STUDIES TOWARD THE DEVELOPMENT OF *IN VITRO* CULTURE SYSTEMS FOR *OCOTEA BULLATA* (Burch) E. May (Lauraceae).

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ABSTRACT

This study investigates the potential to propagate an endangered Afro-montane forest tree, *O. bullata* (Burch) E. May, using micropropagation methods.

During the course of the experiment, a dramatic decrease in levels of microbial contamination was achieved. The procurement of clean explants can be attributed to the various pre-treatments of the mother material that were undertaken, and indicates that the potential to use field-grown material for *in vitro* propagation of this species does exist.

A repeatable protocol for shoot development was achieved using MS macro- and micronutrient formulations, 0.2mg/l BAP and 0.05mg/l NAA and 1g/l casein hydrolysate. Successful rooting of these explants was not achieved due to time constraints, but some relevant observations were made.

The attempted initiation of callus cultures was unsuccessful, but many important observations were made with regard to the growth regulators required, phenolic exudation and the conditions of culture, and these will be of use in further studies of micropropagation of *O. bullata*.

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ABBREVIATIONS USED

AC	activated charcoal
BAP	benzyl amino purine
CH	casein hydrolysate
cm	centimeter
2,4-D	2,4-dichlorophenoxyacetic acid
IBA	3-indole butyric acid
l	litre
mg	milligram
MS	Murashige and Skoog (1962) nutrient formulation
NAA	l-naphylacetic acid
NaOCl	sodium hypochlorite
pH	hydrogen ion concentration
w/v	weight per volume
%	percent

1. INTRODUCTION

The black stinkwood, *Ocotea bullata* (Burch.) E. Mey. is a medium to large evergreen tree that is restricted to moist afro-montane forests in South Africa. The wood is very highly prized due to its fine texture, natural lustre and beautiful colour. Since its discovery by the early settlers in South Africa, this tree has been in constant demand for timber. As early as 1812, the Knysna Forests had been seriously depleted of all easily accessible specimens, and felling of *O. bullata* was forbidden (Coates Palgrave et al. 1977).

Today, the most serious threat facing the black stinkwood is the demand for its bark for use in traditional healing, where it is widely used for magical and medicinal purposes. It is thought to ward off evil and to make the user popular, and is used as a component of many purgatives or emetics to treat a great variety of ailments (Watt and Breyer-Brandwijk 1962, Cunningham 1988).

The over-exploitation of medicinal plant populations is becoming a widespread problem which is due mainly to the increasing numbers of commercial gatherers who exploit what is regarded as a common property resource. Recent reports document that heavy debarking is occurring, with up to 95% of all stinkwoods in certain areas of Kwazulu being exploited for their bark and 40% of these are ring-barked and dying. In the Transkei, 51% of stems have more than 50% of the bark removed. Higher levels of debarking are experienced in afro-montane forests which are close to urban areas where commercial exploitation has continued for longer periods (Cunningham 1988).

As trees become scarcer, they are stripped at progressively earlier ages, and thus locating mature specimens capable of providing seed is becoming increasingly difficult (G. Nichols, pers. comm.). The seeds of this species are reported to remain viable for approximately five days after dehiscing from the mother plant, and although seed viability under cultivation is high (approximately 80%), viability in nature is 0.01%

(Cunningham 1988). The seeds are heavily predated by a weevil and the flowers are subject to a fungal disease (Coates Palgrave 1977).

The heavy demand for the species' timber and bark in combination with its poor natural regeneration from seed have led to *O. bullata* being rated as vulnerable (Cunningham 1988). It therefore has high priority conservational status in addition to its considerable aesthetic value (Coates Palgrave et al. 1977).

Problems relating to Propagation

Obtaining plantlets from seed would be the favoured route of propagation of *O. bullata*, but it is becoming increasingly difficult obtain seeds due to the scarcity of mature specimens (G. Nichols, pers comm.), and this is exacerbated by seed predation and flower pathogens. There is an urgent need for the development of alternative methods of propagation of *O. bullata*.

Attempts have been made to vegetatively propagate this species, but, as is the case with many forest hardwoods, the rooting systems which are produced using established horticultural techniques are weak or inadequate, resulting in plants regenerated by these methods displaying poor growth or failing to reach maturity (M. Thomas, pers. comm.).

Micropropagation methods offer a viable alternative, as these frequently result in superior root systems to those that traditional horticultural methods can produce. Entirely normal root systems develop in plantlets regenerated from somatic embryos, as these follow the same morphological developmental sequence as do seeds (Carron et al. 1984, George 1993).

Species that are slow or difficult to propagate vegetatively may also respond much better *in vitro* due to the finer adjustment of factors influencing regeneration, such as nutrient levels, light, temperature and growth regulator levels (George 1993).

Micropropagation can be successfully used to rapidly increase the amount of healthy plant material available and so is ideal for the multiplication of rare or endangered species.

Apart from tree breeding and propagation, tissue culture technology has widespread application in disease eradication, germplasm storage and genetic manipulation, and has become the focus of considerable research in recent years (Razdan 1993).

The present study was undertaken in order to determine protocols for the *in vitro* culture of *O. bullata* primarily to facilitate the rapid multiplication of existing genotypes, with the long-term survival of the species as the major goal. Direct organogenesis encompasses the emergence of adventitious organs directly from pre-existing meristems on the explant without an intervening callus phase is the cheapest and simplest micropropagation route. It is also associated with the least risk of genetic abberations (Pierik 1987, Krikorian et al. 1993, George 1993).

In addition, direct organogenesis is the best method that may be passed on to other institutions interested in propagating the species, as these may not have the sophisticated laboratory facilities necessary to undertake some of the more demanding micropropagation procedures. In this study, direct organogenesis was therefore attempted prior to any other micropropagation system.

Due to high levels of internal contamination in field-grown material, an alternative protocol was investigated, that of indirect organogenesis via callus production. Calli were initiated from very young leaf material with the intention of avoiding internal contaminants.

If callus cultures could be obtained for *O. bullata*, many possibilities would exist for its propagation and survival. Somatic embryos produced from all remaining genotypes could be cryopreserved in genebanks, ensuring against the genetic decline of the species. Somatic embryogenesis has been described as probably the most

powerful micropropagation pathway in plants (Petiard et al. 1992). The list of species and genera for which it has been reported increases in length every year (George 1993), and *in vitro* plantlet regeneration via somatic embryogenesis of *Ocotea catharinensis*, an endangered Brazilian forest tree, has recently been reported (Moura-Costa et al. 1993).

Although the genus *Ocotea* is extremely large and heterogeneous (Rohwer et al. 1991, van der Werff 1991), this has implications for the regeneration of *O. bullata*. Embryogenesis in *O. catharinensis* was an extremely slow process, requiring two and a half years before hardened plantlets were obtained. Moura-Costa et al. (1993) reported extreme difficulty in controlling the early stages of embryogenesis, and state that improved control may be accomplished by manipulation of media formulations.

Thus it was unlikely that somatic embryos would be obtained, given the time constraints of the present study. Rather, some preliminary investigation with regards to callus initiation from young leaf tissues was attempted. Seed germination under aseptic conditions is another widely recognised method of obtaining contaminant-free leaf material for further studies, particularly callus initiation. Due to continuing problems with internal contaminants, an attempt was made to germinate seeds of this species aseptically.

The development of protocols for indirect organogenesis and the initiation and maintenance of cell suspension cultures has potential value for studies on genetic transformation of *O. bullata*. The possibility of genetically engineering resistance to the pathogens that plague this species could be investigated, and overall, the species would have a better chance of surviving into the future.

2. MATERIALS AND METHODS

2.1 SOURCE OF MATERIAL AND PRE-CULTURE TREATMENTS

2.1.1 Saplings

Eleven young saplings of *O. bullata* were obtained from the National Botanical Gardens, Kirstenbosch, and a further six saplings obtained from Silverglen Nursery, Durban. These plants were placed in a Phytotron Unit in the Department of Botany at the University of Cape Town for two and a half months and one month respectively prior to being used as source material for culture. This was effected due to previous reports of considerably lower levels of contamination being recorded for cultures initiated from material housed in a phytotron than for those initiated from greenhouse material, which in turn was reported to be much cleaner than field-grown material (Bourne 1977).

The phytotron was set for a photoperiod of 14h:10h light/dark with light intensity of 350 μmol.m^{-2}.s^{-1}, and a relative humidity of 65%. The temperature was set to vary throughout the day (as set out in Appendix 1). These conditions were found to be appropriate to induce bud break and formation of new shoots (Figure 1).

In order to further minimise the amount of associated microflora prior to culturing, the plants were treated with systemic fungicides and insecticides. Doses were as prescribed by the manufacturers recommendations (information given in Appendix 2). These treatments were applied separately and were alternated weekly, in order to avoid chemical burn of the leaves. Plants were fertilised monthly with 4mM NO_3^- Long Ashton solution (Hewitt 1966), and watered twice weekly.

FIGURE 1: **New shoot development seen on the mother material subject to the conditions in the phytotron unit.**

2.1.2 Seeds

Fifty nine seeds of *O. bullata* were obtained from Mr G. Nichols of Silverglen Nursery, Durban. Approximately half showed signs of weevil damage, while many were cracked.

2.2 SURFACE STERILISATION PROTOCOLS

2.2.1 Sapling Material

Shoots approximately 5cm long were removed from the saplings and placed into distilled water immediately. These were rinsed well in running deionised water for two minutes, after which they were sterilised using one of the sterilisation protocols outlined below. These procedures were performed in a laminar airflow cabinet and all glassware and equipment was autoclaved prior to use.

Solutions of NaOCl (JIK, Reckitt and Colman) and ethanol were used as sterilants. These contained 1 drop Tween 20 per 100ml. The sterilisation protocols used were:

1. Shoots were placed into 1% NaOCl solution for 5 minutes after which they were rinsed three times in sterile distilled water.
2. Shoots were placed into 70% ETOH for 1 minute before being transferred to 1.5% NaOCl solution for 7 minutes after which they were rinsed three times in sterile distilled water.
3 Shoots were placed into 70% ETOH for 1 minute before being transferred to 2% NaOCl solution for 10 minutes, after which the explants were rinsed three times in sterile distilled water.
4. As above, but immersed in 2% NaOCl for 15 minutes.

The choice of sterilization protocol depended on the age of the material (as older leaves were more tolerant of sterilisation), the degree of bud break exhibited by the explant (as young unfurling buds were damaged by the sterilisation procedure, particularly the ethanol rinse, whereas dormant buds were not) and the source of the material used (the material originating from Kirstenbosch was less prone to infection than the material from Durban).

The bleached cut ends were aseptically removed, and the leaves of explants used for organogenesis were trimmed to approximately 3 cm^2, with two to five leaves remaining, depending on the explant shape and size (Figure 2). These explants were cultured as described in 1.4.1.

A portion of the excised leaf material was used for callus initiation (section 2.2.2 below).

2.2.2 Callus initiation

Very young leaf material from healthy shoots which had been subjected to sterilisation protocol number one were used for callus induction. Leaf material was trimmed into squares of approximately 6mm by 6mm, avoiding primary vasculature which may be a source of infection. These explants were cultured as described in 2.4.2.

2.2.3 Seed Material

The seeds had been immersed in 2% NaOCl for 5 minutes prior to being transported to Cape Town in a paste of the fungicide Benlate. Upon arrival, seeds were surface sterilized (for 10 minutes each) in 70% ethanol followed by 2% NaOCl, both of which contained 1 drop Tween 20 per 100ml. Seeds were then rinsed three times in sterile distilled water and cultured as described in 1.4.3

2.3 MEDIA PREPARATION

All media used in this experiment had the following in common. They contained MS (Murashige and Skoog 1962) macro- and micronutrients solidified with 0.8% agar. Water added to the media was purified through a Milli-Q^{50} ultra pure water system (Millipore). The pH was adjusted to 5.8 prior to the media being autoclaved at 115° C and 1.5 lb/in^2 for 15 minutes. Variations in additions to this basal composition are described and justified below, and the various media compositions tested in this study are given in Table 1.

Culture vessels used in all experiments were Consol Glass jars (100ml volume) with opaque plastic lids. Standard tissue culture procedures were followed in glassware preparation (George 1993).

Media composition

In order to elicit a particular response from a given species, trial and error experimentation is required to find the appropriate balance between the plant growth regulators cytokinin and auxin. Cell division is regulated by the joint action of auxins and cytokinins, each of which appears to influence different phases of the cell cycle. DNA replication is affected by auxins, whilst cytokinins seem to exert control over the events leading to mitosis, and cells are thought not to enter mitosis unless cytokinins are present (George 1993). In general, a high cytokinin: low auxin ratio is used to induce shoot formation and the reverse ratio is used to induce root formation. Intermediate or equal ratios frequently induce callus formation, depending upon the species and responsiveness of the tissue or organ used (George 1993).

High levels of applied auxins seem to be capable of erasing the genetically programmed physiology of whole plant tissues which had previously determined their differentiated state (George 1993). Reversion to a dedifferentiated state and the resumption of cell division occurs in those cells which are responsive to auxins, so an auxin is used in callus initiating media; this is usually the highly active synthetic auxin 2,4-D (Razdan 1993, George 1993).

Some plant taxa, particularly tropical species, naturally contain high concentrations of phenolic compounds. These are strong reducing agents which are oxidized when cells are wounded or senescent. The tissue subsequently blackens and fails to grow (George 1993). The inclusion of activated charcoal (AC) in the media can absorb phenolics and other toxic substances (George 1993, Razdan 1993). However, activated charcoal has been implicated in a variety of subtle effects, including altering the pH of the medium, excluding light from the medium and greatly increasing the hydrolysis of sucrose to glucose and fructose during autoclaving (Druart and De Wulf 1990). Thus, when AC was included in the media, it was used at low concentrations of 1g/l.

TABLE 1: The composition of media used in this study.

MEDIA CODE	MACRO- AND MICRO- NUTRIENTS	SUCROSE g/l	AUXIN (l^{-1})	CYTO- KININS (l^{-1})	OTHER ADDITIVES (l^{-1})
SHOOT INDUCTION MEDIA					
FSMS	MS	3	---	0.2 mg BAP	---
HSMS	1/2 MS	3	---	0.2 mg BAP	---
EMS	MS	3	0.05 mg NAA	0.2	1g CASEIN HYDROLYSATE
ROOT INDUCTION MEDIA					
RMS1	MS	3	2mg IBA	---	1g CASEIN HYDROLYSATE
RMS2	MS	3	5mg IBA	---	1g CASEIN HYDROLYSATE
CALLUS INDUCTION MEDIA					
CMS1	MS	3	2mg 2,4-D	---	---
CMS2	MS	3	2mg 2,4-D	---	1g ACTIVATED CHARCOAL
CMS3	MS	3	5mg 2,4-D	---	---
CMS4	MS	3	5mg 2,4-D	---	1g ACTIVATED CHARCOAL
SEED MAINTENANCE MEDIA					
SMS1	MS	3	---	---	---
SMS2	MS	5	---	---	---
SMS3	MS	3	1mg NAA	1mg BAP	1mg GA_3

2.4 CULTURING PROCEDURES

2.4.1 Organogenesis

Surface sterilized shoots were aseptically transferred into 25ml of the appropriate medium being tested for shoot development. These were incubated at 25° C with a 16h:8h light/dark regime with a photon flux density of 45 μmol.m^{-2}.s^{-1} and monitored for indications of bud break.

In all shooting trials undertaken, infected material was repeatedly surface sterilised by immersion in 70% ethanol for 1 minute (depending on the severity and type of the infection) and subcultured onto the same media. In general, this procedure eliminated slight bacterial infection but had little effect on fungal infection.

Explants that were either below the observed critical minimum size (1 cm in length with no leaves) or deemed no longer recoverable due to tissue damage were discarded. In the last three trials (K5, M1 and M2) explants were discarded at the first sign of infection or loss of vigour due to time constraints, thus the mortality reported is slightly higher.

Experimental methods were modified in accordance with observations made in the previous trials, hence subtle changes in protocol were constantly being undertaken. The initial three sets of cultures were routinely subcultured every 14 days, but the results suggested that this should be reduced, so that subsequent sets were routinely subcultured every 10 days.

The entire experiment was conducted over a period of six months

2.4.2 Callus initiation

Trimmed squares of young leaf tissue were aseptically transferred onto 10 ml of callus initiation media. In order to determine the effect of leaf orientation on callus formation, sections were placed such that either the abaxial surface was in contact with the medium (AB) or such that the adaxial surface was in contact with the medium (AD). Each jar contained 3-4 sections of tissue from the same leaf, all with the same orientation. In each trial, half of the explants were AB orientated and half were AD orientated.

Jars were incubated in a Golencamp Mycological Incubator at 29°C (±4°C) in the dark, and were not subcultured. Due to phenolic accumulation observed in the first two sets of calli initiated, subsequent calli were initiated onto media with the same hormonal concentration as previous sets, but also containing activated charcoal (AC).

2.4.3 Aseptic Seed Germination

Three seed germination media outlined in Table 1 were tested. One third of the seeds were aseptically transferred onto 20ml of one of these media. The seeds were orientated horizontally and pressed gently half-way into the medium. Culture jars were subsequently incubated at 25°C in the dark.

2.5 IDENTIFICATION OF BACTERIA

Preliminary bacterial identification of the contaminants present in four badly infected explants was performed by Ms Katrina Downing of the Microbiology Department, U.C.T.

3. RESULTS

Due to the limited availability of plant material suitable for initiation of shoot cultures, the number of explants in any one trial was unavoidably small. Statistical analyses could therefore not be performed.

After removal of the young shoots, the sapling mother material required at least a month to regenerate new shoots. This led to staggered initiation of shooting trials and the last few trials had only spent ten weeks in culture. In addition, an initial loss of some explants is unavoidable in all tissue culture protocols (Razdan 1993, George 1993).

Early experiments were hampered by severe contamination, possibly due to insufficient time for pre-treatment of mother plants (2.5 months for the Kirstenbosh material, and approximately 1 month for the Durban material).

Results will be presented in the form of a short verbal description of each trial and the modifications that were included therein. The most successful trials are tabulated in Table 2, and graphs indicating the rate of mortality are given in Figures 3 and 4. Finally, as an overview, a brief summary of the observations is presented.

3.1 SHOOT INITIATION TRIALS

3.1.1 Kirstenbosch material

Trial K1

8 mature explants, FSMS, sterilisation protocol 3.

Severe bacterial contamination ultimately led to the loss of all explants. Despite this, bud scale break occurred in 25% of explants prior to their demise. This was an indication that MS nutrient formulation and the use of 0.2mg/l BAP was capable of inducing bud break should clean explants be available.

Trial K2

7 mature explants, HSMS, sterilisation protocol 3.

This batch also experienced severe bacterial contamination which led to the loss of all explants, but the demise of the explants was more rapid than that seen in trial K1, and bud-break did not occur. This gave an indication that half-strength MS nutrients were insufficient to support growth.

Trial K3 [1]

13 young explants, 2 subculture cycles of FSMS then transferred to EMS, sterilisation protocol 2.

This batch showed a decrease in levels of contamination. Within 5 weeks in culture, 31% were abandoned due to possible endophytic bacterial contamination.

It was noted that cultures showed a substantial deterioration during the latter three days of the subculture cycle, with the tissues losing turgor and chlorosing (Figure 5). This indicated that subculturing should be conducted more frequently than at 14 day intervals. As a result, subsequent subculture cycles were reduced to 10 days.

Within 3 weeks after initiation into culture, just over 50% of the explants has abscised all of their leaves, especially the youngest ones, and some has experienced necrosis of their terminal buds. These showed no further growth, indicating the importance of leaf retention to maintain the supply of photosynthate, which is crucial for subsequent growth.

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[1] The most successful trial, therefore lengthy.

Thirty-one percent of cultures, all of which had retained their leaves, showed bud-break and leaf expansion after 6 weeks in culture (Figure 6). These also experienced callusing at the base of the stem, presumably as a reaction to the auxin in the medium.

Additional bud break was observed from one static explant after 11 weeks in culture (Figure 7). After 10 weeks in culture leaf expansion was profuse (Figure 8) and an attempt was made to root the explants which broke bud at five weeks (See rooting trials).

Trial K4

9 young explants, EMS, sterilisation protocol 1.

Thirty three percent of explants were lost to bacterial contamination. Forty-four percent of explants exhibited bud break, although further shoot growth and leaf expansion did not occur. All explants of this batch ultimately lost their leaves and growth ceased.

Trial K5

16 young explants, EMS, sterilisation protocol 2.

A large proportion of explants were lost due to chlorosis, leaf abscission and necrosis of tissues (Figure 9). This may have been caused by chemical burn, as the parent material was treated with Funginex two days prior to culturing.

Survival was 31% after 10 weeks in culture and leaf abscision had occurred in nearly all cultures. After twelve weeks, 12% of explants exhibited bud-break (Figure 10), these being the two explants that had retained their leaves.

Trial K6

12 mature explants, EMS, sterilisation protocol 3.

The level of bacterial contamination observed in this trial was minimal relative to previous trials. Fifty percent of the explants retained their leaves and remained uncontaminated. These all formed callus at their bases after approximately one month in culture. This was probably due to the presence of auxin in the medium, and was not observed in the very young material from other trials. Bud-break occurred in 50% of explants after only 5 weeks (Figure 11).

3.1.2 Durban material

Trial D1

14 mature explants, FSMS, sterilisation protocol 3.

Despite numerous subcultures and resterilisation attempts, fungal contamination resulted in 100% mortality after 6 weeks in culture. The progress of the infection was extremely rapid, resulting in severe tissue damage, especially to the meristems (Figure 12). In one culture shoot growth occurred after 5 weeks, but this explant was ultimately lost to fungal contamination soon thereafter (Figure 13).

Trial D2

21 stem sections without leaves, EMS, sterilisation protocol 4.

After 8 days, mortality due to fungal infection was 95.3% despite 4 resterilization and subculture attempts. The remaining explant exhibited shoot

growth after 4 weeks, and showed callus growth at the base of the stem in response to the auxin-containing medium (EMS). Leaves and shoots continued to develop and expanded well (Figure 14). After 11 weeks, an attempt was made to root this explant (see rooting trials).

Trial D3

2 young stem sections, EMS, sterilisation protocol 1 without the ETOH rinse.

Bud break (with greening of the buds) was initiated from both terminal and auxiliary buds, but did not progress to shoot growth. Auxiliary buds became translucent and the stems blackened. There was no further development after 11 weeks. This apparent quiescence was a commonly observed phenomenon.

3.1.3 Cultures initiated from mixed Kirstenbosch and Durban material

Trial M1

22 young explants, EMS, sterilisation protocol 1 without the ETOH rinse.

While infection was minimal in this batch, leaf abscission and necrosis resulted in substantial mortality. After 7 weeks, 32% of explants were still considered viable, with 18% being healthy and having retained their leaves. Shooting was considered imminent in these cultures.

Trial M2

8 young explants, EMS, sterilisation protocol 2 without the ETOH rinse.

One week after initiation, mortality of explants was 64% from both infection and necrosis. Those explants that succumbed to infection were mostly from the Durban material.

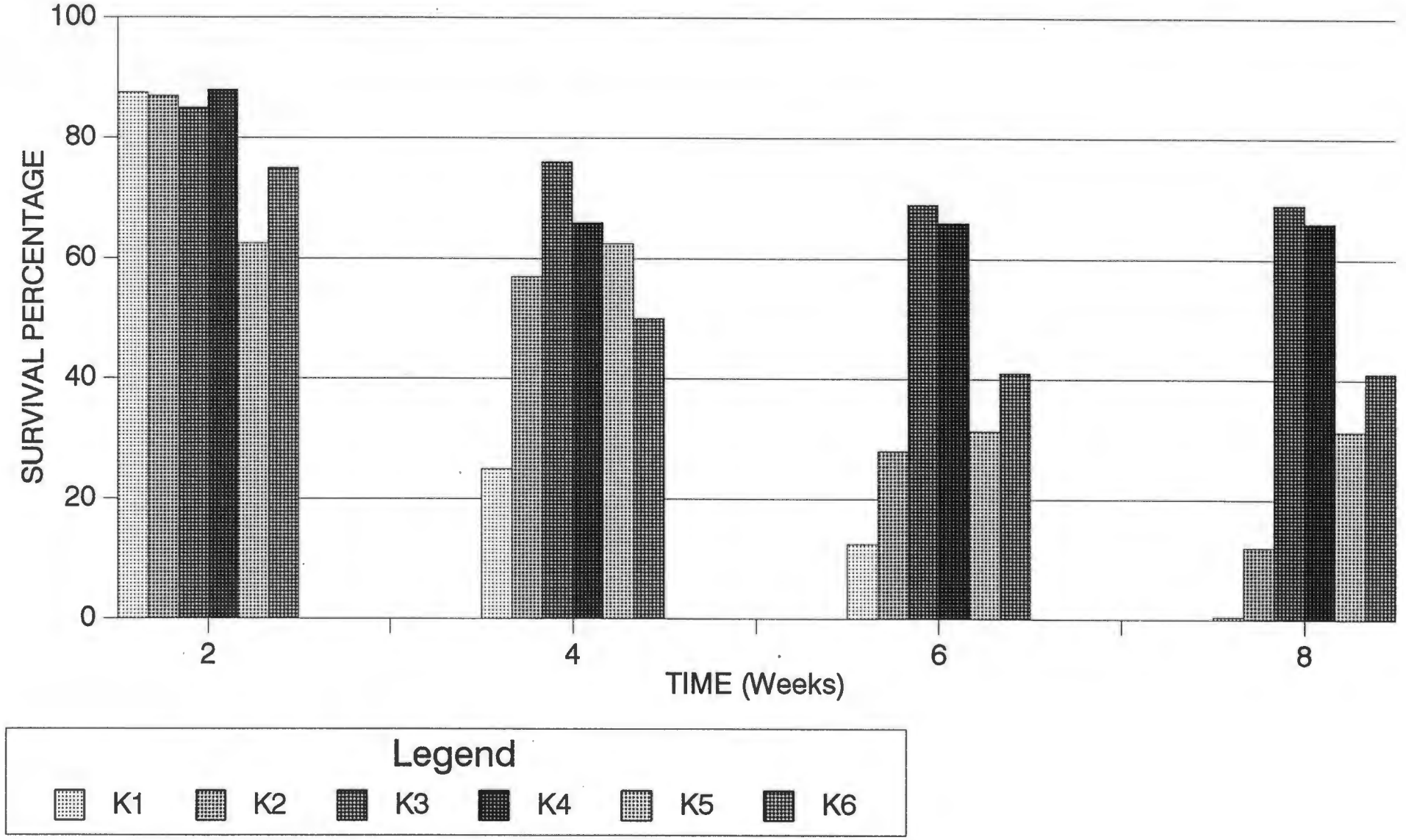

FIGURE 3 : Percentage survival with time of explants in trials initiated from the Kirstenbosch material, showing lower survival after 8 weeks of the first 2 trials (K1 and K2) which were placed onto FSMS and HSMS, respectively. Subsequent trials used supplemented with the Auxin NAA and CH, and these display enhanced survival after 8 weeks.

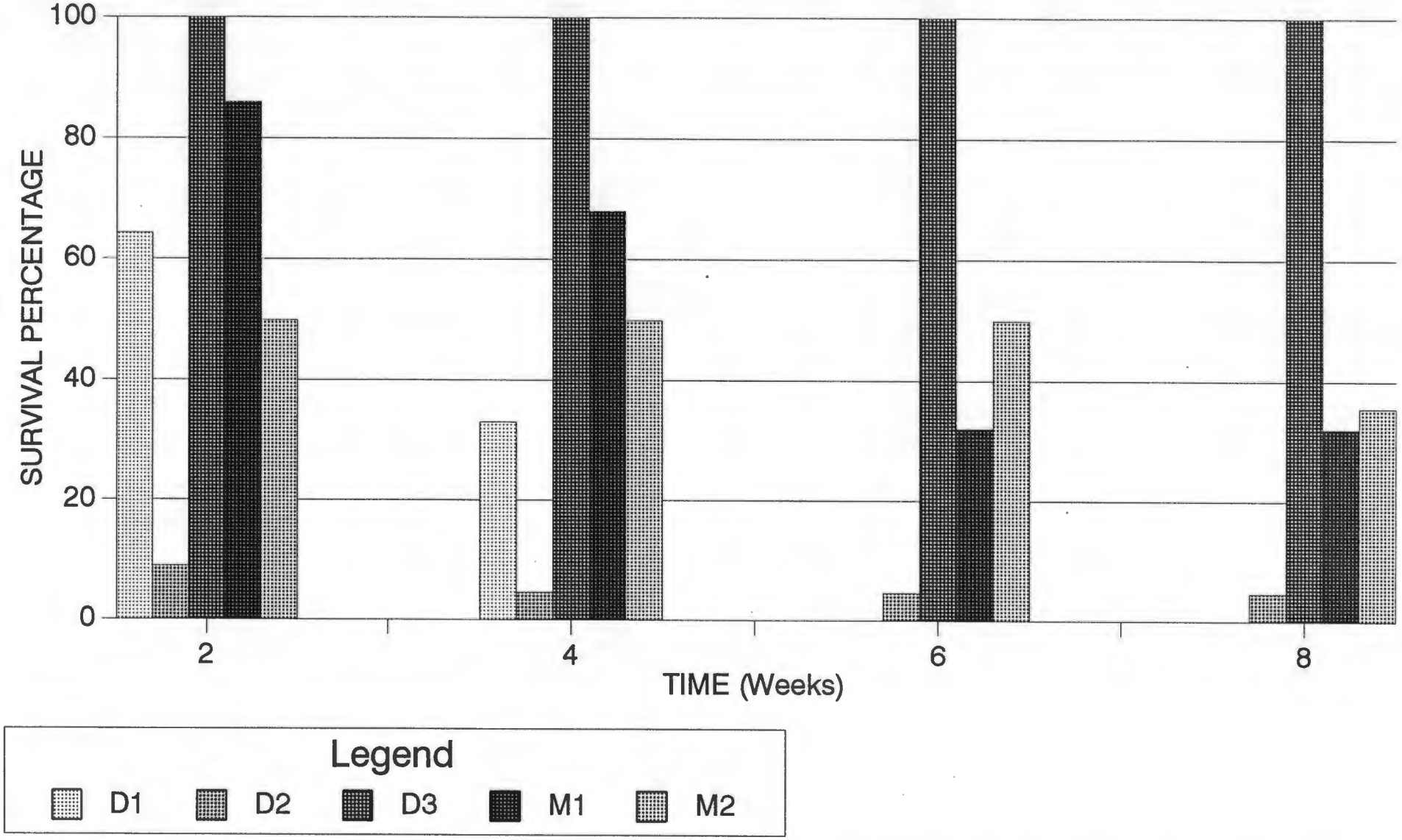

FIGURE 4: Percentage survival with time of explants in trials initiated from Durban material and in trials initiated from both sources, showing a similar trend as seen in Figure 3. Note high survival in trials D3 (2 out of 2 explants survived) compared to D2 (1 out of 21 explants survived), which distorts this trend.

FIGURE 2: **Photograph showing the extent of trimming of explants prior to initiation into culture.**

FIGURE 5: **Explant derived from young leaves showing a loss of turgor accompanied by leaf curl, which was followed in most instances by leaf abscision.**

FIGURE 6: **Bud-break exhibited by an explant on EMS medium from trial K3 after six weeks in culture.**

FIGURE 7: **Additional bud-break exhibited by an explant on EMS medium from trial K3 after 11 weeks in culture.**

FIGURE 8: **Leaf expansion exhibited by an explant on EMS from trial K3 after 11 weeks in culture. Note that original leaves have abscissed; these leaves formed *in vitro*.**

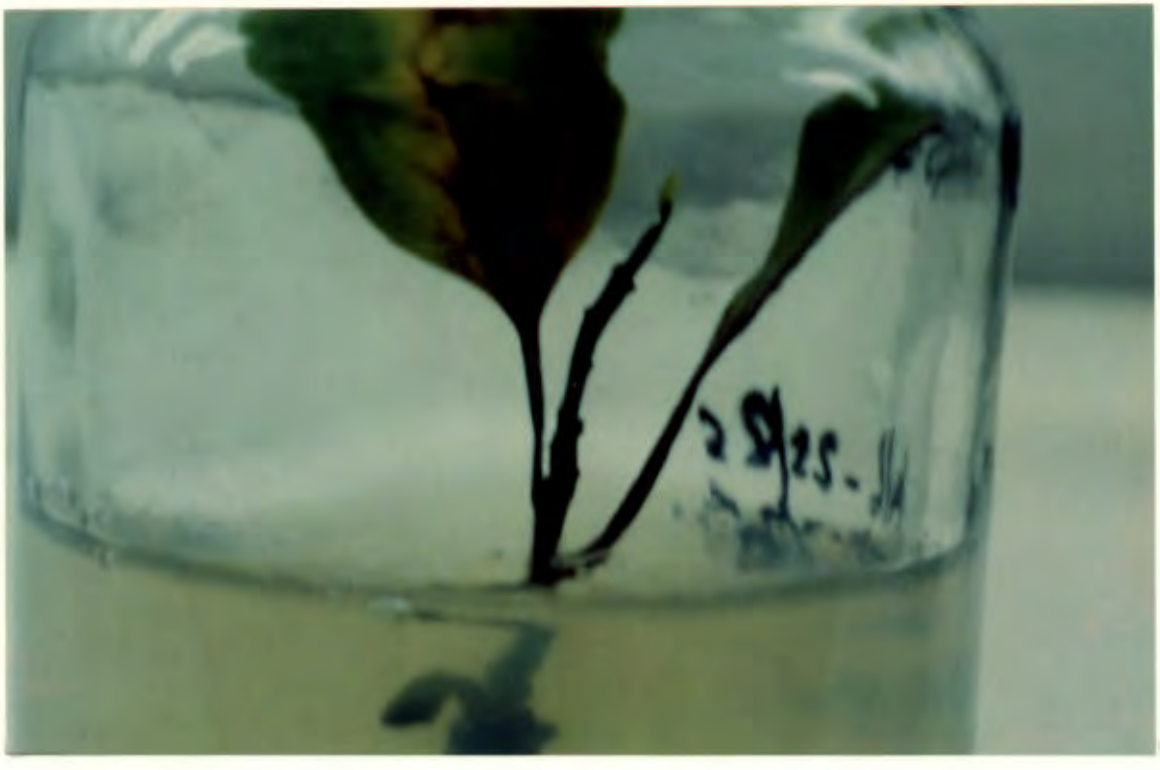

FIGURE 9: **Chlorosis of the leaves followed by necrosis of the midvein and petiole.**

FIGURE 10: **Bud-break observed after 12 weeks in culture on an explant from trial K5. Note prolific callusing at the base of the stem.**

FIGURE 11: **Bud-break observed after 5 weeks in culture on an explant from trial K6.**

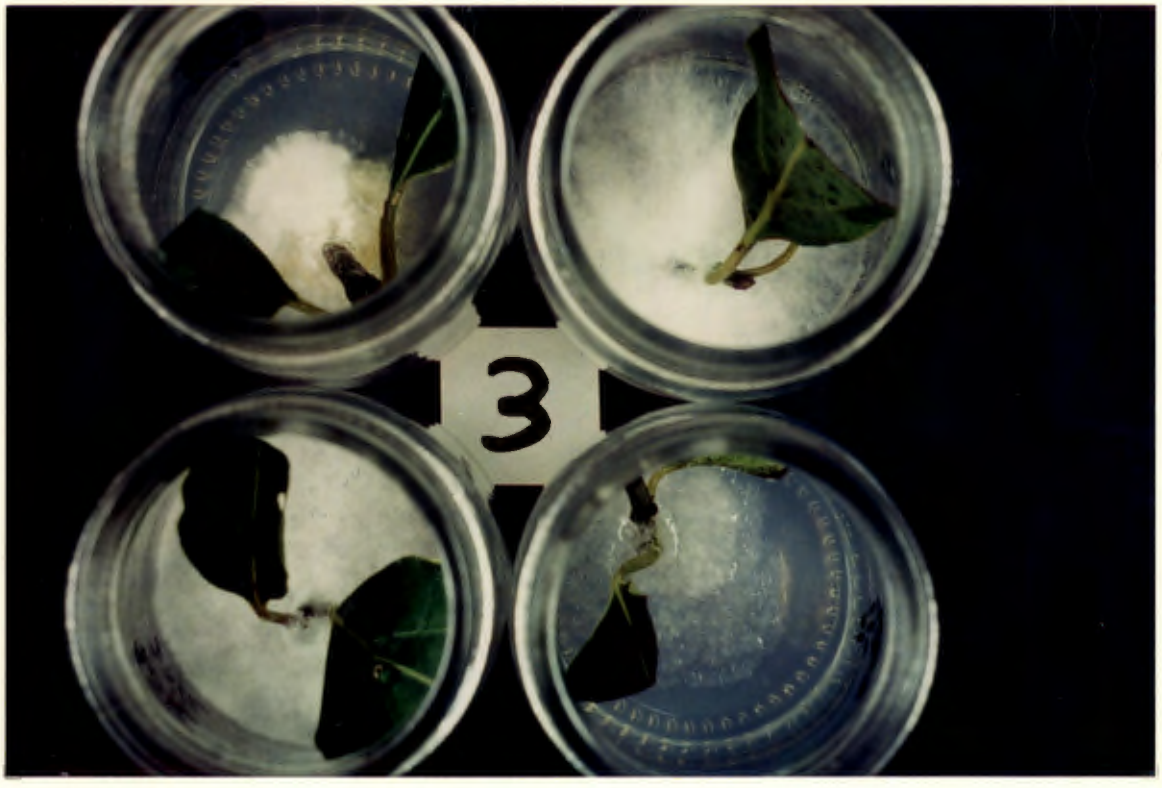

FIGURE 12: **Fungal infection after 3 days, showing the rapid progress of the infection and the extent of hyphae overwhelming the meristematic regions.**

FIGURE 13: **The last surviving explant of trial D1 showing shoot growth accompanied by the fungal infection which led to its demise.**

FIGURE 14: **The surviving explant initiated from a leafless stem section in trial D2, showing prolific shoot growth and good leaf expansion.**

FIGURE 15: **Rapid chlorosis and mature leaf abscission observed in explants that had developed new shoots from trial K3 soon after they were placed onto rooting medium containing 2mg/l IBA. Young shoots had already abscissed.**

FIGURE 16: The extent of phenolic exudation into the callus inducing medium from leaf segments, note the black colouration of the explants.

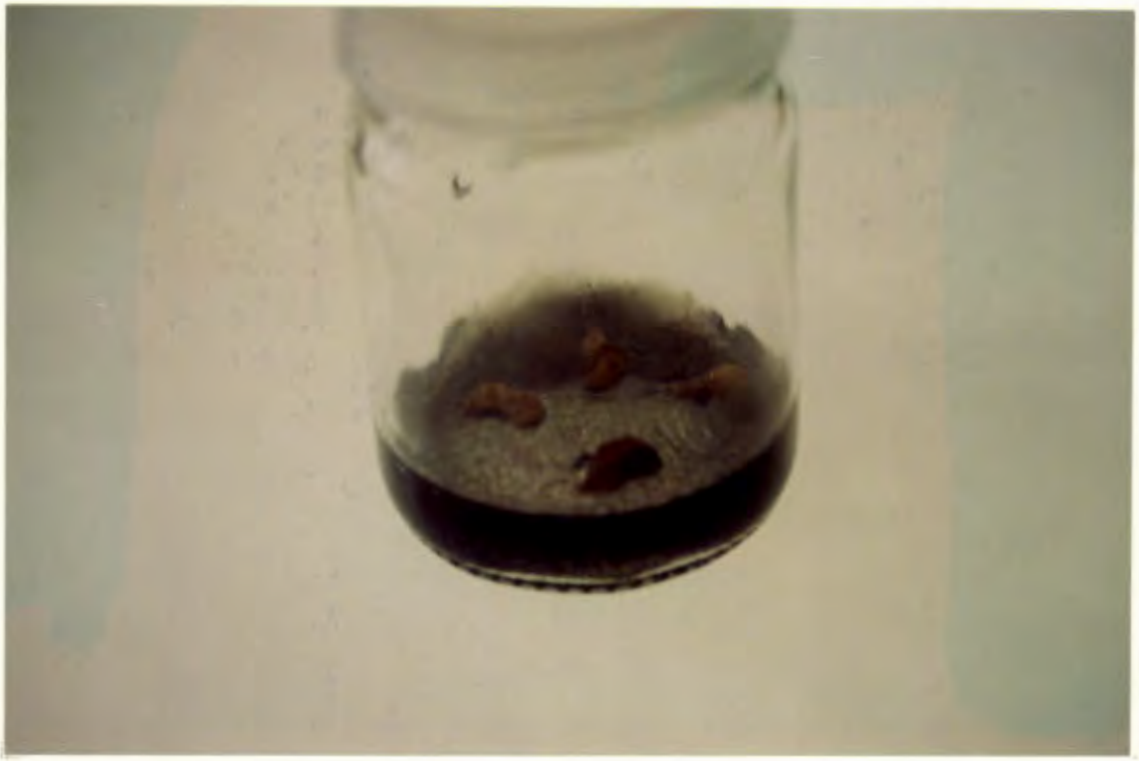

FIGURE 17: Leaf explants initiated onto media containing AC and 2mg/l 2,4-D. In this photograph, the tissue was still dividing and was brown in colour, but growth subsequently ceased and the tissue blackened, as seen in media without AC.

TABLE 2: Summary of the most successful trials.

TRIAL NO.	SOURCE	NO. OF EXPLANTS	% BUD BREAK	STERILIZATION PROTOCOL USED	COMMENTS AND OBSERVATIONS
K1	K	8	25	3 + ETOH	Older material, no leaf expansion due to high mortality from infection.
K3	K	13	39	2 + ETOH	Young leaf material, best survival of new shoots. 50% of explants retained leaves.
D2	D	21	4.7	4 + ETOH	Stem sections, high mortality due to fungus, but prolific shoot growth and leaf expansion observed in the surviving explant.
K4	K	9	44	1	Young material, all shooting explants eventually experienced abscision of older leaves after attempt at rooting.
K6	K	12	50	3 +ETOH	Older material, minimal contamination observed. Callusing at base after 4 weeks in culture.

3.1.4 Summary of observations from experiments on direct organogenesis

Frequency of sub-culture:

This was found to be around 10 days, due to the loss of vigour observed after this period had elapsed.

Infection:

This showed a substantial decrease with the course of the experiment, and was more severe in material from Durban than in that from Kirstenbosch. The alcohol rinse was important in preventing infection, but resulted in tissue damage to young developing shoots and adversely affected the survival of young shoot material.

Age of Material:

Older shoots were found to be more suitable for initiation *in vitro*, as these were better able to withstand sterilisation and did not lose turgor nor suffer leaf abscission to the extent that the young shoot material did.

Leaf necrosis and abscission:

A high proportion of explants abscised their leaves within six weeks of initiation *in vitro*. In cultures that had lost their leaves, bud-break would frequently commence with the scales unfurling, followed by expansion of the auxiliary buds, but shoot growth would then cease and the explant would enter a period of stasis. Frequently, these new shoots would subsequently chlorose and vitrify, which was followed by abscission of the developing organ. Concurrent with these events, infection frequently overcame the explants, suggesting a drop in resistance to infection.

Addition of auxin and casein hydrolysate:

These media additives were found to greatly enhance the growth and maintenance of explants in culture, as can be seen from Figures 3 and 4. These graphs show that explants from the initial trials (using FSMS and HSMS) had suffered considerably higher mortality after eight weeks than the latter trials (using EMS containing auxin and CH). However, the effect of contamination cannot readily be separated from this. The medium containing these additives (EMS) usually facilitated bud-break within six weeks, and was capable of supporting leaf expansion.

Phenolics:

These were negligible in explants initiated for direct organogenesis, but phenolic oxidation could have been responsible for sealing the bases in some cultures, leading to flaccidity.

Callusing at the base of the stem:

This was frequently observed in the medium containing auxins, mostly in more mature explants; young shoot explants experienced this to a lesser extent and did so only after a lengthy period in culture.

3.2 ROOTING TRIALS

De Novo Rooting Trials

4 mature explants from Kirstenbosch, RMS1, sterilisation protocol 4.

Explants were placed directly into rooting media (with no previous attempt at obtaining shoot growth) to determine the length of time that is required for rooting to occur. While callus formed at the stem bases due to the auxin in the medium, all explants were ultimately lost to bacterial contamination after 5 weeks in culture. Repeat trials using RMS2 resulted in the same outcome.

Rooting of successful shoot cultures

4 explants from trial 4 and 1 from trial 5, RMS2.

After successful leaf expansion, rooting was attempted by transferring cultures to RMS2. This medium resulted in leaf abscission and chlorosis after 1 week (Figure 15). The explants were transferred to hormone-free media (SMS1) for two weeks before being returned to EMS. Three explants recovered and continued leaf expansion, but two showed no further growth.

3.3 CALLUS INITIATION

Results from all trials are summarised below. The numbers of culture vials initiated per trial is shown in Table 3.

TABLE 3: Number of culture vials initiated onto the respective callus initiating media.

MEDIUM USED	NUMBER OF VIALS
CMS1	10
CMS2	13
CMS3	16
CMS4	44

No growth was observed in explants placed on media with 5mg/l 2,4-D. This treatment resulted in rapid tissue necrosis.

The explants placed onto medium containing 2mg/l 2,4-D fared substantially better, with cell proliferation from the cut surfaces being observed 10 days after initiation. This growth was initially more rapid in material placed such that the adaxial surface of the leaf was in contact with the medium (AD orientation), but after approximately one month, the explants that were initiated with the abaxial surface in contact with the medium (AB orientation) had progressed to the same extent.

However, it was noted that AD orientated cultures turned brown due to phenolic accumulation more rapidly than AB orientated cultures, but as time passed, phenolic accumulation became just as severe in explants of AB orientation as it was in AD orientated explants.

Within one month all explants, regardless of orientation, had turned dark brown and ceased cell proliferation. The addition of activated charcoal to the media did not prevent phenolics accumulating in the explants; this was as severe in tissue on media with AC as in tissue on media without AC. (Figures 16 and 17). No visible cellular dedifferentiation occurred, only slight proliferation.

Contamination was minimal, with only 2.4% infection being recorded after five months in culture.

3.4 SEED GERMINATION

Despite rigorous initial sterilisation procedures, fungal and bacterial infection was extremely severe. Seeds were subcultured three times in three days, again using rigorous sterilisation procedures, before embryo rescue was attempted. After a further subculture, minimal contamination (with only slow-growing bacteria present) was obtained in 15 embryos, but these did not appear viable and were discarded after 6 weeks.

4. DISCUSSION

Throughout the discussion, potential factors that could explain the results are presented, and recommended courses of action for future studies on *O. bullata* are proposed.

4.1 DIRECT ORGANOGENESIS

4.1.1 Contamination of cultures

In initial experimentation, cultures were severely infected with fungal and bacterial contaminants. Bacteria present in this study include *Bacillus* spp. and *Pseudomonas* spp, which are commonly encountered bacterial genera in tissue culture (George 1993). Fungal infection was particularly severe in material from Durban, and generally resulted in the death of the explant via tissue damage, particularly to the meristems (Figure 12). Bacterial infection was more prevalent in material from Kirstenbosch, but did not result in such severe necrosis of tissues as fungal infection did. Bacteria were occasionally eliminated via frequent subculturing, with explants subsequently continuing growth.

Even though initial contamination levels seem high, they were in fact lower than those which have been experienced in field-grown material by other workers. As an example, Blakeway (1992) found that contamination in field grown material of *Eucalyptus grandis* after only five days in culture was approximately 80% to 90% when antibiotics and fungicides were not included in the media.

Although bactericides and fungicides can reduce infection, the effective doses of these compounds are usually so high that the plant tissues may be poisoned or injured. The growth and development of plant tissues is frequently altered

by antimicrobial compounds as they interfere with growth regulator metabolism (Pierik 1988, George 1993); this makes identifying the exact causes of plant reactions to particular regulators difficult to establish. In addition, antibiotics frequently result only in a transient stasis of microbial growth, and contaminants frequently develop resistance to them (George 1993).

General antibiotics are frequently not effective, and the use of specific antibiotics requires that the identity of the microbes be known. This is time-consuming and requires intricate tests to be performed, and specific antibiotics are usually expensive. This project was aimed at obtaining a simple and cost-effective means of micropropagation, so alternative means of curtailing contamination were attempted.

During the course of the experimentation, there was a dramatic decrease in levels of contamination. This may be attributed to the increasing amount of time that the mother material spent in the phytotron, where it was isolated from external sources of contamination and subject to regular treatment with systemic fungicides and bactericides.

Naturally, the elimination of endophytic contaminants, which are not affected by surface sterilization procedures, is extremely difficult. These infections are usually latent or hidden, and may only become evident after many successful subculture cycles, or when the culture is transferred to another medium which is more favourable to the contaminant's growth (George 1993). An example of an endogenous contaminant discovered in this study was *Pseudomonas pikettii*, which was isolated from four culture vessels containing explants originating from the Kirstenbosch material (by Ms K. Downing). This bacterium was dormant for at least five weeks before it began to proliferate in culture. Species of the genus *Pseudomonas* are well known in tissue culture and microbiological laboratories, and are unaffectionately referred to as 'white ghosts' (Pierik 1987).

Plants can be successfully cultured in the presence of endophytic bacteria, provided that they remain relatively dormant and do not proliferate to the extent of outcompeting the explant for resources. Contamination is frequently the major cause of failure in tissue culture experiments (George 1993), and the eventual procurement of contaminant-free explants in this study should be regarded as a major success.

4.1.2 Phenolic exudation into the media

Phenolic accumulation in the media can be a substantial problem in hardwood micropropagation. In her previous attempt at *in vitro* culture of *O. bullata*, S. Lennox found that phenolic exudation severely hampered root formation (S. Lennox, pers. comm.). In the experiments on direct organogenesis conducted in this study, phenolic excretement was negligible. This can be partially attributed to the young age of the mother material. Slight phenolic exudation did occur in a few cultures with larger stem diameters, but was not severe enough to affect their growth noticeably.

If the explants were not subcultured for periods of time in excess of a month, the level of phenolic exudation escalated, which may have been a result of nutritional stress (discussed further in section 3.2.1.).

4.1.3 Chlorosis, necrosis and leaf abscission

Substantial chlorosis and necrosis was observed in explants used for direct organogenesis, and this was invariably accompanied by leaf abscission (Figure 9). S. Lennox (pers. comm.) reports that the loss of leaves was also a major problem encountered in her previous attempt to culture *O. bullata*.

Leaf abscission was observed both in newly initiated explants and explants that had been in culture for some time, and it was particularly prevalent in newly initiated cultures that utilized very young shoot tips, which indicates that older shoots may be preferable for initiation into culture.

Leaf abscission can be partially attributed to stress associated with the excision of explants and repeated subculture. However, additional factors may have also contributed, notably the temperature at which the explants were incubated. This was kept constant at 25° C, which possibly was slightly high for this moist afro-montane forest species. The explants may also have benefited from a diurnal fluctuation in temperature, as has been reported to be necessary in some species *in vitro* (Pierik 1987, George 1993). Further research is necessary to establish the optimum temperature and light intensity for shoot growth in *O. bullata*, and to determine if these factors are influential in causing this extensive leaf abscission *in vitro*.

From Figures 3 and 4, it can be seen that the first trials, initiated onto media that did not contain auxin or casein hydrolysate (FSMS and HSMS), suffered higher mortality than subsequent trials, where these additives were included. Although contamination has had a greater influence on mortality in initial trials than in subsequent trials, the addition of auxin and casein hydrolysate did markedly increase leaf retention, leading to improved survival of the explants in the subsequent trials.

The observed leaf abscission could possibly have been induced by a deficiency of some essential compound in the medium, which was alleviated by the addition of casein hydrolysate. It is usually not necessary to add amino acids in the form of casein hydrolysate to media supporting shoot cultures, but is a convenient method of ensuring against medium deficiencies, and provides a source of nitrogen that is immediately available to cultured tissues (Razdan 1993). Casein hydrolysate contains a mixture of up to 18 amino acids,

calcium, phosphate, and several microelements and vitamins (George 1993). In the media used in this study, only phosphorus may have been limited, as its levels are low in MS nutrient formulations (George 1993).

In the culture of certain species, the addition of cytokinins alone is sufficient to induce shoot multiplication in large explants, as they usually contain sufficient endogenous auxins, and this was the initial procedure followed here. In other species, the addition of a small quantity of auxin relative to the concentration of cytokinins is sometimes required to promote cell growth and elongation in shoots (George 1993).

The cessation of growth that occurred soon after initiation of explants could be an indication of the exhaustion of endogenous auxin in the explants. In order to assess whether the application of exogenous auxin was required to maintain explants of *O. bullata*, auxin at a concentration of one-quarter that of the cytokinin was added to the revised shooting media (EMS). This caused prolific callus growth at the bases of the stems of shoots originally from more mature shoots (Figure 10), but in general, the inclusion of auxin and casein hydrolysate in the media markedly enhanced the maintenance of *O. bullata* explants *in vitro* and promoted shoot growth and development.

Murashige and Skoog (1962) observe that casein hydrolysate may allow organ development over a broader range of auxin and cytokinin concentrations, and this may have been of significance here, with casein hydrolysate ameliorating the effects of the high auxin concentration. Thus, these two additives probably acted synergistically to facilitate shoot growth and development.

4.1.4 Loss of turgor and leaf curling

In many of the explants derived from very young shoots, leaf curling and the appearance of being flaccid occurred approximately 10 days after initiation (Figure 5). In older material, lignification of tissues had occurred, which may have prevented turgor loss. It is also possible that turgor loss occurred as a result of an inability to absorb water from the medium, possibly due to the sealing of the cut surface at the base of the stem by phenolic oxidation. Cutting bases under a solution of ascorbic acid or other antioxidants may alleviate this (Razdan 1993).

4.2 CALLUS INITIATION

Callus initiation was unsuccessful in these experiments, and possible explanations for this will be addressed.

4.2.1 Phenolics and pH changes

In this study, the exudation of phenolics into the callus initiation medium and accumulation of these in leaf segment tissues was profuse, leading to the tissue appearing black (Figures 16 and 17). Neither the conditions during harvesting nor harsh sterilization procedures could be implicated as the cause of phenolic excretement via the wound response, as leaf material was treated very gently and the least rigorous sterilization protocol was employed. The tissue used for callus initiation was juvenile, and so tissue age is unlikely to be the problem.

It is more reasonable to suggest that phenolic excretement in *O. bullata* cultures is a result of high levels of naturally occurring phenolics in this species, as is seen in related *Ocotea* species. Phenolics can alter the pH of the media, which may in turn have a regulatory role in growth and

morphogenesis similarly to that of plant growth regulating chemicals, particularly auxins (George 1993).

It is known that nutritional stress enhances the activities of polyphenoloxidases (George 1993) thus increasing the release of phenols, and these are exuded into the medium where they contribute to a rise in pH. The presence of both NO_3^- and NH_4^+ in MS medium has a stabilizing effect on pH, as the uptake of one provides a better pH environment for the uptake of the other (George 1993). However, with long periods between subculturing, the NH_4^+ is depleted more rapidly than NO_3^- and the utilisation of only NO_3^- results in an increase in media pH (Moura-Cousta et al.1993, George 1993).

Phenolics and nutritional stress may thus have contributed to the necrosis of tissues kept for long periods of time in the same batch of media via pH changes. Subculturing leaf explants on a monthly basis would probably alleviate the problems associated with media pH fluctuations.

Alternatively, fluctuations in the medium pH could be prevented by the addition of buffers to the medium, but the optimum pH for *O. bullata* callus growth needs to be determined prior to this. Activated charcoal was used in some of the callus initiating media, but leaf segments initiated onto media containing 1g/l activated charcoal did not show reduced phenolic accumulation in the tissues over those on media without AC.

In their work on *O. catharinensis* somatic embryogenesis, Moura-Costa et al. (1993) used 3g/l AC, but calli turned brown regardless. These authors reported the successful procurement of somatic embryos of *O. catharinenesis* from callus after it had turned brown, and a similar results could reasonably be expected in *O. bullata*. In light of the negative effects of the addition of activated charcoal to medium, the benefits associated with increasing the AC content of medium need to be carefully considered.

4.2.2 The orientation of tissue

It is probable that stress caused by inverting the polarity of the leaf segments may account for the initial differences in growth and phenolic exudation detected between AD and AB orientated leaf segments, although a clear long-term preference of one orientation over the other in *O. bullata* could not be discerned.

4.2.3 The effects of plant growth regulators on callus initiation

The optimum level of the synthetic auxin 2,4-D which is required for callus initiation in dicotyledonous plants is usually between 1.0-3.0 mg/l. At high concentrations, 2,4-D is phytotoxic to these broadleafed plants, and the rapid blackening and lack of any growth in the leaf explants placed with either orientation onto callus initiating media containing 5 mg/l 2,4-D indicates that this concentration was too high for successful callus initiation in *O. bullata* leaf tissue.

In leaf explants placed on media containing 2mg/l 2,4-D, all observable cell growth ceased after four weeks in culture, which is possibly a result of the material being kept for too long on media containing the 2,4-D; rather than initiating dedifferentiation in the tissues, the 2,4-D probably became phytotoxic, even at this lower concentration. Nutrient stress could also have been responsible for the cessation of visible growth.

Inhibition of growth by ethylene accumulation in the culture vessels is unlikely to have been the cause of this, as large culture vessels with a substantial volume of air above the media (relative to the size of the explants) were used. In addition, no difference in growth was detected between explants on media containing activated charcoal and those on media without this compound. Activated charcoal is thought to absorb gasses from the air above the media (George 1993).

The addition of a cytokinin to the media may have brought about the formation of calli from explants (Krikorian et al. 1990, George 1993). In this work, reliance was placed on endogenous cytokinin levels for the completion of the cell cycle, which were perhaps insufficient to support prolonged growth of the explants.

If the tissues remain recalcitrant after the addition of a cytokinin, an alternate strategy may be to use more than one auxin at a time to initiate and sustain callus growth, as there may be different sites of action or target molecules (Krikorian et al. 1990).

4.3 SEED GERMINATION

Due to the brief period of viability of seeds of *O. bullata*, it is recommended that embryo rescue be the initial course of action when attempting to germinate these seeds *in vitro*. The rigorous sterilization procedures conducted here may have killed the embryos, and the time wasted in procuring clean embryos possibly exceeded the period of seed viability in this species.

4.4 ROOTING OF EXPLANTS

4.4.1. De novo rooting trials

Unfortunately, no conclusions can be drawn from the attempted rooting of newly excised shoot material, due to bacterial contamination which ultimately resulted in the loss of all explants. Callus formation did occur at the bases of the stems as a reaction to the auxin in the media, indicating that this species does react to the auxin IBA. However, callusing around the bases of the stems was also observed in shoot cultures, which were on media supplemented with the auxin NAA.

4.4.2 Rooting of successful shooting trials

The rapid chlorosing and abscission of both old leaves and newly-formed shoots (Figure 15) indicates that the explants were physiologically shocked by the transfer to media containing 2mg/l IBA. The concentration of IBA in the media may have been too high. High concentrations of auxin in the media acting together with high levels of endogenous cytokinins may have resulted in an inappropriate ratio of these two hormones, causing leaf abscission.

This might have been avoided by placing the explants on a hormone-free medium for a minimum of one subculture cycle, prior to the explants being transferred to rooting media (George 1993). This would have permitted the normalisation of plant growth regulator levels in the tissue, prior to exposure to new exogenous plant growth regulators. This procedure was effective in rescuing two of the explants in which rooting was attempted.

Rooting may also have been attempted prematurely, and perhaps the explants should have been kept in shoot proliferation media for a much longer period.

5. CONCLUSION

5.1 DIRECT ORGANOGENESIS

The protocol developed here repeatedly resulted in the growth of auxiliary buds and the media composition was shown to be capable of supporting shoot growth and leaf expansion. The causative factors leading to leaf abscission still require further investigation, and this may be overcome by slight adjustments to the composition of the media, or alterations in incubation temperatures.

The results of this study suggest that slightly older material is preferable for initiation of shoot cultures for direct organogenesis in *O. bullata*, although younger material usually has less contaminants, so both of these factors need to be taken into consideration and a compromise reached.

The major successes of these experiments on direct organogenesis are undoubtedly the elimination of contaminants by pre-treatment of the mother material, the avoidance of severe phenolic accumulation in shoot inducing media and, most importantly, the repeatable production of new shoots. These results are conducive to the development of *in vitro* culture systems for *O. bullata*.

5.2 CALLUS INITIATION

Callus initiation was unsuccessful in these experiments and this may be attributed to various factors. It was established that subculturing of leaf segments is required, and that the concentration of 2,4-D necessary to initiate callus proliferation is closer to 2mg/l than to 5mg/l. The addition of a cytokinin is recommended for future studies. Callus initiation may also be enhanced by the inclusion in the media a variety of auxins and casein hydrolysate.

Infection was minimal, indicating that young leaves of field-grown material, if subject to stringent pre-treatments, could successfully be utilised to initiate callus growth.

5.3 ROOTING TRIALS

Further investigation into *in vitro* rooting of *O.bullata* is required.

5.4 OVERVIEW

In summary, the potential for the *in vitro* propagation of *O. bullata* has been demonstrated, but experiments were hampered by the scarcity of material available. The development of micropropagation systems requires a fair amount of material in order to produce statistically rigorous and repeatable results.

Populations of rare and endangered species should be monitored and, as soon as the species start to decline, conservation strategies need to be initiated. Potentially vulnerable species need attention ***before*** they become so rare that material is sufficiently scarce to frustrate conservation efforts such as *in vitro* propagation.

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APPENDIX 1

PHYTOTRON TEMPERATURE AND LIGHT CONDITIONS

Temperatures

The temperature in the phytotron was set as follows:

06H00 - 07H00 22° C
07H00 - 16H00 25° C
16H00 - 17H00 22° C
17H00 - 06H00 18° C

Light sources

The sources of the light were:

06H00 - 07H00 Incandescent bulbs
07H00 - 18H00 Metal halide and sodium bulbs
18H00 - 20H00 Incandescent bulbs
20H00 - 06H00 No light

Light intensity

At canopy height, the irradiance in the phytotron was 350 - 400 μmol.m^{-2}.s^{-1}.

APPENDIX 2
TREATMENTS APPLIED TO SAPLINGS.

Dithane M-45: Applied every 10-14 days.
Dosage (as recommended on package) 1.5 g/l
Active ingredient: Mancozeb (dithiocarbamate) 800g/Kg
Manufactured by: Kudu for Starke Ayres.

Metasystox R:Applied every 14 days for aphids
Dosage (as recommended on package) 1 ml/l
Active ingredient: Oxydemeton-methyl (organophosphate) 250g/l
Manufactured by: Bayer S.A.

Oleum: Applied twice for scale insects and red spider mites.
Dosage (as recommended on label) 20 ml/l
Active ingredient: mineral oil
Manufactured by: Efekto

Long Ashton nutrients: Fed sparingly after cutting to induce shooting at a concentration of 4mMNO_3 (Hewitt 1966).

Funginex: Systemic fungicide applied via spraying
Active ingredient: Triforine 190g/l
Manufactured by: Efekto

Benlate: Fungicide applied to seeds.
Active ingredient: Benomyl (benzimadole)
Manufactured by: Dupont Agricura