Early Development of Ponderosa Pine (Pinus ponderosa Laws.) Embryos on a Defined Culture Medium

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Introduction

Tissue culture techniques with somatic cells and tissues originating from various organs have been carried out on both gymnosperm and angiosperm plants in many aspects of botany. During the past 10 to 20 years, emphasis has been place on developing coniferous tissue in vitro from various vegetative parts of the plant. HARVEY and GRASHAM (1969) reported successful callus cultures of 12 gymnosperm species, usually requiring complex culture media and carefully controlled aspetic technique. BETHEL (1972) reported differentiation of roots on Douglas-fir callus, and KONAR (1972) has developed roots or shoots from Pinus gerardiana callus.

Development of coniferous tissue into differentiated structures such as roots and shoots has generally been regarded as difficult. However, SOMMER, BROWN, and KORMANIC (1975) have been successful in producing plantlets from adventitious buds formed along the cotyledons of longleaf pine embryos when grown on a defined medium.

This study was concerned with the development of excised embryos of ponderosa pine (Pinus ponderosa LAWS.) on a defined agar medium, specifically observing the early morphological and histological changes in the young embryo.

Materials and Methods

The procedures followed primarilly parallel those of HARVEY and GRASHAM (1969) and SOMMER, BROWN, and KORMANIC (1975). Seeds of ponderosa pine were used from several trees located near Crown Point Road in Larimer County, Colorado. Viable seeds were first surface sterilized in a 1 : 1 solution of sodium hypochlorite and water, rinsed in sterile-distilled water, and allowed to imbibe for about forty hours in sterile-distilled water. To maintain sterile conditions, the seeds were quickly subjected to a 1 : 2 dilution of sodium hypochlorite and water and once again rinsed in sterile-distilled water before being placed in sterilized petri dishes for dissection. Dissection was carried out in an aseptic transfer chamber. The imbibition time resulted in easy dissection of the embryos from the female gametophyte tissue.

The embryos were transferred intact to either 20 X 150 mm test tubes or 90 mm diameter petri dishes which contained 25 ml of nutrient medium (medium 3, SOMMER, *et* al., 1975). This medium had previously been sterilized in an autoclave at 1.14 kg/cm^2 for 20 minutes.

Test tubes and petri dishes (47 tubes and 10 petri dishes each with one embryo per container) were placed in a growth chamber regulated for a 13-hour light period at 24^0 C and an 11-hour dark at 17^0 C. The tubes were sealed with cotton plugs and foil covers. Observations of the embryos were carried out on a daily basis for five weeks.

At two-day intervals, two embryos were removed from the growth chamber and subsequently both killed and fixed in FAA (formalin-acetic acid per JOHANSEN, 1940). Dehydration was carried out in a tertiary-butyl alcohol series and the embryos were embedded in paraplast for sectioning. Sections were made on a rotary microtome at 10 microns and mounted on standard 2.54 X 7.62 cm microscope slides using Haupt's adhesive. A modified safranin-aniline blue staining schedule was used on the sectioned material as it proved to be more precise than the standard safranin-fast green type stain (JOHANSEN, 1940). Photomicrographs were taken using a Nikon-F camera attached to a Leitz-Wetzlar binocular scope in conjunction with an Orthoilluminator light source.

Results and Observations

This experiment consisted of 57 total embryos grown on a chemically defined nutrient medium. All embryos ranged in size from 0.45 to 0.55 cm averaging 0.50 cm at the start. After five days on the culture medium, the cotyledons showed distinct swelling with a yellowish-green color. Total embryo length at this time averaged 0.70 cm with individual cotyledons measuring between 1.5 to 2.0 mm in length. The radical end of the embryo had started to form a callus plug of undifferentiated tissue which eventually deteriorated to a brown, slimy mass in the following days.

After 8 days in culture, the average cotyledon length had grown to 4.0 mm and a bright green color accompanied divergence of the cotyledons. Histological observations at this point showed distinct leaf primordial buttresses flanking the apical dome (jigure I). In addition, primary xylem tissue development was apparent in the pro-cambial region with distinct spiralling and pit formation in the secondary walls (figures 1 *and 2).*

Distinct bumpiness along the cotyledons was noticed by the 11th day (jigure 3). It is difficult to speculate on meristematic significance of these bumps after only about two weeks in culture. Organization into meristematic centers was quite indistinct.

By three weeks, elongation of the cotyledons had ceased. Numerous wrinkles had developed on the cotyledons next to the medium and the average cotyledon length was 1.5 cm.

Primary leaves subsequently had developed from the primordial buttresses and they were apparent in the apical region on the embryos by the end of the 5-week period (jigures 4 *and* 5).

Discussion and Conclusions

Development of the ponderosa pine embryo in vitro begins as soon as the dissected embryo is subjected to the agar. One of the first events of occur is the establishment of a primary vascular system as evidenced by the xylary tissue in the vicinity of the transition zone of the apical region (ESAU, 1967). Flanking the apical dome itself appear the first leaf primordia which are derived from the peripheral zone as defined by ESAU (1967). Cells of this zone are smaller, darker staining, and not well differentiated implying active division in the area.

As in the general case for the pines, the vascular system in the cotyledones of these embryos was of a collateral

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nature (*figure 6*). An exception to this general case has been noted by SOMMER, BROWN and KORMANIC (1975) for longleaf pine embryos cultured *in vitro*. Their study reported concentric vascular arrangement and a closer look at their photographs revealed an amphivasal system (xylem surrounding in the phloem) to this author.

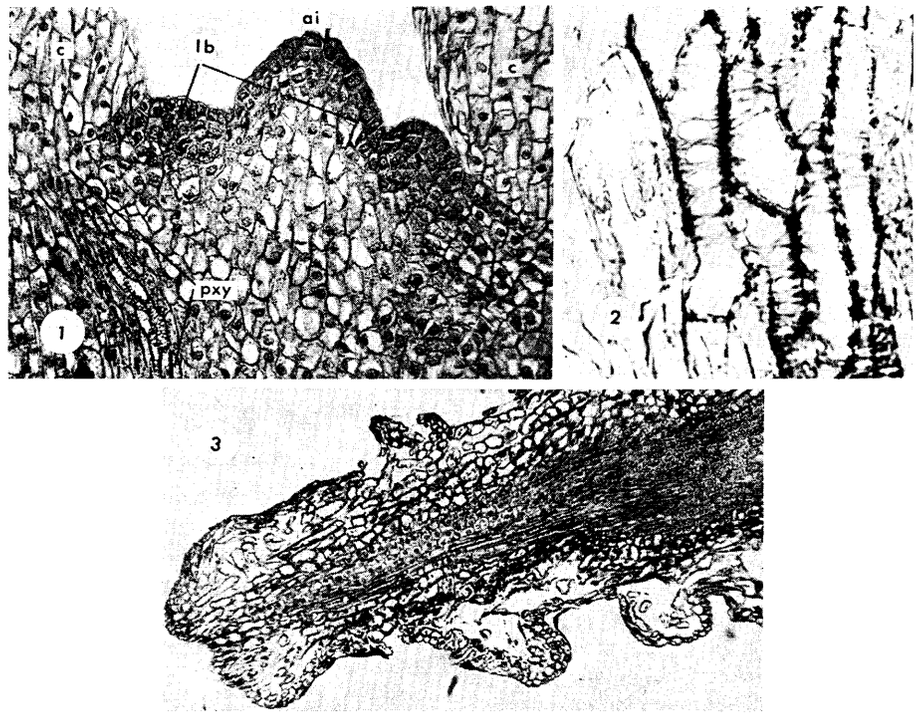

Figure 1. — Longitudinal section of embryo after 8 days on culture medium. (ai — apical initials, c — cotyledons, 1b — primordial leaf buttresses, pxy — primary xylary tissue) ×100; *Figure 2*, primary xylem in apical meristem region after 8 days on culture ×450; *Figure 3*, section through cotyledon showing numerous bumps and cellular differentiation after 11 days in culture ×35.

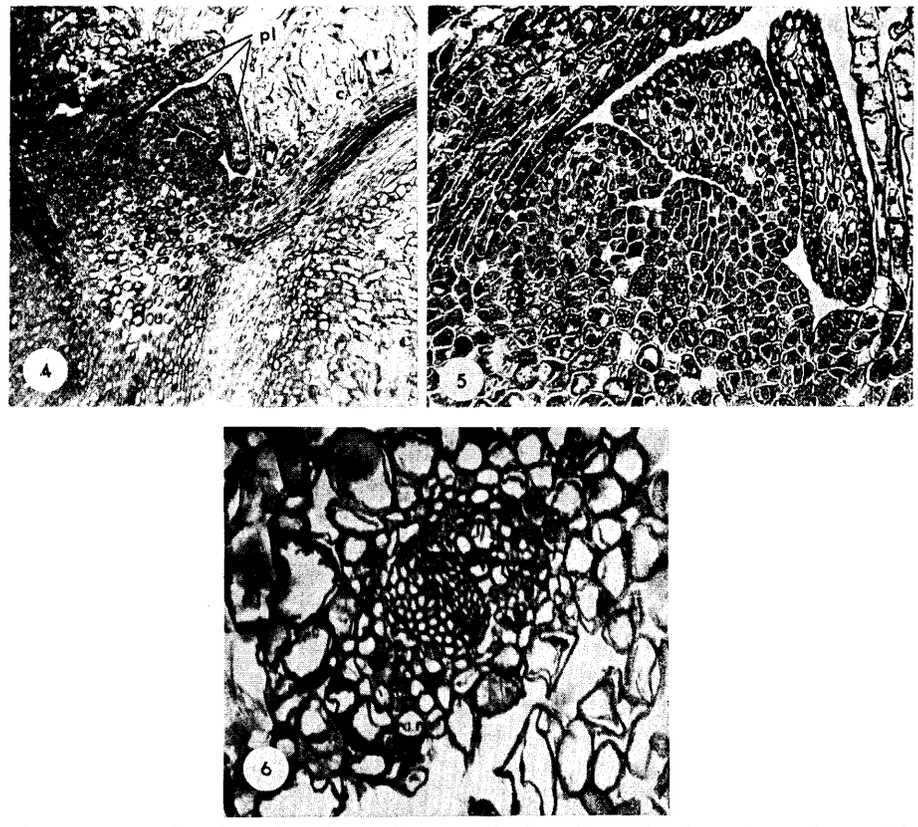

Figure 4. — Section through embryo after 5 weeks in culture, showing primary leaves (pl), and cotyledons (c) with well defined vascular system ×35; *Figure 5*, Close up of figure 4 emphasizing apical meristem region ×100, *Figure 6*, Collateral vascular bundle in cotyledons ×450.

It is apparent that the nutrient medium and controlled environment of this experiment did not necessarily enhance bud formation or rapid development of apical-type meristematic centers on other parts of the embryo (i.e., the cotyledons). However, formation of a cotyledonary vascular system was rapid as well as initial leaf primordia evidence about the apical dome.

Prospects for further tissue culture work on the excised embryos of ponderosa pine look promising. Embryos are relatively easy to work with as compared to other gymnosperm material and they react rapidly to culture media. Further work is planned to determine possibilities and factors involved with plantlet formation from excised embryos cultured *in vitro*. To be able to develop many plantlets of ponderosa pine from a single embryo would allow rapid clonal propagation of genetically identical individuals for genetic studies and tree improvement work.

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Abstract

Early stages of growth were studied on excised embryos of ponderosa pine (*Pinus ponderosa* Laws.) grown on a chemically defined nutrient medium. Morphological and histological studies were made throughout a five week study period. Rapid differentiation of a primary vascular system and primary leaves were noted.

Key words: Tissue culture, *Pinus ponderosa* Laws.

Zusammenfassung

Bei der Kultur von Embryonen von *Pinus ponderosa* Laws. konnte Kallusbildung beobachtet werden, wobei solche undifferenzierten Gewebepartien blattprimordiale Auswölbungen hervorbrachten. Die zum Teil erfolgte Weiterentwicklung solcher Auswölbungen gibt Anlaß, darin eine Knospenbildung zu vermuten, die evtl. zur Regeneration ganzer Pflanzen geeignet erscheint.

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Viable Seed from a Shortleaf Pine 13 Months Old

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Scientists studying tree improvement would like to shorten the maturation period of southern pine cones, since the lengthy ripening (normally about 21 months) causes major delays in obtaining seed from the offspring of controlled crosses. Cones have reportedly matured during their first year (Katsuta 1970, McLemore, in press), but there is no record that such cones have produced viable seed. This note describes a technique to induce shortleaf pine *Pinus echinata* Mill.) cones to mature and yield viable seed in 13 months.

Procedures

Pine trees usually produce no cones before the age of 10 to 15 years, when tree heights of 30 to 40 feet make potting and indoor testing impractical. Therefore, it was necessary to obtain samples by grafting. Fifteen soft-tissue branches of shortleaf pine were collected and placed in a greenhouse in early April 1974. Attached were a total of 45 conelets, which had been wind pollinated immediately before collection. On April 18, the branches were cleft-grafted onto 2-year-old potted slash pine seedlings (*P. elliottii* Engelm.) about 50 cm tall. By May 13, all but one of the scions were dead, and all but three conelets on the successful graft had aborted.

From early June through mid-July (45 days) the surviving ramet with its three conelets was subjected to treatments simulating abbreviated natural seasons. At the beginning and end of the treatment period, the graft was placed in a growth chamber for 7 days and subjected to alternating 8-hour photoperiods (15,000 lux) at 13° C and darkness at 7° C. During the 31 intervenings days, the ramet was moved to a cold room (1° C), where fluorescent lights (1,000 lux) were turned on for 8 hours on weekdays but were omitted on weekends. On July 18, the graft was placed in an air conditioned greenhouse (23° C), where the cones remained until harvesting.

The conelets remained bright green throughout the summer, when they normally turn brown. In late September, they had begun to enlarge, and by mid-November, they appeared fully grown and measured approximately 3.5 cm long and 1.5 cm in diameter, slightly smaller than normal for mature shortleaf pine cones. The diminished size was probably caused by insufficient nutrition due to grafting and to the fact that the seedlings did not have enough rootstock to maintain full-sized cones.

One cone was removed from the ramet on December 31, 1974, one on February 28, 1975, and one on May 1, 1975. Immediately after harvesting, each cone was kiln-dried for 48 hours at 38° C and opened mechanically for seed removal. The seeds were left unstratified and were sown on a moist sand-peat medium for germination tests.

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