ENHANCED GERMINATION OF NORWAY SPRUCE SOMATIC EMBRYOS

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Abstract.-- Prompt germination and vigorous development of somatic embryos is prerequisite to their efficient utilization in mass clonal propagation of forest trees. A number of factors: e.g., growth regulators, media composition, light versus dark culture, and position of embryos on media, have been shown to affect germination. In experiments evaluating these and other factors, improved germination was obtained by exposing Norway spruce (Picea abies) embryos to darkness for 10 days and then transferring them to light culture conditions. Incubation under low intensity yellow light also enhanced germination, as did laying embryos horizontally on media surfaces. Half-strength DCR medium seemed to give the best germination, although differences among media treatments were small and highly variable.

Keywords: Picea abies, germination, growth regulators, light quality and intensity, somatic embryogenesis

INTRODUCTION

Somatic embryogenesis holds much promise for rapid and inexpensive mass clonal propagation of the best genetically improved forest trees. The process evokes a vision of a revolutionary regeneration system capturing larger genetic gains, greater product uniformity, and more versatility than the traditional seed orchard and nursery approach. The many advantages and recent progress are summarized for conifers by Becwar (in press).

Somatic embryogenesis has been obtained in a number of commercially important conifers, but conversion of embryos to seedlings remains a limiting step. Considerable research has been done on treatments to stimulate germination, especially with Norway spruce (Becwar et al. 1987a, von Arnold and Hakman 1988), but yields of seedlings remain low in proportion to numbers of available embryos. In addition, much labor is required and methods are far from reliable.

Norway spruce somatic embryos produced in our laboratory resemble their zygotic counterparts in terms of most outward characteristics. Even so, we have had variable success in germinating them and securing healthy seedlings in quantity. Factors affecting germination have therefore been the focus of much research in recent times. In this report, we summarize results from a series of five experiments undertaken to promote somatic embryo gernination.

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MATERIALS AND METHODS

Norway spruce somatic embryos used in all experiments were obtained from embryogenic callus line, (NS-1)5. This line was initiated from an immature zygotic embryo, has been maintained in our laboratory for a number of years, and has behaved predictably in extensive tissue culture and biochemistry research. Protocols for initiation and maturation are given elsewhere (Becwar et al., 1987b).

To evaluate treatment effects on germination, presumably mature embryos (cotyledonary stage) were removed from callus clumps growing on maturation media and aseptically placed on 25 mL of germination media in 15 X 100 mm plastic Petri plates. Embryos were laid horizontally on media surfaces in all experiments; however, the fifth trial also tested effects of inserting cotyledons in media.

Germination media consisted of either modified half-strength DCR medium (Gupta and Durzan, 1985) or half-stength MS medium (Murashige and Skoog, 1962) without growth regulators. Modified DCR medium differs from the original formulation in that nitrogen sources were altered to include 25 mg/L glutamine and 25 mg/L NH_4NO_3. Media pH's were adjusted to 5.7 prior to autoclaving. Both media were solidified with 0.7 percent agar and supplemented with 3 percent sucrose.

All experiments included cultures grown in a light culture room, intensity = 24 µE/m2/s from cool white florescent and incandescent lights, photo- = 16 hr light and 8 hr dark, temperature = 21°C and relative humidity = 70 percent. In trials testing exposure to darkness, cultures were maintained in a totally dark culture room for 10 days (same temperature and humidity as the light room) and then transferred to the light room.

Tests of different light qualities and intensities were performed in the light culture room, with spectra altered by wrapping individual Petri plates in a single layer of yellow, red or blue acetate paper (#12, 26 and 65; Rosco Lux Gel, Reimer Photomaterial Co.). Low intensities were achieved by covering plates with a layer of "Kimwipes" tissue. Incident energies were measured at the top of culture plates with a Licor, Intregrating Quantum Photometer/Radiometer (Model #Li-188B).

Germination was scored 21 or more days after treatments were initiated, depending upon when primary roots were 2 mm long. Root growth (mm) was measured at the end of two experiments. Individual replications (Petri plates) contained 20 embryos, and each experiment included two or more replications. Where applicable, percentages or means per replication were subjected to Analyses of Variance, with differences among means contrasted via Duncan's New Multiple Range Test. All tests of significance were at P = 0.05.

Detailed descriptions of designs and treatments are given in the discussion of individual Experiments.

RESULTS AND DISCUSSION

A variety of approaches have been used to stimulate germination of Norway spruce somatic embryos. Media composition has been tested by many workers, light vs. darkness by von Arnold and Hakman (1988), and positioning of embryos on media by Becwar et al. (1987a). Useful leads are also available from other crops. In research on soybean (Glycine max); e.g., workers have used indolebutyric acid (IBA) (Ranch et al. 1986), gibberillic acid (Ghazi et al. 1986), and other growth regulators, either singly or in combination (Lazzeri et al. 1985) as well as desiccation (Hammatt and Davey 1987, Parrott et al. 1988). Furthermore, pulse applications of IBA have been shown to stimulate root initiation on shoots from cultures of Norway spruce cotyledons (Bornman 1983).

Given this background, our first two experiments tested effects of continuous incubation with and 12 hr pulses of varying concentrations of IBA. The experiments were performed on half-strength DCR medium in the light.

Both continuous and pulse application of IBA reduced germination below that observed in the absence of IBA (Tables 1 and 2). Percent germination declined markedly as IBA levels increased. In addition, continuous incubation produced multiple malformed roots at concentrations above 1 mg/L.

Table 1. Germination of Norway spruce somatic embryos after 21 days of continuous incubation with and without IBA*.

IBA (mg/L)	germination (%)
0.0	76.1 a **
0.1	50.5 b
1.0	25.5 c
10.0	18.5 c

* Number of embryos per treatment = 80, 20 in each of 4 replications.
** Means followed by different letters are significantly different, P = 0.05.

Table 2. Germination of Norway spruce somatic embryos at 21 days in response to 12 hr pulses with four IBA concentrations*.

IBA (mg/L)	germination (%)
0	45.0a
1	35.0a
10	21.0b
100	16.0b

* Number of embryos per treatment = 80, 20 in each of 4 replications.
** Means followed by different letters are significantly different, P = 0.05.

Though contrary to expectation, such results are not without merit. Auxins, such Ns IBA, typically stimulate adventitious root initiation and inhibit subsequent root elongation. Depression of germination by IBA therefore provides indirect evidence that the embryos had developed more or less normally. The considerable variability among experiments (76 versus 45 percent) in the absence of IBA, however, suggests that embryos were not all at the same stage of development or that some treatment is necessary to maximize germination.

Our third experiment tested the effects of incubation on different media or combinations thereof in the light, either continuously or after exposure to darkness for 10 days. Work elsewhere (von Arnold and Hakman 1988) indicated that exposure to darkness for three weeks or less fostered germination, and preliminary trials in our laboratory suggested that 10 days of darkness was an appropriate exposure length.

Percent germination peaked 38 days after the experiment began, and major differencs were noted between embryos exposed to darkness and those maintained in the light (Table 3). Results in the light were similar to those from control treatments in the earlier IBA experiments (Tables 1 and 2), and comparable to the best obtained under similar conditions by Becwar et al. (1987a).

Table 3. Percent germination and root growth of Norway spruce somatic embryos at 38 days as affected by various combinations of media and light/dark culture conditions*.

treatments	germination (%)	root length (mm)
Darkness, 10 days, then Light		
1/2 DCR, Only	82	4.0 ± 2.5**
1/2 MS, Only	94	7.6 ± 4.7
1/2 MS (15 days), then 1/2 DCR	100	6.8 ± 4.1
Light Only		
1/2 DCR, Only	50	3.4 ± 3.2
1/2 MS (15 days), then 1/2 DCR	65	2.6 ± 2.2

* Number of somatic embryos = 40, 20 in each of 2 replications.
** Mean ± stan. dev.

Exposure to darkness substantially increased germination, and effects were consistent across media (Table 3). Though smaller, media effects suggested that half-strength MS medium aided germination.

Darkness also tended to increase root growth, but effects were quite variable. Media composition had lesser effects, suggesting that both media are suitable and that transfer among them is unnecessary.

Although differences were not especially large, exposure to darkness and half-strength MS medium gave the best overall combination of germination and root growth. Moreover, such treatments tended to hasten germination, promote root growth, and produce healthier seedlings.

In general, these findings confirm earlier observations about the merits of exposure to darkness (von Arnold and Hakman 1988), and responses exceeded those to various treatments tested earlier in our laboratory (Unpublished data, The Institute of Paper Chemistry, and Becwar et al. 1987a). That germination peaked at 38 days, however, indicates need for even better treatments or improved maturation protocols to ready embryos for germination.

The beneficial impact of dark exposure led to questions concerning possible effects of differing light qualities and intensities. Our fourth experiment thus evaluated responses to yellow, red, and blue light at two levels of intensity in the light room. Tested also were two levels of white light, and dark exposure for 10 days followed by transfer to the lower level of white light. Embryos were cultured on half-strength MS medium, and germination was scored at ²5 days.

Differences among treatments were significant (Table 4), with low yellow light producing the best results. Yellow light and exposure to darkness gave the next best germination, and had approximately equal outcomes. The only other significant difference between intensities within light qualities occurred for the red treatment. Red and blue light produced results more or less equivalent to those observed for white light. Germination both in white light and after exposure to darkness was less than in preceding experiments. This continuedvariability among experiments most likely reflects irregular embryo maturation.

Although not measured, root and shoot growth of embryos given yellow light appeared greater and more uniform than in other treatments. In terms of overall development and vigor, however, seedlings derived from both yellow light treatments and the dark exposure were judged rougbly equivalent.

Collective results from our experiments with darkness and light quality or intensity, viewed another way, suggest that Norway spruce somatic embryo germination is inhibited by certain light qualities. This behavior contrasts with that of Norway spruce seed, which germinate readily in light or darkness (U.S.D.A. Forest Service 1974).

The fifth and last experiment was designed to re-examine some of the best treatments from previous trials - culture on half-strength MS medium and exposure to darkness. Also tested were two ways of positioning embryos on media surfaces; laying them horizontally on the surface (Horizontal) and inserting their cotyledons into the media (Inverted). Positioning was included as a factor inthat numerous workers have found that agar inhibits root growth. Inserting cotyledons in media, with embryos either inverted or harging, was

expected to exploit ability of cotyledons to absorb and convey nutrition to embryos. Earlier work by Becwar et al. (1987a) showed that embryos placed on agar slants or hanging by their cotyledons averaged roughly 50 percent germination, almost twice that of embryos with radicles embedded in the medium. Embryos on agar slants, however, had the best root growth.

Table 4. Germination of Norway spruce somatic embryos as affected by light quality and intensity*.

treatment	germination (%)	irradiance
Darkness for 10 Days, then Low white Light	6².5 c**	5.86
Light Only		
White	²3.8 a	9.13
Low white	31.3 a	5.86
Blue	²1.3 a	1.71
Low Blue	²5.8 a	1.²²
Red	36.3 b	3.73
Low Red	²5.0 a	².51
Yellow	66.3 c	4.86
Low Yellow	8².5 d	².70

* Number of embryos per treatment = 60, ²0 in each of 3 replications.
** Means followed by different letters are significantly different, P = 0.05.

Results largely reflected those from preceding experiments, and clearly confirmed the considerable benefits of exposure to darkness (Table 5). Though significant, media effects were smaller than those of darkness, and somewhat at variance with earlier results. That is, half-strength DCR medium proved significantly better than half-strength MS medium. Once again, however, transfer from the former medium to the latter gave no benefit, indicating that this laborious step is unnecessary.

Embryo positioning effects were also significant, with Horizontal embryos having roughly 10 percentage points greater germination than Inverted ones. Such findings conflict with those of Becwar et al. (1987a) who reported that embryos with cotyledons embedded in medium germinated best. In their experiment, however, embryos were hanging rather than inverted as in the present experiment. Exposure to darkness thus seems the factor of greatest importance, with media composition and embryo position having beneficial but lesser effects.

Table 5. Germination of Norway spruce somatic embryos at 21 days as influenced by various combinations of media, light/dark culture conditions, and embryo position on media surfaces':.

treatments	germination (%) inverted	horizontal	mean
Darkness, 10 days, the, Light			
1/² DCR, Only	86.3a **	93.8a	90.0a
1/² MS, Only	76.3a	67.8b	72.0b
1/² MS (15 days), then 1/² DCR	48.8b	68.0b	58.4c
Light Only			
1/² DCR, Only	43.8b	68.8b	56.3c
1/² MS, Only	30.0c	35.0c	3².5d
Mean	57.0	66.7	61.8

* Number of embryos per treatment = 80, ²0 in each of 4 replictions.
** Means within columns followed by different letters are significantly different, F = 0.05.

CONCLUSIONS

Results of the present work indicate that germination of Norway spruce somatic embryos can be stimulated and enhanced via manipulation of media composition, dark versus light culture conditions, and positioning of embryos on germination media. While such manipulations apparently provide appropriate stimuli, continues variability among experiments, relatively low germination percentages, and long periods of time to maximum germination all suggest that embryos may not have developed uniformly, or that they are not sufficiently mature to respond to the stimuli.

Somatic embryos of Norway spruce and other conifers produced in our laboratory appear to develop normally, and to recapitulate most stages in zygotic embryogenesis. Though smaller, they closely resemble their zygotic counterparts in terms of general morphology and anatomy. Some important differences, however, are apparent, and these may bear on germination and conversion to seedlings. Given current methodology, somatic embryos typically accumulate lesser quantities of storage compounds than zygotic embryos (Feirer et al. 1989), and lack access to these and other compounds from female gametophytes. In addition, they do not experience changes that occur in and around zygotic embryos during the final phases of seed ripening and drying.

In sum, our manipulations apparently provided needed stimuli, but did not substitute effectively for complete maturity, accumulated reserves, or other factors governing readiness for germination. Possible avenues to ensure maturity and readiness include incubation with abscisic acid during the maturation phase (Becwar et al. 1987a), and dessication before germination to simulate natural seed ripening and drying. Research on these and other approaches is underway.

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