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OPTIMUM CONE COLLECTION PERIOD IN ARKANSAS FOR ESTABLISHING IN VITRO CULTURES OF LOBLOLLY PINE (*PINUS TAEDA* L.)

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ABSTRACT

This study was conducted to determine the optimum period to collect loblolly pine (*Pinus taeda* L.) cones that contain embryos with the greatest capacity to produce embryogenic callus for establishing somatic embryogenesis. Cones were collected from trees at the University of Arkansas Agricultural Experiment Station, Fayetteville, in 1991 during four consecutive months: May, June, July, and August. Seeds were extracted and disinfected in 70% ethanol for 1 min, 50% Clorox for 20 min, followed by four water rinses. Seed coats were removed, and embryos were cultured on DCR medium adjusted to pH 5.7 and supplemented with 3 mg/L 2,4-D, 0.5 mg/L 6-BAP, 30 g/L sucrose, and 6 g/L agar. Callus was induced and maintained in the dark at 20 ± 5 C. Embryos collected in May failed to proliferate; however, 55%, 88%, and 66% of the embryos cultured produced callus from June, July, and August collections, respectively. Suspension cultures were initiated from pine callus and maintained for over 6 months in MSG medium for subsequent investigation of the conversion of embryogenic complexes into mature embryos and eventually into plantlets. Evidence for embryogenesis was observed with double-staining techniques.

INTRODUCTION

Tissue culture technology is recognized as an important means for rapid vegetative propagation, forest improvement, and increase in productivity (Karnosky, 1981; Farnum *et al.*, 1983; Haissig *et al.*, 1987). Hasnain *et al.* (1986) explained that to fully utilize tissue culture methods for forestry, the development of micropropagation methods from juvenile and mature tissue of commercially important conifers must be achieved before commercialization of forest tree micropropagation. Another important priority is the development of improved techniques for micropropagation and somatic embryogenesis including research toward a better understanding of the biochemical and developmental basis of plant regeneration from cell and tissue culture.

Loblolly pine (*Pinus taeda* L.) is an economically important coniferous species in forests of the southeastern United States, and it is the main species growing on about 12 million hectares of plantations (Brender *et al.*, 1981). Research with tissue culture of loblolly pine has been the focus of several investigators (Gupta and Durzan, 1987a, 1987b; Gupta *et al.*, 1987, 1988; Becwar *et al.*, 1988; Becwar and Feirer, 1989; Durzan, 1988; Teasdale *et al.*, 1986). Although embryogenic callus of loblolly pine can be induced, conversion of the immature somatic embryos to the mature somatic embryos and the recovery of plantlets remains difficult (Becwar and Feirer, 1989). Becwar *et al.* (1988) stated that the explant developmental stage is the most important factor for the initiation of embryogenic callus from loblolly pine immature embryos. The developmental stage is dependent upon the time the cones are collected for explant extraction. Because of regional differences in the developmental stage due to climatic differences, it is essential to define the optimum date (developmental stage) in our region to collect cones that will initiate embryogenic callus. Our goal was to evaluate the effect of this factor on explants obtained from Arkansas-grown loblolly pine trees.

The objectives of the present study were 1) to determine the climatic effect on the formation of callus, namely to identify the optimum period for collecting pine cones that would produce embryogenic callus and 2) to establish and maintain cell suspension cultures from this callus for conducting year-round research focused on the improvement of somatic embryogenesis, particularly to circumvent the problem associated with the availability of viable immature embryos, which may be restricted to a short period of the year.

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MATERIALS AND METHODS

CONE COLLECTION

Loblolly pine cones were collected from the end of branches of four trees grown at the Arkansas Agricultural Experiment Station, Fayetteville, Arkansas. During 1991, four collections were made at the end of May, June, July, and August. Four cones were collected from each tree providing 16 cones at each collection. The 16 cones, however, were mixed as a way of randomizing the samples.

SEED EXTRACTION, DISINFECTION, AND EMBRYO REMOVAL

Scales were removed by pealing them away from the axis of the cone with a knife. The seeds were removed after being exposed, placed in a plastic bag, and kept in a refrigerator (4 C) for several days until all the seeds from the sample were extracted. The seeds were surface disinfected in 70% ethanol for 1 min, followed by immersion for 20 min in 50% Clorox solution containing 0.01% Tween 20, and rinsed four times in sterile water. Seed coats were removed with a scalpel to expose the immature embryos.

CALLUS INDUCTION

The embryos were cultured individually in culture tubes (16x100 mm) containing 5 ml of DCR medium (Gupta and Durzan, 1985) supplemented with 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/L benzylaminopurine (BAP), and 30 g/L sucrose, solidified with 6 g/L agar, and adjusted to pH 5.7. Callus was induced and maintained in the dark at 20 ± 5 C. The cultures were maintained by transfer to fresh medium at 4-week intervals.

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CELL SUSPENSION ESTABLISHMENT AND MAINTENANCE

Suspension cultures were initiated from pine callus by cutting the callus into 1- to 2-mm pieces and placing 0.25 g of callus per 125-ml flask containing 25 ml of liquid MSG medium (Becwar *et al.*, 1988). The medium was supplemented with 0.5 mg/L BAP, 0.5 mg/L kinetin, and 1 mg/L 2,4-D. The cultures were maintained by shaking at 100 rpm in darkness and subcultured at 2-week intervals. Callus from suspension cultures was retrieved after a month of culturing in liquid medium and cultured for 4 weeks on a medium solidified with 6 g/L agar to interrupt continuous culturing in liquid medium.

REDIFFERENTIATION OF CALLUS

To induce redifferentiation of the callus into somatic embryos, the callus was transferred to solid MSG medium supplemented with 0.5 mg/L kinetin, 0.5 mg/L BAP, and 0.2 mg/L 2,4-D. Callus cultured on this medium was kept in the dark for 4 weeks and then transferred to the light (50 $\mu E/m^2/s$). The cultures were transferred to fresh medium every 2 to 3 weeks.

CYTOCHEMICAL STAINING

Staining procedures used were after Gupta and Durzan (1987a). Samples obtained from cell suspension, which contained cells and cell aggregates, were stained in 2% acetocarmine by mixing 1:1 (v/v) of cells and acetocarmine and heating slightly for 15 s. This stain was filtered out, and the tissue was stained again in 0.5% Evan's blue, also at 1:1 (v/v) ratio. Excess of stain was removed by washing the tissue in liquid medium, and to increase optical clarity of the cells, 100% glycerol was added. The double-stained cells were observed through an inverted microscope, and microphotographs were obtained.

RESULTS AND DISCUSSION

CALLUS INDUCTION

The number of seeds collected per cone was variable, ranging from about 10 to 30 seeds. From each collection period, 200 seeds were used. Immature embryos collected in May failed to proliferate callus. These early collected seeds had soft seed coats, which may have promoted the penetration of the disinfectant to the embryos, consequently killing them. Embryos from the other collections, however, resulted in callus formation within 5 to 10 weeks after culturing on callus induction medium. The number (and percentage) of immature embryos that formed callus were 110 (55%), 176 (88%), and 132 (66%) obtained from June, July, and August collections, respectively. These results suggest that the best collection date for pine cones in northwest Arkansas for callus induction in 1991 was around the end of July. June and August, however, were also suitable for cone collection if culturing a maximum number of embryos was desired.

Although the percentage of callus induction gave an indication of the capacity of the immature embryos to form callus, the quality of the callus produced, as expressed in its capacity to regenerate plants, was not realized. Therefore, a relationship between the collection date and the capacity to regenerate has not been established. This subject is the objective of further investigation, which will be conducted on 1992 cone collections. Cell suspension established from callus was maintained viable for over 6 months. Initially, the cell suspension appeared white but gradually changed to brown after about 30 days in suspension, even though subculturing at 2-week intervals was carried out. This problem was circumvented by periodic interruption during the growth in liquid cultures and the transfer of callus to solid medium. When the brown callus was placed on agar medium, proliferation of white callus resumed. This new callus proliferation was useful in establishing new cell suspension cultures. Using this method, we achieved a continuous source of callus for studying the differentiation process. Evidence of redifferentiation and somatic embryo formation was observed based upon staining techniques.

CYTOCHEMICAL STAINING OF REDIFFERENTIATING CALLUS

Medium designed to induce differentiation and somatic embryo formation contained a lower level of 2,4-D. This alteration in the medium was sufficient to induce differentiation of non-differentiated callus into various stages of development. These stages were observed with the staining method described by Gupta and Durzan (1987a). This double-staining provides a means for the identification of certain structures based upon their color rendered after staining; for example, red indicates embryonic tissue, and blue indicates suspensor. Some stages of development observed in our study are presented in Fig. 1. Although the colors are useful indicators, in the black and white photos only the shapes of the structures are visible. Differentiation began after the non-differentiated callus (Fig. 1, A) was placed on regeneration medium where it formed an early proembryonic complex, which continued to develop, forming proembryo masses and leading to the formation

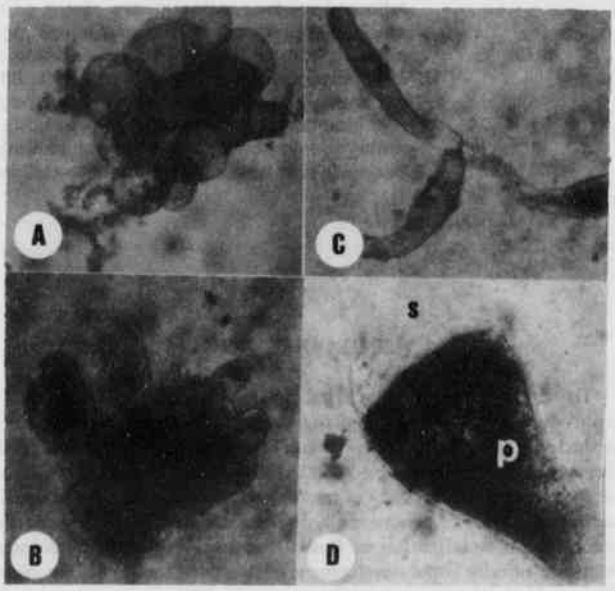

Figure 1. Stages of differentiation in pine callus. A) non-differentiated callus before transfer to regeneration medium, 200 x; B) proembryo mass, 200x; C) cell elongation of prombryos, 200x; D) formation of a dark red-stained proembryo (p) with light blue-stained suspesor (s) region, 400x.

proembryos (Fig. 1, B). The proembryo development was followed by cell elongation (Fig. 1, C) and the formation of a dark red-stained proembryo with a light blue-stained suspensor region (Fig. 1, D). This was the last stage of development observed in this study, and more research is needed to induce conversion of the proembryos to mature embryos. The manipulation of the growth regulators will be our tool to achieve this conversion and the subsequent regeneration of plantlets.

CONCLUSION

Our investigation showed that July was the optimum period of the year to harvest cones for the purpose of pine callus induction in northwest Arkansas. Defining this time is critical for investigations related to pine tissue culture and the development of improved methods for plant regeneration. In addition to achieving this prerequisite for further research, we have demonstrated the establishment and maintenance of callus and cell suspension cultures and observed early stages of somatic embryogenesis.

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