# *In vitro* plantlet regeneration of *Ocotea catharinensis*, an endangered Brazilian hardwood forest tree.

**P.H. MOURA-COSTA[1], A.M. VIANA[2] AND S.H. MANTELL[1*]**

[1] Unit for Advanced Propagation Systems, Department of Agriculture, Horticulture and the Environment, Wye College, University of London, Wye, Ashford, Kent TN25 5AH, UK.

[2] Horto Botânico, Universidade Federal de Santa Catarina,
CEP 88000, Florianópolis, SC, Brazil.

* to whom correspondence should be addressed

**Keywords:** cell aggregate differentiation, Lauraceae, *Ocotea*, pH effects, somatic embryogenesis

**Abstract.** A successful system of somatic embryogenesis is described for the forest tree *Ocotea catharinensis* Mez., which used mature zygotic embryo explants cultured on a modified Murashige and Skoog (MS) medium with activated charcoal, at 25°C in the dark. A medium composed of MS supplemented with 2% (w/v) sucrose, 0.3% (w/v) activated charcoal (AC), 362 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.8% (w/v) Technical Agar Grade III was used for multiplication of embryogenic cultures. Development up to the globular-stage was achieved using Lloyd and McCown woody plant medium (WPM) with 2.0% sucrose, 0.3% AC, 181 μM 2,4-D and 0.8% Technical Agar Grade III. Significant effects of media pH on differentiation of early pro-embryogenic *Ocotea* cell aggregates were found. Low pH of media (*ca.* 3-4) appeared to prevent differentiation of pro-embryogenic cell aggregates whereas higher pH levels (*c.a.* 5-5.5) favoured the formation of globular structures. Once globular structures formed, those developed further to form cotyledonary somatic embryos, under the same set of culture conditions. Successful that conversion of these somatic embryos to plantlets was achieved after culture on a medium composed of ½-strength WPM (minerals only) with 2% sucrose, 0.3% AC, 0.8 % Technical Agar Grade III and 90.5 μM 2,4-D, followed by transfer to a medium composed of ½-strength WPM (minerals only) with 2% sucrose, 0.8% Technical Agar Grade III and 0.905 μM 2,4-D and 1.4 μM gibberellic acid 3, in a 16-h photoperiod regime.

## Introduction

*Ocotea catharinensis* Mez (Lauraceae) is a hardwood tree species of S. Brazil, logged extensively from the Atlantic forests for timber production. Propagation is by seeds but these have brief viability. Also, this tree exhibits erratic fruiting, limiting the possibility of relying on the production of seeds for propagation programmes as well as for botanical studies. Other problems are the presence of germination inhibitors in the fruit (Randi, 1982) and consequently poor germination responses in nursery seed beds. There is an urgent need for development of alternative methods of propagation considering that vegetative propagation systems are not available and the tree is on the verge of extinction. In this paper we report the results of investigations in which somatic embryogenesis of this forest tree species was achieved using mature zygotic embryos as explants. The process of development of the induced pro-embryogenic cell aggregates into globular, heart-shaped and cotyledonary-stage embryos and plantlets is also described.

## Materials and methods

### 1. *Origin and multiplication of Ocotea cultures*

Mature zygotic embryos of *Ocotea catharinensis* were excised from seeds surface-disinfested with 1.0% (v/v) sodium hypochloride solution for 10-15 min and washed thrice in sterile distilled water. Sterilized embryos were placed on Murashige and Skoog (1962)(MS) medium supplemented with 2% (w/v) sucrose, 0.3 % (w/v) activated charcoal (AC) and 0.8% (w/v) agar (Technical Agar Grade III, Oxoid, UK), henceforward *Ocotea* Embryo Initiation Medium (OEIM). Media prepared for all experiments described here were dispensed in culture tubes (7.5 cm-long, 2 cm-diam), 10 ml per tube. Cultures were incubated at 25°C in the dark on a 3-week subculture cycle over a period of 4 months. Globular structures produced on the surfaces of zygotic embryos were separated from explant tissues and transferred to a medium of the same formulation but with the addition of 18.1 µM 2,4-dichlorophenoxyacetic acid (2,4-D). After incubation for 2 months at 25°C in darkness, these cultures proliferated giving rise to more globular structures and to structures resembling cotyledonary-stage somatic embryos. Portions (*ca.* 150 mg per culture tube) of cultures containing globular and cotyledonary-shaped structures were inoculated into tubes containing MS medium with 2% (w/v) sucrose, 0.8% (w/v) agar (Technical Agar Grade III), 0.3% (w/v) AC and 362 µM 2,4-D (henceforward MS-80). Cultures were incubated at 25°C in darkness. Observations of growth and development were carried out monthly, during transfer of structures to fresh MS-80 medium.

### 2. *Differentiation of cell aggregates to globular and cotyledonary-shaped structures*

Different media were tested for their ability to promote differentiation of cell aggregates of *Ocotea catharinensis*. These were MS, woody plant medium (WPM; Lloyd and McCown, 1981) or B5 (Gamborg *et al.*, 1968) prepared with 2.0% sucrose, 0.3% activated charcoal, 0.8% Technical Agar Grade III and 181 µM 2,4-D. Portions of 200 mg cell aggregates were inoculated per culture tube. Five replicates were used per treatment and kept at 25°C in the dark. After incubation for 21 days, 0.5 µg samples of cultures were taken from each tube and diluted in 2.5 ml distilled water in a 5 cm diam petri dish. The relative numbers of different types of cell aggregates (CA1 and CA2) per standard x200 field of view on an IMT-2 Olympus inverted microscope were determined. Results were expressed as mean percentages of total number of cell aggregates, after one-way analysis of variance following arc-sin transformation of data.

A batch of cultures of each of the three media above was maintained without disturbance for a longer time (up to 50 days) and media pH values were recorded in these at 3-10 day intervals following media preparation. Three tubes of each treatment were assessed at each sampling time and their media passed through a stainless steel sieve (0.38 µm mesh size) to

liquidise the gel for pH measurements, using a standard pH probe.

### 3. *Conversion of cotyledonary-shaped structures to plantlets*

Cotyledonary-shaped structures were inoculated into a medium composed of ½-strength WPM salts (vitamins excluded) supplemented with 2% sucrose, 0.3% AC, 0.8% Technical Agar Grade III and 90.5 µM 2,4-D, henceforward *Ocotea* Plant Conversion 1 medium (OPC1). Cultures were incubated for 1 month at 25°C in the light (16-h photoperiod, 55-62 µmol $m^{-2}$ $s^{-1}$, provided by Philips `TDL 84' fluorescent tubes). After roots formed, these structures were transferred to a medium composed of ½-strength WPM salts (vitamins excluded), 2% sucrose, 0.905 µM 2,4-D and 1.4 µM gibberellic acid 3 ($GA_3$) and monthly subcultured under the same conditions described above.

## Results and discussion

### 1. *Origin and multiplication of Ocotea cultures*

After 4 months of culture on OEIM medium, *Ocotea* zygotic embryos turned brown. However, *ca.* 5% gave rise to globular structures that were separated from necrotic tissues of the initial explants and these were reinoculated onto fresh OEIM medium containing 18.1 µM 2,4-D. After 1 month, globular structures expanded forming structures resembling cotyledonary-stage somatic embryos. After a further 1 month, these cotyledonary-shaped structures browned, yet numerous globular structures developed directly on their surfaces. These globular structures were subcultured further to maintain stocks of *Ocotea* cultures for experimentation.

Following results of preliminary investigations on the culture of *Ocotea* globular structures (Viana *et al.*, 1990), the level of 2,4-D in media used for routine multiplication of *Ocotea* cultures (MS-80) was raised 20-fold to 362 µM. This was done in order to compensate for the adsorption properties of the AC used in media (Ebert & Taylor, 1990). When cultured on MS-80, globular structures developed through to cotyledonary-shaped structures which then turned brown and gave rise directly to more globular structures on the surfaces of their hypocotyl regions (Fig. 1), in the period of 1 month. Regular subculturing of globular and cotyledonary-shaped structures of *Ocotea* was repeated successfully once every month for 2.5 years without any losses in the multiplication capacities of cultures.

After 4 subcultures of 1 month duration each on MS-80, masses of cell aggregates formed on the surfaces of the cotyledonary-shaped structures. These cell aggregates were classified into two types, CA1 and CA2. CA1 consisted of aggregates of few cells, with *ca.* 75 to 250 µm diam (Fig. 2 A), and had transparent appearance when viewed under an Olympus IMT-2 inverted microscope. By contrast, CA2 were more differentiated aggregates, containing more cells, with a more compact appearance and larger size (*ca.* 100-300 µm diam, Fig. 2 B). When viewed with through the microscope, CA2 were darker than their CA1 counterparts. These masses of cell aggregates were separated from the cotyledonary-shaped structures and cultured separately. When grown on MS-80, both types of cell aggregates did not differentiate but proliferated further at a steady rate (x4 in 3 weeks) and the proportion of CA1 and CA2 types remained at a ratio of *ca.* 3:1. These cultures were maintained for 2 years on regular 3-week subculture cycles without any detectable loss of multiplication capacity.

### 2. *Differentiation of cell aggregates to globular and cotyledonary-shaped structures*

Cultures growing on MS medium containing 181 µM 2,4-D maintained a proportion of CA1 and CA2 types of aggregates at the ratio of 75:25%. Further differentiation of CA1 cell aggregates to the next developmental stage (CA2) was observed more frequently in cultures growing on B5, in which *ca.* 58% of the cell aggregate population was of the CA2 type as against

25% in MS. By contrast, WPM produced only 28% CA2 but *ca.* 70% of globular structures, thus demonstrating the latter medium superiority for promoting differentiation of cultures of cell aggregates of *Ocotea catharinensis* (Fig. 3). Once formed, globular structures developed further to form heart-shaped structures which expanded forming cotyledonary-shaped structures, taking *ca.* 1 month for globular structures to form cotyledonary-shaped structures. No intermediate stage of development between heart-shaped and cotyledonary-shaped structures was found.

One possible explanation for the observed differences in development of *Ocotea* cells grown on the various media described above is that the media used contained different relative concentrations of $NH_4^+$ and $NO_3^-$. In previous studies on somatic embryogenesis of other species (Veliky & Rose, 1973; Sargent & King, 1974; Rose & Martin, 1975; Kirby *et al.*, 1987; Minocha, 1987), uptake of $NH_4^+$ was preferential, and cell aggregates developed in an undifferentiated manner when $NH_4^+$ was available in large amounts in culture media (Kirby *et al.*, 1987). These findings may explain why *Ocotea* cultures grown on MS, which contains high $NH_4^+$ (20 meqs $l^{-1}$), remained predominantly in an undifferentiated form (CA1). By comparison, further differentiation to CA2 and globular structures was observed on cultures growing on WPM, which contains 5 meqs $l^{-1}$ $NH_4^+$.

The differential utilisation of $NH_4^+$ and $NO_3^-$ by cells invariably leads to shifts in medium pH (Kirby *et al.*, 1987; Minocha, 1987). During the current study, when cell aggregates were transferred to fresh medium, an initial decrease in pH took place, possibly due to preferential $NH_4^+$ uptake, followed by a period of stable pH (Fig. 4). The pH levels at which the culture media were self-regulated appeared to be inversely correlated with the amount of $NH_4^+$ present since the pH of MS (which contains high $NH_4^+$) was usually found to be in the range 3 - 4, and remained at this level for *ca.* 3 weeks, whereas the pH of WPM remained consistently within the range 5 - 5.5. If cell aggregates were kept in culture for an extended time (> 30 days), a final rise in pH took place (possibly due to the depletion of $NH_4^+$ and the utilisation of only $NO_3^-$ by the cells). Also, it is known that nutritional stress enhances the activities of polyphenoloxidases (George & Sherrington, 1984) thus increasing the release of phenols from cells into culture media, which can also contribute to rises in media pH. Such effects may have been involved in the browning and death of cell aggregates observed when *Ocotea* cultures were kept for long periods of time in the same medium. By contrast, the pH shifts that occurred in the case of B5 medium were different. An initial fall in pH was followed by a rise without passing through a self-regulated stable phase. B5 medium contains low levels of $NH_4^+$ (2 meqs $l^{-1}$) and these may have been used during the initial culture phase, which was followed by a period in which $NO_3^-$ would be expected to have been utilised as a sole nitrogen source, thereby causing an overall increase in media pH levels. Differences in the self-regulated pH levels of the three culture media tested may also have led to the contrasting behaviours of cell aggregates grown in these media. According to Smith and Krikorian (1990b), low pH maintains pro-embryogenic cell clumps of carrot in a highly proliferative, undifferentiated state, whereas increases in pH to 5 caused these clumps to differentiate to globular stage embryos.

One possible explanation for the low levels of callus observed in the *Ocotea* cultures described here was that any non-totipotent cells present as potential calluses were susceptible to some selection pressure such as the low pH (3-4) of the MS-80, which may have resulted in the death of these cells and an accumulation of more substantial numbers of totipotent cells. This observation is similar to the one made by Smith and Krikorian (1990a, b, c) who described a system for carrot cells in which low media pH caused nonembryogenic cells to die, while pro-embryogenic cell aggregates remained. This observation was supported by the finding that secondary embryogenesis was extremely commonplace in the *Ocotea* cultures described here. Alternatively, another explanation for the lack of callus production in the *Ocotea* cultures was that the structures obtained *in vitro* from mature zygotic embryo explants developed in the absence of growth regulators, in a manner reminiscent of nucellar embryo formation from ovule and nucellar embryo explants of polyembryogenic *Citrus* spp. (Kochba *et al.*, 1972). *Citrus* somatic embryos can be induced *in vitro* using plain mineral media without growth regulator supplementation. Once embryos were formed, secondary embryogenesis was induced by the 2,4-D supplements in MS-80 and other media.

### 3. *Conversion of cotyledonary-shaped structures to plantlets*

Successful *Ocotea* plantlet conversion was achieved albeit at low frequency (2.5%) after culturing cotyledonary-shaped structures on media containing reduced concentrations of nutrients. After culture for 1 month on a low nutrient medium, the OPC1, root formation was observed in 10% of the inoculated structures (Fig. 2 E). These rooted structures were transferred to another low nutrient medium, the OPC2 medium, containing lower 2,4-D concentration (0.905 μM) and $GA_3$, and after 1 month in culture some of the structures produced a short shoot (*ca.* 1 cm long) showing leaf primordia. After another subculture in OPC2 medium, plantlets then grew to 4 cm in height, producing three to four leaves and a single long radicle (*ca.* 10 cm long, Fig. 2.F). Interestingly, in most cases, cotyledons remained on the surfaces of media and eventually browned, in a manner reminiscent of the process of hypogeal germination of *Ocotea* seeds (Sturion & Iede, 1982)(Fig. 2 F, note similarity to seedlings - Fig. 2 G). In view of the fact that cotyledonary-shaped structures (Fig. 2 C) closely resembled dormant and germinating zygotic embryos of *Ocotea catharinensis* (Fig. 2 D), and that there was development of both shoot and root structures in single differentiating units within cultures, it was concluded that the process of morphogenesis occurring in the described culture systems was indeed somatic embryogenesis. The fact that germination of *Ocotea* somatic embryos was possible when weaker strengths of mineral media were used was consistent with observations that somatic embryos of woody species benefit from a reduction in nutrients in the culture medium for germination (Tulecke, 1987). It is also known that high nutrient contents of media are inhibitory for seed germination of many woody species (Hartmann *et al.*, 1990), and that excised zygotic embryos cultured *in vitro* require less nutrients at early stages of development than they do later on (Hu & Wang, 1986).

The results obtained from these experiments are encouraging for the further development of *Ocotea* culture systems in which the control of early stages of embryogenesis might be successfully accomplished by manipulation of media formulations. Although plantlet conversion has been achieved, further experimentation is necessary to increase the frequency of germination of somatic embryos, as in the celery system described by Redenbaugh *et al.* (1988). The improvement of this embryogenic system, so that plants can be consistently regenerated, would enable its application for propagation of elite trees of *Ocotea catharinensis* and its related species. Furthermore, the availability of an efficient embryogenesis system will greatly facilitate basic experiments on *Ocotea* embryogenesis, since at the present time living zygotic embryo materials of this genus are only available for a few weeks once every 4-5 years, when gregarious flowering and fruit set occurs in natural stands of this forest tree.

**Acknowledgements**

We wish to acknowledge the financial support of the Brazilian Government (CNPq) for a postgraduate studentship held by Dr P.Moura-Costa and the International Foundation for Science (Sweden) for a research grant held by Dr A.Viana. We are grateful for the excellent technical support provided by Shelagh Reardon, Fiona Holt, Sue Farris and Kodi Kandasamy.

**References**


Ebert A & Taylor HF (1990) Assessment of the changes of 2,4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. Plant Cell Tissue Organ Culture **20**:165-172.

Gamborg OL, Miller RA & Ojima K (1968) Plant cell cultures. I. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res **50**:151-158.

George EF & Sherrington PD (1984) Plant Propagation by Tissue Culture. Exegetics Ltd, Basingstoke. 709 pp.

Hartmann HT, Kester DE & Davies FT (1990) Plant Propagation: Principles and Practices. Prentice-Hall International Ltd., London. 647 pp.

Hu C & Wang P (1986) Embryo culture: technique and application. In: Evans DA, Sharp WR & Ammirato PV (Eds) Handbook of Plant Cell Culture, Vol.4 (pp. 43-96). Macmillan Publishers, London.

Kirby EG, Leustek T & Lee MS (1987) Nitrogen nutrition. In: Bonga JM & Durzan DJ (Eds) Cell and Tissue Culture in Forestry, Vol.1 (pp. 67-88). Kluwer Academic Publishers, Lancaster.

Kochba J, Speigel-Roy P & Safran H (1972) Adventive plants from ovule and nucelli in *Citrus*. Planta **106**:237-245.

Lloyd G & McCown B (1981) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Comb Proc Int Plant Prop Soc (1980) **30**:421-427.

Minocha SC (1987) pH of the medium and the growth and metabolism of cells in culture. In: Bonga JM & Durzan DJ (Eds) Cell and Tissue Culture in Forestry, Vol.1 (pp. 125-141). Kluwer Academic Publishers, Lancaster.

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

Randi AM (1982) Estudo preliminar sobre inibidores de germinação em frutos de *Miconia cinammomifolia* e *Ocotea puberula*. In: Anais do Congresso Nacional Sobre Espécies Nativas, Campos do Jordão, Setembro 1982. Silvicultura em S. Paulo **16**(1):238-242.

Redenbaugh K, Fujii JA & Slade D (1988) Encapsulated plant embryos. In: Mizrahi A (Ed) Biotechnology in Agriculture. (pp. 255-248). Alan R.Liss Inc., New York.

Rose D & Martin SM (1975) Effect of ammonium on growth of plant cells (*Ipomea* sp) in suspension cultures. Can J Bot **53**:1942-1949.

Sargent PA & King J (1974) Investigations of growth-promoting factors in conditioned soybean root cells and in the liquid medium in which they grew: ammonium, glutamine and aminoacids. Can J Bot **52**:1747-1755.

Smith DL & Krikorian AD (1990 a) Low external pH replaces 2,4-D in maintaining and multiplying 2,4-D-initiated embryogenic cells of carrot. Physiol Plant **80**:329-336.

Smith DL & Krikorian AD (1990 b) pH control of carrot somatic embryogenesis. In: Nijkamp HJJ, Van der Plas LHW & Van Aartrijk J (Eds) Progress in Plant Cellular and Molecular Biology. (pp.

449-453). Kluwer Academic Publishers, Lancaster.

Smith DL & Krikorian AD (1990 c) Somatic embryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. Plant Cell Rep **9**:34-37.

Sturion JA & Iede ET (1982) Influência da profundidade de semeadura, cobertura do canteiro e sombreamento na formação de mudas de *Ocotea porosa* (Nees) Liberato Barroso (Imbuia). Documentos, Unidade Regional de Pesquisa Florestal Centro-Sul, Embrapa **10**:71-79.

Tulecke W (1987) Somatic embryogenesis in woody perennials. In: Bonga JM & Durzan DJ (Eds) Cell and Tissue Culture in Forestry, Vol.1. (pp. 61-91). Kluwer Academic Publishers, Lancaster.

Veliky IA & Rose D (1973) Nitrate and ammonium as nitrogen nutrients for plant cell cultures. Can J Bot **51**:1837-1844.

Viana AM, Moura-Costa PH & Mantell SH (1990) High frequency somatic embryogenesis of *Ocotea catharinensis*. In: Abstracts VIIth International Congress on Plant Tissue and Cell Culture, Amsterdam, 1990, p. 261.

**Figure 1:** Longitudinal section through a cotyledonary-shaped structure of *Ocotea catharinensis* cultured on MS-80 showing several secondary globular structures arising directly from the basal "hypocotyl" end.

**Figure 2:** Overall morphology of somatic embryos and plantlets of *Ocotea catharinensis* compared to zygotic embryos and seedlings of the same species. **A:** CA1, the least differentiated cell aggregates found on cultures; **B:** CA2, further differentiated cell aggregates; **C:** cotyledonary-shaped structures; **D:** excised zygotic embryos resembling structures shown in C; **E:** germinating cotyledonary-shaped structure resembling a zygotic embryo with radicle and cotyledonary development; **F:** *in vitro* plantlet from a cotyledonary-shaped somatic embryo as in E; **G:** two seedlings of *Ocotea catharinensis* six months after seed germination.

**Figure 3:** Percentages of different cell aggregates and structures found in *Ocotea catharinensis* cultures after 21 days on three different media (MS, WPM or B5). Results are given as the angles (in degrees) corresponding to the mean percentages of total number of embryogenic structures, after one-way analysis of variance using arc-sine transformation. CA1 = the least differentiated cell aggregates found in cultures; CA2 = the next state of differentiation.

**Figure 4:** Changes in pH of media during culture of differentiating cell aggregates of *Ocotea catharinensis*. Data points are the means of three replicates. MS = Murashige and Skoog medium; WPM = Woody Plant Medium; B5 = Gamborg B5.