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Factors Influencing *in vitro* Micropropagation of *Pinus strobus* L.

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Abstract. An *in vitro* procedure for micropropagation of *Pinus strobus* L., consisting of the following four steps has been established: shoot induction, shoot growth, root induction, and root growth. Influence of certain selected factors on each of these steps was determined. Shoot induction was found to be influenced by the age of the explant as well as the concentration of 6-benzyladenine in the medium. Best shoot growth was obtained on the medium of Litvay *et al.* without any growth regulators. Addition of activated charcoal to shoot growth medium resulted in fewer shoots. Root induction on several media was compared. Best root induction occurred when shoots were put on a half strength Gresshoff and Doy's medium supplemented with 0.5 to 1.0 mg l^{-1} α-naphthylacetic acid (NAA) for two weeks. Shorter exposure of shoots to higher concentrations of NAA or indol-3-ylbutyric acid (IBA) was not as effective. Low temperature seemed to be a requirement for root growth.

The need to develop procedures for rapid mass vegetative propagation of forest trees has been recognized for many years. *In vitro* micropropagation of conifers *via* shoot bud induction is one such procedure described for several species (BOULAY 1987). Micropropagation of eastern white pine (*Pinus strobus* L.), a species of considerable economic value in North America, has been reported within the last two years (KAUL 1987, SCHWARZ *et al.* 1988, WEBB *et al.* 1988). In an attempt to optimize the micropropagation procedure, effects of several modifications in a procedure for micropropagation of white pine from cotyledon/hypocotyl (C/H) explants (KAUL 1987) were examined.

MATERIALS AND METHODS

Mature seeds of *Pinus strobus* L. were cold treated, surface sterilized, and germinated as described earlier (KAUL 1987). The following protocol for micropropagation was used as the control:

Step 1. Shoot induction on one week-old C/H explants on modified (KASPERBAUER and REINERT 1967)Murashige and Skoog's medium (MS) + 0.2 mg l^{-1} α-naphthylacetic acid (NAA) + 2.0 mg l^{-1} 6-benzyladenine (BA) for 2–3 weeks.

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Step 2. Shoot growth on the medium of LITVAY *et al.* (1981) without any growth hormones (LM) for 12 weeks.

Step 3. Root induction on half strength GRESSHOFF and DOY's (1972) medium (GD) + 0.5 mg l^{-1} NAA for 2 weeks.

Step 4. Root growth on half strength GD without any growth hormones (1/2 GD).

Incubation for steps 1–3 was done under continuous light at 26 °C while step 4 was carried out under 16 h light, 8 h dark cycle at 17 °C. Any one of the steps was modified to study the effects of specific factors as described below. In order to minimize the effects of genetic variability between clones, shoots from a given clone were distributed over various media in an experiment.

Shoot Induction and Growth

Shoot induction step (step 1) in the micropropagation protocol was modified in two separate ways. In one modification C/H explants were taken either within a week after emergence of the cotyledons from the seed coat or five weeks after the cotyledons had emerged. In the other modification C/H explants were put on a shoot induction medium containing basal MS supplemented with 0.2 mg l^{-1} NAA and 0.5, 1, 2, 4, or 8 mg l^{-1} BA.

To study the effects of medium composition on shoot growth (step 2) C/H explants were put on the shoot induction medium for 2 to 3 weeks and then transferred to one of the following shoot growth media: (1) MS without any growth hormones, (2) LM without any growth hormones, (3) LM without any growth hormones + 1% (m/v) activated charcoal (LMC). Shoot growth was allowed for 12 weeks.

Root Induction and Growth

Shoots were obtained by steps 1 and 2 of the control micropropagation protocol. Effects of two kinds of auxin treatments were tested on root induction (step 3). Low auxin treatment consisted of planting the *in vitro* produced shoots for two weeks on 1/2 GD supplemented with 0.2, 0.5, 1.0, or 2.0 mg l^{-1} NAA. The high auxin treatments were adopted from those described by GRONROOS and ARNOLD (1987) for root induction on *Pinus contorta* hypocotyls. High auxin treatments consisted of planting the shoots on 1/2 GD medium supplemented with an appropriate high concentration of auxin. A total of 8 high auxin treatments were tested: 80 μM auxin [14.9 mg l^{-1} NAA or 16.3 mg l^{-1} indol-3-ylbutyric acid (IBA)] for 4 or 8 days and 1.25 mM auxin (232.8 mg l^{-1} NAA or 254.2 mg l^{-1} IBA) for 7 or 24 h. All auxin treatments were carried out at 26 °C under continuous light. After the specified low or high auxin treatment shoots were transferred to 1/2 GD without any growth regulators and the cultures were incubated at 17 °C under a 16 h light/8 h dark cycle.

To study the effect of incubation temperature on root growth (step 4), root

induction was allowed to occur on 1/2 GD + 0.5 mg l^{-1} NAA for 2 weeks. Shoots were then transferred to 1/2 GD without any growth hormones and the cultures were incubated at either 26 °C or 17 °C.

All media were prepared with analytical grade reagents. For high auxin (14.9 or 232.8 mg l^{-1} NAA and 16.3 or 254.2 mg l^{-1} IBA) treatments for root induction, media without auxin were sterilized by autoclaving at 121 °C, 98 kPa for 15 min and then appropriate volumes of filter-sterilized auxin solutions were added. All other media were sterilized by autoclaving after addition of all ingredients. All media were gelled with 0.8 or 1.0% Difco bacto-agar and pH of all media was adjusted to 5.8 before autoclaving. All experiments were repeated at least twice.

RESULTS

Shoot Induction and Growth

The potential for adventitious bud induction decreases with the increase in the age of the seedling from which the C/H explant was taken. As shown in Fig. 1 the average

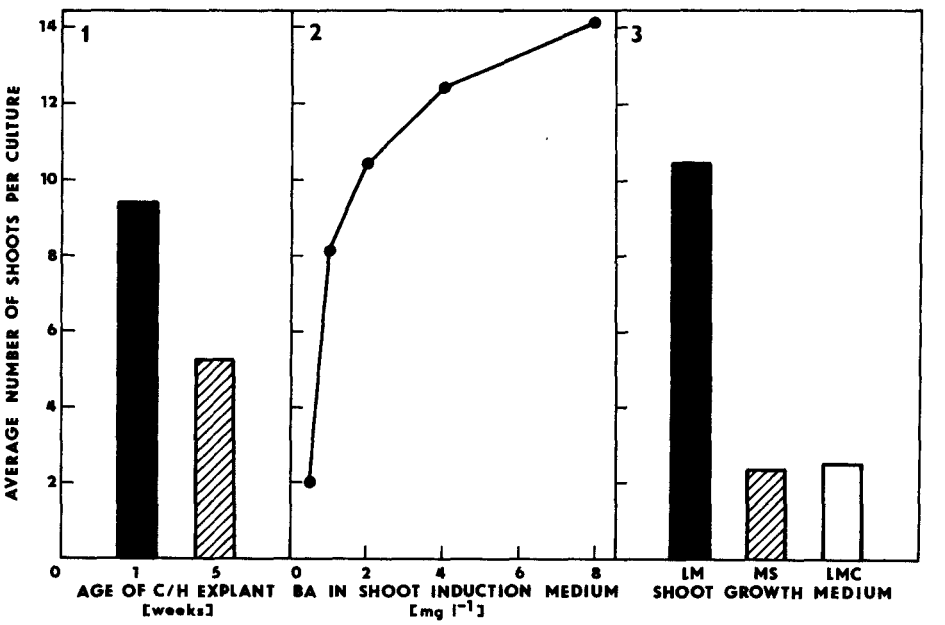

Fig. 1. Effect of age of cotyledon/hypocotyl (C/H) explant on the number of shoots produced during *in vitro* micropropagation of *P. strobus*. Bars represent average from 10 cultures.

Fig. 2. Effect of benzyl adenine (BA) concentration in shoot induction medium on the number of shoots produced during *in vitro* micropropagation of *P. strobus*. Data points represent average values from at least 10 cultures for each BA concentration.

Fig. 3. Effect of three kinds of shoot growth media on the number of shoots produced. Data represent averages from 10 cultures on each medium. LM, conifer cell culture medium of Litvay *et al.*; LMC, LM + 1% activated charcoal; MS, modified Murashige and Skoog's medium.

number of shoots produced per explant was 38.7% lower if the C/H explants were taken 5 weeks after the emergence of cotyledons instead of within a week of the emergence. The number of shoots produced per culture increased with the increasing concentration of BA in shoot induction medium (Fig. 2). However, shoots produced from explants induced on a medium containing 8 mg l^{-1} BA were stunted, difficult to separate from each other, and difficult to root. Media containing 2 or 4 mg l^{-1} BA were found to be the optimal for shoot induction.

Three media were evaluated for their ability to bring about shoot growth. Of these LM without any growth regulators and activated charcoal was decidedly better for shoot growth than LM + 1% activated charcoal or MS (Fig. 3). Addition of 0.2% or 0.5% activated charcoal to LM also resulted (data not presented) in a similar suppression of shoot production.

Root Induction and Growth

Auxin in the root induction medium was found to be of great significance in rooting of *in vitro* produced *P. strobus* shoots. Treatment with 0.5 or 1.0 mg l^{-1} NAA for 2 weeks resulted in rooting of about 50% of the shoots within 4 weeks after the end of treatment (Fig. 4) and caused 75% rooting after 12 weeks. Treatment with 0.2 or 2.0 mg l^{-1} NAA for 2 weeks resulted in lower root induction (Fig. 4). Apparently a critical range of auxin concentration (0.5–1.0 mg l^{-1} NAA) is needed for optimal root induction in *P. strobus* shoots. Higher as well as lower than critical auxin concentration results in lower root induction.

High auxin treatment results have been summarized in Fig. 5. High auxin treatment, in general, suppressed rooting. The suppression was only temporary and after 12 weeks on auxin-less medium significant amount of rooting had occurred. Among the two auxins tried, NAA seemed to be a better root inducer than IBA.

The incubation temperature was found to greatly influence root growth in *P. strobus*. After optimal induction (0.5 mg l^{-1} NAA, 2 weeks) only 10.5% shoots rooted after 12 weeks at 26 °C compared to nearly 80% if the incubation temperature was 17 °C (Fig. 6).

DISCUSSION

Shoot induction during micropropagation was found to be dependent on age of the seedling from which C/H explant was taken and the concentration of BA in the shoot induction medium. A decrease in the potential for shoot formation with the increase in seedling age was observed during the present study (Fig. 1). This is consistent with the fact that almost all successful procedures for micropropagation of conifers through shoot bud induction have employed embryonic or very young seedling explants (THORPE and BIONDI 1984, KAUL 1987, SCHWARZ *et al.* 1988, WEBB *et al.* 1988). WEBB *et al.* (1988) reported a similar decrease of caulogenic potential in cotyledons derived from *P. strobus* seedlings of increasing ages.

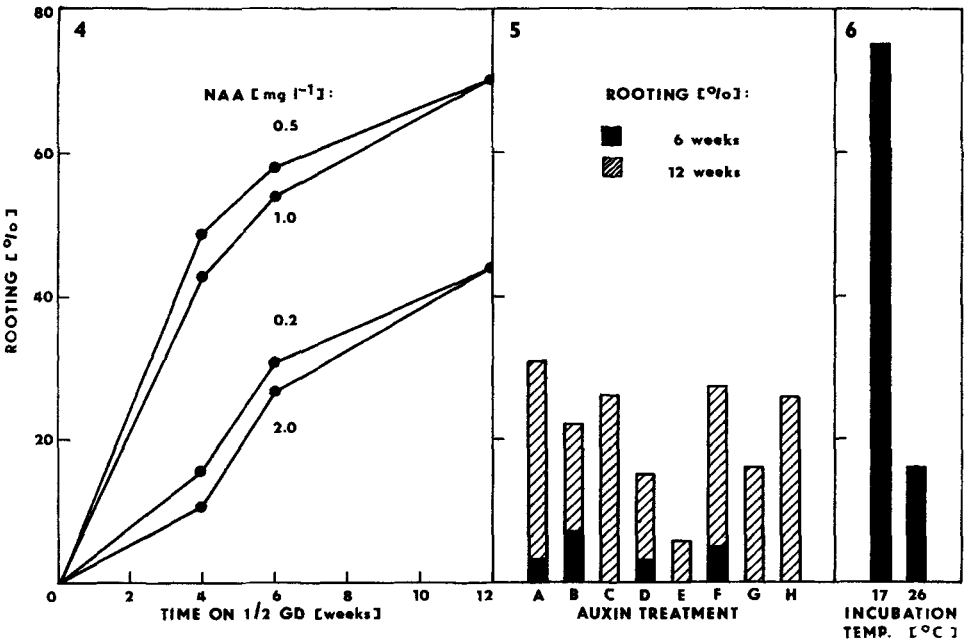

Fig. 4. Effect of low auxin treatment (0.2 to 2.0 mg l^{-1} NAA for 2 weeks) during root induction on rooting of *in vitro* produced *P. strobus* shoots. Values were calculated from 16 cultures for each treatment.

Fig. 5. Effect of high concentration of NAA or IBA in root induction medium on rooting of *in vitro* produced *P. strobus* shoots. Extent of rooting was determined 6 and 12 weeks after the auxin treatment. Values are means from the three experiments – each with 10 shoots per treatment. The auxin treatments were – A: 14.9 mg l^{-1} NAA for 4 days; B: 14.9 mg l^{-1} NAA for 8 days; C: 232.5 mg l^{-1} NAA for 7 h; D: 232.8 mg l^{-1} NAA for 24 h; E: 16.3 mg l^{-1} IBA for 4 days; F: 16.3 mg l^{-1} IBA for 8 days; G: 254.2 mg l^{-1} IBA for 7 h; H: 254.2 mg l^{-1} IBA for 24 h.

Fig. 6. Effect of two incubation temperatures during root growth on rooting of *in vitro* produced *P. strobus* shoots. Values were calculated from 40 cultures incubated at each temperature.

Effects of BA concentration in shoot induction medium on number of shoots produced, reported in the present study, is in agreement with similar effects reported by SCHWARZ *et al.* (1988) for shoot formation on *P. strobus* embryos. It seems that a critical concentration of cytokinin is needed in the shoot induction medium in order to obtain healthy, well developed shoots which can be successfully rooted. In the present study this critical concentration was 2 to 4 mg l^{-1} BA. Lower concentrations resulted in fewer shoots whereas higher concentration resulted in a few more but hard-to-root shoots.

The suppression of shoot production by inclusion of activated charcoal in shoot growth medium, observed in the present study is in contrast with observations reported in several other conifer micropropagation systems (KARNOSKY *et al.* 1984, THORPE and BIONDI 1984, AMERSON *et al.* 1987). WEBB *et al.* (1988) reported inhibition of caulogenesis on *P. strobus* cotyledons when activated charcoal was

included in shoot induction medium. In the same study enhancement of shoot elongation occurred when activated charcoal was included in the shoot elongation medium. The differences between the results reported from the present study and those from the study of WEBB *et al.* (1988) may be due to the difference in activated charcoal used in the two studies.

Until recently root induction on *in vitro* produced conifer shoots was a major hurdle in developing micropropagation procedures (THORPE and BIONDI 1984). Use of appropriate auxin treatments has helped overcome this problem in some systems (KARNOSKY *et al.* 1984, KAUL 1987). SCHWARZ *et al.* (1988) reported no or only poor (28%) rooting of *in vitro* produced shoots of *P. strobus*. WEBB *et al.* (1988) reported high (80%) but extremely slow rooting of such shoots. The present study clearly demonstrates that type and concentration of auxin in root induction medium and incubation temperature during root growth phase are critical factors for rooting of *P. strobus* shoots *in vitro*. Enhancement of rooting at lower temperature reported here is in agreement with results obtained with other conifer species (CHALUPA 1987). Root induction with high concentrations of auxin observed in the present study was not as good as that with low auxin concentrations. However, better induction with a moderately high NAA concentration (5 to 10 mg l^{-1}) for a short period of time may be possible which may further reduce the time required for obtaining rooted *P. strobus* plantlets.

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