GROWTH PARAMETERS OF CELL SUSPENSION CULTURES OF PSEUDOTSUGA MENZIESII AND EFFECTS OF NITROGEN SOURCES ON GROWTH

MOON SOO LEE*
and
EDWARD G. KIRBY†
Department of Botany, Rutgers University,
Newark, New Jersey 07102, United States

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ABSTRACT

Systematic analysis of factors affecting proliferation of cell suspension cultures of **Pseudotsuga menziesii** (Mirb.) Franco (Douglas fir) derived from cotyledonary callus indicated that an initial inoculum of 30 mg dry weight per 10 ml medium resulted in optimal growth on a medium containing full-strength salts, 15μM NAA and 500 nM BAP. Study of the effects of specific nitrogen sources on dry weight accumulation revealed that 30 mM KNO_3 as a sole nitrogen source was superior to ammonium and nitrate medium and produced a 120% increase in growth after 8 days in culture. Although a low level of ammonium (10 mM) when supplied as a sole nitrogen source could promote modest growth for 5 days, ammonium at either higher levels (30 and 50 mM), or for longer periods, was toxic. Cells grown on glutamate exhibited a pattern of growth similar to controls for the first 7 days, after which growth decreased sharply. When grown on glutamine (10, 30, and 50 mM) as a sole nitrogen source, cell cultures of **P. menziesii** grew rapidly with a shortened lag phase, and exhibited exponential growth rates approaching twice that of control cells grown on ammonium and nitrate.

Keywords: cell culture; glutamine; **Pseudotsuga menziesii**.

INTRODUCTION

The effects of various nitrogen sources on plant cell growth *in vitro* have been examined. Cell cultures of soybean, rice, bean, and Paul's scarlet rose proliferate in the presence of both nitrate and ammonium, whereas tobacco, sweet clover, wheat, and *Vicia* cell cultures can be maintained on nitrate alone (Sargent & King 1974; Mohanty & Fletcher 1978). A source of reduced nitrogen, such as ammonium, glutamine, asparagine, or alanine is required as a supplement to nitrate for maximum growth of bindweed callus (Harvey *et al.* 1973) and wild carrot cell cultures (Wetherell &

* Present Address: Massachusetts Eye and Ear Infirmary, Howe Laboratory, 243 Charles Street, Boston, Massachusetts 02114, United States.
† To whom all correspondence should be addressed.

Dougall 1976). *Acer platanus* L. (sycamore) cell cultures can be established with either nitrate or glutamate as a sole source of nitrogen (Jessup & Fowler 1976). Behrend & Mateles (1975) and Gamborg (1970) reported that tobacco cells are capable of growing on ammonium alone, provided citrate, malate, or pyruvate was added as an organic counter ion.

For *in vitro* growth of cells of conifer species, glutamine has been shown to stimulate growth of spruce callus (Steinhart *et al.* 1961; White & Gilbey 1966) and glutamine is required for sustained cell division in *P. menziesii* protoplast cultures (Kirby 1980). The growth of *Pinus banksiana* Lamb (jack pine) cell suspensions was stimulated by the addition of arginine and glutamine (Durzan & Chalupa 1976). Glutamine has been shown to markedly increase growth of *Pseudotsuga menziesii* cell cultures in the presence of ammonium and nitrate (Kirby 1982). At present little has been reported regarding techniques enabling rapid growth of gymnosperm cells in culture. The present study was, therefore, undertaken to establish procedures enabling maximum growth of cell cultures of *P. menziesii* and to determine the effects of various nitrogen sources on growth.

MATERIALS AND METHODS

Cell cultures: Seeds from open-pollinated cones of *P. menziesii* were a gift from Weyerhaeuser Company, Tacoma, Washington. Surface-sterilised seeds were germinated on a half-strength modified Murashige & Skoog (1962) medium containing the following: 825 mg NH_4NO_3/l, 475 mg KNO_3/l, 220 mg $CaCl_2.2H_2O/l$, 185 mg $MgSO_4.7H_2O/l$, 85 mg KH_2PO_4/l, 12 mg $FeSO_4.5H_2O/l$, 14 mg Na_2EDTA/l, 3.1 mg H_3BO_3/l, 5.25 mg $ZnSO_4.7H_2O/l$, 0.415 mg KI/l, 0.125 mg $Na_2MoO_4.2H_2O/l$, 0.013 mg $CuSO_4.5H_2O/l$, 0.013 mg $CoCl_2.6H_2O/l$, 250 mg myo-inositol/l, 2.5 mg thiamine HCl/l, 0.5 mg nicotinic acid/l, 0.5 mg pyridoxin/l, 30 000 mg sucrose/l. The seed germination medium was adjusted to pH 5.5 prior to autoclaving, and solidified with 0.6% Bacto-agar. Cotyledons (age 3–4 weeks) obtained from sterile seedlings were excised and transferred to the same agar medium supplemented with 15 μM α-naphthaleneacetic acid (NAA) and 500 nM N_6-benzylaminopurine (BAP). Callus was obtained after 6–8 weeks and 1–2 g fresh weight was placed in 125-ml flasks containing 25 ml liquid callus medium, as described. Cultures were maintained on a gyratory shaker at 120 rpm and subcultured every 12–14 days. For experimental cultures, cells were first collected on a nylon screen (110 μm opening) and washed repeatedly with nitrogen-free medium. Washed cells (4–5 g fresh weight) were placed in 250-ml flasks containing 100 ml nitrogen-free medium supplemented with various nitrogen sources. Potassium nitrate, ammonium sulphate, L-glutamic acid, and L-glutamine were separately dissolved in nitrogen-free medium at concentrations of 10, 30, and 50 mM. Experimental media were adjusted to pH 5.5 and filter-sterilised prior to use.

Culture conditions: Cultures were maintained in a constant temperature facility (24°C) and received approximately 10 watts/m^2 supplied by 40-watt Sylvania Cool-White bulbs with a photoperiod of 18 h followed by 6 h darkness.

Growth measurements: Fresh weight determinations were made after 0, 3, 5, 7, and 12 days in culture on 10-ml aliquots of cell suspensions. Cells were collected on pre-

weighed nylon screens mounted on a manifold collector and repeatedly rinsed with distilled water. Fresh weights were directly determined. Dry weight determinations were made by drying cells collected for fresh weights at 60°C for 12 h, according to the procedure of Street (1977).

RESULTS

Initial analysis of suspension cultures of *P. menziesii* was performed using one-half the concentration of a modified Murashige & Skoog (MS) salt formulation, a medium initially utilised for protoplast culture (Kirby & Cheng 1979; Kirby 1980). Under these conditions an inoculum size of 40 mg dry weight per 10 ml suspension gave optimal growth (unpubl. data). Growth of cell cultures on medium containing full-strength salts was examined (Fig. 1). Cells grown on full-strength salts exhibited a shortened lag phase of approximately 1 day, with rapid growth for 8 days starting from Day 1 before a gradual decline in growth. During the 12-day culture period the dry weight of cells grown on half-strength salts increased 50% (20 mg/10 ml), while cells grown on full-strength salts increased 93% (37 mg/10 ml) during the same period. Cells grown on full-strength salts increased by 4.8 mg dry weight per day per 10 ml during exponential growth, whereas cells cultured on half-strength salts increased by 3.3 mg dry weight per day per 10 ml. In all further experiments full-strength salts were utilised.

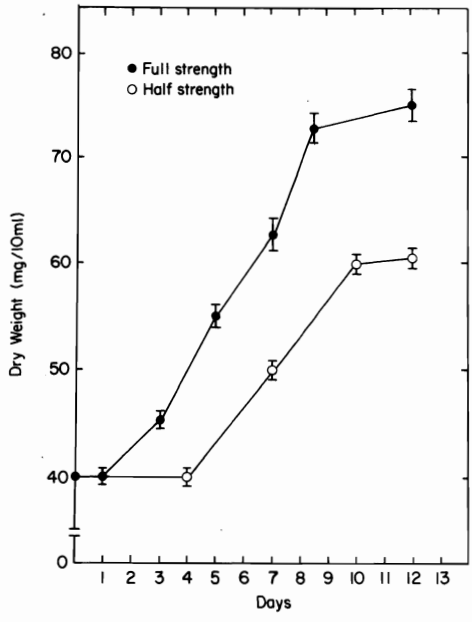

FIG. 1—The influence of nutrient salt concentration on the growth of **P. menziesii** cells in suspension culture. Inocula of 40 mg/10 ml were grown on full-strength and half-strength salts. The reported values are means calculated from triplicate 10-ml samples. Vertical bars represent standard errors.

Cells cultured on 10 mM KNO_3 as a sole source of nitrogen (Fig. 2) exhibited a 1-day lag followed by an initial period of slow growth (Days 2–4). Exponential growth (Days 5–7) was followed by stationary phase at Day 12. The total dry weight increase for cells grown for 12 days on 10 mM KNO_3 was 116%, as compared to 90% for control cells during the same period. Cells grown on 30 mM KNO_3 behaved similarly to control cells until Day 9, when control cells reached stationary phase. Stationary phase was not reached until Day 12 by cells grown on 30 mM KNO_3 and the total dry weight increase at Day 12 was 120% of the initial inoculum. Cells grown on 50 mM KNO_3 exhibited a 3-day lag phase followed by linear growth until Day 12. These cells achieved a 96% increase in dry weight.

Cells cultured on 10 mM ammonium sulphate increased in dry weight only slightly between Day 0 and Day 5, followed by a gradual decrease in dry weight (Fig. 3).

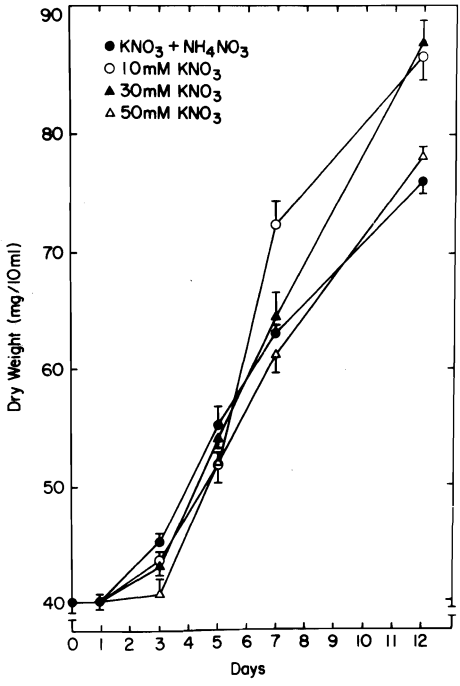

FIG. 2—The effect of nitrate as a sole nitrogen source on the growth of **P. menziesii** cells in suspension culture. Inocula of 40 mg/10 ml were grown on 10, 30, and 50 mM KNO_3 and on nitrate plus ammonium medium as control. The reported values are means calculated from triplicate samples. Vertical bars represent standard errors.

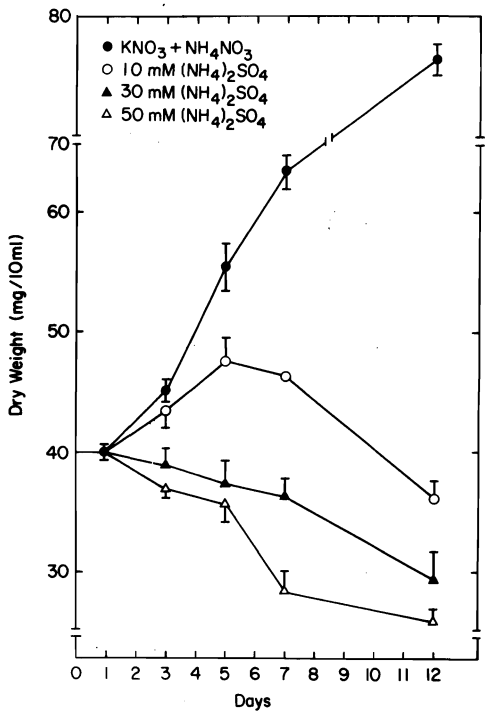

FIG. 3—The effect of ammonium as a sole nitrogen source on the growth of **P. menziesii** cells in suspension culture. Inocula of 40 mg/10 ml were grown on 10, 30, and 50 mM $(NH_4)_2SO_4$ and on nitrate plus ammonium medium as control. The reported values are means calculated from triplicate samples. Vertical bars represent standard errors.

Decreases in dry weight are interpreted as an indication of cell lysis and death. Cells grown on 30 and 50 mM concentrations exhibited decreases in dry weight immediately after subculture and dry weights continued to decrease during the culture period. Cytological observations of cell cultures grown on 30 and 50 mM ammonium revealed severe cellular damage and extensive browning.

During the first 7 days of culture on 10, 30, and 50 mM glutamate, cells exhibited a pattern of growth similar to controls (Fig. 4). After 7 days, dry weights decreased sharply.

During the early logarithmic phase of growth (Day 3), cells grown on 10 and 30 mM glutamine increased in dry weight at a rate almost twice that observed in controls (9.4 and 10.2 mg/10 ml per day *v.* 5.3 mg/10 ml per day) (Fig. 5). After Day 3, cells grown on 10 and 30 mM glutamine showed similar growth to controls. Although cells grown on 50 mM glutamine began growth more slowly than the lower

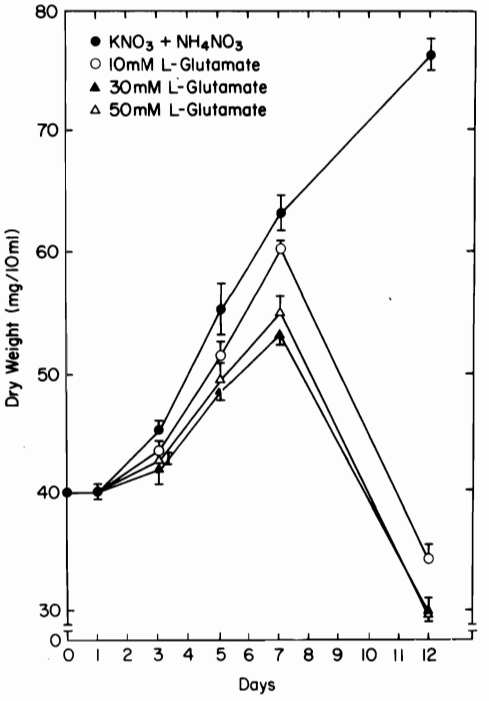

FIG. 4—The effect of glutamate as a sole nitrogen source on the growth of **P. menziesii** cells in suspension culture. Iniocula of 40 mg/10 ml were grown on 10, 30, and 50 mM glutamate and on nitrate plus ammonium medium as control. The reported values are means calculated from triplicate samples. Vertical bars represent standard errors.

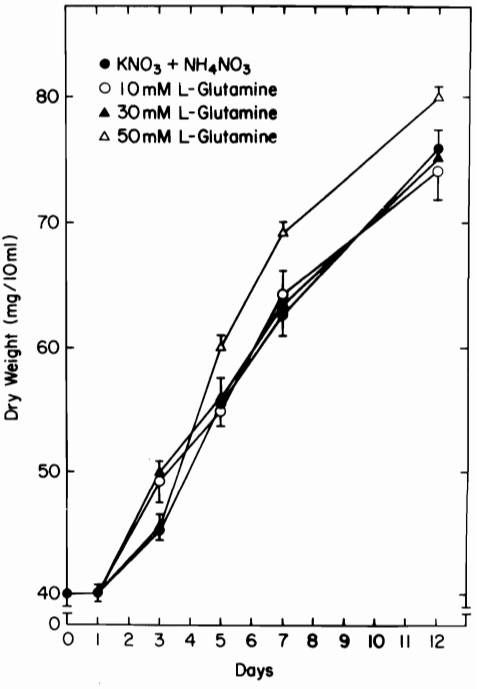

FIG. 5—The effect of glutamine on the growth of **P. menziesii** cells in suspension culture. Inocula of 40 mg/10 ml were grown on 10, 30, and 50 mM glutamine and on nitrate plus ammonium medium as control. The reported values are means calculated from triplicate samples. Vertical bars represent standard errors.

concentrations, they grew rapidly after Day 3 – 6 mg dry weight per day per 10 ml between Days 3 and 7 and 3.4 mg per day per 10 ml for the entire period. Among the concentrations of glutamine tested, 50 mM displayed optimal growth for *P. menziesii* cell cultures, showing a doubling time of 12 days. Cells grown on glutamine were healthy, green, small, and compact.

DISCUSSION

When cells were grown on half-strength salts, a lag phase of 4 days was exhibited and growth ceased abruptly on Day 10 (Fig. 1). Cells grown on full-strength salts showed a 1-day lag period followed by 8 days of linear growth after which growth gradually decreased. Based on slopes of the exponential phase, the growth rate of cells on full-strength salts was approximately 1.5 times the rate observed on half-strength salts, indicating that medium components are limiting growth. This observation is supported by work on cell cultures of *A. platanus* (Young 1973) in which the culture biomass was doubled when the nitrogen level in the medium was increased two-fold. Similar results have been reported by King *et al.* (1973).

Results of studies of effects of various nitrogen sources on growth of *P. menziesii* cells *in vitro* indicate that the form and level of nitrogen limit growth. As a sole nitrogen source, nitrate supported growth (Fig. 2). Although in earlier stages of growth there were no statistical differences between control cells and cells grown on nitrate alone, after Day 6 nitrate cultures continued to increase in dry weight. Initially, however, control cells grew faster than nitrate cells.

Gamborg (1970) has shown that cell cultures of soybean, wheat, flax, and horseradish were able to grow on nitrate as the sole nitrogen source. Yields, however, were higher when ammonium or glutamine was added. A similar study (Craven *et al.* 1972) showed that nitrate medium supplemented with ammonium led to increased growth of carrot cells. In these studies ammonium did not influence fresh weight accumulation during early growth, although its presence resulted in a two-fold increase in growth during the later stages. In *P. menziesii* cell cultures growth was maintained for longer periods when a medium containing nitrate alone was utilised. Ammonium is not suitable as a sole source of nitrogen. Gamborg (1970) has suggested that ammonium causes formation of compounds which, in turn, increase total nitrate reductase activity. Although the identity of such compounds is not known, there is some indication that glutamine may be involved, since cultures which respond to ammonium also respond to glutamine (Nesius *et al.* 1972). Further evidence comes from tobacco cell cultures in which ammonium-stimulated growth results in the accumulation of glutamine (Bergman *et al.* 1976).

Ammonium, when used as a sole nitrogen source for *P. menziesii* cell cultures, was not able to support growth (Fig. 3). Among the concentrations tested, 10 mM ammonium supported growth until Day 5 and the increase in dry weight at that time was approximately 20% – about half that of control cells. At higher concentrations of ammonium (30 and 50 mM) growth was inhibited by Day 3. Such inhibition may stem from acidification of the medium at high ammonium levels. Although low levels of ammonium have not been examined in the present study, our results indicate that

cell cultures of *P. menziesii* are sensitive to ammonium as a sole nitrogen source when supplied at levels present in MS medium.

Supplementing ammonium and nitrate medium with glutamate stimulated growth of *A. platanus* cells (Young 1973) and, interestingly, this treatment also resulted in an increase in growth of cell cultures of *P. menziesii* (Kirby 1982). The present study, however, showed that glutamate, when supplied as a sole nitrogen source, cannot support growth of *P. menziesii* cell cultures (Fig. 4). This may be due in part to the inability of glutamate to be taken up in sufficient levels to maintain growth. Poor growth may also be a result of the lack of an available nitrogen source for the synthesis of glutamine and asparagine (Lea *et al.* 1979). In contrast to the present work, glutamate has been shown to function well as a sole nitrogen source for the growth of *A. platanus* cells (Jessup & Fowler 1976) and exogenous glutamate stimulated protein synthesis in bean cotyledons (Lea *et al.* 1979).

The present work demonstrates that glutamine can not only serve as a sole nitrogen source for *P. menziesii* cell cultures, but can also account for the highest rates of growth of the supplemented media examined. Stimulatory effects of glutamine have been reported for *P. menziesii* cells (Kirby 1982) and soybean cell cultures (Gamborg 1970). These stimulatory effects, however, were observed when media containing ammonium and nitrate were supplemented with glutamine. In *P. menziesii* cell cultures the enhancement of the rate of growth by glutamine alone can be explained on the basis that glutamine provides a source of nitrogen which can be directly taken up and channeled for protein synthesis and growth.

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