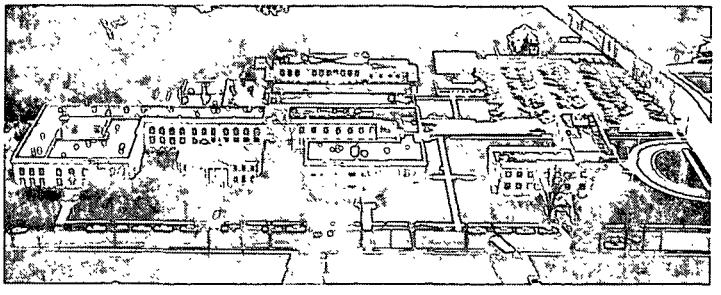

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

IPC TECHNICAL PAPER SERIES

NUMBER 342

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JUNE, 1989

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This manuscript is based on results obtained in IPC research and is to be presented at the Southern Forest Tree Improvement Conference in June, 1989

FACTORS REGULATING LOBLOLLY PINE (Pinus taeda L.) SOMATIC EMBRYO DEVELOPMENT

M.R. Becwar[1] and R.P. Feirer[2]

Abstract. The effect of medium components and culture conditions on somatic embryo development and the level of storage lipids (triglycerides) was studied in embryogenic callus of loblolly pine. A factorial experimental design was employed to test the effects of three factors on somatic embryo development: 1) basal medium, DCR (Gupta and Durzan, 1985) and MSG (Becwar et al., 1988); 2) culture condition, light versus dark; and 3) level of ABA. After 28 days on the treatments embryogenic calli were collected for somatic embryo counting and determination of triglyceride levels. Basal medium had a significant effect on the number of somatic embryos per gram of callus. Similarly, the level of ABA significantly affected somatic embryo development. Averaged across light and dark treatments, MSG medium with 30 µM ABA produced about 600 total somatic embryos per gram of callus and 15% of these reached stage 2 of embryo development (Hakman and von Arnold, 1988). Basal medium and ABA also influenced the level of triglycerides. The highest level of triglycerides accumulation, about 15 µg/mg protein, occurred on the MSG medium containing 10 µM ABA. Light versus dark culture conditions did not significantly affect the measured parameters. These results suggest that the beneficial effect of ABA on somatic embryo development in loblolly pine may in part be related to ABA-enhanced accumulation of lipids. This ABA effect is strongly influenced by the basal medium on which the cultures are grown.

Keywords: Pinus taeda L., somatic embryogenesis, abscisic acid, triglycerides

INTRODUCTION

Although embryogenic callus of loblolly pine can be initiated reproducibly, development of the resulting immature somatic embryos remains difficult. Treatment with abscisic acid (ABA) has recently been reported to enhance development of somatic embryos of Norway spruce and white spruce (von Arnold and Hakman, 1988; Dunstan et al., 1988). ABA has also been shown to induce the accumulation of storage lipids in somatic embryos of Norway spruce (Feirer, et al., in press). Preliminary experiments in our laboratory have demonstrated the positive effects of ABA on the development of loblolly pine somatic embryos (Becwar et al., in press). These studies suggested that the transfer of embryogenic calli to MSG basal medium (Becwar et al., in press), sucrose levels of 3 to 6%, and 10 µM ABA promoted embryo development.

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The objectives of the study described here were to optimize conditions for maturation of somatic LP embryos. The experimental design employed in this study afforded the opportunity to obtain quantitative data and statistical evidence for the effect of several factors on somatic embryo development and levels of storage lipids. Variables tested included basal medium, ABA treatments and the presence or absence of light.

MATERIALS AND METHODS

Embryogenic callus of loblolly pine (Pinus taeda L.) was initiated from an immature embryo explant collected August 3, 1987 (clone #7-34; see Becwar et al., in press). The callus was initiated and maintained on DCR basal medium (Gupta and Durzan, 1985) containing 2,4-D and 6-benzylaminopurine (3 mg/l and 0.5 mg/l, respectively), hereafter referred to as DCR 3/0.5 medium. The embryogenic callus was maintained in the dark at 23 °C and subcultured every two weeks.

The factorial design employed tested the effects of three factors on somatic embryo development: 1) basal media (DCR and MSG, both containing 3% sucrose). MSG medium was formulated/described by Becwar et al.(in press). 2) levels of ABA (0, 1, 10, 30 μM). and 3) culture conditions (light and dark). Light intensity was 80 μEm^{-2} sec^{-1} from cool-white fluorescent and 20 μEm^{-2} sec^{-1} from incandescent bulbs. Three embryogenic calli (100 mg each) were transferred to each culture plate which contained 10 ml of one of the above 16 treatments. Each treatment was replicated 4 times (4 plates). After 28 days of growth on these developmental media, embryogenic calli were collected for somatic embryo counting and determination of triglyceride levels.

To determine the number of somatic embryos per callus, a modification of the technique described by Becwar et al. (1987) was used. Each piece of callus was weighed and added to 1 ml of water in a 10 cm test tube, which was stoppered and vigorously agitated for 10 sec. One ml of 1.2 % low melting point agarose containing 1 % Merthiolate, which had been tempered to 40 °C, was added to the dispersed callus in the test-tube. After mixing, the 2 ml suspension was poured onto a 10 ml base layer of 0.8% agarose in 100 mm plastic petri plates. After cooling, the somatic embryos were counted using a dissecting microscope at 15X magnification. The plates were placed on a background grid to facilitate counting. Both the total number of somatic embryos and the number of "large" well-formed embryos having dense, smooth-surfaced embryonal heads (stage two according to the description of Hakman and von Arnold, 1988) were counted.

Triglycerides were quantified by biochemical analysis. A method used to determine triglyceride levels in human serum, commercially available in kit form, was adapted for use with plant tissues (Feirer et al., 1989).

Data were analyzed by factorial ANOVA, this approach being useful for identifying the variables within a complex, multifactorial experiment which significantly affect culture response.

RESULTS AND DISCUSSION

The effects of the media components and light on the production of loblolly pine somatic embryos appear in Figure 1. To illustrate statistical results, abbreviated ANOVA tables are also included (Table1). Both the basal medium and ABA treatments significantly affected total embryo production in the calli. MSG medium led to greater embryo production than DCR. The enhancement of embryo production by ABA was also evident in both basal media, with optimal ABA concentrations being 10 to 30 μM, perhaps higher. This is within the range previously reported to augment somatic embryo formation in Norway spruce

Figure 1. Effect of ABA, basal medium and light on somatic embryo production in loblolly pine. The total number of somatic embryos per gram of callus was determined.

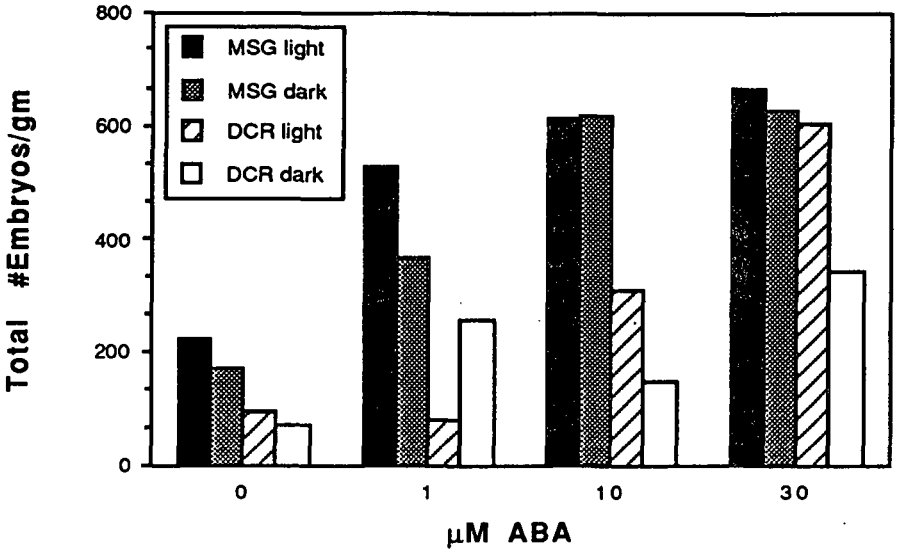

Table 1. Analysis of variance of the effect of media, light and ABA on total production of loblolly pine somatic embryos.

SOURCE	DF	MS	F	Prob.	
Treatment	15	192296	6.57	< 0.05	*
Media	1	891542	30.49	0.000	*
Light	1	65712	2.25	0.140	ns
ABA	3	507073	17.34	0.000	*
media x light	1	179	0.01	0.938	ns
media x ABA	3	57638	1.97	0.131	ns
light x ABA	3	17218	0.59	0.626	ns
media x light x ABA	3	60411	2.07	0.117	ns
Error	47	29236			
Total	62				

* = significant
ns = nonsignificant

and white spruce (von Arnold and Hakman, 1988; Dunstan et al., 1988). The effect of light (presence or complete absence) on embryo production was not significant (Table 1).

While a simple count of all somatic embryos within a callus is representative of embryo initiation and early development, it may not be relevant to or accurately reflect the degree of development or maturation of the somatic embryos. In order to better determine the effects of culture parameters specifically on somatic embryo development, a subpopulation of the embryos was counted. "Large", well formed, smooth surfaced embryos were counted to determine the production of further developed somatic embryos by the calli. In this case, the effects of basal medium were more pronounced (Figure 2, Table 2). Of the parameters tested, basal medium had the greatest effect on embryo development. MSG medium was clearly superior to the DCR formulation used. The effect of ABA on embryo development was evident, especially when used in conjunction with MSG basal medium (Figure 2). It again appeared that the optimal ABA concentration was 30 μM or higher. Obviously, future experiments must use concentrations above 30 μM in order to ensure that the optimal ABA response is being attained. ABA additions to DCR medium did not enhance development. As was found for total embryo production (Figure 1), light also failed to influence somatic embryo development (Figure 2).

ABA has been shown to enhance the development of somatic embryos of a number of plant species. The maturation of angiosperm somatic embryos to more advanced stages of development is promoted by ABA, and more importantly, the treated embryos appear to have a more normal morphology (Ammirato, 1974, 1983). The enhancement of conifer somatic embryo development by ABA has been reported in a number of recent publications. Development of both Norway spruce and white spruce somatic embryos was improved by treatment of cultures with ABA (Becwar et al., 1987; Dunstan, et al., 1988; Feirer et al., in press; von Arnold and Hakman, 1988). Our results show that ABA similarly improves the development of loblolly pine somatic embryos. It appears, then, that future studies on the promotion of loblolly pine somatic embryo development should include ABA in the MSG basal medium as a starting point or a baseline against which to judge and test the effects of other variables. At this point, the effect of light appears to be insignificant, but experiments having photoperiods and light quality as variables cannot be dismissed and should be considered.

The effects of culture variables on triglycerides, a biochemical parameter theorized to be important to the normal development of conifer embryos (Feirer et al., 1989), were also determined. Once again, basal media and ABA significantly influenced the response. Tissues grown on MSG contained significantly higher levels of triglycerides than those grown on DCR (Figure 3, Table 3). Triglyceride accumulation was affected by ABA treatments, optimal triglyceride accumulation being at (or near)10 μM ABA. Light had no effect on triglyceride levels in these tissues.

In their report describing the enhancement of morphological development of Norway spruce somatic embryos by ABA, von Arnold and Hakman (1988) noted that intracellular lipids were also affected. Using a histochemical stain specific for lipids, more of these reserve compounds were observed in the ABA-treated tissues. This finding complements and corroborates our biochemical results on the enhancement of triglyceride accumulation by ABA. Clearly, then, ABA promotes the accumulation of reserve lipids. These findings are consistent with the ability of ABA to suppress precocious germination, allowing the formation of embryo-specific storage proteins (Finkelstein et al., 1985; Quatrano, 1986). Low levels of storage or reserve compounds may be characteristic of somatic embryos of many plant species. In addition to

Figure 2. Effect of ABA, basal medium and light on somatic embryo maturation in loblolly pine. The number of "large" well formed somatic embryos having smooth, dense embryonal heads per gram of callus was determined.

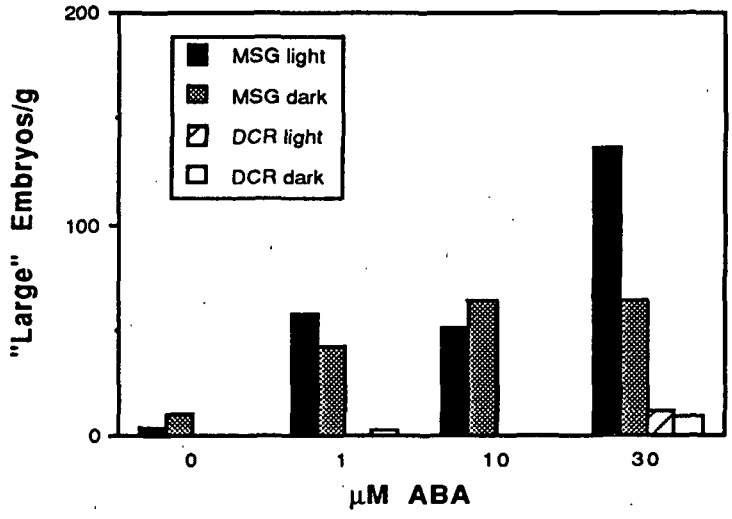

Table 2. Analysis of variance of the effect of media, light and ABA on production of "large" loblolly pine somatic embryos.

SOURCE	DF	MS	F	Prob.	
Treatment	15	5861	2.07	< 0.05	*
Media	1	40054	14.17	0.000	*
Light	1	1220	0.43	0.514	ns
ABA	3	7244	2.56	0.066	
media x light	1	1255	0.44	0.508	ns
media x ABA	3	4745	1.68	0.184	ns
light x ABA	3	1638	0.58	0.631	ns
media x light x ABA	3	1501	0.53	0.663	ns
Error	47	2826			
Total	62				

* = significant
ns = nonsignificant

Figure 3. Effect of ABA, basal medium and light on triglyceride levels in cultured loblolly pine tissues.

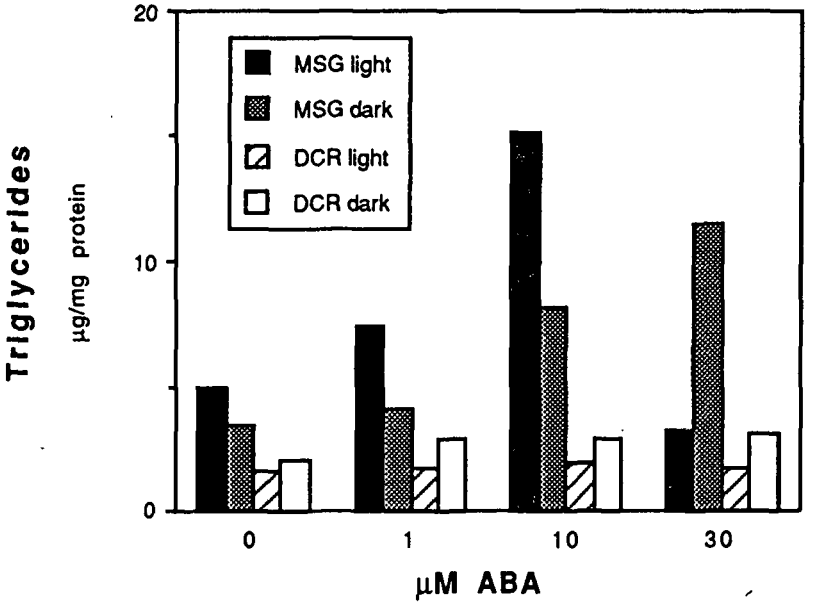

Table 3. Analysis of variance of the effect of media, light and ABA on loblolly pine triglycerides

SOURCE	DF	MS	F	Prob.	
Treatment	15	61.87	7.66	.0001	*
Media	1	401.40	49.67	.0001	*
Light	1	0.07	.009	0.927	ns
ABA	3	48.01	5.94	0.002	*
media x light	1	14.41	1.78	0.188	ns
media x ABA	3	36.92	4.57	0.007	*
light x ABA	3	45.88	5.68	0.002	*
med. x L x ABA	3	39.95	4.94	0.005	*
Error	48	8.081			
Total	63				

* = significant
ns = nonsignificant

the low levels of reserve lipids (triglycerides) in cultured conifer tissues, somatic embryos of rapeseed and cotton have been reported to accumulate storage proteins at reduced levels (Crouch, 1982; Shoemaker et al., 1987). This failure to accumulate adequate storage reserves may hinder continued development and germination of somatic embryos. The beneficial effect of ABA on somatic embryo development may then be related to the ABA-enhanced accumulation of storage compounds.

Since a good correlation exists between triglyceride content and somatic embryo development (compare Figures 2 and 3), studies attempting to further enhance the triglyceride content will continue. The role of ABA in this process will continue to be explored, along with other means to improve triglyceride biosynthesis and accumulation.

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