

INDUCTION OF VITRIFICATION IN PICEA SITCHENSIS CULTURES

A. JOHN and DEBORAH J. PEARSON

Forestry Commission, Northern Research Station, Roslin, Midlothian, EH25 9SY,
Scotland, United Kingdom

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ABSTRACT

The flooding of *Picea sitchensis* (Bong.) Carr. (Sitka spruce) cultures on hormone-free medium with water resulted in promotion of both apical extension and bud and shoot proliferation. New growth became vitrified within 14 days and the cultures reverted to normal after the water was poured away, with the cultures treated for the longer periods (28–56 days) reverting more quickly. Mean bud and shoot production in cultures submerged for 56 days followed by 28 days in air was 15 compared to two in untreated cultures. Retreatment of the apical portions of the vitrified and reverted cultures by 49 days' submergence followed by 28 days' air resulted in a further increase in mean bud and shoot proliferation to 28. The increases in bud and shoot numbers after submergence were due mainly to the initiation and development of adventitious structures on the vitrified and reverted portions of the stems. Rooting *in vitro* was higher in treated cultures.

Keywords: vitrification; micropropagation; *Picea sitchensis*.

INTRODUCTION

Vitrification is a phenomenon that has been described in many species in numerous publications over the last 10 years. The generalised symptoms for vitrified cultures are translucent stems with leaves that are thickened, turgid, and brittle, with an abnormal glass-like appearance to the culture surfaces. Such cultures are regarded as unsuitable for micropropagation because of poor growth of the shoots, generally accompanied by low rates of multiplication, rooting, and survival on transfer to soil (Vieitez *et al.* 1985).

Culture conditions are usually manipulated to prevent vitrification or to revert cultures to normal if it appears. However, it would seem that vitrification is a condition that might be of benefit during the multiplication of some conifer species *in vitro*. Aitken *et al.* (1981) found that the translucent shoots that formed on vitrified *Pinus radiata* D. Don cultures were slow to elongate but grew into normal shoots if left on the same medium for 2 to 3 months. Aitken-Christie & Thorpe (1985) described three types of shoot that could be formed, i.e., waxy shoots which were similar to seedling shoots, wet shoots which were dark green with the needles stuck together and lacked abundant epicuticular waxes, and translucent shoots which were water soaked and did not survive transfer from *in vitro* conditions. Proliferation was much higher in cultures

in which wet shoots were present although survival after transfer from *in vitro* conditions was poor (Aitken-Christie *et al.* 1985). Bornman & Vogelmann (1984) found that vitrified *Picea abies* (L.) Karst. cultures could produce 10 times as many buds and shoots as normal cultures.

Ziv *et al.* (1983) observed that parts of carnation cultures that were in contact with condensed water droplets became vitrified whereas other portions of the cultures not in contact were quite normal. This suggests that free water within the culture vessel could be part of the induction process.

It was found that vitrification in *Picea sitchensis* shoot cultures on hormone-free medium could sometimes be induced by surface sterilisation, could arise spontaneously, or could be induced by flooding the cultures with water (John 1985a). Regardless of the method of induction, vitrification resulted in enhanced growth and development that was manifested as an increase in bud and shoot proliferation. The cultures spontaneously reverted to normal growth.

The aim of this study was to examine the conditions that would enable induced vitrification to be used during the growth and multiplication of *P. sitchensis* cultures.

MATERIALS AND METHODS

Seedlings were produced and all cultures were maintained in a growth room at 20°C with a daylength of 16h and lit from above at an intensity of 10.3 W/m² (natural fluorescent).

Picea sitchensis seeds 83(1012) were soaked in water for 2 days at 2°C and stored moist in a polythene bag for 21 days at 2°C. The stratified seeds were germinated in compost (John Innes No. 1) in seed trays.

Cultures were established and maintained on full-strength Murashige & Skoog type medium (MS salts; Flow Laboratories) containing 3% sucrose and 1% Oxoid Bacteriological Agar No. 1 at pH 5.5. The medium was dispensed in 25-ml aliquots into 150 × 25-mm flat-bottomed soda glass tubes, completely sealed with an aluminium cap (Oxoid), and sterilised at 103 kPa for 20 min.

Plant material was selected for culture when the hypocotyl was fully extended, the cotyledons were open, the seed coat had fallen, and the epicotyl was just visible. The cotyledons, epicotyl, and 20 mm of the hypocotyl were excised and surface sterilised by stirring gently for 15 min in a 7% filtered calcium hypochlorite solution containing two drops of Tween 80 and rinsed three times with sterile distilled water. The basal 10 mm of the hypocotyl was excised and the culture pushed into the medium until the base of the cotyledons just touched the medium. The tubes were recapped and sealed with "cling-film". The primary cultures were incubated for 42 days and any which were contaminated, dead, or vitrified were rejected.

Normal growth in the cultures was identified by needles that were blue-green, straight or slightly curved, and tapered to a fine point with white stomatal plugs visible to the naked eye on both surfaces. The stem was light green near the apex and light brown near the base. Vitrified growth was identified by the surfaces of the cultures appearing wet. The needles were dark green, bent, twisted, and short, with surface protuberances and no visible stomatal plugs. The stem was more swollen than normal.

Reverted-to-normal cultures were identified as those with the new growth having normal symptoms, proximal to vitrified growth.

The primary cultures were transferred to fresh tubes of basal medium, after excision of the cotyledons and hypocotyl to leave an apical culture 5–10 mm in length. The cultures were inspected for contamination and spontaneous vitrification after 16 days and abnormal cultures were rejected.

Three hundred and sixty non-vitrified cultures were selected at random for treatment. Sterile distilled water was added to each treatment culture to a depth of approximately 10 mm above the apical meristem; control treatments had no water added. The treated cultures were submerged for 2, 4, 6, or 8 weeks, after which the water was poured away and the tubes resealed. Untreated cultures were opened and resealed. Growth and development of the cultures were monitored for 84 days from the start of treatment. Cultures of the four submerged time periods and unsubmerged controls were randomised within each of 72 blocks.

Cultures were selected at the end of the first treatment cycle to give examples of vitrified and reverted-to-normal apices from within each of the previous time treatments. Previously untreated cultures were also selected from the first cycle. The apical 5–10 mm of each culture was transferred to fresh medium. Half of the cultures were selected at random and sterile distilled water was added to each to a depth of 15 mm above the apical meristem, with the remainder of the cultures untreated (Table 1). Replicates of the six culture types ((normal, vitrified, reverted) \times (submerged, air)) were completely randomised within a 10×14 matrix with a double buffer layer of empty capped tubes around the outside.

The sterile distilled water was poured away after 47 days and the tubes resealed. Untreated cultures were also opened and resealed. The growth and development of the cultures were monitored for 76 days from the start of treatment.

The length of the normal, vitrified, and reverted-to-normal zones was measured within each culture and the number and type of buds and shoots present in the various zones were counted in both cycles.

A variety of statistical methods was used to analyse the data.

First treatment cycle

The data were analysed both before and after a $\log_{10}(x+1)$ transformation. The significance of differences in the data was assessed by analysis of variance. However, chi-squared tests were used to make individual comparisons of the spontaneous rooting in the different treatments.

Second treatment cycle

The data for the rate of bud production and extension in the period before the water was poured away and after were analysed after log transformation. Dead or contaminated cultures were ignored in the analysis. Analysis of variance tables were formed using regression statements in GENSTAT that gave adjusted treatment sums of squares for the six treatments not evenly represented (Table 1).

TABLE 1—Treatment of cultures in the second treatment cycle

Apex type	Previous treatment (weeks submerged)	No. of cultures re-submerged	No. of cultures not re-submerged
Normal	0	10	10
	Vitrified	2	8
Reverted	4	4	4
	6	3	2
	8	5	5
	2	10	10
	4	10	10
	6	10	10
	8	10	10

RESULTS

The shoot cultures continued extension at the apex before, during, and after submergence treatment to form three zones, i.e., the normal zone present before treatment and having all the characteristics of normal growth, the vitrified zone induced by submergence, and the reverted zone formed after the water was poured away and having the morphological characteristics of normal growth. The various shoot meristems that developed were the apical meristem that grew to form the three zones, axillary buds and shoots that were initiated in the apical meristem, and pseudobuds and pseudo-shoots (described below) that were initiated on the main stem after submergence treatment.

First Treatment Cycle

The apical regions of 90% to 95% of submerged cultures were vitrified after 14 days and there was some spontaneous vitrification (c. 2%) of axillary shoots in untreated cultures after 42 days. There was no significant difference in vitrification in the 2-, 4-, 6-, and 8-week treatments. Shoots treated for 2 weeks were the slowest to revert to normal growth after the water was poured away (Fig. 1) and the rates of reversion in the 4- and 6-week treatments were also significantly ($p < 0.05$) different.

The promotion of linear extension of the main stem by all submergence treatments was very significant ($p < 0.01$) although extension in the 2-week treatment was significantly ($p < 0.05$) less than in the other treatments (Fig. 2). Untreated cultures were composed of a normal zone only that was significantly ($p < 0.05$) longer than in treated cultures (Fig. 3). The vitrified zone varied in length in treated cultures, with the 4-week treatment significantly ($p < 0.05$) shorter than the 6- and 8-week treatments. The length of the reverted zone was very significantly ($p < 0.01$) shorter in the 2-week treatment than in the other treatments.

Bud and shoot production was promoted by submergence and there was a very significant ($p < 0.01$) increase in production with each increase in duration of treatment

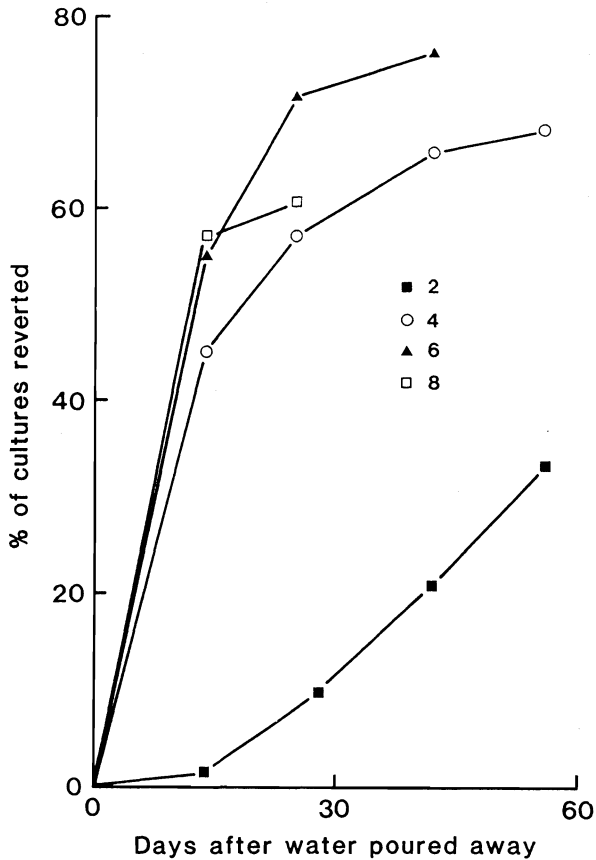


FIG. 1—Reversion to normal growth of the apical meristem of cultures submerged for 2, 4, 6, and 8 weeks, after the water was poured away.

(Fig. 4). Bud and shoot production within the various zones was analysed in detail for the final assessment only, i.e., 84 days from the start of treatment. Axillary bud production was low over-all and within the various zones (Fig. 5). Axillary shoot production was significantly ($p < 0.05$) promoted by submergence but the duration of treatment had no significant effect either over-all or within the various zones. Pseudobud and pseudoshoot formation did not occur either in untreated cultures or in the normal zone of treated cultures. However, with each increase in the duration of treatment, there was a very significant ($p < 0.01$) increase in pseudobud and pseudoshoot proliferation over-all and within both the vitrified and reverted zones. The visual appearance of treated and untreated cultures was different after 84 days. Untreated cultures were similar to seedling shoots with axillary buds and shoots formed at fairly regular intervals along the main stem (Fig. 6a). Treated cultures, in general, showed the symptoms of vitrification with both the vitrified and reverted zones covered with vitrified and normal buds and shoots (Fig. 6b).

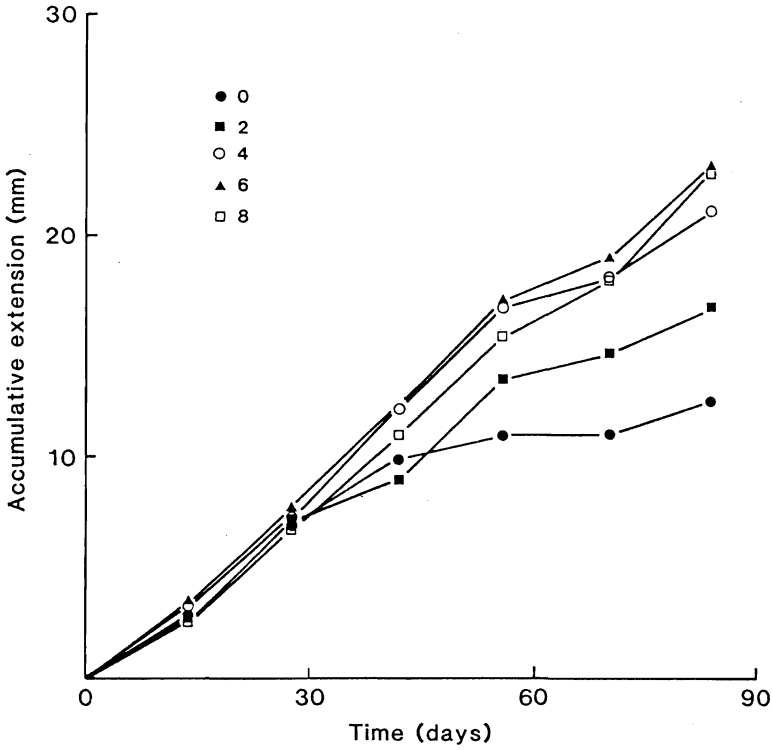


FIG. 2—Accumulative extension of the apical meristem of cultures submerged for 0, 2, 4, 6, and 8 weeks.

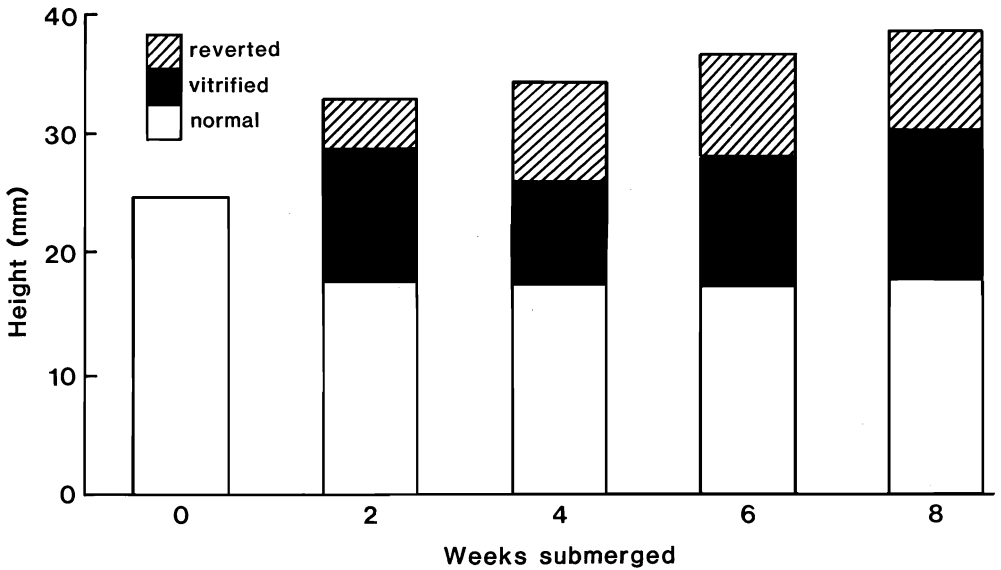


FIG. 3—Mean height of the normal, vitrified, and reverted zones of cultures submerged for 0, 2, 4, 6, and 8 weeks, 84 days after the start of treatment.

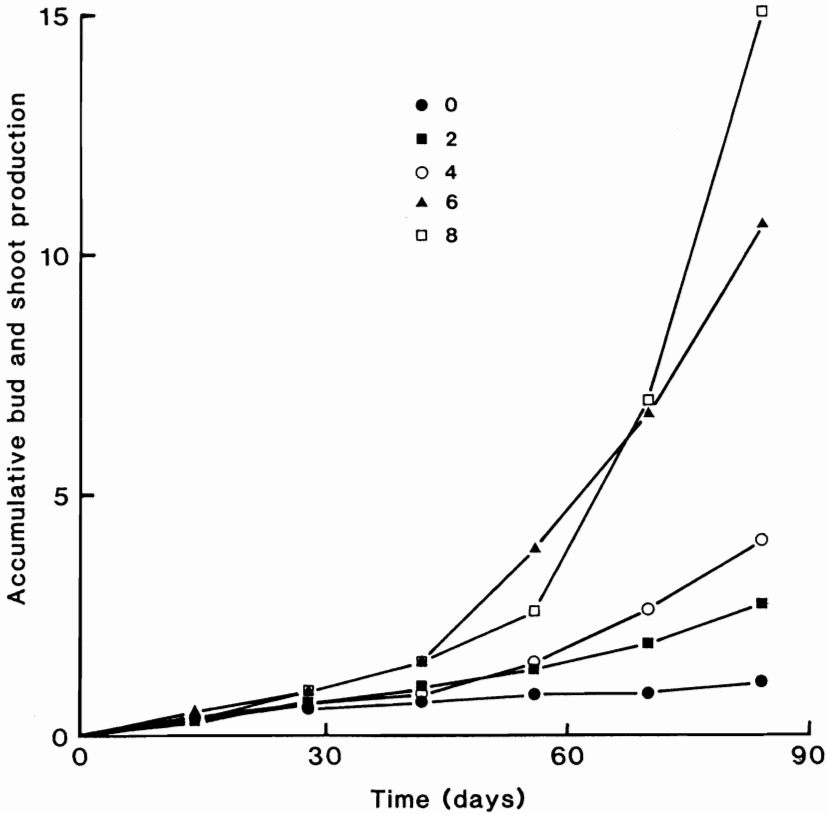


FIG. 4.—Accumulative over-all bud and shoot production in cultures submerged for 0, 2, 4, 6, and 8 weeks.

Axillary buds and shoots were formed throughout the duration of the experiment, whereas there was a lag period of about 4 weeks after the start of treatment before pseudostructures were visible, regardless of whether or not the water in the flooded treatments had been poured away.

The pseudostructures appeared to arise adventitiously from the outer layers of the cortex of the stem, probably in the region of the phellogen. The buds appeared in or near the axils of needles as green globular structures. The bud was composed of a small shoot meristem consisting of small, non-vacuolated cells with large nuclei, with the whole protected by scale-like leaves (Fig. 7a). More scale-like leaves were formed during the early stages of development. However, when the axis of the bud began elongation, the apical meristem initiated primordia that developed into needles typical of those found in cultured plants (Fig. 7b). The initial stages of vascularisation could be seen during the early stages of bud development.

Pseudobuds could arise as normal or vitrified structures. Pseudostructures that appeared under water were always vitrified whereas those that appeared after the water had been poured away tended to be normal.

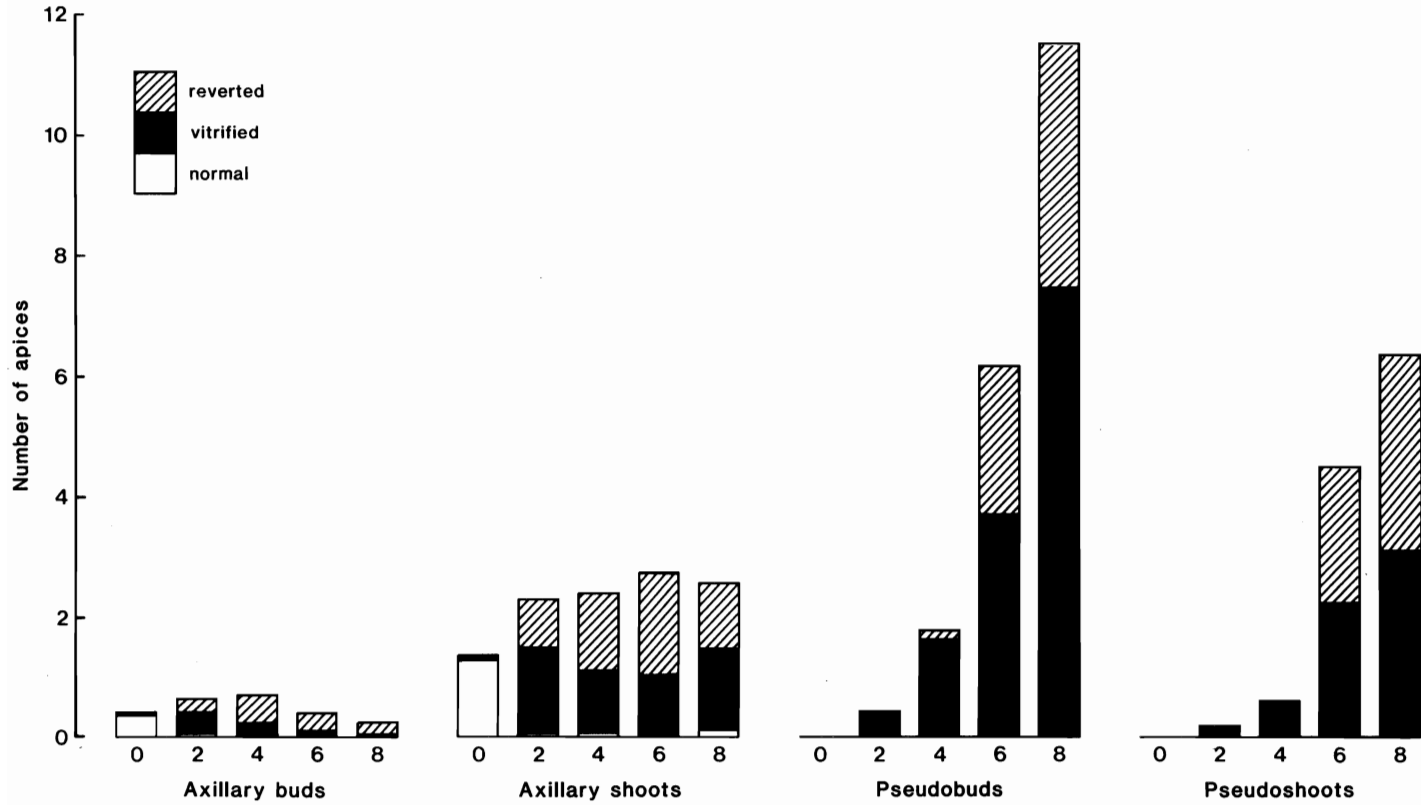


FIG. 5—Mean number of axillary buds, axillary shoots, pseudobuds, and pseudoshoots in the normal, vitrified, and reverted zones of cultures submerged for 0, 2, 4, 6, and 8 weeks, 84 days after the start of treatment.

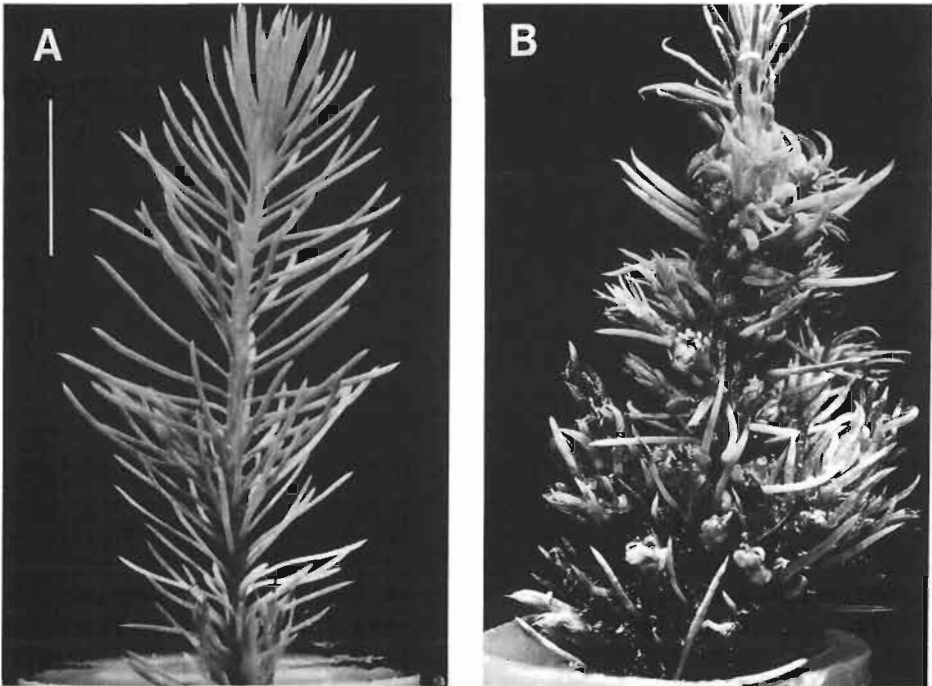


FIG. 6—A: Control, unsubmerged culture after 84 days (bar = 10 mm).
B: Culture treated for 6 weeks, after 84 days.

The data for the classification of cultures as rooted or not rooted after 84 days were analysed using chi-squared tests. Rooting was significantly promoted by submergence and the level of rooting observed in the 2-week submergence treatment (17.1%) was significantly ($p < 0.05$) greater than in the other treatments, i.e., 4 weeks 11.6%, 6 weeks 1.5%, and 8 weeks 5.7%. There was no rooting in untreated cultures.

Second Treatment Cycle

Submergence had no over-all effect on the mean rate of extension of the cultures before the water was poured away (Fig. 8a). The mean rates of extension of initially reverted cultures were not significantly different from each other but both were significantly ($p < 0.05$) different from initially normal cultures. However, both the culture type and the submergence treatment had effects on extension after the water was poured away (Fig. 8b). The mean rate of extension of submerged cultures was significantly greater than that of unsubmerged cultures. Initially vitrified cultures extended at a significantly ($p < 0.05$) faster rate than initially normal cultures but initially reverted cultures were not significantly different from either.

Initially normal and initially reverted cultures had no vitrified zone and when untreated extended to produce apparently normal growth throughout the experiment. Initially vitrified cultures that were untreated reverted to normal growth before the

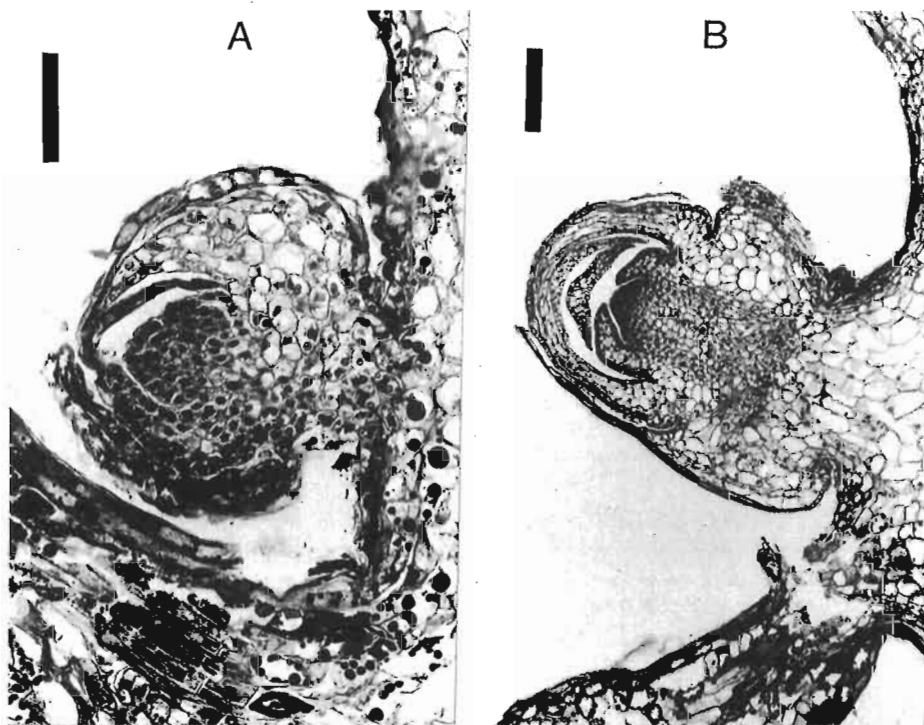


FIG. 7—A: Pseudobud in the axil of a needle with the apex composed of small cells with large nuclei, protected by scale-like leaves (bar = 0.1 mm).

B: Pseudobud in the axil of a needle with the main axis just beginning extension. The apical meristem is producing primordia that will develop into normal, culture-type needles. The beginnings of vascularisation can be seen in the stem axis (bar = 0.2 mm).

water was poured away from treated cultures. All treated cultures became vitrified and reverted to normal growth after the water was poured away.

Initially reverted and vitrified cultures proliferated buds and shoots at a significantly ($p < 0.05$) faster rate than initially normal cultures in the period before water was poured away, regardless of treatment (Fig. 9). However, after the water was poured away, bud and shoot proliferation was significantly higher in submerged cultures, with the condition of the apical meristem at the start of treatment having no significant effect. There were differences in the types and numbers of shoots produced in the various zones before and after the water was poured away (Fig. 10). Bud and shoot proliferation in untreated cultures, before the water was poured away from treated cultures, was low. However, in the vitrified zone of initially vitrified cultures pseudobuds and pseudoshoots were formed and in the normal zone of reverted cultures there was a significant increase in axillary shoot formation. Bud and shoot proliferation was higher in the vitrified zones of all treated cultures owing to increases in pseudobud and pseudoshoot formation in initially normal and vitrified cultures and to an increase

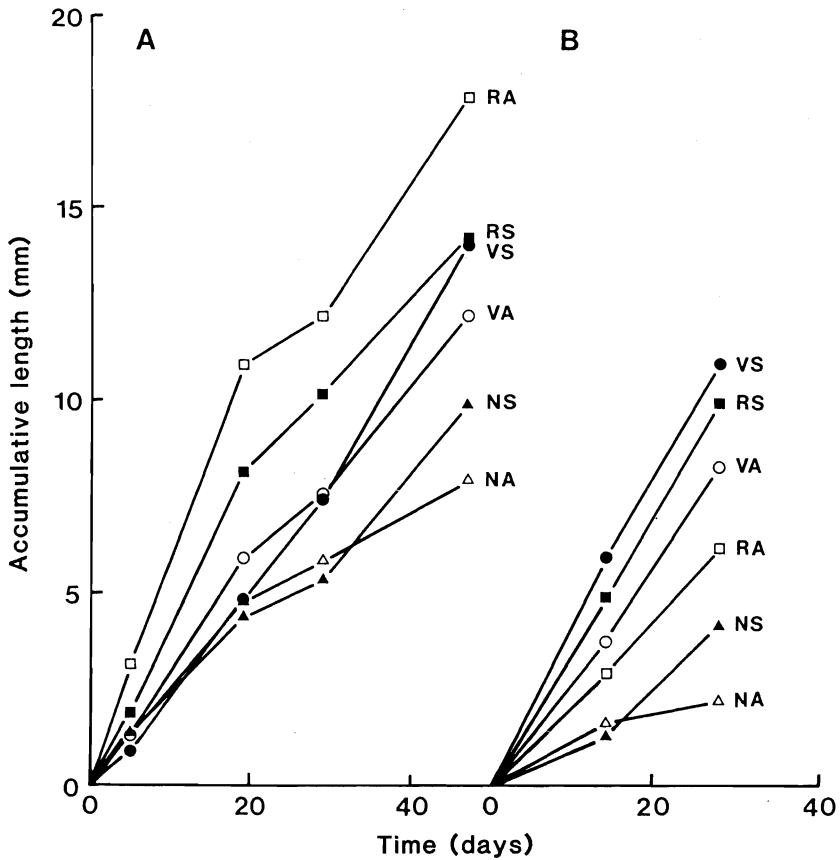


FIG. 8—Accumulative extension of initially normal (N), vitrified (V), and reverted (R) cultures, submerged (S) or in air (A), in the period —
 A: Before the water was poured away.
 B: After the water was poured away.

in axillary shoot formation in initially reverted cultures. Bud production was still relatively low in untreated cultures except in initially vitrified cultures because of the production of pseudobuds and pseudoshoots in the vitrified zone and axillary buds and shoots in the reverted zone. The large increase in bud and shoot production in treated cultures after the water was poured away was due to pseudobud and pseudoshoot production in the reverted and vitrified zones of these cultures.

DISCUSSION

The submergence of shoot cultures of *P. sitchensis* under water resulted in physiological changes that successfully induced vitrification. Growth and development within the various zones formed on the cultures were complex but resulted in increases in both linear extension of the main axis and bud and shoot proliferation. In the first treatment cycle the vitrified zone developed rapidly after the start of treatment and most cultures

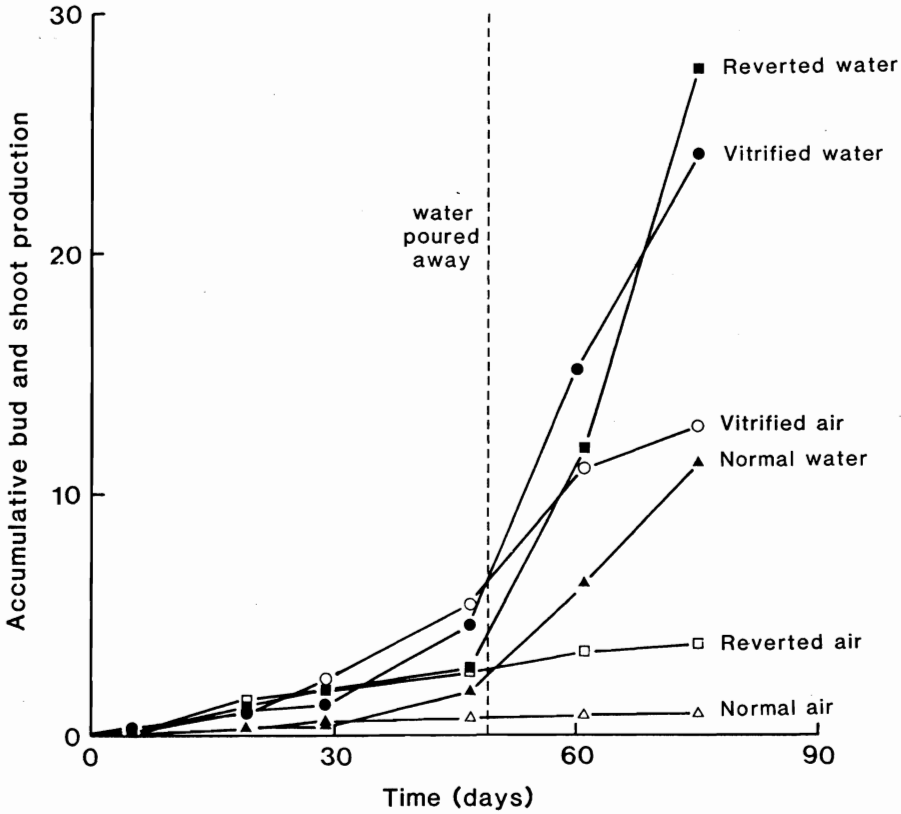


FIG. 9—Accumulative over-all bud and shoot proliferation in treated and untreated, normal, vitrified, and reverted cultures in the second treatment cycle. The water was poured away after 49 days.

reverted to normal growth after the water was poured away. Reversion was more rapid after the longer periods of treatment. Bud and shoot proliferation increased dramatically with increases in the duration of treatment. The greatest contribution to the increased proliferation rate was made by pseudostructures that arose adventitiously on the main stem after prolonged treatment. In general, the increased proliferation occurred on stem tissue that was formed after the start of treatment with little or none on the stem tissue present at the start.

Apex type had a significant effect on the proliferation of buds and shoots in the second cycle. Initially vitrified and reverted cultures proliferated at faster rates after the second treatment than after the first. However, after previously untreated cultures had been treated, the proliferation rate was much lower and comparable with rates observed for the 6- and 8-week submergence in the first treatment cycle. The increase in proliferation rates with retreatment suggests that more treatment cycles may further increase bud and shoot production.

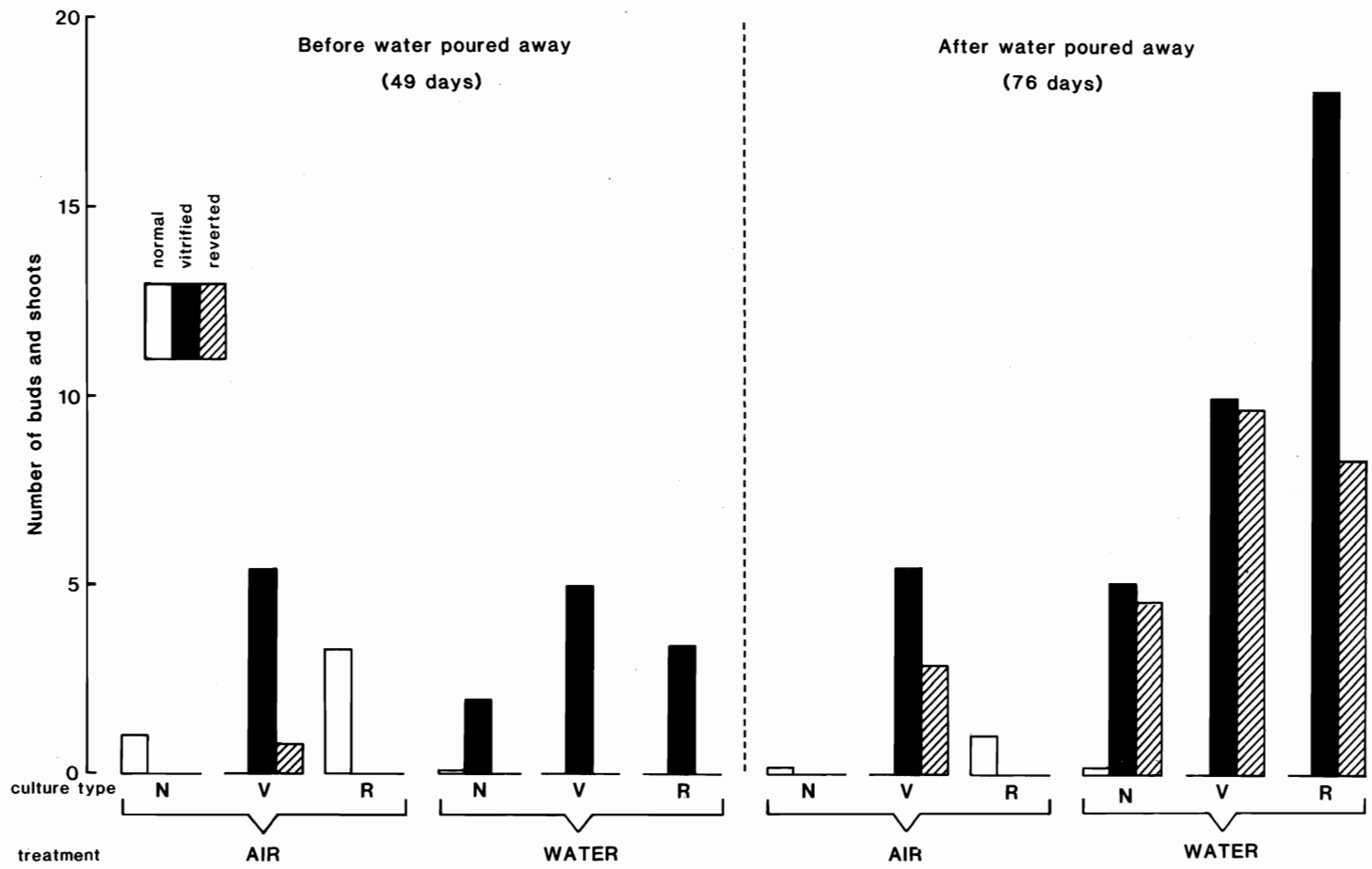


FIG. 10—Mean bud and shoot proliferation in the normal, vitrified, and reverted zones of treated (water) and untreated (air), normal (N), vitrified (V), and reverted (R) cultures in the periods before and after the water was poured away.

Bud and shoot proliferation in initially vitrified and reverted cultures was promoted even without further treatment in the second cycle. This "carry-over" effect from one culture period to the next has been described previously (John 1985a). The increased proliferation in the initially vitrified cultures could have been due, in part, to the outgrowth of pseudostructures that were initiated in the previous treatment cycle whereas that in the initially reverted cultures was due to axillary buds and shoots that developed in the second cycle. The effect of submergence on detached axillary shoots and pseudoshoots has yet to be determined.

The anatomical development of induced pseudobuds and pseudoshoots was similar to that of adventitious buds induced by auxin and cytokinin treatment (von Arnold & Eriksson 1978; Jansson & Bornman 1981). However, the position in which the buds developed was different. Submergence resulted in the *de novo* formation of pseudobuds and shoots along the length of the main stem whereas adventitious bud formation in response to hormone treatment in *P. sitchensis* cultures tends to be on or near the basal callus that forms in response to treatment (Selby & Harvey 1985; A. John, unpubl. data).

Picea sitchensis cultures growing on hormone-free medium are strongly apically dominant. Axillary buds form in the apex and axillary shoots of increasing length are found with increasing distance from the apex (Fig. 6a). Submergence resulted in axillary buds, long and short axillary shoots, large and small pseudobuds, and long and short pseudoshoots that formed all over the vitrified and reverted portions of the main stem. It was not clear whether this apparent disruption of apical dominance was the cause or effect of induced vitrification.

The physiological condition of the cultures becomes extremely important after the transfer from *in vitro* conditions. Aitken-Christie *et al.* (1985) demonstrated that up to 40% of the vitrified shoots of *Pinus radiata* die after transfer from *in vitro* conditions. All cultures rooted outside the culture vessel would need to be reverted to ensure high survival. Apices and axillary shoots of *Picea sitchensis* revert to normal growth after the treatment water has been poured away and many of the pseudoshoots arise as normal. Vitrified pseudoshoots revert to normal growth after excision and transfer to new medium (John 1985b). At present, no information is available on the survival of vitrified or reverted rooted cultures after transfer from *in vitro* conditions. This system of *in vitro* reversion, if it could be applied to other species, would be invaluable, as a high proportion of normal shoots could then be rooted.

The high potential multiplication rates and the reversion to normal growth of the various shoots formed enable this new technique of induced vitrification to be used during the multiplication phase of a micropropagation scheme for *P. sitchensis*. Plant material would be submerged for 7 weeks and maintained in air for 4 weeks. The shoots formed would be transferred to fresh medium and immediately resubmerged. Rooting of reverted cultures, probably under non-sterile conditions, would occur as the last stage of the scheme. Experiments are now in progress to study the effect of up to five cycles of treatment on potential multiplication rates. One of the merits of the system is that only a single, readily available medium is required.

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