Development of *Ocotea catharinensis* Mez. cell aggregates cultured on media containing different concentrations of nitrogen and with pH buffered at different levels.

P.H. MOURA-COSTA AND S.H. MANTELL[1,2]

Abstract: Experiments were conducted to assess the effects of media pH levels and nitrogen concentrations on growth and differentiation of embryogenic cell aggregate cultures of *Ocotea catharinensis* Mez., a Brazilian hardwood tree species. Since media with low levels of pH were utilised, and it is difficult for agar to set at such levels, liquid media was used with a membrane raft support system. It was tested the effects of three media (Murashige and Skoog - MS; woody plant media - WPM; and B5) prepared with 2% (w/v) sucrose, 0.3% (w/v) activated charcoal (AC), 181 µM 2,4-D and 9.4 mM MES (2-(N-morpholino)ethane-sulfonic acid), with pH adjusted to either 3.5 or 5.5. Cultures growing on WPM exhibited faster biomass production and differentiation than those on MS or B5 media, and these effects were more pronounced on media with pH 5.5. Other experiments were conducted in which cell aggregates were cultured on WPM prepared with different concentrations of nitrogen (0, 1.5, 15 and 60 meqs l^{-1}), 2% sucrose, 0.3% AC, 181 µM 2,4-D and pH buffered at 3.5 or 5.5 using 9.4 or 18.8 mM MES. Cultures on media with pH 5.5 exhibited higher proportion of further differentiated cell aggregates than those on media with pH 3.5, for any nitrogen concentration, suggesting that medium pH has a stronger effect on differentiation of *O.catharinensis* cell aggregates than nitrogen concentrations. Higher rates of fresh weight increase were achieved in media with pH 5.5 containing at least 15 meqs l^{-1} nitrogen, demonstrating that both pH and nitrogen concentrations affect growth of cell aggregate cultures of *Ocotea catharinensis*.

Keywords: cell aggregate differentiation, Lauraceae, MES, pH effects, somatic embryogenesis

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.

A system of somatic embryogenesis of *O.catharinensis* was developed in which large number of embryogenic cell cultures can be multiplied and manipulated to differentiate, and successful conversion of embryos to plants was achieved (Viana *et al.*, 1992; Moura-Costa *et al.*, 1993). In this system, the early stages of embryogenesis (*ie.* from embryogenic cell aggregates to globular somatic embryos) can be controlled by manipulation of mineral media composition. Contrasting patterns of differentiation were observed when cell aggregates were cultured in either Murashige and Skoog (MS; 1962); B5 (Gamborg *et al.*, 1968) or woody plant medium (WPM; Lloyd & McCown, 1981), with WPM promoting the highest levels of differentiation and MS the lowest. It was inferred that these differences were probably due to the contrasting concentrations and forms of mineral nitrogen in the above media. In a further paper we reported that growth and differentiation of *O.catharinensis* cell aggregates is affected by nitrogen concentrations and forms, as well as the balance between ammonium and nitrate in culture media (Moura-Costa & Mantell, 1993). However, it was also noticed that medium pH fluctuations during a culture cycle were markedly different between media containing different concentrations of ammonium and nitrate.

[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] Plant Cell, Tissue and Organ Culture

It is known that nitrogen uptake is closely linked with the pH levels of culture media (Rose & Martin, 1975; Durzan, 1987; Kirby *et al.*, 1987; Minocha, 1987). In culture media containing ammonium and nitrate, NH_4^+ seems to be preferentially utilized by plant cells causing an overall reduction in pH (Veliky & Rose, 1973; Sargent & King, 1974; Rose & Martin, 1975; Kirby *et al.*, 1987; Minocha, 1987). Therefore, it was not possible to ascertain whether the developmental differences were only due to media composition or if the resulting contrasting pH levels in the media resulting from differential nitrogen uptake during culture have an important role. There is much evidence suggesting that media pH levels are important for the uptake of nutrients, growth regulators, activity of enzymes, metabolism of hormones (Minocha, 1987), permeability of cell membranes (Fry, 1988), and many other cellular processes, and therefore it most likely affects development and growth of cells. Smith and Krikorian (1989; 1990a, b, c) reported a system for the control of carrot embryogenic cell differentiation based on regulation of media pH.

This paper describes experiments which investigated the response of *Ocotea catharinensis* cell aggregates cultured on media with different nitrogen contents and pH levels. An organic buffer, MES, was used to avoid pH fluctuations and determine the isolated effects of pH and N on growth and differentiation of *O.catharinensis* cell aggregate cultures.

Materials and methods

Origin of plant material

The plant material used for these experiments were embryogenic cell-aggregate cultures of *Ocotea catharinensis* Mez. produced according to the method described by Moura-Costa *et al.* (1993). 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, 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). When viewed with through the microscope, CA2 were darker than their CA1 counterparts. Before the beginning of these experiments, cell aggregates had been cultured for 1 year on a medium consisting of MS supplemented with 2% (w/v) sucrose, 0.8% (w/v) agar (Technical Agar Grade III, Oxoid Ltd., UK), 0.3% (w/v) neutralized activated charcoal (AC, Sigma Chemical Co., UK) and 362 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and pH 5.8, on a 3-week subculture cycle at 25°C in the dark. When grown on this medium, both types of cell aggregates did not differentiate but proliferated at a steady rate (x4 in 3 weeks) and the proportion of CA1 and CA2 types remained at a ratio of *ca.* 3:1.

Medium preparation and culture conditions

Since media with low levels of pH (3.5) were utilised, and it is difficult for agar to set at such levels, a Membrane Raft system (Sigma Chemical Co., UK) was utilised. This system consists of a polypropylene permeable membrane (0.075 x 0.25 μm pore size) placed on the surface of liquid media. Plant material is placed on the Membrane Rafts and can absorb nutrients that are diffused through the membrane at a diffusion rate similar to that of agar. The Membrane Rafts were used in Magenta vessels (Sigma Chemical Co., UK) containing 40 ml of media. Adjustments of media pH were carried out before autoclaving, using a standard temperature compensated combination electrode (Gallenkamp, UK) attached to a AGB-4000 (UK) digital pH meter. All media utilised were autoclaved for 20 min at 121°C and 1.6 kg cm^{-2}. After inoculation with cell aggregates, cultures were incubated at 25°C in the dark.

Assessments and statistical analyses

Experiments were assessed for biomass production (relative increase in fresh weight of cell aggregate cultures), final media pH and cell aggregate differentiation. Data was analyzed by one-way

analyses of variance. All experiments used five replicates per treatment. Differentiation was expressed as mean percentages of total number of differentiated cell aggregates (*ie.* CA2 and globular somatic embryos), after arc-sin angular transformation of data (Zar, 1984).

Experimental

Three experiments were conducted to assess the effects of media pH levels on cell aggregate differentiation and growth using MES, an organic buffer.

Experiment 1: Development of cell aggregates of *Ocotea catharinensis* cultured on three media buffered at two levels of pH

MS, WPM and B5 media were prepared with 2% sucrose, 0.3% AC, 181 µM 2,4-D and 4.7 mM MES (2-(N-morpholino)ethane-sulfonic acid - Sigma Chemical Co., UK). Media pH were adjusted to levels of either 3.5 or 5.5. A portion (400 mg) of *Ocotea catharinensis* cell aggregate cultures containing CA1 and CA2 types of aggregates (71 and 29%, respectively) was inoculated into each culture vessel. Assessments were carried out after 28 days in culture. This experiment was repeated but using media without AC, since this compound might have adsorbed MES. The concentration of 2,4-D was reduced to 4.5 µM (since no AC was used) and the concentration of MES raised to 9.4 mM.

Experiment 2: Development of cell aggregates of *Ocotea catharinensis* cultured on media containing two contrasting concentrations of nitrogen and buffered at two levels of pH

This experiment aimed to compare the effects of media pH on the development of *Ocotea catharinensis* cell aggregates cultured on two media with contrasting levels of nitrogen. A low concentration of 15 meqs l^{-1} of nitrogen (equivalent to the original WPM concentration) was compared to a higher level of 60 meqs l^{-1} (the same concentration as MS medium). A medium consisting of WPM supplemented with 2% sucrose, 4.5 µM 2,4-D and 9.4 mM MES was prepared and pH adjusted to either 3.5 or 5.5. A second medium was prepared and had identical formulation but for the addition of 15 mM NH_4NO_3 and 15 mM KNO_3, to raise total nitrogen concentrations to 60 meqs l^{-1}. A portion (400 mg) of cell aggregate cultures with both CA1 (76 %) and CA2 (23 %) types of aggregates was inoculated per culture vessel. Assessments were carried out after 24 days in culture.

Experiment 3: Development of cell aggregates of *Ocotea catharinensis* cultured on media containing four contrasting concentrations of nitrogen and buffered at two levels of pH

This experiment was conducted to confirm the results of Experiment 2 and test the effects of media containing very low or even no nitrogen on *Ocotea catharinensis* cell aggregate development. A medium composed of WPM minerals and vitamins excluding nitrogen was prepared with 2% sucrose, 4.5 µM 2,4-D and 18.8 mM MES and supplemented with four concentrations of nitrogen: 0.0, 1.5, 15 or 60 meqs l^{-1}. Nitrogen was supplied as 0.5 mM NH_4NO_3 and 0.5 mM $Ca(NO_3)_2$; 5 mM NH_4NO_3 and 5 mM $Ca(NO_3)_2$; or 20 mM NH_4NO_3 and 20 mM $Ca(NO_3)_2$, respectively. Each of these different media had their pH adjusted to either 3.5 or 5.5. Media were inoculated with 200 mg cell aggregate cultures containing CA1 and CA2 types of aggregates (69 and 31 % respectively). Assessments were carried out after 28 days in culture.

Results and discussion

Experiment 1: Development of cell aggregates of *Ocotea catharinensis* cultured on three media buffered at two levels of pH

After 28 days in culture, pH of media containing activated charcoal changed considerably from the initial buffered levels, altering the conditions required for this experiment (Table 1). Activated charcoal is known for its capacity to adsorb organic compounds (Reinert & Bajaj, 1977; Weatherhead *et al.*, 1978; Pierik, 1987; Ebert & Taylor, 1990) and it may have reduced the availability of MES, an organic

buffer (Partiff *et al.*, 1988). For this reason this experiment was repeated excluding AC from culture media and the levels of MES were increased to 9.4 mM to provide a better control of pH, although some variation still occurred as seen in Table 1.

Significant differences ($P < 0.05$) on differentiation of *Ocotea catharinensis* cell aggregates were observed on cultures growing on MS and WPM media buffered at low and high pH levels (Fig.1). Cell aggregates cultured on media with pH 3.5 remained in a more undifferentiated form (higher percentage of CA1's) than those cultured on media with pH 5.5. Cultures on WPM exhibited higher proportions of differentiated cell aggregates than those on MS, at any level of pH. This suggests that apart from pH levels, another factor(s) intrinsic to the media may have contributed favourably to the conditions necessary for cell aggregate differentiation. One possible explanation may be that WPM contains lower concentrations of nitrogen (especially NH_4^+), as compared to MS. Cell aggregates cultured on B5 did not show any significant differences in differentiation at either of the pH levels tested.

Significant differences ($P < 0.05$) in biomass production were observed between cultures growing at different pH levels on the three media (MS, WPM, B5). Cultures growing on media with pH 5.5 showed a 1.5 - 2.2 fold increase in fresh weight while the ones on media with pH 3.5 showed only 0.85 - 1.12 fold increase in fresh weight (Fig.2).

Experiment 2: Development of cell aggregates of *Ocotea catharinensis* cultured on media containing two contrasting concentrations of nitrogen and buffered at two levels of pH

No significant differences in cell aggregate differentiation were observed between cultures growing on media with the two contrasting concentrations of nitrogen (15 and 60 meqs l^{-1}), although there were significant differences ($P < 0.05$) between cultures growing on media with different levels of pH (Fig.3). These results suggest that the factors leading to *O.catharinensis* cell aggregate differentiation are more dependent on media pH levels than on nitrogen concentrations and that the differences in behaviour observed between cultures on MS and WPM (Experiment 1) might have been due to media constituents other than nitrogen.

A faster increase in fresh weight was observed in cultures growing on media with pH 5.5 than with 3.5, and cultures containing 15 meqs l^{-1} N showed faster increase in fresh weight than those containing 60 meqs l^{-1} N (Fig.4). There were still some variations in pH at the end of the culture cycle (Table 2), and for this reason the levels of MES were increased to 18.8 mM for Experiment 3.

Experiment 3: Development of cell aggregates of *Ocotea catharinensis* cultured on media containing four contrasting concentrations of nitrogen and buffered at two levels of pH

No significant differences in cell aggregate differentiation were observed between cultures on media with pH 5.5, for any level of nitrogen in the media (Fig.5). However, significant differences ($P < 0.05$) were observed in cultures on media with pH 3.5: high nitrogen concentrations (15 or 60 meqs l^{-1}) induced cell aggregate differentiation while low concentrations ($\leq$ 1.5 meqs.l^{-1}) prevented it.

Cultures growing at pH 5.5 showed significantly higher biomass production ($P < 0.05$) than the ones at pH 3.5, with the exception of cultures on media without nitrogen (Fig.6), in which growth was much reduced. Larger variations in the values of media pH at the end of this experiment occurred in media containing higher concentrations of nitrogen (Table 3).

General discussion

It appeared that pH levels played a more important role than nitrogen concentrations or media components in the control of differentiation of *Ocotea catharinensis* cell aggregates. The importance of pH in processes related to cell differentiation has been widely reported in the literature. Gradients between intracellular and medium pH are important for determining membrane charges, which play an essential role in the co-transport processes across the plasmalemma (Minocha, 1987), affecting

nutrients and growth regulator uptake. Although cytoplasmic pH is tightly regulated within a range of 6.5 - 7.5 (Minocha, 1987, and references cited therein), Smith (1984) suggested that this fine control works less efficiently when cells are exposed to media pH below 4.5, due to a net influx of H^+ transport across the plasmalemma, probably affecting many of the metabolic processes (especially enzymatic reactions) taking place in cells. The results reported in the current study showed that *O.catharinensis* cell aggregates remained in an undifferentiated condition when cultured in media with low pH (*ca.* 3.5). It has been reported that low pH levels in culture media favour the uptake of auxins and therefore cells may accumulate supraoptimal levels of this growth regulator (Minocha, 1987, and references cited therein) which might inhibit differentiation due to an increase in the capacity of plastic extension of cells (Fry, 1988). However, Smith and Krikorian (1989) described an auxin-free system for multiplication of pro-embryogenic cultures of carrot using NH_4^+ as a sole nitrogen source, and in further publications (Smith & Krikorian, 1990a, b, c) suggested low pH as the essential factor for maintaining cells in an undifferentiated, proliferative condition. According to Fry (1988), differentiation of cells is related to the tightening of cell walls due to, mainly, the formation of phenolic dimer cross-links. Many of the wall-loosening enzymes have low pH optima and, furthermore, there is evidence of a direct action of low pH on wall polymers, both processes leading to loosening of cell walls and prevention of differentiation, whereas raising pH causes wall tightening due to the increase in formation of Ca^{++}-bridges in the cell walls (Baydoun & Brett, 1984).

Although nitrogen concentrations played an important role in the control of growth and differentiation of *O.catharinensis* cell aggregates (Moura-Costa & Mantell, 1993), other media components appeared to be also responsible for the differences observed in development of cell aggregates growing on MS, WPM and B5 media. WPM contains considerably more Ca^{++} than does MS. This element is centrally involved in the process of cell wall tightening leading to differentiation of pro-globular somatic embryos due to the formation of Ca^{++}-bridges (Fry, 1988), and its effects on the activity of peroxidases in lignification processes (Sticher *et al.*, 1981).

Media with pH around *ca.* 5.5 promoted faster biomass production than those with pH *ca.* 3.5, regardless of the nitrogen concentrations in the media, and therefore a positive relationship between differentiation and biomass production became evident. A positive correlation ($P < 0.001$) between these factors was also reported by Moura-Costa and Mantell (1993). It may be that as embryogenic structures differentiated, cells became more meristematic, containing more cytoplasmic contents and were therefore more dense. The fresh weights of the differentiating cell aggregates probably increased dramatically due to this one factor alone. Differences in cell growth related to pH levels are reported by many authors (Veliky & Rose, 1973; Rose & Martin, 1975; Minocha, 1987), although the reasons accounting for these differences are still uncertain. Further studies on effects of media pH on cytoplasmic pH levels, membrane charges, cell wall processes, nitrogen uptake and metabolism and enzymic activity, as well as growth regulator uptake in the *Ocotea catharinensis* system are necessary to provide a more clear understanding of the underlying reasons why medium pH has such a marked effect on growth and differentiation of embryogenic cell aggregates.

Acknowledgements

We wish to acknowledge the financial support of the Brazilian Government (CNPq) for a research grant held by Dr P.Moura-Costa. We are grateful for the excellent technical support provided by Sue Farris and other colleagues in UAPS.

References

Baydoun EA-H & Brett CT (1984) The effect of pH on the binding of calcium to pea epicotyl cell walls and its implications for the control of cell expansion. Journal of Experimental Botany 35:1820-1831.

Durzan DJ (1987) Ammonia: its analogues, metabolic products and site of action in somatic embryogenesis. In: Bonga JM & Durzan DJ (Eds) Cell and Tissue Culture in Forestry, Vol.1 (pp. 92-136). Kluwer Academic Publishers, Lancaster.

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.

Fry SC (1988) The growing plant cell wall: chemical and metabolic analysis. Longman Scientific and Technical, London. 333 pp.

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.

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.

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.

Moura-Costa PH & Mantell SH (1993) Effects of inorganic nitrogen supply in culture media on development of embryogenic cell aggregate cultures of *Ocotea catharinensis* Mez. Plant Cell, Tissue and Organ Culture (submitted).

Moura-Costa PH, Viana AM & Mantell SH (1993) *In vitro* plantlet regeneration of *Ocotea catharinensis*, an endangered Brazilian hardwood forest tree. Plant Cell, Tissue and Organ Culture (in review).

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

Parfitt DA, Almehdi AA & Bloksberg LN (1988) Use of organic buffers in plant tissue-culture systems. Scientia Horticulturae 36:157-163.

Pierik RLM (1987) *In vitro* culture of higher plants. Martinus Nijhoff Publishers, Dordrecht. 345 pp.

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: 238-242.

Reinert J & Bajaj YPS (1977) 1. Anther culture: haploid production and its significance. In: Reinert J & Bajaj YPS (Eds) Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. (pp. 251-267). Springer-Verlag, Berlin/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 FA (1984) Regulation of the cytoplasmic pH of *Chara corallina*: response to changes in external pH. Journal of Experimental Botany 35:43-50.

Smith DL & Krikorian AD (1989) Release of somatic embryogenic from excised zygotic embryos of carrot and maintenance of proembryonic cultures in hormone-free medium. American Journal of Botany 76:1832-1843.

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.

Sticher L, Penel C & Greppin H (1981) Calcium requirement for the secretion of peroxidases by plant cell suspensions. Journal of Cell Science 48:345-353.

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.

Weatherhead MA, Burdon J & Henshaw GG (1978) Some effects of activated charcoal as an additive to plant tissue culture media. Z. Pflanzenphysiol 89:141-147.

Zar JH (1984). Biostatistical analysis. Second edition. Prentice-Hall Inc, New Jersey. 717 pp.

Figure 1. Percentages of differentiated cell aggregates (CA2 and globular somatic embryos) of the total population of cell aggregates of *Ocotea catharinensis* after culture for 28 days on three media (MS, WPM or B5) at two levels of pH (3.5 or 5.5) buffered with 9.4 mM MES. Figures are the means of five replicates after arc-sin transformation.

Figure 2. Relative fresh weight increase of *Ocotea catharinensis* cell aggregate cultures grown for 28 days on three media (MS, WPM or B5) at two levels of pH (3.5 or 5.5) buffered with 9.4 mM MES. (N = 5).

Figure 3. Percentages of differentiated cell aggregates (CA2 and globular somatic embryos) of the total population of cell aggregates of *Ocotea catharinensis* after culture for 24 days on media containing different concentrations of nitrogen (15 or 60 meqs l^{-1}) at two levels of pH (3.5 or 5.5) buffered with 9.4 mM MES. Figures are the means of five replicates after arc-sin transformation.

Figure 4. Relative fresh weight increase of *Ocotea catharinensis* cell aggregate cultures grown for 24 days on media containing different concentrations of nitrogen (15 or 60 meqs l^{-1}) at two levels of pH (3.5 or 5.5) buffered with 9.4 mM MES. (N = 5).

Figure 5. Percentages of differentiated cell aggregates (CA2 and globular somatic embryos) of the total population of cell aggregates of *Ocotea catharinensis* after culture for 28 days on media containing different concentrations of nitrogen (0, 1.5, 15 or 60 meqs l^{-1}) at two levels of pH (3.5 or 5.5) buffered with 18.8 mM MES. Figures are the means of five replicates after arc-sin transformation.

Figure 6. Relative fresh weight increase of *Ocotea catharinensis* cell-aggregate cultures grown for 28 days on media containing different concentrations of nitrogen (0, 1.5, 15 or 60 meqs l^{-1}) at two levels of pH (3.5 or 5.5) buffered with 18.8 mM MES. (N = 5).

Table 1: Final pH of media containing 0.3% AC and buffered at different initial pH values using different concentrations of MES after supporting growth of cell aggregate cultures of *Ocotea catharinensis* for 28 days. (N = 5). SE for 4.7 mM MES = 0.120; SE for 9.4 mM MES = 0.056.

MES (mM)	Initial pH	MS[1]	WPM[2]	B5[3]
4.7	3.5	3.2	3.4	6.1
4.7	5.5	4.6	5.1	5.6
9.4	3.5	3.2	3.4	3.3
9.4	5.5	4.8	5.1	5.6

[1] Murashige and Skoog medium; [2] woody plant medium; [3] Gamborg B5 medium.

Table 2: Final pH of media containing two contrasting concentrations of nitrogen and buffered at different pH values with 9.4 mM MES after supporting growth of *Ocotea catharinensis* cell-aggregate cultures for 24 days. (N = 5; SE = 0.076).

Initial pH	15 meqs l^{-1} N	60 meqs l^{-1} N
3.5	3.4	3.0
5.5	5.0	4.9

Table 3: Final pH of media containing four concentrations of nitrogen and buffered at different pH values with 18.8 mM MES after supporting growth of cell-aggregate cultures of *Ocotea catharinensis* for 28 days. (N = 5; SE = 0.049).

	Nitrogen concentration (meqs l^{-1})			
Initial pH	0.0	1.5	15.0	60.0
3.5	3.5	3.0	2.8	2.8
5.5	5.4	5.2	4.4	4.3