TISSUE CULTURE AND GREENHOUSE PRACTICES FOR THE PRODUCTION OF LOBLOLLY PINE PLANTLETS

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Abstract.--Methodology is presented for tissue culture propagation of Pinus taeda. Pulse stimulation of adventitious shoot production on a cytokinin rich medium, is followed by shoot organization and growth on medium devoid of growth regulators. Rooting is initiated with a high concentration auxin pulse and completed on a medium free of exogenous auxins. When roots are about 5 mm long the plantlets are transferred to a fine textured soil mix of peat, vermiculite, and perlite under intermittent mist in the greenhouse. Leach tubes are used to promote a strong, well developed root system for transfer to the field.

Additional keywords: Pinus taeda, rooting, in vitro, vegetative propagation.

Conifer tissue culture has received great interest in the last 10 years and is now realistically viewed as a potential method for vegetative propagation. Early work on adventitious bud formation in Pinus palustris (Sommer et al. 1975) paved the way for research on many different species (Mott and Amerson 1981). Despite the high level of effort, only about 10 species have produced rooted plantlets which could be carried to soil media (Mott 1981), and detailed greenhouse and field studies have not been reported for any species.

The tissue culture program for loblolly pine (Pinus taeda L.) at N.C. State University was initiated in 1974 and expanded in 1979 to produce and thoroughly evaluate plantlets in the laboratory, greenhouse, and field. Methods developed by Mehra-Palta et al. (1978) and Mott et al. (1977) provided an initial building point for propagation work on about 40 open-pollinated families in an effort to produce several thousand plantlets for evaluation. Early studies by Kelly (1978) and Leach (1978, 1979) served as initial guides for greenhouse and field work. Many changes and improvements have been made from these starting points both in the laboratory and the greenhouse. All phases of the culturing process; shoot initiation, elongation, rooting, plantlet transfer to the soil, and greenhouse growth have developed to the point that plantlets can be routinely obtained from most families. This paper reports these improvements and developments.

TISSUE CULTURE PROCEDURES AND RESULTS

The sequence of steps which constitutes the process used for clonal plantlet production from seed embryos of Pinus taeda as diagrammed by Mott and Amerson (1981) is given in figure 1. This process outlines overall steps, but more importantly it recognizes a number of substeps which must be accomplished

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in the proper sequence via judicious addition and removal of growth regulators (i.e. pulse techniques) (Mott and Amerson 1981). Examination of the process will follow on a step by step basis, with particular emphasis on step 3, rooting of shoots.

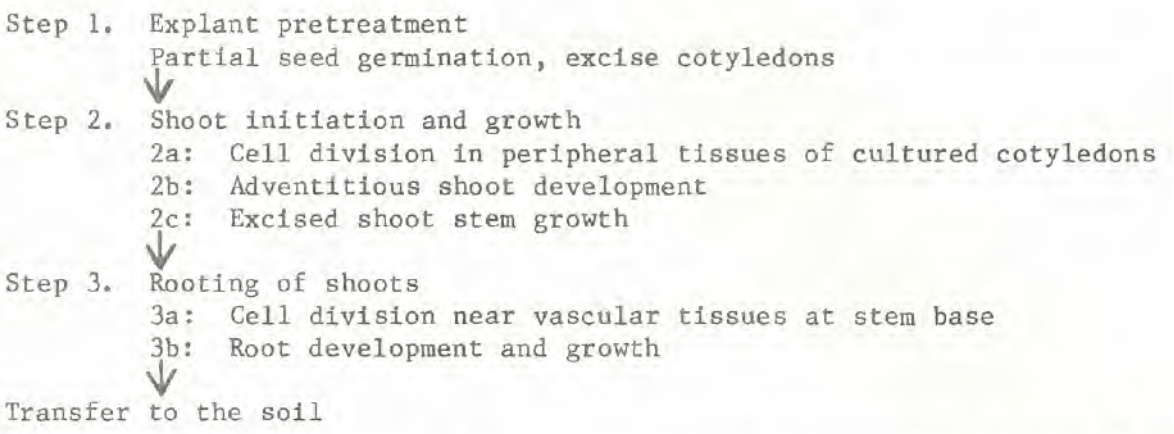

Figure 1.--A diagrammatic process for tissue culture propagation of pine from cotyledons of excised embryos.

Examination of the Process

Step 1. Explant pretreatment.--Seeds are scarified at the micropylar end and pretreated in 1% H_2O_2 (changed daily) at 28° to 32°C to stimulate germination (Ching and Parker 1958). After 4 to 5 days of H_2O_2 treatment, the seed coats are removed. Next, the embryos and the female gametophyte tissues are surface sterilized in 15% clorox for 5 minutes and then rinsed 3 times (3-5 minutes per rinse) in sterile distilled water. Following surface sterilization, the embryo is separated from the gametophyte and the cotyledons are excised and placed on a modified GD_1 (Gresshoff and Doy 1) shoot initiation medium (Mehra-Palta et al. 1978, Mott and Amerson 1981) containing 10 mg/l BAP (6-benzylamino-purine) and 0.01 mg/l NAA (a-naphthaleneacetic acid). All media used throughout the process are adjusted to pH 5.5 prior to autoclaving and solidified with 1% agar.

Step 2. Shoot initiation and growth.--There are three substeps necessary for shoot initiation and growth: a. stimulation of cell division, b. shoot development, and c. shoot growth. This tricotomy is necessary since the treatment which stimulates cell division is antagonistic to shoot development and growth.

Substep 2a: Shoot initiation.--The newly excised cotyledons (Step 1) are placed on the shoot initiation medium and maintained at 21±2°C under 1000 to 2000 lux mixed incandescent and fluorescent light. After 2 weeks, numerous cell divisions are visible in the epidermal region, and after 4 to 6 weeks cotyledons are ready for removal from the high cytokinin initiation medium. Cotyledons ready for transfer to substep 2b typically appear swollen, shiny, dark green, and bumpy due to surface divisions (fig. 2a).

Substep 2b: Shoot organization.--Cotyledons entering this substep are placed on half strength GD_1 medium (GD1/2) which contains 1% activated charcoal and no added growth regulators. The charcoal facilitates removal of the

regulators used in substep 2a. Cotyledons typically remain on charcoal medium for 4 weeks at 21 ± 2°C under 8000-9000 lux mixed incandescent and fluorescent light. During this period, small shoot apices begin to differentiate. Continued exposure to the charcoal medium is considered detrimental to further development; thus, the cotyledons with newly differentiated shoots are transferred to GD1/2 without charcoal. Shoots, still attached to the cotyledons, remain on this medium for 4 to 8 weeks (1 or 2 transfers). During this 4 to 8 week period, the developing shoots become crowded on the cotyledons and when shoots are 2-5 mm long they are transfered to substep 2c.

Substep 2c: Shoot growth.--All shoots and developing shoot clusters are excised form the cotyledons and placed individually on GD1/2 medium. Shoots remain on this medium for 4 to 12 weeks (1 to 3 transfers) in the same environment used in substep 2b to allow for elongation. Individual shoots typically grow well, but shoot clusters often fail to grow or grow slowly. Shoots that reach a total height of 0.5 cm are acceptable for rooting, but those shoots >.1.5 cm in height (fig. 2b) are better since preparation for the rooting step requires a fresh basal cut of the stem.

With loblolly pine, adherence to the above methods generally results in shoot initiation from more than 80% of the embryos tested. One can expect an average of 35 to 40 shoots per embryo, but embryos producing as few as 1 or as many as 100 shoots are not uncommon. Currently, about 37% of the shoots produced elongate to a height of 0.5 cm or greater, but higher percentages for individual embryos are common.

Step 3: Rooting of shoots.--As with shoot initiation, the rooting process requires substeps, since the treatment which stimulates cell division at the basal end of the stem is antagonistic to further root development.

Substep 3a: Stimulation of cell division.--Shoots which reach a height of 0.5 cm or more may be rooted. These shoots receive a fresh basal cut on the existing stem and are placed on root initiation medium (GD1/2 ± BAP 0.1 mg/l and NAA 0.5 mg/l) for 6-13 days. The shoots are maintained at 23 ± 3°C under 1000 to 2000 lux mixed incandescent and warm white fluorescent light. Cell division begins at the stem base within a few days and continues in the presence of auxin. Mott and Amerson (1981) indicated that the shoots were ready for transfer to substep 3b when cell division had progressed to a point where the stem base was swollen, the epidermis was split, and extruded basal callus was evident. That point typically occurred within 10 to 12 days. More recently, we modified our evaluation of substep 3a and no longer seek extruded callus as a signal for passage to substep 3b. The degree of basal swelling and very slight epidermal splitting now sought (fig. 2c) is less than that described by Mott and Amerson (1981) and generally occurs within 8 to 9 days on the above root initiation medium.

Substep 3b: Root formation and growth.--Shoots transferred to substep 3b are placed on CIA medium devoid of growth regulators, and are maintained under the environmental conditions described in 3a. Root primordia typically emerge from the basal callus pad within 14 to 21 days, but occasionally they appear earlier. Once root primordia are observed, the rooted shoots should be maintained on GD1/2 medium until roots are at least 3 mm long, and preferably

5 mm or more long (fig. 2d). Plantlets at this stage are suitable for transfer to the soil.

Examination of the Factors Influencing Rooting

Many different parameters and shoot characteristics are emerging as important factors that influence rooting. Shoot age, shoot size, basal medium, growth regulators (types and concentrations), pulse vs. continuous stimulation, previous exposure to rooting trials, and environmental factors such as light quality and quantity all exercise some influence on the rooting efficiency of loblolly pine. All of these factors have been or currently are being evaluated. Two of these factors, shoot size and pulse vs. continuous auxin treatment will be considered here.

Shoot size has an influence on the rooting efficiency of shoots which are pulse treated with GD1/2 BAP 0.1 mg/l + NAA 0.5 mg/l. Generally, with other parameters equal, larger shoots root better than smaller shoots. This trend has been seen in a number of experiments and table 1, a data set from one experiment, demonstrates the degree of influence. All shoots included in this table were vigorous and of equal age (6.5 months old at the time of rooting). Environmental treatment for all size categories was the same.

Table 1.--Rooting performance by size categories for loblolly pine shoots pulse treated for 9 days with GD1/2 BAP 0.1 mg/l + NAA 0.5 mg/l

Shoot size	Number Tested	Number Rooted	Percent Rooted[1/]
< 0.5 cm	106	57	54a
> 0.5 cm but < 1.5 cm	145	94	65ab
> 1.5 cm	14	12	86 b

1/ Percentages followed by different letters indicate a significant X^2 difference at a = 0.05.

Comparisons of pulse vs. continuous treatments should consider the rooting percentages obtained, but also should note the time required for rooting. Thus far, the best continuous auxin exposure treatment tested utilized GD1/2 BAP 0.1 mg/l + NAA 0.1 mg/l medium. This treatment produced an average of 37% rooting in 12 weeks. In contrast, pulse treatments with GD1/2 BAP 0.1 mg/l + NAA 0.5 mg/l for 6-13 days followed by transfer to GD1/2 medium routinely produced 60-85% rooting. Rooting via pulse treatments typically is completed 5 to 6 weeks after the pulse, thus the total process requires only 6 to 8 weeks. In comparison to continuous exposure treatments, pulse treatments have given higher rooting percentages in a shorter time period. However, further work is needed before continuous exposure treatments are abolished, since constant exposure treatments were conducted early in the research program before many of the parameters influencing rooting were recognized. In contrast, the pulse treatments were conducted more recently and thus may have some advantage. Experiments are now in progress with shoots of equal quality

in both pulse and continuous treatments to see if continuous treatments can match both the high percentage and rapid rooting obtained with pulse treatments.

GREENHOUSE PRACTICES

There is little published information concerning the growth of tissue cultured conifer plantlets in soil, since few species have been successfully rooted and transferred to soil. Research dealing with transfer of plantlets to the soil has been largely neglected not only for conifers (Thorpe 1977) but also for most crop species (Murashige 1974). However, early studies with loblolly pine by Kelly (1978) and Leach (1978, 1979) indicated that plantlets could be successfully transferred from culture medium to the soil with a reasonable degree of success.

Current Greenhouse Practices

Rooted shoots with total top length (including needles) of 1-2 cm and root length of 3-5 mm or greater are most suitable for transfer to the soil. Plantlets are transplanted directly from the agar medium to soil mix in 164 ml Leach tubes on a greenhouse bench under shade cloth with intermittent mist.

Soil.--The texture of the soil mix is critical for the survival of the small plantlets. A very fine mix of peat, vermiculite, and perlite is necessary since the roots on the plantlets are initially so small that poor soil-root contact results if a coarse mix is used. The fine grades of perlite and vermiculite are used, and the peat is sifted through a 3 mm mesh screen. The pH of the soil is raised to 5.5 by the addition of $Ca(OH)_2$ at approximately 1.5 g/1 peat.

Mist.--A Mist-O-Matic® is used to control the amount of mist applied to the plantlets by spraying a fine mist when water droplets evaporate from the control mechanism. This is preferred to an arbitrary misting schedule using time clocks. The mist bench is covered only with 47% shade cloth so that the amount of mist applied fluctuates depending upon temperature and humidity. Plantlets remain in the mist bench until new needle growth is apparent, usually in 10 days to 3 weeks. New needle growth generally indicates that the plantlet's roots are growing and that it is acclimated to greenhouse conditions. The first few weeks of growth in the greenhouse are very critical to survival of the plantlets. Once they are established with well developed root systems (at about 2 months of age) survival to transplanting size is almost assured. The most recent greenhouse experiment started in March, 1981, had a survival of 136 out of 144 plantlets or 94% after two months.

Containerization.--Choice of container is very important for survival and growth of plantlets in the field. Unlike seedlings, tissue culture plantlets do not tend to form a strong taproot. The plantlets usually develop one or two main roots which grow laterally rather than down. If plantlets are grown in conventional pots, the main root circles within the pot and forms a coiled root system (fig. 2e and 2f). Apparently this is a less serious problem for seedlings since the taproot grows straight down and the laterals circle in the pot. Seedling roots can be pot-bound and still grow outward to give support.

The Leach tubes which we are now using force the tissue culture roots to grow downward like a taproot. The ribs in the tube prevent coiling, and the air pruning at the bottom promotes more lateral branching (fig. 2e). The general root configuration of plantlets in tubes is much superior to that in pots.

CONCLUSIONS

Tissue culture and greenhouse procedures for vegetative propagation of loblolly pine have progressed to a point where plantlets are routinely obtained from embryonic material. Pulse application of growth regulators is fundamental to the tissue culture process. Initial greenhouse survival of plantlets is highly dependent upon soil, temperature, and moisture regimes.

Information obtained from and methods developed with plantlets from embryonic origin have experimental value, and value as aids in developing methods for mass propagation of plantlets from callus of mature trees. The steps in callus propagation should be essentailly the same as those from embryonic culture once shoots are obtained. Shoots will be elongated followed by rooting, transfer to soil, and growth in the greenhouse.

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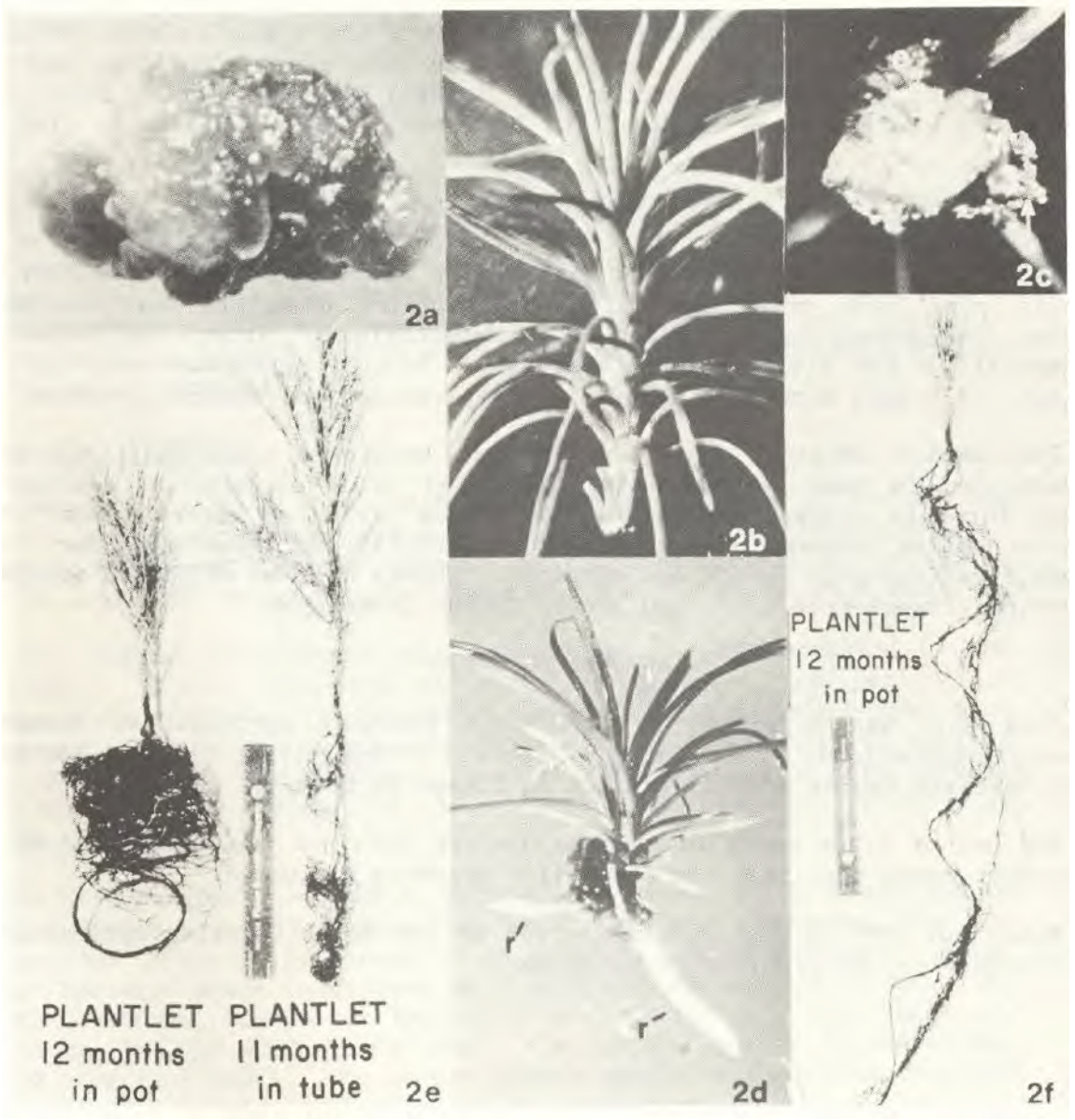

Figure 2.--Various stages of loblolly pine plantlet development.

2a.* Cotyledon ready for transfer to substep 2b. Note shiny, bumpy surface. 13 X

2b.* An adventitious shoot ca. 1.5 cm long. 4X

2c. An end view of a shoot base showing the proper degree of basal swelling (cell division) sought in pulse stimulated rooting. Note: split epidermis (arrow), and intact epidermis (e). 16X

2d.* A rooted propagule ready for transfer to soil medium. 2X

2e. Root systems of a potted plantlet (severely coiled roots) and a tubed plantlet after about one year in soil.

2f. Uncoiled roots of the potted plantlet shown in Figure 2e.

* Pictures from Mott and Amerson 1981.

LITERATURE CITED

Kelly, C.W., III. 1978. Intra-clonal variation in tissue cultured loblolly pine (Pinus taeda L.). M.S. Thesis. North Carolina State University, Raleigh, NC.

Leach, G.N. 1978. Early growth of tissue culture produced loblolly pine (Pinus taeda L.) plantlets in soil. M.S. Thesis. North Carolina State University, Raleigh, NC.

Leach, G.N. 1979. Growth in soil of plantlets produced by tissue culture, loblolly pine. Tappi 62:59.

Mehra-Palta, A., Smeltzer, R.H., and Mott, R.L. 1978. Hormonal control of induced organogenesis: Experiments with excised plant parts of loblolly pine. Tappi 61:37.

Mott, R.L. 1981. Tree propagation. In: Principles and Practices of Cloning Agricultural Plants Via In Vitro Techniques. (B.V. Conger Ed.). Forthcoming CRC Press Inc. Bocaa Raton, FL.

Mott, R.L., Smeltzer, R.H., Mehra-Palta, A., and Zobel, B.J. 1977. Production of forest trees by tissue culture. Tappi 60:62.

Mott, R.L., and Amerson, H.V. 1981. A tissue culture process for the clonal production of loblolly pine plantlets. N.C. Agr. Res. Service. Tech. Bul. No. 271. 14 p.

Murashige, T. 1974. Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. 25:135.

Sommer, H.E., Brown, C.L., and Kormanik, P.P. 1975. Differentiation of plantlets in longleaf pine (Pinus palustris (Mill.)) tissue cultured in vitro. Bot. Gaz. 136:196.

Thorpe, T.A. 1977. Plantlet formation of conifers in vitro. Vegetative propagation of forest trees -- physiology and practice. Lectures from a symposium in Uppsala, Sweden. p. 27-33.