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POLYETHYLENE GLYCOL AND MALTOSE ENHANCE SOMATIC EMBRYO MATURATION IN LOBLOLLY PINE (*PINUS TAEDA* L.)

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SUMMARY

A culture medium that can efficiently produce mature somatic embryos was developed for loblolly pine (*Pinus taeda* L.). The medium contained maltose as a carbohydrate source and polyethylene glycol as an osmoticum. This medium formulation significantly enhanced embryo maturation efficiency compared to a medium with only maltose, or with sucrose combined with polyethylene glycol. Maltose at 4% and polyethylene glycol at 6% resulted in the highest embryo maturation efficiency; an average of around 100 cotyledonary embryos were produced from 1 g of embryogenic tissue. These results suggested that previous ineffective embryo maturation in loblolly pine may be due to the lack of the proper combination of osmoticum and carbohydrate source. This embryo maturation method also improved morphology of cotyledonary embryos of loblolly pine.

Key words: carbohydrate; conifers; cotyledonary embryos; osmoticum; somatic embryogenesis; tissue culture.

INTRODUCTION

Loblolly pine (*Pinus taeda* L.) is an economically important coniferous species in the southeastern United States (Gupta and Durzan, 1991). Somatic embryogenesis has promising potential to produce genetically uniform plant stocks of loblolly pine on a large scale. Although this technique has been very successful with some other coniferous species, especially in *Picea*, most *Pinus* species, including loblolly pine, showed high recalcitrance to somatic embryogenesis and progress has been very slow (Tautorus et al., 1991). Difficulty in inducing embryo maturation is one of the main barriers for using this technique commercially in loblolly pine. However, as soon as a protocol of somatic embryogenesis is successfully developed, it will not only have high commercial value but also interface with genetic engineering (Becwar et al., 1995).

To improve embryo maturation efficiency in loblolly pine, a high concentration of maltose has been used to replace sucrose in maturation media, and maltose at 6% was proven optimum (Uddin, 1993; Becwar et al., 1995). Plant tissue culture media generally include 2–3% carbohydrate. The high concentration of maltose suggests that maltose may have served as both carbohydrate source and osmoticum, but exactly how maltose affected somatic embryogenesis has not been determined. Our previous study demonstrated that polyethylene glycol (PEG) enhanced somatic embryo maturation by increasing medium osmolarity, although the development of some stage 2 embryos was arrested on the medium containing PEG and sucrose (Li et al., 1997). The objectives of this study were to investigate whether PEG and maltose act synergistically to enhance somatic embryo maturation in loblolly pine and to determine the best combination of PEG and maltose.

MATERIALS AND METHODS

Plant materials. Two embryogenic cell lines, H_{10} and H_{20}, initiated from an Arkansas source of loblolly pine, were used in this study (Li and Huang, 1996). The embryogenic cultures were initiated in 1993 and have been maintained and proliferated on BM_3 medium (prepared just before using) in darkness at 21–22° C and subcultured biweekly (Gupta and Pullman, 1991) since then. Embryogenic ability of the two cell lines has been evident on a maturation medium containing PEG and sucrose (Li et al., 1997).

Maturation medium. As a maturation medium, the modified basal medium (Li et al., 1997) was supplemented with 40 mg/l (151.3 μM) abscisic acid (ABA), 10 m*M* KCl, 1.5 g/l charcoal (KC Biological, Lenexa, KS), 0–6% PEG (MW 3350), 0–6% maltose, 0–3% sucrose, and 8 g/l agar (Sigma Chemical Co., St. Louis, MO). The pH was adjusted to 5.8 after the charcoal was added but before the addition of agar. The media were then autoclaved at 121° C and 15 psi for 15 min. L-glutamine and freshly prepared ABA solutions (dissolved by 1 *N* NaOH) were filter-sterilized and added to the culture media after being cooled to about 50° C. After about 20–25 ml culture medium was poured into petri dishes, the medium was solidified and dried in a Bioflow chamber without the lid for 3 h. Thus, condensation on the dish lids and walls was prevented. Because ABA is sensitive to light, the Bioflow chamber and room were kept dark during the solidification process.

Culture condition. About 200 mg (fresh weight) embryogenic tissue, which was white to translucent, was excised from the surface of the proliferating embryogenic tissue and cultured in a petri dish with maturation media in darkness at 22° C. Each petri plate contained two pieces of embryogenic tissue from each cell line. The embryogenic tissues had to be subcultured on the same maturation medium every 2 wk. Each petri dish was considered a replication and each treatment included a minimum of four dishes.

Experiments and statistical analysis. In experiment 1, the effect of combinations of PEG, maltose, and sucrose on embryo maturation was determined. Among sucrose, maltose, and PEG, the maturation medium containing only sucrose was used as control. In experiment 2, we determined the best combination of PEG and maltose for embryo maturation. In experiment 3, the

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TABLE 1

SOMATIC EMBRYO MATURATION OF LOBLOLLY PINE (*PINUS TAEDA* L.) ON MEDIA CONTAINING POLYETHYLENE GLYCOL (PEG), MALTOSE, AND SUCROSE

Treatment[1]			
Maltose (%)	PEG (%)	Sucrose (%)	Embryo maturation frequency[2,3] (No.)
0	0	3	0.9 c
0	3	3	8.8 c
0	6	3	8.1 c
2	5	1	28.1 bc
3	4	0	51.0 b
4	3	0	95.3 a
6	0	0	10.6 c

[1]All treatments contain 40 mg/l (151.3 μ*M*) abscisic acid.

[2]Means followed by same letter are not significantly different at $P < 0.05$.

[3]Number of cotyledonary embryos per gram (initial fresh weight) of embryogenic tissue at the proliferation state.

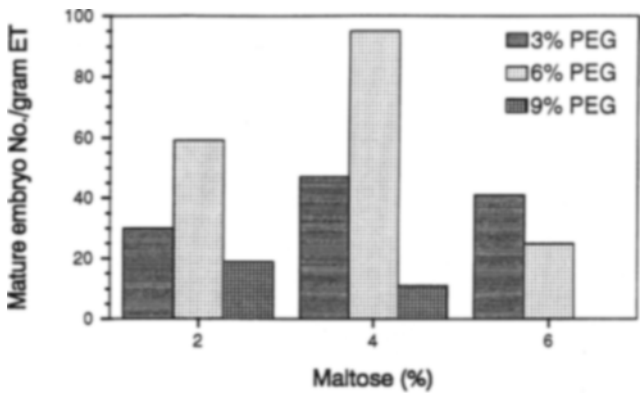

FIG. 1. Effect of the concentrations of maltose and polyethylene glycol (PEG) on the somatic embryo maturation efficiency in loblolly pine (*Pinus taeda* L.). ET = embryogenic tissue at proliferation stage.

optimized combination was then confirmed with a larger number of embryogenic tissues. In each experiment, the maturation frequency (i.e., the number of cotyledonary embryos produced from 1 g of embryogenic tissue at the proliferation stage) was determined at 15–16 wk. Cotyledonary stage embryos are embryos with an elongated embryonic axis and distinct cotyledons.

Experiments 1 and 2 were considered as completely randomized one-factor and two-factor factorial design, respectively. The means of maturation frequency in both experiments were subject to analysis of variance (ANOVA) and were separated by a protected Least Significant Difference (LSD) at $P < 0.05$. Because there were no embryos produced on the medium containing 6% maltose and 9% PEG in experiment 2, this data was not included in the statistical analysis in order to allow a more reliable statistical interpretation. The data from experiment 3 were subjected to a two-sample *t*-test to compare means. Based on results of a preliminary *F* test for equality of variance, degrees of freedom for this *t*-test were adjusted with Satterthwaite's approximation (Steel and Torri, 1980). The significance level was set at $P < 0.05$.

RESULTS

Initial formation of cotyledonary embryos was observed at 6–8 wk on most of the maturation media. Embryogenic cultures on maturation media then showed various stages of embryos. Somatic embryo maturation of loblolly pine was a slow process and might be continuous up to 20 wk before embryogenic tissues declined.

In experiment 1, significantly improved embryo maturation occurred on media containing both maltose and PEG (Table 1). With sucrose alone, the embryo maturation efficiency was less than one embryo produced from 1 g of embryogenic tissue. About a 10-fold enhancement was achieved by either using maltose to replace sucrose or adding PEG to the medium containing sucrose. The highest embryo maturation frequency resulted from the medium containing 4% maltose and 3% PEG. Significantly higher maturation frequency also resulted from the medium with 3% maltose and 4% PEG. However, the maturation frequency was decreased when sucrose was added into the medium with maltose and PEG. Maltose and PEG seemed to act synergistically to promote somatic embryo maturation, while sucrose was deleterious.

The optimal combination of maltose and PEG was then determined in experiment 2. PEG at 6% and maltose at 4% resulted in the highest embryo maturation frequency (Fig. 1). Statistically, no interaction existed between PEG and maltose, and only the amount of PEG significantly affected the embryo maturation frequency. PEG at 3 and 6% achieved significantly higher maturation frequency than PEG at 9%. There was no statistical difference between PEG at 3 and 6%. Maltose at 2, 4, and 6% did not result in statistical differences in terms of embryo maturation frequency.

Although 6% PEG and 4% maltose showed the highest embryo maturation efficiency in experiment 2 (Fig. 1), this efficiency was almost identical to the result achieved by 3% PEG and 4% maltose in experiment 1 (Table 1). So both combinations were used in a larger scale experiment (64 pieces of tissues in 16 petri dishes for each treatment). The result from experiment 3 confirmed that efficient embryo maturation in loblolly pine can be achieved with the presence of PEG and maltose (Fig. 2 *A* and *B*). Statistical difference was found between 6 and 3% PEG (109 vs. 60 cotyledonary embryos per gram of embryogenic tissue, $P = 0.005$). This improved maturation medium achieved embryo maturation more efficiently and consistently than the previously used medium containing PEG and sucrose (Li et al., 1997).

Using maltose as the carbohydrate source, the conversion from immature to cotyledonary embryos was also rapid, and the transition stages could be overlooked if not very closely monitored. In contrast, embryos had very clear developmental stages on the medium in which sucrose alone served as the carbohydrate source, from a very small dense embryo head, then slow elongation forming a bullet-shaped embryo region, followed by forming a flat head, and finally crown-shaped cotyledonary region and further elongation of cotyledons. Morphology of cotyledonary embryos induced on the medium with sucrose or maltose was also different from each other. The cotyledonary embryos induced by sucrose were short and had a swollen hypocotyl region (Li et al., 1997). In contrast, the typical embryos induced by maltose were longer and had a better defined root cap (Fig. 2 *C* and *D*).

With maltose replacing sucrose as a carbohydrate source, the development of somatic embryos and suspensors was also altered. Maltose seemed to inhibit the elongation or growth of the suspensor compared to sucrose (Fig. 2 *A* and *E*), although the degree of inhibition varied somewhat from tissue to tissue or time to time. We directly observed that maltose was inhibitory for the growth of suspensor when the embryogenic tissue with extensive suspensor growth on the medium containing sucrose was transferred to the medium

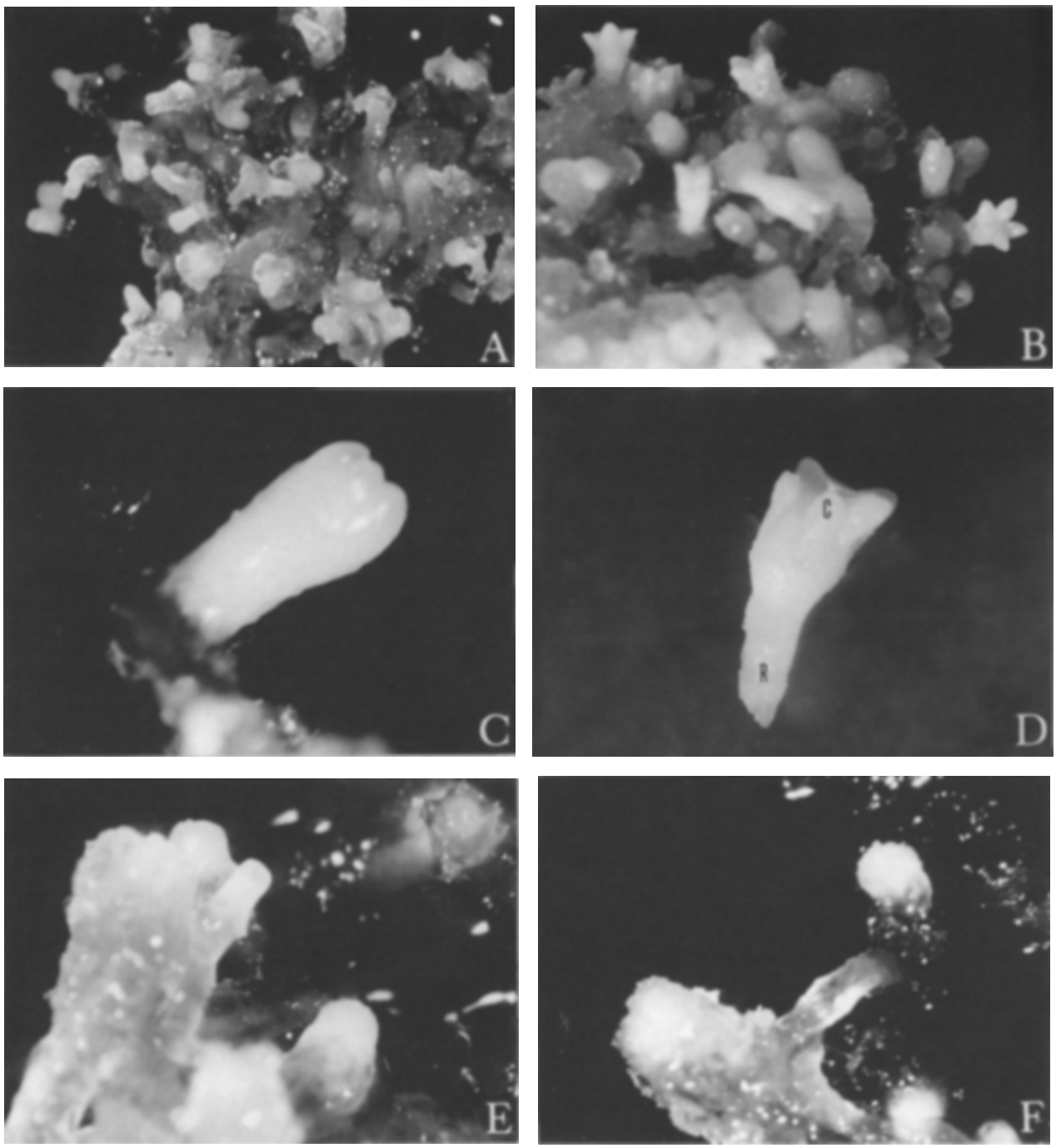

FIG. 2. Enhanced somatic embryo maturation and germination by maltose and/or polyethylene glycol (PEG). *A*, Early cotyledonary stage embryos were formed on the medium containing 4% maltose and 6% PEG, but they were not supported by vigorously growing suspensor (×7). *B*, Late cotyledonary stage embryos were induced on the medium with 4% maltose and 3% PEG (×13). *C*, Early cotyledonary stage embryo induced by maltose and PEG was long and without swelling in the hypocotyl region (×28). *D*, A typical long cotyledonary embryo, formed on the medium with 4% maltose and 6% PEG, was long and had well-developed cotyledons (*C*) and a better defined root cap (*R*) (×8.5). *E*, Early cotyledonary stage embryos were formed on the medium containing 3% sucrose and 6% PEG, and were supported by the vigorously growing suspensor (×7). *F*, Embryo development was arrested on the medium with sucrose and PEG, with the formation of suspensorlike cells around the embryos.

with maltose. The suspensors immediately showed browning after the transfer, but somatic embryo development continued.

Maltose also significantly reduced the arrest frequency of embryo development compared to sucrose. PEG combined with sucrose induced a large number of stage 2 embryos, but most of these embryos failed to develop further (Fig. 2 *F*). And even the development of cotyledonary embryos was arrested many times on this medium. A similar phenomenon was not observed after maltose was employed to replace sucrose as the carbohydrate source.

Discussion

Although the lowest maturation frequency resulted from the maturation medium with sucrose alone, this was the first time that cotyledonary embryos were induced on this maturation medium in our laboratory. Compared to the previous experiments, the maturation medium was dried for 3 h with an open lid before being used. Condensation is considered to have a deleterious effect on inducing cotyledonary somatic embryos (Aitken-Christie, personal communication). The current study confirmed the beneficial effect of a medium-drying treatment. This simple method for medium solidification is now a routine procedure for maturation media in our laboratory.

Although the importance of maltose for somatic embryo maturation has been recognized in loblolly pine, the reported embryo maturation frequency induced by maltose was significantly different between the U.S. patent 5,187,092 (Uddin, 1993) and 5,413,930 (Becwar et al., 1995). In our laboratory, maltose at 6% did not efficiently induce cotyledonary embryos with the different conditions (Li et al., 1997), although 6% has been demonstrated to be the best concentration for embryo maturation if no other osmoticant was involved (Uddin, 1993; Becwar et al., 1995). Plant tissue culture medium generally contains 2–3% carbohydrate; 6% maltose may have served as both carbohydrate source and osmoticum. In this study, only 10.6 cotyledonary embryos per gram embryogenic tissue were produced from the medium containing 6% maltose (Table 1). This embryo maturation frequency was similar to the results reported by Uddin (1993).

PEG, with a molecular weight above 3000, is a nonplasmolyzing osmoticum and may be superior to sucrose or maltose, which induce cell plasmolysis at high concentrations (Attree and Fowke, 1993). This has been demonstrated in white spruce, *Picea glauca* (Attree et al., 1991). Our previous study also showed that the PEG promoted somatic embryo maturation in loblolly pine. PEG combined with sucrose as a carbohydrate source produced a large number of stage 2 embryos, although the precotyledonary embryos had a low conversion frequency to the cotyledonary stage (Li et al., 1997).

Embryo maturation efficiency was significantly enhanced on the culture medium containing both PEG and maltose, which seemed to act synergistically in embryo development. Therefore, the proper combination of carbohydrate source and osmoticum is probably the main factor limiting efficient embryo maturation in loblolly pine. PEG combined with a low concentration of maltose resulted in more efficient embryo maturation than the 6% maltose. This may indicate that PEG is superior to maltose as an osmoticum. Maltose seems to be a better carbohydrate than sucrose for somatic embryo development in loblolly pine. Pullman and Webb (1994) used a maturation medium including PEG (13%) and maltose (2%) to produce cotyledonary embryos, but no detailed explanation and embryo maturation frequency were disclosed. Maltose at 4% and PEG at 6% induced the highest embryo maturation efficiency in this study. Based on the maturation data and our observations, we recommend that maltose at 3–4% and PEG at around 6% be used for embryo maturation in loblolly pine. Although the two embryogenic cell lines were not cryopreserved and were about 3 yr old, this newly developed medium still resulted in high embryo maturation frequency.

Considerable variation of maturation frequency existed between replicates, sometimes this variation was very large. Although an average of 109 cotyledonary embryos per gram embryogenic tissue were produced on the medium with 4% maltose and 6% PEG, maturation frequencies up to 300 embryos per gram tissue were observed. A specific reason for this variation was not explored in this study but may be related to the quality of the initial embryogenic tissues. Despite the variation on maturation frequency, cotyledonary embryos are routinely and consistently produced in our laboratory. The maturation frequency presented here is a significant improvement compared to some published results in *Pinus* species.

Besides embryo maturation frequency, the quality of mature embryos is another criterion for the optimization of an embryo maturation protocol. Relatively short embryos are generally considered low quality, which may lead to low efficiency of embryo germination and plant establishment (Liao and Amerson, 1995). With sucrose, most cotyledonary embryos were short and showed swelling in the hypocotyl, whereas most embryos matured on the maturation medium with maltose were generally longer and had less swelling in the hypocotyl and a better defined root cap region. PEG affected the maturation frequency but had no obvious effect on the morphology of somatic embryos. As a carbohydrate source, maltose showed superiority to sucrose in both maturation frequency and embryo morphology.

We also noticed a correlated phenomenon between the embryo and suspensor development. If the suspensor had grown vigorously, embryo development would probably be arrested at the mid to late stage (Fig. 2 *F*). Another striking difference between sucrose and maltose on embryo maturation was the stimulation of suspensor growth. Suspensor development was inhibited to some extent by maltose compared to sucrose. The arrested development of stage 2 embryos on the medium with sucrose was often accompanied by the formation of suspensorlike cells around the embryo and might result from the overgrowth of suspensors (Li et al., 1997). This phenomenon was observed much less often when sucrose was replaced with maltose. Therefore, maltose was also superior to sucrose from this standpoint, but research on this topic is lacking. On the other hand, maltose did not always fully inhibit the growth of the suspensors. It is unknown if any other methods inhibiting suspensor growth can further promote embryo maturation in loblolly pine.

Conclusions

Compared to our previous research (Li et al., 1997), three changes in somatic embryo maturation were observed when maltose replaced sucrose in the maturation medium containing PEG. First, maturation frequency was enhanced significantly. Second, morphology of somatic embryos was improved. Third, growth of suspensor could be inhibited, and the frequency of arrested embryo development was reduced. In contrast, PEG seemed to mainly affect maturation frequency. The enhancement of embryo maturation by maltose and PEG together may indicate that the previous failure of ineffective embryo maturation in loblolly pine is due to the lack of osmoticum and/or an inappropriate carbohydrate source. We consider that PEG is poten-

tially a better osmoticum than maltose, while maltose is superior to sucrose as a carbohydrate source. It needs to be further investigated if limited suspensor development had a role in inducing efficient embryo maturation. The combination of about 4% maltose and 6% PEG is generally recommended to induce efficient embryo maturation in loblolly pine.

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