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Influence of a loblolly pine (*Pinus taeda* L.). Culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.)

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ABSTRACT

A new culture medium, originally designed and shown to grow cell suspensions from a variety of loblolly pine (Pinus taeda L.) explants, was used to study growth and somatic embryogenesis of the wild carrot (Daucus carota L.) in cell suspensions. The new loblolly pine medium (LM) differed from the standard wild carrot medium (WCM) in having very low Ca^{2+}, very high Mg^{2+}, and enrichment with PO_4^{3-} and microelements. When WCM was altered to contain levels of Ca^{2+} or Ca^{2+} and Mg^{2+} equivalent to LM, it supported neither growth nor embryogenesis of the wild carrot. However, growth and embryogenesis in LM was superior to WCM. The phosphate level in WCM was found to be suboptimal.

INTRODUCTION

In this laboratory, a new culture medium for conifers was developed, based in part on the chemical composition of the developing ovule prior to fertilization (Litvay et al. 1981). This medium, herein referred to as LM, has demonstrated its value in obtaining and maintaining cell suspensions from a variety of explants of loblolly pine (Verma et al. 1982) and it has been used or cited by several other workers (e.g., Biondi and Thorpe 1982, Bornman 1983, Rugini 1984, Thorpe and Biondi 1984, George and Sherrington 1984). Although the quality of cell suspensions so generated is far better than at any time before, at no time have we been successful in inducing organogenesis or somatic embryogenesis in any of these suspension cultures. This raised an important question, i.e., does LM per se or the proportions of any of its characteristic constituents foil processes related to embryogenic capacity of cell suspensions?

We addressed the above question somewhat indirectly. An embryogenic cell suspension culture of wild carrot was chosen as an experimental model system for this study. It was considered that if any component(s) of the LM proved deleterious to growth and/or embryogenesis in wild carrot, such information could lead to further refinement in the culture medium for loblolly pine.

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MATERIALS AND METHODS

An embryogenic cell line, WC-8, of wild carrot (Daucus carota L.), derived originally from a petiole explant, was a gift from Don F. Wetherell of the University of Connecticut, Storrs. The WCM composition used to grow wild carrot has been described by Wetherell (1969), whereas the LM composition originally derived by Litvay et al. (1981), appears in Table 1. All media were adjusted to pH 5.8 prior to autoclaving. Growth curve studies were begun by subculturing (1 to 20) wild carrot cell suspensions from WCM into the various media treatments of Table 3 for a number of biweekly passages (Erlenmeyer flasks) in the presence of 0.5 mg/L of 2,4-D as the growth regulator. Following this, data for growth curves were obtained by inoculating (1 to 20) into 10 mL of the respective medium in roller drum tubes (20 x 195 mm). At intervals, triplicate tubes were filter-harvested for determination of tissue weights after oven-drying at 60°C overnight. The effectiveness of various media in supporting embryogenesis was determined in triplicate by standard procedures (Brown et al. 1976; Verma and Dougall 1978). A screened (63–125 µm) and washed cell population at 0.5 µL packed-cell volume per mL of culture medium was used in screw-cap tubes (16 x 150 mm), each containing 2 mL culture. All cultures grew in darkness at 23°C on roller drums at 30 rpm.

Table 1. Composition of the loblolly pine medium (LM)

	Compound	mg/L		Compound	mg/L
a.	NH_4NO_3	1650	c.	$FeSO_4 \cdot 7H_2O$	27.80
	KNO_3	1900		$Na_2EDTA \cdot 2H_2O$	37.30
	$MgSO_4 \cdot 7H_2O$	1850	d.	Sucrose	30,000
	KH_2PO_4	340	e.	myo-inositol	100
	$CaCl_2 \cdot 2H_2O$	22		Nicotinic acid	0.5
				Pyridoxine HCl	0.1
b.	H_3BO_3	31			
	$MnSO_4 \cdot H_2O$	21		Thiamine HCl	0.1
	$ZnSO_4 \cdot 7H_2O$	43	f.	2,4-Dichlorophen-	0.5
	$Na_2MoO_4 \cdot 2H_2O$	1.25		oxyacetic acid	
	$CuSO_4 \cdot 5H_2O$	0.50			
	$CoCl_2 \cdot 6H_2O$	0.125			
	KI	4.15			

RESULTS AND DISCUSSION

WCM and LM Compositions

The standard WCM and LM are compared in Table 2. Perhaps the most notable difference is a 10-fold reduction in the Ca^{2+} level and a corresponding increase in the Mg^{2+} level in LM compared with WCM. Another important feature of LM is that it is enriched in P and micronutrients. Sucrose and nitrogen supplements are also higher in LM than in WCM. The reader may refer to the last column of Table 2 for a comparison of the various media components.

Growth

This investigation was carried out in the presence of 2,4-D, a growth regulator widely implicated in the generation and maintenance of embryogenically competent cell lines in wild carrot and many other species (Wetherell 1978; Sharp et al. 1980). Table 3 gives the experimental design. Treatments 2 through 12 represent changes where concentrations of only specific components of the WCM were altered to match LM levels. Treatments 1 and 13 denote WCM and LM, respectively.

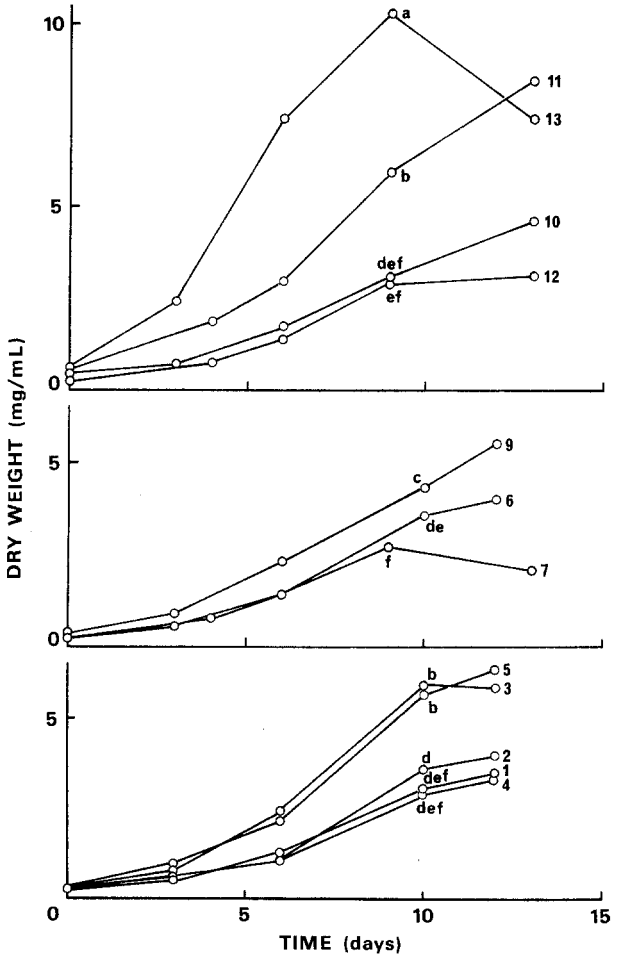

Fig. 1. Growth curves of wild carrot cell suspensions in the various media formulations of Table 3. The numbers on the curves correspond to the treatment numbers of Table 3. ANOVA and Duncan's Multiple Range Tests (Snedecor and Cochran 1967) were run for treatment differences at each time point, but only the results for day 9/10 are shown; treatment yield means for day 9/10 with a common letter were not significantly different at the 95% confidence level.

Table 2. Comparison of WCM and LM[a]

Component	in LM, mM	in WC, mM	Ratio $\frac{LM}{WCM}$
Sucrose	87.6	58.4	1.5
Nitrogen	60.0	49.6	1.2
P	2.50	0.50	5.0
K	21.3	40.0	0.5
Ca	0.15	1.50	0.1
Mg	7.5	0.75	10.0
Fe	0.1	0.05	2.0
	µM	µM	
Mn	124	41	3.0
Zn	150	14	10.7
B	500	39	12.8
Mo	5.17	0.056	92.3
I	25	2.3	10.9
Cu	2.0	0.06	33.3

[a]With respect to such factors as vitamins, WCM contains 3.0 mg/L thiamine·HCl only, while LM contains (in mg/L) 0.1 thiamine·HCl, 100 myo-inositol, 0.5 nicotinic acid and 0.1 pyridoxine·HCl. Both are adjusted to pH 5.8.

Table 3. Influence of LM components on wild carrot embryogenesis

Treatment No.[a]	Δ In WCM To Equal LM[b]	Growth (as Embryos) at 21 days mg Dry wt/2 mL[c]
1	None (WCM)	10.7^{e}
2	Sucrose (Su↑)	10.6^{e}
3	$KH_2PO_4(PO_4^{3-}\uparrow)$	18.7^{d}
4	NO_3^- (↑)	10.7^{e}
5	Su, PO_4^{3-}, NO_3^- (↑)	25.2^{c}
6	Mg^{2+} (↑)	6.5^{f}
7	Ca^{2+} (↓)	0.4^{i}
8	Mg^{2+} (↑) Ca^{2+} (↓)	4.4^{g}
9	Δ5 + Δ8	25.2^{c}
10[d]	Micro, Fe, Vit Inos	8.2^{f}
11	Δ5 + Δ10	31.0^{a}
12	Δ8 + Δ10	2.4^{h}
13	LM	29.0^{d}

[a]Wild carrot suspensions were subcultured for two biweekly passages in the various media in the presence of 2,4-D prior to embryogenesis in auxin-free media.
[b]Δ = change; ↑ = increase; ↓ = decrease.
[c]ANOVA and Duncan's Multiple Range Test (Snedecor and Cochran 1967) were run for differences in embryogenic growth means among treatments; values with a common letter superscript were not significantly different at the 95% confidence level.
[d]Micronutrients, iron, vitamins, and myo-inositol levels equivalent to LM.

A wild carrot cell suspension line growing totipotently in WCM was subcultured (1:20) at least 5 times in each Table 3 medium, prior to construction of a growth

curve. However, treatments 7 and 12 underwent only two subcultures, beyond which they could not be maintained. No growth curve could be generated with treatment 8, since the cells did not maintain adequate growth even beyond the first passage. Clearly, modification of WCM so that it has the same level of Ca^{2+} or Ca^{2+} + Mg^{2+} as LM rendered WCM unfit for growing wild carrot suspensions. Cells could, however, be maintained in WCM modified to include the LM level of Mg (see treatment No. 6, Fig. 1), and the growth pattern was similar to the WCM control.

Increasing sucrose or nitrogen alone did not improve WCM. However, increasing PO_4^{-3} alone was definitely advantageous, and simultaneously increasing sucrose, nitrogen, and PO_4^{-3} was even better (see Fig. 1).

The maximum and the fastest growth occurred in the standard LM where, in eight days, three times as much dry weight was produced as in WCM (see treatments 13 and 1, Fig. 1). This is intriguing, since Ca^{2+} and Mg^{2+} levels in LM are the same as in treatments 8 and 12, but these levels in LM were compatible with growth of wild carrot, due presumably to the presence of counteracting factors in LM. The fact that treatment 9 was better than 8 and 12 suggests some involvement of sucrose, KH_2PO_4, and nitrogen supplements.

Embryogenesis

The same cell line was used in this part of the investigation except that embryo development was allowed to proceed as usual in the absence of 2,4-D. The data on embryo growth appear in Table 3. Various treatments bear a direct correspondence between their ability to support proliferative growth on one hand and embryogenesis on the other. Clearly, the worst embryogenesis media are those that were judged unsatisfactory for growth (No. 7, 8, and 12). LM supports three times as much embryo growth as the WCM control. Although embryos were not counted but only weighed in the results presented in Table 3, in other experiments increases in embryo dry weight in LM over WCM have been paralleled by similar increases in embryo counts. For other apparently desirable intermediate combinations such as treatments 3, 5, 9, and 11, correlations between embryo weights and embryo counts are not firmly established except for treatment 3.

A photographic record of embryos developed in a few selected treatments appears in Fig. 2. The presented data were, however, derived from an independent experiment. They not only show the compatibility of LM for wild carrot embryogenesis but also LM's superiority over the standard WCM in embryo number yield as well as mass yield.

Note that the high levels of trace elements in LM are not accompanied by chelating agents. However, to the extent that micronutrients were sorted out (treatments 10, 11, and 12), they appear to have little positive influence on growth or embryogenesis until sucrose, phosphate, and nitrate are also elevated. The major single deficiency of WCM relative to LM for growth and embryogenesis of wild carrot *per se* would appear to be phosphate.

The findings reported herein support the view that a medium (LM) originally designed for conifers has exceeded the performance of a commonly used wild carrot medium (WCM) when applied to wild carrot suspensions. It is to be noted that the wild carrot medium in question has proven its usefulness in a number of nutritional and metabolic studies (Brown *et al.* 1976; Wetherell and Dougall 1976; Verma and Dougall 1977). We recommend that LM be tried with other species. As regards proliferative growth of conifers, LM has performed far better than WCM or the well known Murashige and Skoog medium (1962) (authors, unpublished). Fast growing cell suspensions from both Douglas-fir and loblolly pine have been generated and maintained for prolonged periods of time (> 2 years for pine) using LM.

CONCLUSIONS

In summary, for both growth and embryogenesis of wild carrot, LM outperformed WCM. One should note, however, that if quantities of certain components of LM (e.g., Ca^{2+}, Mg^{2+}) are copied singly in WCM, the

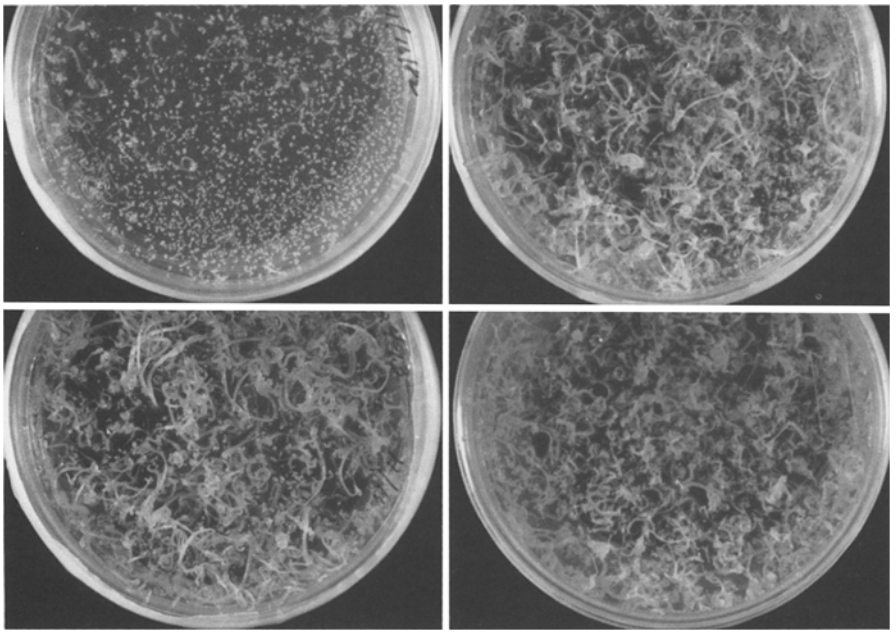

Figure 2. Development of wild carrot somatic embryos in treatments 1, 3, 9, and 13 (clockwise from top left) of Table 3. In this experiment, subsequent to preculturing, wild carrot cells were plated in various media solidified with Gelrite (0.2% w/v).

ability of the latter to support growth and embryogenesis can be severely limited. This finding will be kept in mind as our media development efforts continue toward generation of totipotent cell lines in conifers.

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