar using Millepore filtration.

Appendix 1[d]: Zoospore production (modificated from Morgan, 1991).

* place miracloth mats, 7 cm in diameter, on 10% V8 agar
* place *Phytophthora cinnamomi* or *P. citricola* on each mat
* place mycelial mats, 4 to 8 days old, in sterile 9 cm petri dishes containing 10 ml sterile 2% V8 broth
* incubate the petri dishes overnight on a shaker at roomtemperature
* the following morning rinse the mats twice with mineral salt solution while they are stored under light on a shaker
* every hour for the next 4 hours rinse the mats with a mineral salt solution while they are stored under light on a shaker
* mature sporangia are plentiful after 20 hours, zoospore release is stimulated by washing the mats twice in distilled water and then incubating the mats in 20 ml, 18°C distilled water for 15 minutes
* The mats are removed and kept at 24°C until zoospore release occurs (usually from 30 to 60 minutes)

Appendix 2: The seedling growth of *Banksia grandis* seedlings counted after 22, 29, 35 and 42 days (1-21: treatment 1, 1-16: treatment 2).

	22	29	35	42
1	0.6	1.1	1.8	2.3
2	0.9	1.2	1.5	1.9
3	1	1.3	1.6	2.2
4	0.8	1	1.2	1.9
5	1	1.4	1.7	1.8
6	1	1.6	2	2.5
7	1.1	1.6	2	2.4
8	0.6	1	1.6	2.2
9	1	1.2	1.7	2.3
10	0.8	1.1	1.5	2
11	1	1.4	1.9	2.5
12	1	1.5	2	2.2
13	0.7	1.3	1.4	1.7
14	0.9	2.2	2.5	2.7
15	0.8	1.5	2	2.7
16	0.8	2.3	2.7	3
17	0.7	1.1	1.4	2
18	1	1.3	1.5	1.5
19	1.4	2.6	2.6	2.8
20	1.1	2.3	2.6	2.8
21	0.6	1	1.8	2.4
1	0.6	1	1.4	1.6
2	1	1.2	1.6	2.2
3	1	1.2	1.4	2.2
4	0.5	0.7	1.1	1.5
5	0.7	1.1	1.7	2.5
6	0.9	1.3	1.7	1.8
7	0.6	1	1.5	2
8	1.6	2	2.2	2.5
9	1	2	3	3.2
10	0.7	1.2	1.5	1.7
11	1.3	2.5	3	3.2
12	1.4	2.1	3.1	4
13	0.6	1.3	1.5	1.6
14	0.2	0.9	1.1	1.1
15	1.1	2.4	3	3.4
16	0.5	1.5	2.6	2.8

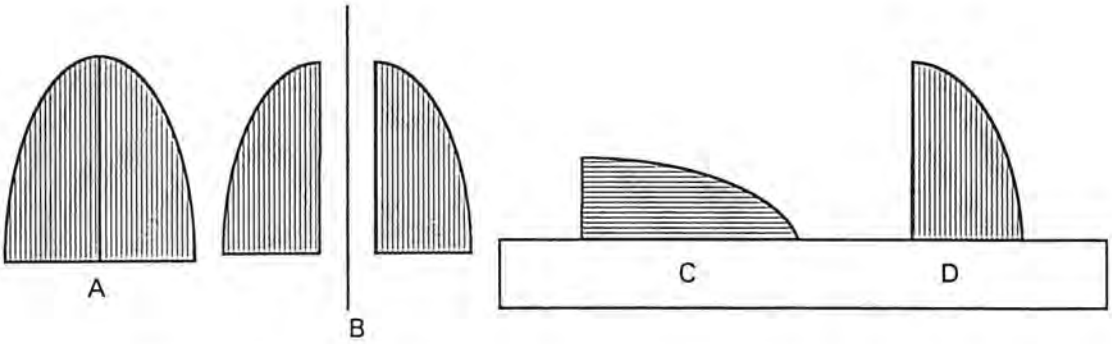
Appendix 3[a]: The results of the control media 0 counted after 7, 14 and 27 days.

days	nothing	something	sum
7	6	2	6
14	8	0	0
27	5	3	3

Appendix 3[b]: The results of the trial with the cotyledons of *Banksia grandis* started on 15 October counted after 12, 18 and 31 days.

days	media	nothing	something	sum
12	1	0	4	4
	2	0	3	4
	3	0	3	3
	4	0	3	7
	5	1	1	1
	6	2	1	2
18	1	0	4	4
	2	0	3	4
	3	1	2	2
	4	0	3	4
	5	1	1	1
	6	2	1	2
31	1	0	4	4
	2	0	3	4
	3	0	3	3
	4	0	3	7
	5	1	1	1
	6	2	1	3

Appendix 4: The methods used to culture *Banksia* cotyledons.



A: the normal way with a complete cotyledon with its cutting edge in the agar
B: cotyledon cut in 2 parts
C: cotyledon cultured with its long edge in the medium (lying position)
D: cotyledon cultured with its small edge in the medium (upright position)

Appendix 5: Staining methods

A) Ethanol method:

* Wash roots
* Fix in ethanol (abs.) overnight for pigmented roots (or half an hour for fine roots)
* Transfer to Lactophenol Cotton Blue (toxic when heated) and place in 60°C overnight.
* Cool down.

B) KOH method (Davidson, pers.comm.)

* Stand in 10% KOH overnight and simmer at 90°C the following day (if necessary) roots are soft and cleared (1 hour).
* Remove KOH and wash roots at least 3 times with water. Remove pigment by standing in 10 vol. % H_2O_2 for 10-60 min at room temperature.
* Acidify roots with 5% HCl .
* Pour off HCl.
* Add stain[1] and simmer at 90°C for 2-3 min only and cool down.
* Pour off stain.
* Add Lactic acid (destaining) and simmer for 10-15 min.
* Cool down.

[1]) Lactophenol Trypan Blue:

50 ml	Technical grade lactic acid
25 ml	glycerol
2.5 g	phenol
20 ml	distilled H_2O
5 ml	1% Trypan Blue