GENERATION OF A SUSTAINABLE PINUS RADIATA CELL SUSPENSION CULTURE AND STUDIES OF CELLULAR NITROGEN NUTRITION

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ABSTRACT

A **Pinus radiata** D. Don cell suspension culture was developed from excised embryos using a Schenk and Hildebrandt medium modified by addition of 2.6 mM ammonium phosphate and 10 mM arginine hydrochloride. Initiation of the culture involved selection of initially rare meristematic cells from the dominant mass of differentiating cells with limited mitotic capacity. The chromosomally normal culture was found to be capable of long-term culture and amenable to quantitative growth experiments using dry-weight yields of suspension-cultured cells. Responses to nitrogen nutrients provided growth contour plots illustrating the interaction between ammonium and nitrate nutrients, with optimal growth in the vicinity of 3 mM ammonium and 15 mM nitrate ions. Arginine was able to replace ammonia entirely, nitrate was necessary for good growth. Glutamine and asparagine were also growth effective, whereas little benefit was found with lysine, ornithine, or glutamate, and urea yielded an intermediate response. Higher levels of lysine (3 mM), glutamate (10 mM), or casein hydrolysate (10 mM amino acids) resulted in growth reduction. The effectiveness of beneficial organic nitrogen supplements is ascribed to endogenous supply of reduced nitrogen for general cellular biosynthesis, and such supplements are considered unnecessary when inorganic nitrogen is optimised.

Keywords: amino acids; cell culture; cell nutrition; **Pinus radiata**.

INTRODUCTION

The availability of reliable cell culture methods for many angiosperms is of undoubted benefit in plant biochemical, physiological, and propagation programmes, and these methods are indeed essential for the realisation of many potential benefits of advanced breeding methods such as genetic engineering and protoplast fusion. With gymnosperms, and conifers in particular, much less has been achieved in cell

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culture, even though these procedures hold promise of very substantial benefits with trees of such long generation times. As discussed by Bornman (1983), one serious factor in conifer tissue-culture is considered to be the use of culture media specifically developed for the growth of angiospermous callus. Increasing recognition that genotypic selection processes may operate, and that genetic variation may be induced in plant cells through nutrient stresses (Cullis 1981; Durrant 1962), further justifies the need to ensure that media formulations are optimised to promote genetic stability of cultures.

In assessing nutritional suitability of media, it is further recognised that with callus growing on solidified media, concentration gradients will be generated; cell suspensions growing in liquid medium permit better definition of the nutrient environment, and also prove amenable to more precise growth measurements, as shown in studies of copper uptake into *P. taeda* L. cells by growth analysis (Teasdale 1984). Further discussion of the utility of cell suspension cultures for many biochemical and genetic studies has been provided by Ludden & Carlson (1980).

The dominant pine plantation species in the Southern Hemisphere is certainly *Pinus radiata.* No cell suspension cultures of *P. radiata* have yet been documented, and the only medium reported to sustain a cell suspension culture of any pine species over many subcultures is the LM formulation (Litvay *et al.* 1981). In preliminary trials LM medium was unsuccessful for initiation of *P. radiata* suspension cultures.

Studies with many plant species, including other gymnosperms, have indicated that nitrogen plays a critical role in cell and callus growth (White & Gilbey 1966; Street 1966; Kirby 1982) as it does for intact plants (Haynes & Goh 1978). Arginine is recognised to play a central role in the natural storage, transport, and metabolism of nitrogen in conifers, as reviewed by Bidwell & Durzan (1975), and has been used with benefit in the nutrition of sugar cane cells (Maretzki *et al.* 1969) and spruce tissue (Steinhart *et al.* 1961). One study of *Picea glauca* Voss found that arginine was unsatisfactory whereas glutamine was beneficial (White & Gilbey 1966). More recently Kirby (1982) has shown that glutamine promotes rapid growth of *Pseudotsuga menziesii* (Mirb.) Franco (Douglas fir). A number of other studies with conifer cells and tissues have incorporated arginine, but without any evaluation of its role (Bornman 1983; Teasdale & Rugini 1983).

It was the aim of this study to examine whether modification of the nitrogen composition of a standard medium would first yield a sustainable cell suspension culture of *P. radiata,* and then to employ this for systematic study of the role of inorganic and organic nitrogen forms towards media optimisation.

MATERIALS AND METHODS

Increase in dry cell mass was used to monitor nutrient responses. This was selected because other healthy pine cell cultures were known to exhibit pure exponential growth when measured in this way (Teasdale & Rugini 1983; Teasdale 1984): nutrient stresses invariably lowered the dry mass of the culture and so this provides a convenient and precise measure of cell-culture response. The base medium selected was that of Schenk & Hildebrandt (1972) which has been used frequently in conifer micropropagation studies (Aitken *et al.* 1981; Mott & Amerson 1981).

Pinus radiata suspension cultures were developed from the embryos of control-pollinated seeds (the cross between parents from the Australian plus-tree register 50048 × 80055). The seeds were disinfected by immersion in 100 volume H_2O_2 for 20 min then rinsed three times in sterile distilled water; after imbibition for 3 days at 2°C the embryos were excised under sterile conditions. Cell suspensions were induced by culturing the excised embryos in wells of 25 compartment trays (sterilin No. 306V) containing 2 ml of liquid SH medium (Schenk & Hildebrandt 1972) which had been modified by addition of 2.6 mM ammonium phosphate plus 10 mM arginine hydrochloride (designated SHR4 medium), a medium shown in preliminary studies to maintain growth of *P. taeda* cells; naphthaleneacetic acid was included at 10.7 μM (2 mg/*l*) as sole auxin in this and all media for growth of *P. radiata* cells. During the initiation phase, subculturing was carried out by diluting two-fold with fresh medium when the wet tissue volume was judged to occupy approximately one-third of the total. After establishment, the cultures were maintained by transferring 10 ml of suspension to 50 ml of fresh SHR4 medium in a 250-ml conical flask every 12–14 days. A shaking speed of approx. 70 orbits p.m. was maintained with a 16-h daily photoperiod. A single culture line was arbitrarily selected for all subsequent work reported here. Inocula for quantitative growth experiments were prepared by vigorously stirring rapidly-growing cell cultures on a 500-μm sieve so as to fragment larger colonies, collecting the filtrate on a 100-μm sieve, washing with sterile water, and then suspending them in the desired medium. This procedure was found to rupture any residual elongated and vacuolated cells, but did not appear to damage compact cells, thereby yielding apparently meristematic cultures which grew rapidly.

To obtain quantitative responses to nitrogen nutrients, sieved cells were suspended in modified SH medium lacking nitrogen and with the macro-element ions adjusted to the following concentrations: K+ 17.5 mM, Mg++ 2 mM, Ca++ 1.3 mM, SO_4= 2 mM, phosphate ions 2.5 mM, Cl– 17.6 mM. Inocula of 1.0 ml volume, typically containing 0.5 mg (d.w.) of cells, were placed into wells of 25 compartment trays which had been preloaded with small volumes (50 μl each) of appropriately concentrated supplements; final volumes were adjusted to 2.0 ml with additional base media. Attention was given to uniform cell dispersion prior to withdrawing and dispensing the inocula. The cells were cultured with shaking at 25°C with a 16-h daily photoperiod for a typical period of 25 days. They were quantitatively filter-harvested (residual colonies were readily seen) and extensively water-washed on prepared glass-fibre discs (Whatman GF/A), then weighed to a precision of 0.01 mg on an electrobalance (Perkin Elmer ADZ2) after drying for a minimum of 24 h at 60°C. Standard deviations for quadruplicate samples were typically 5% of the mean (and invariably below 10%).

The procedure was slightly modified for the assessment of organic nitrogen forms. The base medium contained 0.2 mM NH_4^+ and 3 mM NO_3^-; supplemental inorganic nitrogen was added to obtain 3 mM NH_4^+ and 15 mM NO_3^-. This was added as a concentrated stock in the same manner as the organic supplements. The total amino-acid concentration in casein hydrolysates was estimated using a mean-residue-weight of 110 – thus a solution specified as 10 mM would have a weight concentration of 1.1 g/*l*. Chromosomal counts of cultured cells were made on squash preparations after acid fixing and toluidine-blue staining as described by Marks (1973).

RESULTS AND DISCUSSION

Culture Initiation

Excised *P. radiata* embryos placed in SHR4 medium containing 2 mg NAA/*l* (10.7 μM) became noticeably swollen in the hypocotyl and cotyledons, but not in the radicle, after only 3 days of culture. With continuing growth, embryonic structures became progressively more difficult to distinguish. Elongated and vacuolated cells were profusely sloughed into the medium so that at 5 days such single cells dominated the culture (Fig. 1a). These differentiated cells did not appear to divide further, but remained static without senescence. Colonies of compact non-vacuolated cells were only occasionally observed at 20 days (Fig. 1b) but continued to grow, eventually dominating the culture after a period of 3 months (Fig. 1c). Such cultures have been found sustainable in long-term studies. One has been selected as a standard culture and maintained in SHR4 medium for 12 months with subculturing every 14 days. Examination of the karyotype of these cells after 12 months' culture revealed only cells containing the normal diploid complement of 24 chromosomes (Fig. 1d). No difficulty has been incurred with initiation from embryos in this way. A limited trial with a random selection of (open-pollinated) *P. radiata* seeds produced 16 such cultures. Where suspension cultures were not obtained, the embryos appeared non-viable and did not exhibit any growth in culture.

Inorganic Nitrogen Nutrition

Cellular requirements of inorganic nitrogen by *P. radiata* were estimated from cell yield of batch-grown suspension cultures. A nitrogen-free base medium supplemented to produce the 100 different combinations from the sets of concentrations 0, 2, 5, 10, 15, 20, 25, 30, 35, 40 mM for potassium nitrate and 0, 0.1, 0.4, 1, 2, 5, 7.5, 10, 15, 20 mM for ammonium chloride. The mass increases corresponding to each nitrogen combination were used to construct a contour plot of growth *v.* ammonium chloride *v.* potassium nitrate (Fig. 2). This most clearly illustrates the effect of ammonium and nitrate on cell growth.

It is seen that interaction occurs between the two forms of nitrogen. This cannot be accounted for by simple requirement of a fixed nitrate to ammonium ratio. An optimum is found in the vicinity of 3 mM ammonium and 15 mM nitrate. Toxicity due to ammonium results in obvious cell senescence after only a few days of growth. Increasing the nitrate level alleviated this toxicity in a monotonically increasing but non-linear manner. A control experiment in which ammonium was supplied as ammonium sulphate (data not shown) yielded an identical result, indicating that ammonium rather than chloride is toxic under these conditions.

The progressively declining growth seen with high concentrations of ammonium chloride and potassium nitrate was ascribed, at least in part, to high concentrations of potassium and chloride ions, rather than nitrate and ammonium ions. Potassium chloride toxicity was clearly incurred at concentrations above 30 mM (data not shown) and, in other studies not reported here, potassium deficiency was not incurred unless its total concentration was below 5 mM. Experiments to circumvent possible counter-ion toxicities were considered, but were complicated by lack of the necessary detailed knowledge of the effects of appropriate counter-ions. The optimal combination of

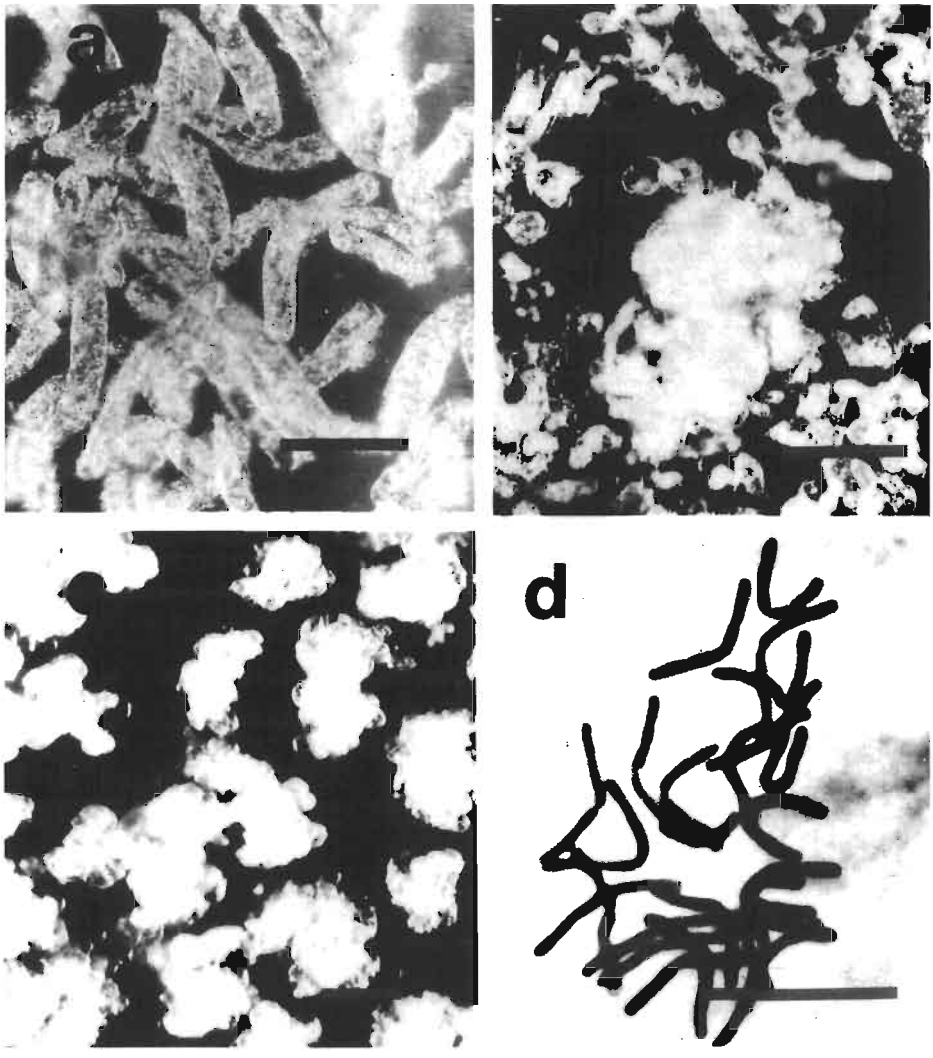

FIG. 1—Stages in the formation of a **Pinus radiata** cell suspension culture in liquid SHR4 medium from an excised embryo.

(a) Initial proliferation of differentiated cells as seen at 5 days (bar = 100 μm).

(b) First appearance at 20 days of colonies of compact non-vacuolated cells which are capable of sustained division (bar = 100 μm).

(c) Culture at 3 months now dominated by apparently meristematic cells (bar = 100 μm).

(d) A squash preparation of a typical cultured cell stained with toluidine blue to show a chromosome count of 2n = 24 (bar = 10 μm).

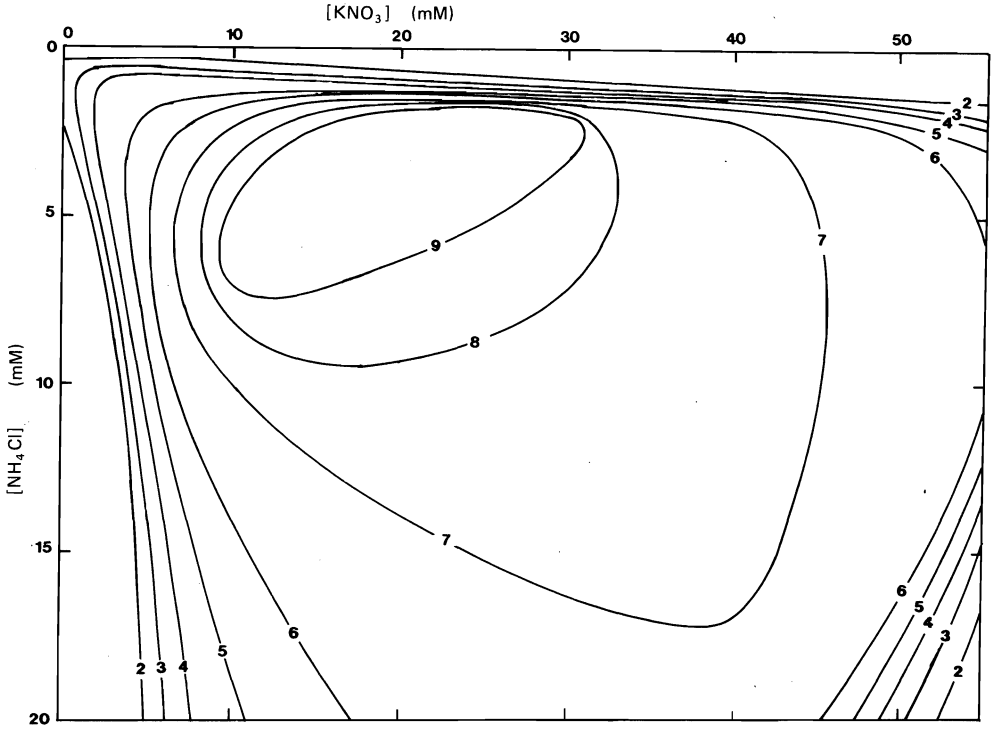

FIG. 2—A contour plot showing the pattern of cell growth in response to varying combinations of ammonium chloride and potassium nitrate added to a nitrogen-free base medium. Contour lines indicate regions of identical growth: the dimensionless numbers are growth multiplication factors obtained by dividing the dry cell mass measured at 24 days by the mass of original inocula.

ammonium and nitrate concentrations indicated in Fig. 2 may well require adjustment when such information in the higher nitrate-ammonium concentration domain is available. Nevertheless, the data at low nitrogen levels are not compromised by counter-ion toxicity considerations, and the potassium (17.5 mM) and chloride (15 mM) levels are quite unlikely to be deficient, so that the form of contour plots can confidently be attributed to ammonium and nitrate ions in the concentration ranges 0–5 mM for ammonium and 0–20 mM for nitrate ions.

Organic Nitrogen Supplements

The role of arginine as a nitrogen source was explored by conducting a study of the ammonium *v.* nitrate interaction in the presence of 10 mM arginine. Comparison of Fig. 2 with Fig. 3 reveals that the pattern obtained in the presence of arginine was quite different from that in its absence. No significant requirement for ammonium is evident; nitrate was highly beneficial, with 2 mM sufficient for maximum growth. High levels of potassium nitrate were, in fact, found more toxic in the presence of arginine hydrochloride than in its absence. Detailed study of this has not been undertaken, but the observation that, in SHR4 medium, 25 mM nitrate is tolerated suggests

that this toxicity is due to the combination of high potassium and arginine ions, conceivably by competitive displacement of some other cation to the extent that deficiency levels occur.

One simple explanation for this effect of arginine is that the transfer of four reduced-nitrogen atoms across the cell for each positive charge requires correspondingly lower nitrate influx for maintenance of cellular charge balance than that required with ammonium ions. Avoidance of a number of energetically significant steps in the assimilation of reduced nitrogen is also a likely benefit. The ammonium-nitrate combinations (0.2, 3 mM) and (3, 15 mM), corresponding to the optima (Fig. 2 and Fig. 3 respectively), were specified for construction of two base media in which a range of organic nitrogen supplements could be assessed. Each supplement to be evaluated was added at the three concentrations of 1, 3, and 10 mM.

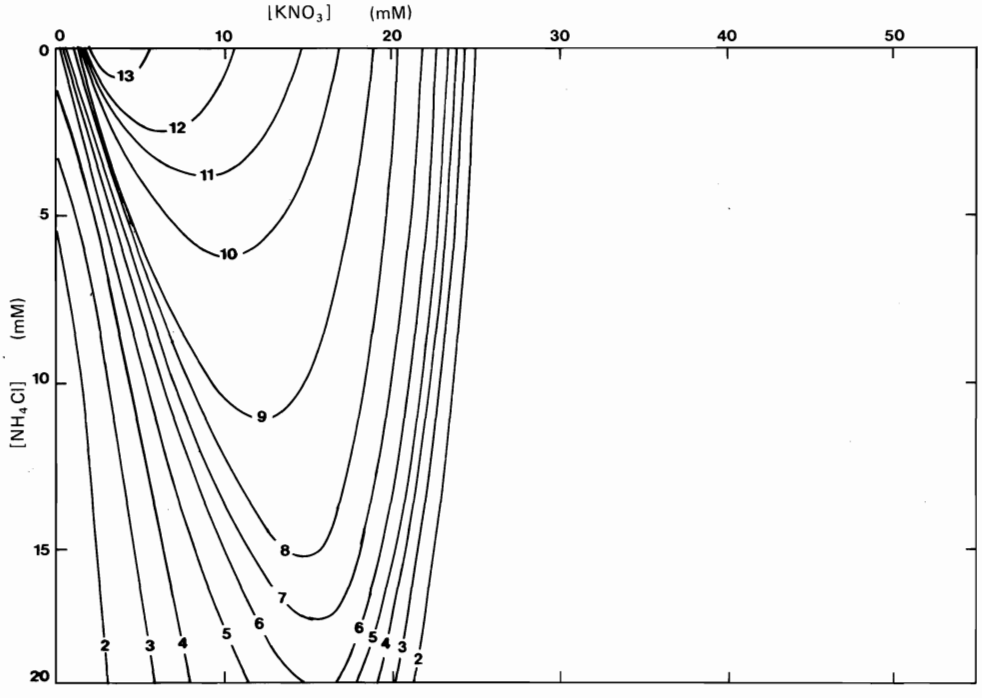

FIG. 3—A contour plot for an experiment analogous to that represented in Fig. 2 with the constant addition of 10 mM arginine hydrochloride to all treatments.

The control experiment in Fig. 4a indicates, as expected, that the 3 mM ammonium, 15 mM nitrate combination without supplements provides good growth, with some arginine, glutamine, and asparagine additions providing only small improvements. Moderate levels of urea, ornithine, and casein had little effect, whereas glycine, lysine, and glutamate were found to be toxic. In the low inorganic nitrogen combination (Fig. 4b) the improvement obtained with arginine, glutamine, and asparagine was quite substantial, with increased growth also provided by casein and moderate urea; the best result in this set was that obtained with asparagine which approaches the

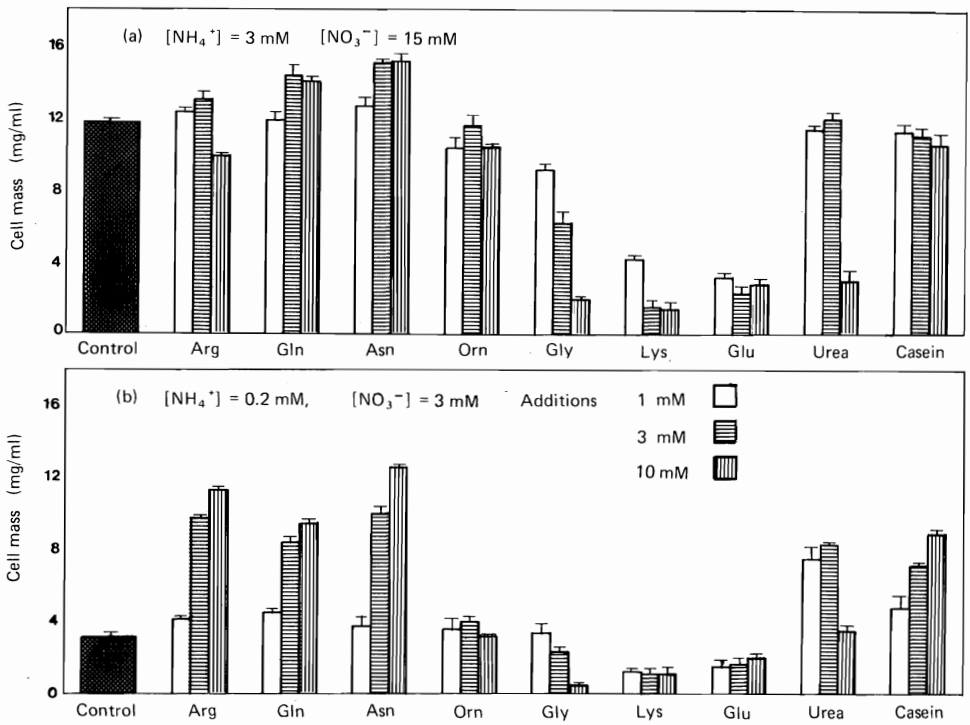

FIG. 4—The growth response obtained by addition of organic nitrogen supplements to base media constructed to have inorganic nitrogen compositions approximately corresponding to the respective optima in Fig. 2 and Fig. 3. Each supplement was added to give final concentrations of 1, 3, and 10 mM. Growth estimated from dry mass measurements after culture for 24 days. Error bars indicate standard errors.
(a) Responses in base media with $[NH_4^+]$ = 3mM, $[NO_3^-]$ = 15mM.
(b) Responses in base media with $[NH_4^+]$ = 0.2mM, $[NO_3^-]$ = 3mM.

control value found in Fig. 4a. Over-all, the best yield was where the higher inorganic nitrogen combination (Fig. 4a) was supplemented with asparagine. Since growth was not quite as good with the lower inorganic nitrogen, either the quantity of asparagine alone did not satisfy total nitrogen requirements or there was some beneficial interaction between nitrogen forms. The observation that glycine is inhibitory at levels above 3 mM is of note as this is the sole amino acid supplement specified in the widely used medium of Murashige & Skoog (1962), albeit at the low level of 25 μM.

The absence of benefit from addition of ornithine is of interest. This amino acid is normally found *in vivo* from arginine and is a direct precursor for cellular biosynthesis of polyamines (Altman & Bachrach 1981). While this result may possibly be explained as a failure of ornithine to penetrate the plasmalemma, the growth-promoting effects of urea and asparagine cannot be construed as a polyamine effect since these molecules cannot provide the necessary carbon skeleton. It is therefore suggested that the growth-promoting effect of amino acids observed in this study is through simple provision of endogenous reduced nitrogen for general biosynthesis of cellular components.

Casein hydrolysate is seen to provide growth benefit when inorganic nitrogen is low. In Fig. 4a, however, the higher levels of casein are seen to be slightly inhibitory when not overshadowed by nitrogen limitation. While casein hydrolysate has been considered a convenient means to provide "balanced" nitrogen nutrition, these results indicate greater benefit can be obtained through selective use of beneficial amino acids and avoidance of those that are inhibitory.

The dramatic variations in cell-culture yield that can be obtained through changes in nitrogen nutrition certainly suggest that nitrogen may be a growth-limiting factor in many media. The sustainable growth of *P. taeda* in LM medium (Litvay *et al.* 1981) indicates that nitrogen nutrition can be met adequately with nitrate and ammonium ions without recourse to organic nitrogen supplements. Supplements such as glutamine, asparagine, and arginine, may well be beneficial with media for which nitrogen has not been optimised. However, the shortest cell doubling time (6 days) obtained in this study was considerably slower than that of under 4 days found with *P. taeda* cells in LM medium (Teasdale 1984). This slower growth of *P. radiata* cells in variants of SH medium reflects operation of growth-limiting factors other than nitrogen nutrition. Further study of growth responses to nutrients other than nitrogen is considered warranted in view of the failure of the LM medium to support *P. radiata* growth, and also to determine toxicity thresholds for counter-ions to nitrate and ammonium so as to facilitate extension of the present study to higher levels of inorganic nitrogen. Studies with other species have long implicated reduced nitrogen supplements as promoters of certain morphogenetic events, including embryogenesis (Halperin 1966; Tazawa & Reinert 1969). It will therefore be of interest to know whether such effects are due to simple removal of nutritional stresses; in particular, will a medium optimised using quantitative cell-yield measurements prove conducive to more efficient micropropagation or, of greater significance, will it promote embryogenesis which has been so elusive in pine species. The initiation and growth of sustainable cell cultures which are amenable to the sieving, washing, and dispensing operations necessary for precise growth studies represents a significant development which will open the way for use of many powerful cell-manipulation procedures with this species.

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