PRODUCTION OF NATIVE PLANTS IN TISSUE CULTURE

STEPHEN GARTON AND MEENA S. MOSES

Native Plants, Inc.
417 Wakara Way
Salt Lake City, Utah 84108

Abstract. Tissue culture propagation methods were successfully applied to the production of these native woody plants; *Mahonia repens*, *Alnus oregona* [syn. *A. rubra*], and *Populus tremuloides*. Successful initiation of cultures was strongly dependent on the conditon of the donor plant. Multiplication in all three species was achieved on low salt media supplemented with only a cytokinin. *In vitro* rooting treatments were applied to microshoots obtained from multiplying cultures; for *M. repens* shoots, the medium was unsupplemented; for *P. tremuloides* shoots, rooting occurred on a medium supplemented with IAA; and, In *A. oregona*, rooting occurred in the presence of either IAA or IBA. After *in vitro* rooting, plants were routinely established in a conventional nursery environment.

REVIEW OF LITERATURE

Native plants have emerged as desirable alternatives for use in the home landscape and have a major role in the revegetation and reclamation of disturbed sites. In the production of native woody landscape materials, there is much variability in growth rate, form, disease susceptibility, and stress tolerance when individuals arise from heterozygous seeds. *Mahonia repens*, *Alnus oregona*, and *Populus tremuloides* are examples of native plants which are typically seed propagated and such propagation results in a variable plant populations. Modern production systems can be more resource efficient and products more uniform when the genetic background of a crop is limited as a result of vegetative propagation. Macropropagation has been achieved in quaking aspen by the use of root sprouts (1) but has been of limited applicability in the production of *Mahonia* and alder. Superior individuals were recognized in populations of *Mahonia repens* plants based on growth rate and plant habit; a superior selection of quaking aspen was made based on bark coloration and leaf shape, and a superior red alder was selected based on growth rate. Having recognized elite plants from heterozygous seedling populations there was a need to rapidly multiply these superior individuals. This paper describes the experimentation which was undertaken with the goal of developing micropropagation methods for the rapid multiplication and commercial exploitation of these superior native plants. Both *Populus* and *Alnus* spp. had been previously investigated and shoot tip propagation methods had been described (2, 8). These systems were reviewed and used as a basis for the work.

MATERIALS AND METHODS

a) *Mahonia repens*

Cuttings were obtained from greenhouse grown stock plants. Defoliated stems were rinsed under running water for 15-min. followed by a 10-min. soak in 0.5% aqueous sodium hypochlorite supplemented with two drops of detergent per liter. The stems were rinsed 3 times with autoclaved distilled water. Under aseptic conditions, at a laminar flow hood, shoot tips and lateral buds ranging in size from 2 to 4 mm were transferred, 1 per vessel, to 25 ml aliquots of nutrient medium contained in test tubes. The tubes were placed under fluorescent lamps which provided a light intensity of 2,000 lux for 16 hours per day. The culture room was maintained at 26 ± 2°C. Cultures were routinely transferred at 28 to 35 day intervals.

The basal nutrient medium was composed of ½ strength Murashige-Skoog (7) salts except for iron which was added at full strength. The following organic supplements were included in mg per liter; i-inositol, 100; thiamine HCl, 0.4; pyridoxine HCl, 0.5; nicotinic acid, 0.5; glycine, 2.0; sucrose, 30,000; and Sigma™ agar, 7,000. The pH of the medium was adjusted to 5.8 ± 0.02 prior to dispensing into culture vessels and autoclaving at 121° C for 20 min. Explants were initiated onto either basal medium with no growth regulator supplement or onto basal medium plus 0.3 mg per liter benzyladenine (BA). Cytokinin response studies were carried out with successfully initiated cultures by transferring explants from 0.3 BA medium to basal medium alone, or supplemented with 0.1, 0.3, 1, or 3 mg per liter BA. The BA experiment was maintained for 4 culture cycles during which time all shoots produced per vessel were separated and transferred to fresh aliquots of a similar medium. The quality of multiplied shoots was determined by visual inspection.

The effects of activated charcoal addenda (0, 0.03, 0.01, 0.1, and 0.3%) to multiplication medium supplemented with 1.0 mg per liter BA were determined. Multiplying explants were also placed on media supplemented with charcoal at either 0.1 or 0.3% and BA at 10 or 30 mg per liter. In order to induce root formation, shoots from multiplication were transferred to basal media alone or supplemented with indolebutyric acid (IBA) at 1, 3, 10, or 30 mg per liter. The shoots were transferred to soil after 4 weeks *in vitro*. Both rooted and unrooted shoots were transferred to a peat, vermiculite, perlite (1:1:1) mix and maintained under warm humid conditions for 2 to 3 weeks. After 8 weeks in the soil, surviving plants were evaluated in the greenhouse.

(b) *Populus tremuloides*

Explants were obtained from 3 sources: an individual tree planted in a landscape, root sprouts from the specimen tree, and greenhouse grown plants resulting from *in vitro* grown buds. Soft vegetative shoots were collected and, after removal of the leaves, were disinfested by soaking for 15-min. in 10% Clorox under a partial vacuum. The stems were rinsed 3 times with sterile water. Shoot tips and lateral buds were placed onto 25 ml aliquots of medium contained in test tubes. The basal medium was a modified woody plant medium (WPM) described by Lloyd and McCown (5) supplemented with FeNa-DTPA in place of $FeSO_4$ and NaEDTA, 30 g per liter sucrose, 6 g per liter Sigma™ agar and 1 micromolar BA. The pH of the medium was adjusted to 5.75 ± 0.01 prior to autoclaving. Cultures were incubated on lighted shelves (2,000 lux, 16 hour photoperiod) in a room maintained at 25 ± 2°C.

A benzyladenine dose response experiment was undertaken by transferring successfully initiated explants to either basal medium alone or supplemented with 0.3, 1, or 3 mg per liter BA. The experiment was initiated with 10 explants per treatment and maintained during 5 subculture cycles of 4 weeks duration. Explants were multiplied by division of clumps and placed in culture vessels containing aliquots of similar media. In those treatments where multiplication rates were high, samples of 12 vessels were taken for detailed observation at each subculture.

Shoots were removed from multiplying cultures and placed on rooting medium consisting of ½ strength MS salts except nitrates which were at ¼ strength and iron at full strength. The medium was supplemented with MS organics, 7 g per liter Sigma™ agar, 30 g per liter sucrose, and 0.03 mg per liter indoleacetic acid (IAA). The pH was adjusted to 5.75 prior to autoclaving as described previously. Shoots were also rooted directly from multiplication medium under high humidity conditions maintained by a low volume mist system in a controlled environment tented bench maintained at 25 ± 2°C by hot water bottom heat and illuminated with fluorescent lamps at 3,000 lux for 16 hours per day. The microcuttings were stuck into a pasteurized 1:1:1 peat:vermiculite:perlite mix contained in plastic flats. Plants from *in vitro* rooting medium were planted, when 50% of the shoots showed roots, in similar media and maintained under similar conditions. The plants were gradually acclimatized to the open greenhouse by manipulation of relative humidity, temperature, and light intensity in the plant environment.

(c) *Alnus oregona*

Shoot tips and lateral buds were collected from several

greenhouse grown stock plants. Disinfestation was achieved as described for aspen and explants were transferred to aliquots of modified WPM enriched with 1 micro-molar BA. The cultures were incubated in conditions similar to those for aspen and were transferred at 2 to 3 week intervals to fresh aliquots of medium.

In vitro derived shoots from two rapidly multiplying genotypes were transferred to either ½ MS or WPM supplemented with either IBA or IAA at either 1 or 3 mg per liter. Shoots and plantlets were transferred to soil and acclimatized at described for aspen plantlets.

RESULTS

(a) *Mahonia repens*

Shoot tips developed into single shoots after 2 or 3 weeks incubation whereas lateral buds developed over 4 to 6 weeks. BA was not necessary for shoot development in initial cultures but in the absence of BA, shoots exhibited a pronounced yellow or red pigmentation. Such pigmentation was not apparent in the presence of 0.3 mg per liter BA.

The response of acclimated initiates to various levels of BA in the medium is represented in Table 1. During the first subculture cycle all treatments produced new shoots; however, a BA concentration of more than 0.1 mg per liter was necessary for continued multiplication. The mean values for multiplication rate averaged over the four subculture cycles would indicate no difference between the 1 and 3 mg per liter treatments; however, the shoots produced on the higher level were stunted in height and often developed a yellow or reddish pigmentation. The 1 mg per liter BA medium produced shoots which were of the highest quality.

Table 1. Influence of BA concentration, and time in culture, on multiplication rate in *Mahonia repens* shoot cultures.

Cycle Number	BA concentration, mg per liter				
	0	0.1	0.3	1.0	3.0
1	1.8	1.3	1.5	2.1	2.3
2	1.1	1.0	1.2	1.5	1.4
3	1.0	1.0	1.1	1.4	1.4
4	1.0	1.0	1.3	1.5	1.3
Mean	1.2	1.1	1.3	1.6	1.6

On continued culture on a medium supplemented with 1 mg per liter BA explants multiplied but some pigmentation appeared and shoot height began to decrease. In an attempt to improve shoot quality activated charcoal was added to the medium. The results of such additions are presented in Table 2 and clearly show that the addition of activated charcoal

inhibits multiplication. The shoots produced on charcoal media were tall with well developed unpigmented leaves and were regarded as good quality shoots. However, the addition of BA at 10 and 30 mg per liter in combination with charcoal at 0.1 and 0.3% did not overcome the inhibitory effect of charcoal on shoot multiplication.

Table 2. Effect of activated charcoal supplements on multiplication in *Mahonia repens* cultures on medium containing 1 mg per liter BA.

Cycle	Percent Activated Charcoal			
Number	0	0.03	0.1	0.3
1	2.4	0.9	1.1	1.7
2	1.6	1.2	1.0	0.9
3	1.6	1.0	1.2	1.1
4	1.5	1.0	1.0	1.0
Mean	1.7	1.0	1.1	1.2

The results of adding IBA to media in order to stimulate rooting are presented in Table 3 and show that IBA did not affect root formation during a four week period. However, increasing the concentration of IBA in the medium drastically influenced the survival of shoots when they were transplanted to soil and maintained in the greenhouse.

Table 3. Effect of IBA on rooting of *Mahonia repens in vitro*.

IBA Conc.	Number of Shoots Cultured	Number of Shoots Rooted at 4 wks	Number of Plants After Transfer to Soil
0 mg/l	10	5	7
1.0 mg/l	10	4	4
3.0 mg/l	10	5	5
10.0 mg/l	10	5	1
30.0 mg/l	10	5	0

(b) *Populus tremuloides*

The influence of the stock plant on successful initiation was pronounced. Initiation phase is concerned with the establishment of clean cultures which are capable of multiplication. However, explants taken directly from the select tree cultivated in the landscape resulted in 100% contamination in all initiation attempts. The culture of explants derived from shoots produced on root cuttings cultivated in moist vermiculite in the greenhouse also resulted in contaminated cultures. When shoots from bacterially contaminated cultures were established in the greenhouse and explant tissue was taken from resulting vigorous soft growth, aseptic cultures were obtained.

The results of the BA concentration experiment with multiplying cultures which are presented in Table 4, clearly show

that the low level of BA, 0.3 mg per liter, stimulated the highest multiplication rate in aspen shoot cultures. The quality of the shoots in this treatment was also superior to other treatments in terms of shoot height, appearance of leaves, and general overall color.

Table 4. Influence of BA concentration and time in culture on multiplication rate in aspen shoot cultures.

Culture Cycle Number	Concentration of BA, mg per liter			
	0	0.3	1.0	3.0
1	1.0	1.0	1.0	1.0
2	1.1	3.0	1.1	1.2
3	0.9	2.5	1.4	0.6
4	1.0	3.6	2.5	1.0
5	1.2	3.6	2.7	0.8
Mean	1.04	2.74	1.74	0.9

Aspen microshoots were successfully rooted *in vitro* on a medium supplemented with IAA and were also successfully rooted *in vivo* by sticking into a conventional rooting medium under controlled conditions. In wet situations aspen microshoots were subject to rapid leaf loss and did suffer a severe check in growth. However, plants were rapidly established after an *in vitro* rooting treatment and were easily handled by nursery staff once the plant was acclimatized to the greenhouse environment.

(c) *Alnus oregona*

Alder shoot tips and buds were more difficult to establish in culture than the aspen explants. Alder explants exuded substances into the medium and these substances seemed to be detrimental to explant growth and survival. This exudation of substances compounded the problem of establishing clean vigorous explants during the initiation phase. Figure 1 shows the fate of 160 initial alder shoot cultures during 6 months *in vitro*. It can be seen that after 10 weeks only a small fraction of original cultures remained and only after 22 weeks had the cultures multiplied to the point at which the original numbers were exceeded. However, once multiplication was established, numbers of cultures increased very rapidly indeed. The alder cultures were allowed to form clumps and the division and reculture of clumps was the basis for multiplication.

Microshoots were harvested from shoot-producing clumps and these were either rooted directly in soil under greenhouse conditions, or were put into culture for *in vitro* rooting treatment. Table 5 shows the results of a trial involving the rooting of alder microshoots. Considering plant A, the WPM stimulat-

ed a higher incidence of rooting than ½ MS medium in the presence of both IAA and IBA. However, in the case of plant B, the inhibition of rooting due to ½ MS salts was overcome by the presence of 1 mg per liter IAA.

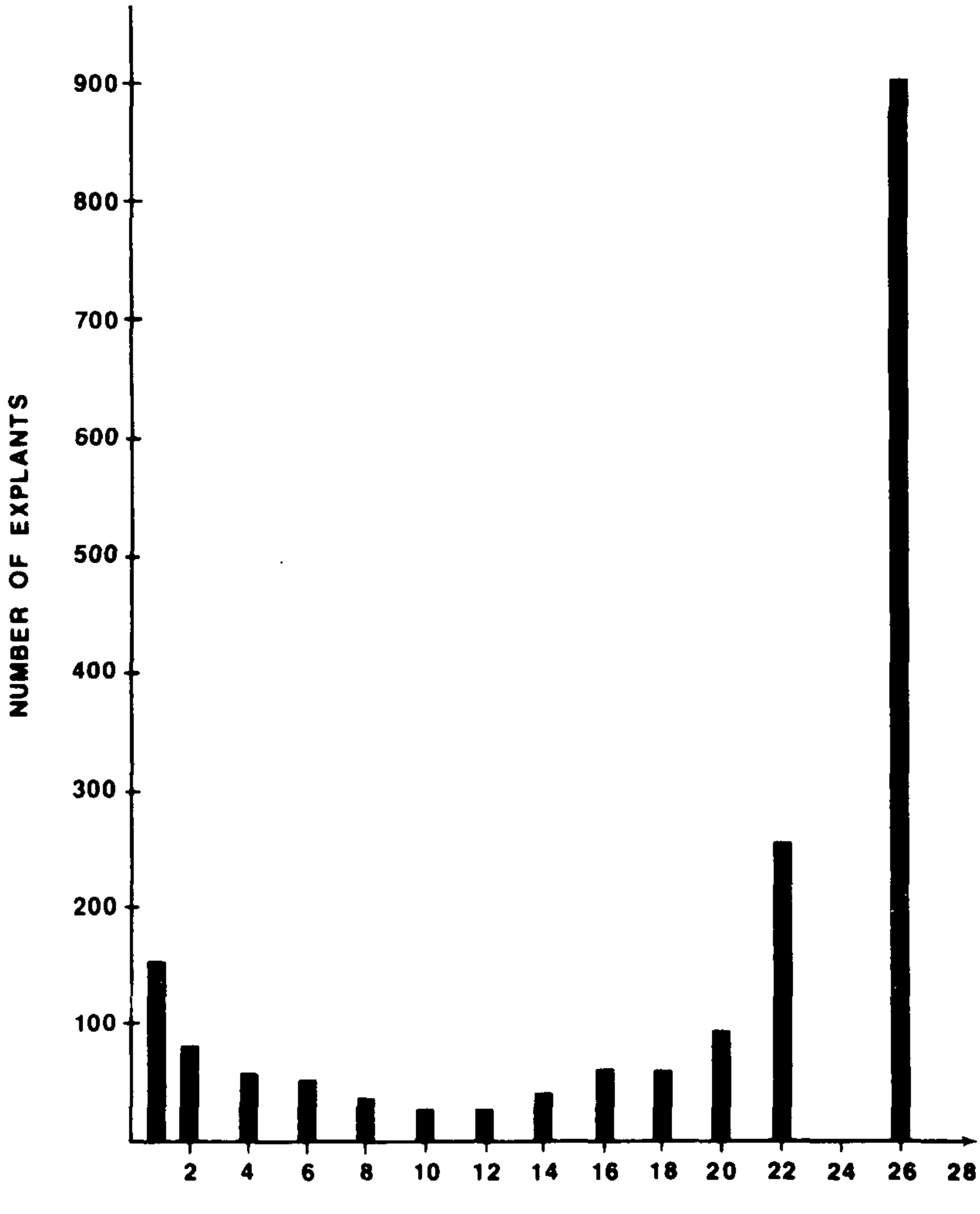

Figure 1. Number of explants vs. time in culture for alder shoot tip and lateral bud cultures.

Greenhouse plants were established from both *in vitro* rooted microshoots and microshoots which had been stuck directly into soil. A higher percent survival was always ob-

tained with *in vitro* rooted plants and such plants required shorter acclimatization periods than did unrooted microshoots. The plant quality was generally superior in plants which had a rooting treatment prior to planting in soil.

Table 5. Effect of basal salt medium and auxin supplements on rooting of alder microshoots from two stock plant sources[1].

	Concentration of IAA, mg per liter		Concentration of IBA, mg per liter	
	1	3	1	3
½ MS Plant A	3	2	3	2
WPM Plant A	9	7	7	9
½ MS Plant B	8	5	6	5
WPM Plant B	9	10	10	8

1 Numbers of microshoots rooted in a sample of 10 from each treatment.

DISCUSSION

The work with the three woody natives resulted in the development of micropropagation systems which were applied to the production of plants for wholesale customers. The observed multiplication rates in *Mahonia repens* cultures were low in comparison to other commercial systems but the alternatives for vegetative propagation of this plant are very limited. In all three species the condition of the stock planted and the presence of new soft, vigorous growth was of paramount importance in establishing not only aseptic cultures but also cultures which maintained themselves during a sometimes protracted acclimation period. Preliminary work involving the initiation of *Mahonia repens* cultures from field-grown material had resulted in either death of explants due to disinfestant damage or contamination of cultures with fungi and bacteria due to incomplete disinfestation. The aspen example serves to illustrate that, if the condition of the original stock plant is not conducive to the establishment of clean cultures, then contaminated shoots, if allowed to grow up as plants in the greenhouse, can produce a stock plant from which clean cultures can be initiated. The aspen tree that was selected as desirable due to its ornamental characteristics was the only tree of its kind from which to begin the propagation program and, therefore, very few alternatives were available other than use of contaminated shoots for the production of new stock plants.

The response of alder initiates to culture *in vitro* serves to illustrate the phenomenon of acclimation which is exhibited when materials are introduced to the *in vitro* environment. This phenomenon has been attributed to a gradual "rejuvenation" of the explant tissues and is typified by almost no multiplication and gradual attrition of initial cultures (4, 6). However, after a 5 month lag period this tissue expressed a rapid

multiplication potential and could be regarded as fully acclimated to the tissue culture system. The lag phase, during which explants slowly adapt to the culture environment, can be a source of concern and is a dramatic contradiction to some of the less informed claims made by inexperienced micropropagators.

In vitro multiplication was achieved in all three species by the addition of only a cytokinin to the multiplication medium. The aspen and alder cultures responded to incubation on a medium containing around 1 micromolar BA whereas the *Mahonia* culture responded to a higher level (approximately 4 micromolar BA) of cytokinin. *Mahonia* cultures were also the least stable during multiplication since these cultures tended to alter in color and form even when cultured on a medium which had previously produced good quality explants. The instability of the *Mahonia* cultures was thought to be due to the presence of autointoxicating substances in the explant since activated charcoal supplements were able to restore shoot quality. However, the presence of charcoal inhibited multiplication probably as a result of adsorption of cytokinin. It is also possible that the instability in these cultures was due to incomplete acclimation.

Shoots which were produced in multiplying cultures were successfully established as plants in the greenhouses either with or without an *in vitro* rooting step. However, under the prevailing conditions, plants which were established after a rooting treatment in culture produced more vigorous plants more rapidly than unrooted shoots under nursery production conditions. The relative merits of the two methods were compared, both horticulturally and economically and the production systems adopted for the three species included the *in vitro* rooting step. The production of roots in *Mahonia* microshoots was unaffected by the level of IBA but subsequent survival of the microshoots was severely prejudiced by an *in vitro* IBA treatment. The effect of IBA on the *Mahonia* microshoots may have functioned to enhance the production of autointoxicants and therefore inhibited future growth, rooting and plant establishment. In alder microshoots there was a strong influence of plant genotype on rooting success and this was particularly apparent if the *in vitro* rooting medium was suboptimal. Such genotypic effects have been noticed previously in the response of plants to *in vitro* culture (3).

The three examples of native plants to which tissue culture methodology was successfully applied serve also to highlight some general principles of commercial micropropagation; i.e. there must be a market for such plants and the market must be willing to bear the added cost of a high technology

propagation system. These added costs can only be justified if the product is competitive with existing alternatives or is superior to existing genotypes in the market place. In the case of aspen the parent plant was superior in landscape value since the tree form and color of bark were selected as outstanding characteristics. In *Mahonia* the limited availability of an alternative vegetatively propagated product made the tissue culture product readily acceptable, and in alder the absence of reliable rapid vegetative propagation systems prompted the use of micropropagation. However, the utility of a particular micropropagation process in an overall commercial enterprise should be carefully evaluated before large investments of resources are committed.

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VOICE: I am interested in the bacterial leaf spot of *Populus*. Is it carried within the tissues of the explants and do your tissue culture techniques give any lasting freedom from this?

STEVE GARTON: I do not think that our techniques would necessarily mean that our plants are disease-free. *Populus tremuloides* in our area is tremendously susceptible to bacterial leaf spot, but our two-year-old tissue-cultured plants seem to be reasonably clean.

STEVE McCULLOCH: *Have you tried meristem culture at* all with quaking aspen?

STEVE GARTON: No, we have not. Meristems are very labor intensive to obtain and they need a considerable time period in culture. For a rapid propagation system we would not want to start with a meristem.

VOICE: Are you propagating for large scale revegetation sites by tissue culture?

STEVE GARTON: No, we are not considering tissue culture at all for revegetation sites, like mines, highways, or pipelines. Such plants would be too costly for those applications. We use tissue culture for perpetuation of selected genotypes for their superior landscape properties. This market, we feel, would carry the added cost of tissue culture propagation.