Effects of inorganic nitrogen supply in culture media on development of embryogenic cell aggregate cultures of *Ocotea catharinensis* Mez.

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Abstract:
Embryogenic cell aggregates of *Ocotea catharinensis* were cultured for 8 weeks at 25°C in the dark on Lloyd and McCown woody plant medium (WPM) with 2.0% (w/v) sucrose, 0.3% (w/v) activated charcoal (AC), 181 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.8% (w/v) Technical Agar Grade III, and initial pH 5.8. Analyses of used media showed that uptake of ammonium is faster than nitrate uptake, and that media were depleted of the former nutrient after a 3-week initial culture period, after which nitrate was the only nitrogen form in media. Medium pH decreased while NH_4^+ was available and increased after it finished. It appeared that AC did not uptake any nitrogen from culture medium. In a second experiment, media consisting of WPM minerals and vitamins lacking nitrogen were prepared with 2% sucrose, 181 µM 2,4-D, 0.8% agar, 0.3% AC, pH 5.2, supplemented with 5 concentrations of NH_4^+ (0, 2.5, 5, 10 or 20 meqs l^{-1}) each in combination with 5 concentrations of NO_3^- (0, 5, 10, 20 or 40 meqs l^{-1}), and used for culturing cell aggregates of *O.catharinensis* for 6 weeks at 25°C in the dark. Results showed that growth and differentiation of *O.catharinensis* cell aggregate cultures were dependent on medium nitrogen concentrations, the balance between different nitrogen forms and medium pH. Biomass production (fresh weight increase) of cultures was positively correlated to total nitrogen and NO_3^- concentrations in culture media, with best growth when NO_3^- was present at a concentration at least 4-fold higher than NH_4^+. Cell aggregate differentiation was positively correlated to media NO_3^- and pH, whereas pH was negatively correlated to the NH_4^+/NO_3^- ratios in media.

Keywords: Ammonium, cell differentiation, nitrate, nutrient uptake, pH effects

Introduction

Plant morphogenesis in tissue culture is affected to a large extent by the composition of nutrient media. A series of different media may therefore be listed for different species and for different objectives. Significant changes in nutrient status occur also during culture as a consequence of cell growth and cell death such that these changes may be closely linked to the developmental phenomena which are observed *in vitro* (R.Williams, unpublished data). Few references about such dynamic aspects of plant tissue culture systems have been presented as yet in the scientific literature.

The control of somatic embryogenesis is affected by exogenous applications of auxins in media. A large number of embryogenesis systems use auxins in an initial phase to induce somatic embryos (SE) or highly meristematic nodular tissues that subsequently become embryogenic, followed by a subsequent phase in which removal or reduction of the auxins in the medium promote further differentiation of the induced proembryogenic structures (Sharp *et al.*, 1980; Ammirato, 1983). Other cultural factors can be employed to effectively control somatic embryogenesis. For example, Smith and Krikorian (1990a, b, c) described an auxin-independent method for manipulation of somatic embryogenesis based on pH control of culture media.

A system of somatic embryogenesis of *Ocotea catharinensis* Mez (Lauraceae) was developed in which differentiation of the early stages of embryogenesis (*ie.* from embryogenic cell aggregates to globular somatic embryos) can be controlled by manipulation of mineral media composition (Moura-Costa *et al.*, 1993). Contrasting patterns of differentiation were observed when cell aggregates were

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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 promoting proliferation of undifferentiated structures. It was inferred that these differences were probably due to the contrasting concentrations and forms of mineral nitrogen in the above media.

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). Absorption of ammonium and nitrate itself is also pH-dependent: high pH levels tend to enhance ammonium uptake and low pH nitrate uptake (Bayley *et al.*, 1972a,b). It has also been suggested that NH_4^+ acts as a buffer preventing any significant rise in pH caused by NO_3^- uptake (George & Sherrington, 1984). Therefore manipulation of the balance of NH_4^+ and NO_3^- affects not only the nitrogen status of the culture medium but also pH, with significant implications for both chemical and physiological mechanisms operating within or on the surface of cells and in the culture medium itself.

There is much evidence that relatively high proportions of NH_4^+ are essential for somatic embryogenesis to occur although this nutrient tends to keep embryogenic cells in a highly proliferative undifferentiated form (Kirby *et al.*, 1987; Smith & Krikorian, 1989) while relatively high proportions of NO_3^- seem to be associated with further differentiation (Kirby *et al.*, 1987). This fact raises the question: are these nutrient alterations a cause or a consequence of developmental changes? Also, is the NH_4^+/NO_3^- balance the important factor or is it changes in pH resulting from their differential uptake that determine pathways of embryogenic development? Some reports show that NH_4^+ can be used as a sole nitrogen source (Dougall & Verma, 1978; Smith & Krikorian, 1989, 1990a, b, c) although in many cases a balance of reduced and non-reduced forms is acknowledged to be more beneficial than sole sources of nitrogen (Kirby *et al.*, 1987).

Considering the dynamic aspect of tissue culture media, it can be inferred that the medium may be at its `optimal' for inciting a particular kind of morphogenic development for only a limited period of the culture cycle on those occasions when the right balance of nutrients and pH is present. For this reason alone, it is important to know the constitution of the medium as well as the developmental changes that occur concomitant with particular parts of the culture cycle.

Furthermore, complications arise when activated charcoal (AC) is used in culture media, since its adsorption properties can alter significantly the relative constitutions of the medium in terms of availability of organic components to plant cells. A recent report by Ebert and Taylor (1990) on the effects of AC on availability of 2,4-D describes dynamic processes which occur in culture media themselves, independently of the effects of nutrient uptake by plant cells. Unfortunately, little is known about the effect of AC on other compounds in the medium, and it is frequently used in tissue cultures of *Ocotea catharinensis* to reduce browning (Viana *et al.*, 1990).

Ocotea catharinensis 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.

This paper describes an experiment to monitor the patterns of uptake of medium nitrogen by embryogenic cell aggregates of *Ocotea catharinensis*, and its effect on medium pH. A second experiment investigated the differences in growth and development of Ocotea cell aggregates cultured in media containing different concentrations of ammonium and nitrate.

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

All media utilized for these experiments were dispensed at 10 ml per culture tube (10 cm-long, 2 cm-diam) and were autoclaved for 20 min at 121°C and 1.6 kg cm^{-2}. Adjustments of media pH were carried out before adding the agar and AC constituents to the media using a standard temperature compensated combination electrode (Gallenkamp, UK) attached to a AGB-4000 (UK) digital pH meter.

Experimental

Two experiments were conducted to assess the changes in nitrogen contents and pH of media during different stages of the culture cycle of *Ocotea catharinensis* cell aggregates; and to investigate the effects of altering the concentrations of medium NH_4^+ and NO_3^- on their growth and differentiation.

Experiment 1: Assessment of medium nitrogen utilisation by *Ocotea catharinensis* cell aggregates during a typical culture cycle in woody plant medium

A medium consisting of WPM prepared with 2.0% sucrose, 0.3% AC, 0.8% agar, 181 μM 2,4-D and pH 5.2 was inoculated with 200 mg portions of cell aggregate cultures (75% CA1 and 25% CA2 types of cell aggregates) and incubated at 25°C, in the darkness. Tubes containing uninoculated media were used as controls. A medium of the same formulation but without AC was prepared and left uninoculated to test if AC adsorbs medium nitrogen. Both these tubes and the controls were incubated under the same culture conditions as the cell cultures. Five tubes per treatment were sampled for each analysis of medium nitrogen content, which were performed at 0, 1, 2, 3, 4, 6, and 8 weeks after media preparation. Samples were prepared by passing media through a fine stainless steel sieve (mesh size 0.38 μm) to separate the liquid phase and centrifuged for 5 minutes at 3500 rpm (Denley BS400 centrifuge, UK) to obtain a solution clean of AC and traces of agar. The pH of the samples were measured after centrifugation.

Samples were analyzed to determine total nitrogen and NH_4^+ contents using the Kjeldahl method. For analysis of total nitrogen, 3 ml aliquots were digested using the Kjeldahl method. To determine the concentrations of NH_4^+, samples were utilised without acid digestion. Since the only nitrogen sources in the media were NH_4^+ and NO_3^-, the concentrations of NO_3^- were calculated from the difference between total nitrogen and NH_4^+ contents of a given sample. Analyses were carried out using a SP6.500 UV Spectrophotometer (Pye Unicam, UK).

Experiment 2: Effects of different concentrations of ammonium and nitrate in media on development of *Ocotea* cell aggregate cultures grown on woody plant medium

Media consisting of WPM minerals and vitamins lacking nitrogen were prepared with 2% sucrose, 181 µM 2,4-D, 0.8% agar, 0.3% AC, pH 5.2 and supplemented with 5 concentrations of NH_4^+ (0, 2.5, 5, 10 or 20 meqs l^{-1}) each in combination with 5 concentrations of NO_3^- (0, 5, 10, 20 or 40 meqs l^{-1}). Nitrogen concentration was treated in terms of meqs l^{-1} to enable the comparison of units of NH_4^+ and NO_3^- present in the media. The concentrations of ammonium and nitrate were chosen deliberately to provide combinations ranging from no nitrogen, the normal WPM formulation (*ca.* 5 meqs l^{-1} NH_4^+ and 10 meqs l^{-1} NO_3^-) up to the concentrations present in MS medium (20 NH_4^+/40 NO_3^-). Ammonium and nitrate were supplied as NH_4NO_3, $Ca(NO_3)_2.4H_2O$ or $NH_4H_2PO_4$. NH_4NO_3 was used whenever possible to reduce the interference of other ions in the final medium formulation.

Tubes were inoculated with 150 mg portions of cultures consisting of *ca.* 75% CA1 and 25% CA2 types of cell aggregates, and incubated at 25°C in the dark. The experiment was designed as a 5x5 factorial with 5 replicates per treatment. Assessments were carried out after 6 weeks incubation, by recording increase in fresh weight of cell aggregates per culture tube, media pH and the relative proportion of different cell aggregates of cultures. This latter parameter was determined by taking 0.5 µg samples of cultures from each tube and diluting in 2.5 ml distilled water in a 5 cm diam petri dish. The relative numbers of different types of cell aggregates (CA1, CA2 and globular somatic embryos) were determined per standard x200 field of view on an IMT-2 Olympus inverted microscope. Results were expressed as mean percentages of total number of differentiated cell aggregates (*ie.* CA2 and globular somatic embryos), after two-way analysis of variance following arc-sin angular transformation of data (Zar, 1984). Correlations between all factors were calculated and levels of significance determined using T-test.

Results and discussion

Experiment 1: Assessment of medium nitrogen utilisation by *Ocotea catharinensis* cell aggregates during a typical culture cycle in woody plant medium

Results of nitrogen analyses showed that AC does not adsorb any of the nitrogen forms present in the media used since the nitrogen concentration of both control media (uninoculated media with or without AC) remained unchanged during the whole period of monitoring (data not shown).

Total nitrogen (TN) uptake was faster in the 3-week initial culture period and continued at a slower rate after that (Fig.1). This was probably due to the rapid uptake of NH_4^+ during the initial period of culture, until its depletion from the culture medium. NO_3^+ uptake, on the other hand, was slow but uniform during the whole culture period. These results reinforce the assumption that cells use NH_4^+ preferentially (Veliky & Rose, 1973; Sargent & King, 1974; Rose & Martin, 1975; Kirby *et al.*, 1987), possibly due to its relatively energy-inexpensive uptake by cells (Kirby *et al.*, 1987). A decrease in medium pH was observed during the initial 2-week period, followed by an increase from the third week until the end of the experiment (Fig.2). These changes occurred concomitantly with the changes in uptake of ammonium and nitrate. Media pH decreased as NH_4^+ uptake was rapid, and increased when the medium was depleted of this nutrient and NO_3^- remained as the sole nitrogen source used by cells (Fig.1 and 2).

Experiment 2: Effects of different concentrations of ammonium and nitrate in media on development of *Ocotea* cell aggregate cultures grown on woody plant medium

The results of this experiment showed that biomass production (fresh weight increase) of *Ocotea catharinensis* cell aggregates is dependent on both total nitrogen concentrations and NH_4^+/NO_3^- ratios in the culture media. Biomass production was positively correlated with both total nitrogen and NO_3^- concentrations in the medium (Table 1). However, it appeared that best rates of biomass production were achieved if the concentration of NO_3^- in the medium was at least 4 fold the concentration of NH_4^+

(Fig.3). The best media for supporting biomass production were those with maximum concentration of NO_3^- (40 meqs l^{-1}) and moderate NH_4^+ (5 - 10 meqs l^{-1}). No biomass production occurred in the absence of NH_4^+ but very low concentrations (2.5 meqs l^{-1}) were sufficient to promote growth as long as NO_3^- was supplied at a concentration equal or exceeding 10 meqs l^{-1}. Low concentrations of NO_3^- (< 10 meqs l^{-1}) did not promote proliferation of cell aggregate cultures, even when NH_4^+ was present at a high concentration (20 meqs l^{-1}).

Initial differentiation of cell aggregates also appeared to be regulated by nitrogen forms in the medium. It was found that NO_3^- concentration was positively correlated to the percentage of further differentiated cell aggregates (CA2 and globular somatic embryos, Table 1). The best medium for differentiation contained 2.5 meqs l^{-1} NH_4^+ and 40 meqs l^{-1} NO_3^- (Fig.4).

Medium pH at the end of the culture period was negatively correlated with NH_4^+ concentrations and to the NH_4^+/NO_3^- ratio in the medium (Table 1). Media containing higher concentrations of NH_4^+ exhibited lower pH values at the end of the culture cycle (Fig.5). However, a higher pH was reached in media containing 2.5 meqs l^{-1} NH_4^+ (2.5 meqs l^{-1} NH_4^+, 10 - 40 meqs l^{-1} NO_3^-) than when NH_4^+ was absent. Media lacking NH_4^+ did not change in pH during the culture period and cultures did not proliferate and/or increase in fresh weight. A positive correlation between medium pH and differentiation was observed. This further supports the correlation between differentiation and NO_3^- concentration, since absorbtion of this nitrogen form promotes a rise in medium pH.

General discussion

The combination of these results suggested that some selective cell-membrane mechanisms, possibly linked to membrane charges and/or nitrate permeases, may have been involved in the initial absorption of nitrogen by cells, regardless of its further metabolism and action of nitrogen reduction enzymes, as has been suggested by Jackson *et al.* (1973). The exact process of nitrogen absorption by cells is still unknown. However, it is known that nitrate uptake is undoubtedly an active process requiring energy-rich compounds to drive it, and the presence of special carrier proteins, nitrate permeases, which catalyse the passage of nitrate ions across cell membranes have been suggested. On the other hand, although ammonium absorption is a process that involves both active and passive components, NH_4^+ uptake is never completely suppressed, as is the case in nitrate absorption (Lewis, 1986). It may be that for *Ocotea* cells to absorb NO_3^-, NH_4^+ must either be present in the medium or have been previously utilised, thus creating the conditions for uptake of nitrate to start. A minimum amount of NH_4^+ appeared to be required for initiating the process of nitrogen uptake (with a resulting change in pH) and promoting cell aggregate proliferation. In the absence of NH_4^+, nitrogen was not absorbed by cells (or only at negligible amounts) and even when NO_3^- was present in high concentrations (up to 40 meqs l^{-1}) no changes in medium pH nor cell aggregate proliferation were observed. According to Kirby *et al.* (1987), the pattern of N-usage by cell cultures appears to be that ammonium is utilised initially, followed by nitrate. This might explain why final pH of *Ocotea* cultures on media lacking NH_4^+ did not change while those on 2.5 meqs l^{-1} NH_4^+ showed significant differences depending on the amount of NO_3^- present: media containing high NO_3^- levels had their pH at high values at the end of the culture period while those containing low levels of NO_3^- exhibited low pH values. As seen in Experiment 1, during the initial culture phase (first 3 weeks after inoculation) NH_4^+ uptake occurs at a fast rate (causing a reduction in medium pH) followed by a phase in which NO_3^- remains as the only nitrogen source in the medium (causing an increase in pH), but after a period of time, cell proliferation ceases and browning takes place. In the case of media with low NO_3^- and NH_4^+ concentration (5 and 2.5 meqs l^{-1}, respectively), pH decreases during the initial culture period, due to NH_4^+ uptake, and NO_3^- uptake follows but its concentration is not sufficient for pH to be brought back to levels of *ca.* 5. Higher concentrations of NH_4^+ at low NO_3^- (5 meqs l^{-1}) maintained pH at low values until the end of the culture period (Fig.5), possibly due to ammonium's preferential uptake (Veliky & Rose, 1973; Sargent & King, 1974; Rose & Martin, 1975; Kirby *et al.*, 1987; Minocha, 1987). Media lacking NO_3^- also had their pH much reduced although cell proliferation and growth did not occur. It may be that since NH_4^+ uptake is relatively energetically inexpensive (Kirby *et al.*, 1987), uptake of this nutrient takes place (causing pH to fall) although cell aggregate proliferation does not occur. It

appeared from these results that a balance of reduced and non-reduced nitrogen forms in the medium is required to support proliferation of cell aggregates of *O.catharinensis*.

As far as cell differentiation was concerned, it appeared that excessive NH_4^+ and/or low pH levels prevented the further differentiation of *Ocotea* cell aggregates. A similar system was described by Smith and Krikorian (1990b), in which 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. A possible explanation for the different patterns of cell differentiation observed may be related to the role of cell walls in controlling cell growth. Differentiation and tissue organisation are usually related to small cells, unlike the large cells of callus or parenchymatous tissue which are conceptually undifferentiated (Fry, 1990; Lyndon, 1990). According to Fry (1988), cell growth is dependent on the structure of the cell wall and its capability of undergoing extension and therefore cell expansion. Cell wall 'tightening' or 'loosening' mechanisms are affected by many factors but H^+ ions appear to play a very important role in these processes. There is evidence that during growth, cells secrete H^+ creating an acidic cell wall that favours the action of indole-3-acetic acid (IAA) on the process of wall loosening and that exogenous H^+ ions mimic the action of IAA on growth. Also, H^+ appears to be involved in activating cell wall-loosening enzymes and, furthermore, many of these enzymes have low pH optima. Confirming these assumptions, the application of buffers to abolish the wall-acidifying effects of secreted H^+ ions blocked the effect of IAA on growth of cells (Fry, 1988). High pH levels are associated with wall tightening, creating the necessary conditions for organisation and differentiation to occur. Low pH favours the effects of auxins and promotes cell wall extension concomitant with cell expansion and division, in a situation of rapid cell proliferation in an unorganised manner. These findings strongly reinforce the assumption that pH is of extreme importance in the control of the process of cell differentiation, and this factor may be more important than the effects of medium nitrogen levels themselves. This possibility was investigated employing organic buffers to determine whether the observations reported here were either due to the specific manipulations of nitrogen concentrations or to the resulting variations in media pH levels and the results are reported elsewhere (Moura-Costa & Mantell, 1993).

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 Sara Brocklehurst and Sue Farris. We would like to thank Dr Jennet Blake and Dr Frank Taylor for their comments on an early version of the manuscript.

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Figure 1. Changes in total nitrogen, ammonium and nitrate contents of media during a 8-week culture cycle of *Ocotea catharinensis* cell aggregates. LSD's ($P < 0.05$) are shown. (N = 5).

Figure 2. Changes in media pH during a 8-week culture cycle of *Ocotea catharinensis* cell aggregates. LSD's ($P < 0.05$) are shown. (N = 5; means ± SE).

Figure 3. Biomass production (fresh weight increase) of cultures of *Ocotea catharinensis* cell aggregates after a 6-week culture cycle on a medium containing different concentrations of ammonium and nitrate. (N = 5; $LSD^{*} = 108.24$)

Figure 4. Percentages of differentiated cell aggregates (CA2 and globular somatic embryos) of the total population of cell aggregates of *Ocotea catharinensis* after a 6-week culture cycle on media containing different concentrations of ammonium and nitrate. (Figures are the means of 5 replicates, after arc-sine transformation. (LSD = 10.05 at $P < 0.05$)

Figure 5. Final pH of media containing different concentrations of ammonium and nitrate after a 6-week culture cycle of *Ocotea catharinensis* cell aggregates. (N = 5; LSD = 0.4187, at $P < 0.05$).

Table 1: Correlations between medium nitrogen forms and concentrations, medium pH, biomass production (BP) and differentiation (D) of *Ocotea catharinensis* cell aggregates. Levels of significance (T-test) are shown under the Pearson product-moment coefficients of correlation. (N = 125).

	BP	D[1]	pH[2]
D	0.738 0.000	-	-
pH	-0.032 0.877	0.398 0.049	-
Total N	0.572 0.003	0.345 0.091	-0.180 0.389
NH_4^+	-0.013 0.952	-0.332 0.105	-0.661 0.000
NO_3^-	0.627 0.001	0.551 0.004	0.129 0.538
NH_4^+/NO_3^-	-0.187 0.370	-0.243 0.242	-0.395 0.050

[1] Proportion of further differentiated cell aggregates in cultures (*ie.* CA2 and globular somatic embryos); [2] Medium pH at the end of a culture cycle.