Mineral Nutrient Requirements of a Loblolly Pine (Pinus taeda) Cell Suspension Culture

EVALUATION OF A MEDIUM FORMULATED FROM SEED COMPOSITION DATA

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ROBERT D. TEASDALE*, PAMELA A. DAWSON, AND HAROLD W. WOOLHOUSE

John Innes Institute, Colney Lane, Norwich NR4 7UH United Kingdom (P.A.D., H.W.W.), and Gippsland Institute of Advanced Education, Switchback Road, Churchill Vic 3842 Australia (R.D.T.)

ABSTRACT

The mineral nutrient requirements of Pinus taeda cells were explored using quantitative cell culture growth measurements. An appraisal was thereby made of the critical features of a novel and successful medium which was developed specifically for this gymnosperm using chemical composition data for developing seeds, and characterized by generally high concentration of all micronutrients, high magnesium, and low calcium. The high magnesium concentration was found not to be detrimental and possibly beneficial whereas the calcium level bordered on a deficiency threshold. Within the microelements high iodide was found to be essential, as was a higher boron level than is present in media developed for angiosperms. High zinc concentrations were also beneficial, with normal levels permitting slower but nevertheless healthy growth. An improved medium was thereby formulated which was stress-free and exhibited broader genotype specificity. This new formulation has proved very successful in maintaining long-term growth of highly uniform and apparently meristematic suspension cultures of Pinus radiata.

There is a general paucity of knowledge of the cellular nutritional requirements of pines. The availability of a P. taeda culture system highly amenable to quantitative growth measurements encouraged us to obtain precise response curves to a range of individual mineral nutrients.

P. taeda cell suspensions have been shown to exhibit rapid exponential growth when transferred to fresh medium, as estimated by dry weight measurements on harvested cultures (16). Nutrient stresses were generally found to diminish the dry mass of harvested cells. While such measurements may not be satisfactory for some more detailed studies of cell nutritional physiology, they were accepted as quite sufficient for the present purpose of defining the limits of nutrient requirements. Dry mass measurements were therefore accepted as a guide for formulation of a medium optimized for pines.

MATERIALS AND METHODS

Culture Initiation. Pinus taeda cell suspensions were obtained as previously described (16). Hypocotyls from a 2 week old P. taeda seedling were surface sterilized (50% Hilex containing 5.25% NaOCl plus 1% Tween 20 for 20 min) and placed on solid MS medium containing 2,4-diphenoxycetic acid at 2 mg/L (9). After subculturing for 15 weeks at 5-7 weeks intervals, the callus was transferred to liquid L medium containing 2,4-di-phenoxycetic acid (2 mg/L), and incubated with shaking at 23°C. The resultant suspension culture consisted of small friable cell clusters. It was maintained by transferring 5 ml of culture to 50 ml of fresh medium every 14 d. The Pinus radiata cell culture was developed from the excised embryo of an elite seed (Australian plus tree register 50048x 80055) cultured with shaking at 25°C in liquid Schenk and Hildebrandt (13) medium modified by addition of arginine (10 mM), additional ammonium phosphate (2.6 mM), and also containing naphthalene acetic acid (2 mg/L) as sole auxin (15).

Growth Experiments. To obtain nutrient response curves, suspension cultured P. taeda tissue was fragmented by stirring on a 500 μm stainless steel sieve. Cellular colonies less than 500 μm in size were collected on a 100 μm sieve, then washed with 500 ml of water, followed by 50 ml of L medium deficient in the nutrient of interest, and finally suspended in the desired medium at a density of 5 μl packed cell volume per ml. Incubation of 950 μl volume were withdrawn with careful agitation of the medium to ensure uniform cell dispersion and placed in wells of disposable trays (24 well, Costar disposable labware No. 1324) which had been preloaded with 50 μl volumes of 20-fold concentrated stocks of the required nutrient. These cells were grown for (typically) 20 d with shaking at 60 orbits/min in the dark at 25°C. The cells were then filtered harvested and washed on preared glass-

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1 Financial support from APM Forests Pty Ltd is gratefully acknowledged.

2 Abbreviations: L, Litvay medium; MS, Murashige and Skoog medium.
fiber discs (Whatman GF/A 25 mm), and finally weighed to a precision of 0.01 mg on an electrobalance after drying at 65°C for 24 h. All treatments were replicated four times, and the means expressed as a percentage of controls similarly grown in L medium. Error bars were calculated as 95% confidence limits in accordance with standard procedures (12).

This growth procedure was modified for study of Fe Na EDTA responses where, in preliminary experiments, cells were found not to recover from brief exposure to totally deplete media. Cells were suspended in L medium containing 20 μM Fe Na EDTA (L/5). To obtain lower concentrations, 2 ml volumes of Fe Na EDTA deplete media were added to 1 ml of inocula in the wells. The cells were then allowed to sediment naturally, and 2 ml of supernatant carefully removed. Sequential repetition of this in situ dilution procedure extended the final Fe Na EDTA concentration range to desired levels without exposure to yet lower concentrations in the treatment history.

RESULTS

Growth responses generally consisted of a plateau region of good growth bounded by domains of deficiency and toxicity where growth drops dramatically with a relatively small change in nutrient concentration. The limits of adequate growth are arbitrarily defined as the highest and lowest nutrient concentrations for which growth is at least 80% of that obtained in complete medium. Table 1 presents these thresholds together with the corresponding nutrient levels found in MS and L media.

Micronutrient Cations. Lowering Zn (as ZnSO₄) below 10 μM resulted in growth dropping below 50% of the control. Growth increased gently up to 200 μM, but the yields at 100 and 200 μM were not statistically different. Toxicity of Zn was pronounced until a concentration of 2000 μM was reached.

Co and Cu each became toxic as their respective concentrations approached 100 μM. This is in accordance with the observation that iron is precipitated as iron phosphate (4, 14), thereby leaving EDTA available to chelate transition metals. Marked deficiency effects were not seen with omission of either Co or Cu, as recognized from the more detailed study of Cu in Teasdale (14). This underscores the need to employ special procedures to avoid and remove trace contaminants if deficiency conditions are sought. A wide range of Co and Cu additions are seen to be adequate, including the MS levels, but the L Cu level is considered unnecessarily high.

A requirement for both Mn and Fe was evident, but this was more marked in the case of Fe. Toxicity occurred gradually with both metals, with concentrations well in excess of the total [EDTA] (100 μM) tolerated. Since phosphate was always in excess of iron, formation of iron-phosphate precipitates was again recognized as an important determinant of free Fe⁺⁺, a consideration not applicable to Mn.

Micronutrient Anions. Omission of molybdate permits reasonable growth. Optimal growth is found with 0.4 μM molybdate, a concentration below the MS level, and well below the L value of 5 μM which borders on toxicity.

The response to borate was of considerable interest in that the MS level was inadequate and induced culture senescence; the L level (5 X MS) was only marginally adequate, although a further 5-fold increase resulted in toxicity.

An unexpected result of some significance was the clear requirement for iodide. As was the case with borate, the MS level was deficient, resulting in senescence. It is noted that the L medium has a low chloride content (0.3 mM); the iodide requirement seen here may therefore not be evident in high chloride media where sufficient trace iodide may be introduced as a chloride contaminant. The low requirement for iodide relative to the chloride concentration present weighs against the possibility that iodide compensates for a chloride deficiency.

 Macronutrients. Responses to ionic nutrients present in relatively high concentrations are complicated by the unavoidable cointroduction of counterions. This study has not considered the most abundant ions K⁺ (21.5 mM), NO₃⁻ (40 mM), and NH₄⁺ (21 mM), for which the concentrations in L and MS media are very similar, but has focused on the nutrients Mg and sulfate, for which the L concentrations are 5-fold higher than MS, and on Ca for which the concentration of L is only a 20th of MS. Study of phosphate was readily included and is also reported.

To obtain a response to SO₄²⁻, a modified L medium was made wherein Mg and K were lowered to 4 and 13.5 mM, respectively, with concomitant omission of SO₄²⁻. The response to addition of K₂SO₄ was attributed to SO₄²⁻ ions. The data indicated that both MS and L levels of SO₄²⁻ support good growth, but that MS borders on deficiency, whereas L Borders on toxicity.

Similarly, an L medium lacking MgSO₄, but containing SO₄²⁻ as K₂SO₄ (1 mM) allowed response to Mg²⁺ to be obtained through addition of MgSO₄ without serious effects of SO₄²⁻ until its level reached 10 mM. Higher levels of Mg²⁺ were then obtained by addition of MgCl₂. Both MS and L levels of Mg are suboptimal, with benefit continuing up to nearly 15 mM.

Addition of Ca back to the L level was inadequate for recovery of cells exposed to the shock of Ca deplete medium. Higher concentrations, up to the level in MS medium, were sufficient. At still higher Ca levels growth declines, so that the MS level was found close to optimal, and the L level is considered hazardously low.

The phosphate response resulted in a broad plateau. Deficiency occurred below 100 μM, the point where phosphate no longer exceeds iron, which may suggest that iron toxicity is occurring, rather than phosphate deficiency per se.

Formulation of an Improved Medium. Changing one nutrient concentration may, through various chemical interactions such as precipitation or binding phenomena, change the response curves for other nutrients. Metabolic interactions will further compound such effects, so that the deficiency and toxicity thresholds will be moved. A nutrient concentration well removed from

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**Table 1. Nutrient Concentration Ranges Suitable for P. taeda Cell Culture Growth in L Medium Compared with the Mineral Compositions of Selected Media**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Concentration Ranges (μM)</th>
<th>Culture Media Compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supporting Adequate Growth</td>
<td>L</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>10 - 200</td>
<td>150</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>0 - 10</td>
<td>0.5</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>0 - 10</td>
<td>2</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>0 - 500</td>
<td>100</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>10 - 100</td>
<td>1000</td>
</tr>
<tr>
<td>MoO₄²⁻</td>
<td>0 - 5</td>
<td>5</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>500 - 1500</td>
<td>500</td>
</tr>
<tr>
<td>I⁻</td>
<td>25 - 500</td>
<td>25</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>500 - 10000</td>
<td>7500</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>2500 - 15000</td>
<td>7500</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>500 - 7500</td>
<td>1500</td>
</tr>
<tr>
<td>Phosphate</td>
<td>100 - 5000</td>
<td>2500</td>
</tr>
</tbody>
</table>

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stress thresholds is less likely to be affected by changes to other medium components. Moreover, a medium which is clear of nutrient stresses (for one culture) can be expected to exhibit wider genotype specificity.

A medium was constructed taking into account this nutrient response information together with the recognized interactions noted in the text. The macronutrient ions K⁺, NH₄⁺, and NO₃⁻ were set at 20, 15, and 41 mm, concentrations similar to the L level within the constraints of ionic charge balance. The other important components of this medium (designated P6) are specified in Table 1 where they may be compared with the L and MS formulations. Organic components of the medium were as specified in MS medium, but with glycine omitted, and NAA included at 2 mg/L as sole auxin. The P6 medium was tested with a suspension culture of P. radiata cells which had been initiated using the arginine supplemented medium SHR4. This culture line has now been maintained in liquid P6 medium for 42 months with subculturing every 14 d. These cells are uniformly compact and nonvacuolated. Upon cytological examination (8), the culture was found to be dominated by normal diploid cells, although occasional tetraploid cells were also sighted (data not shown). These P. radiata cells grew exponentially with a doubling time of 3.6 d, up to a maximum density of 15.6 mg (dry weight)/ml, followed by culture senescence. This growth of P. radiata cells compares favorably with that of the model P. taeda system, where the maximum growth rate gave a doubling time of 3.8 d (14). Additional experiments have shown that cultures can be initiated into P6 medium using excised embryos from a wide range of unrelated families. Moreover, suspension cultures have been successfully developed from various mature P. radiata tissues, and from immature haploid pollen, in this medium.

**DISCUSSION**

The finding that elevated levels of borate, iodide, Mg, and possibly also Zn were beneficial for growth of loblolly pine cells raises the question of whether these represent novel nutrient requirements of pines, or whether they primarily reflect interactive effects in the system. It is feasible that the high Mg requirement may in some way be compensatory for the low Ca, as may another high borate requirement, as both borate (10) and Ca (2) have been implicated in maintenance of membrane integrity.

The chemical speciation of the transition metal cations is important. Precipitation of Fe by phosphate, combined with interaction with EDTA, lowers free [Fe³⁺] to 10⁻¹⁵ M or less; therefore, other iron species may need to be considered as biologically important. As described in greater detail elsewhere (17), the EDTA liberated by this quantitative Fe precipitation will completely titrate Cu²⁺ and Co³⁺, then partially titrate Zn²⁺ with an equilibrium position being reached. The free levels of Cu²⁺ and Co³⁺ are therefore exceedingly low (<10⁻⁶ M) whereas Mn is essentially all found as the free Mn²⁺ ion. Co and Cu are found to become toxic as their respective total concentrations approach 10 μM, corresponding to the total EDTA present, indicating that the unchelated cations are quite toxic. In contrast, Zn²⁺ and Mn²⁺ may be raised to levels entering the macronutrient range before toxicity is encountered.

Beneficial effects of iodide on plant growth have previously been demonstrated (18) but the requirements are believed to be satisfied with concentrations much less than 10 μM (6), so that contamination levels from other salts, particularly chlorides, are expected to be sufficient for maximum growth. The pronounced requirement for higher levels of iodide evident with this system may therefore, at least in part, result from the reduced chloride content of L medium.

The unexpectedly high borate requirement may also reflect some interactive phenomena rather than an elevated requirement per se. The role of borate has not been fully elucidated, but is known to be necessary for mitosis to proceed in root meristems, with deficiency producing symptoms similar to an auxin excess (11); it is also known to play a role in membrane integrity with uptake of ions such as phosphate, chloride, and rubidium suppressed when boron is inadequate (10).

A variety of different medium formulations may, due to interactive phenomena, arrive at similar effective concentrations of particular nutrients, and consideration of which is most biologically appropriate may be difficult. The formulation of the L medium using chemical composition data for the developing embryo may be seen to have some relevance here, although such total elemental analysis data gives little insight into chemical speciation. Nevertheless, such findings as a high Mg to Ca ratio for all embryonic and seed tissues of immature Douglas-fir (Pseudotsuga menziesii) seeds (7) is highly suggestive that a similar ratio may be appropriate with media for culture of gymnosperm cells and tissues.

The response data presented here indicate that nutrient stresses are in fact readily incurred and it seems probable that most standard media developed for angiosperms, including MS, are inappropriate for pines. The formulation of the P6 medium is considered to be a rational step towards amelioration of this situation, as indicated by its ability to support excellent growth of a different pine species.

Formulation of optimized media suitable for P. radiata cell culture is not the only benefit of these studies. Definition of specific stresses for genetic screening or for physiological and biochemical study requires understanding of interactive phenomena. For example, phosphate addition may, through iron precipitation and consequent EDTA liberation, lead to deficiency of a transition metal rather than phosphate toxicity per se. Similarly, trace metal deficiency stresses can be made more accessible by removal of other growth limiting factors, and through a judicious balance of components so as to minimize sources of contamination, optimize its chelation, and increase competitive inhibitions. Homogeneous and apparently meristematic cell suspension cultures of pines have proved highly suited for precise growth studies of cellular nutrition. They are also eminently well suited to many biochemical and cellular studies, particularly those associated with modern in vitro genetic improvement procedures which offer considerable promise for forest species of such long generation times.

It is perhaps understandable that, in the absence of suitable media, little exploration has been made of the promise of these new procedures in the forestry sector. The recalcitrant reputation of conifers with regard to regeneration from disorganized tissues has no doubt dampened research effort in this direction. However, it is entirely reasonable that the use of standard media that apparently is nutritionally stressful can account for much of the lack of success encountered. It is hoped, therefore, that the findings presented here will further the application of in vitro methods in forestry, particularly relating to nutrient stress adaptation in P. radiata.

**Acknowledgments—**The bulk of this work was carried out while the author was a visitor at the John Innes Institute (Norwich, U.K.). Appreciation is expressed for provision of research facilities. Suspension cultures of Pinus taeda were gratefully received from the Institute of Paper Chemistry (Appleton, WI). Dr. G. Creissen is thanked for making chromosome counts.

**LITERATURE CITED**


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