Research Reports:

In Vitro Propagation of *Viburnum opulus* 'Nanum'[1]

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Abstract

Explants of actively growing shoot tips from greenhouse-grown plants of *Viburnum opulus* 'Nanum' initiated new shoots on a modified Murashige and Skoog (MS) revised medium plus 0.1 mg/L indoleacetic acid (IAA). These shoots were transferred for proliferation to the same medium, but with 1 mg/L 6-benzylamino purine (BA) replacing IAA and the addition of 2.5 mg/L 2-iso-pentenyladenine (2iP). Both adenine sulfate AdS) and $NaH_2PO_4.H_2O$ inhibited shoot proliferation, while gibberellic acid (GA_3) and glycine had no effect. The shoots could be rooted either in the basal medium without cytokinin or in vermiculite under mist.

Index words: *Viburnum opulus* 'Nanum,' *in vitro* propagation, tissue culture, micropropagation

Introduction

The number of woody plants that can be propagated using *in vitro* culture has increased significantly in the last few years and includes rhododendrons (1, 5), birch (9), mountain laurel (8), roses (2, 3, 13) and lilac (4). *In vitro* techniques of micropropagation generally involve the culture of shoot tips or isolated buds on cytokinin-rich media and the production of more shoots through the outgrowth of axillary meristems. These shoots can then be used either to produce more shoots, or induced to form adventitious roots *in vitro,* or rooted as softwood cuttings under mist. This paper reports plant generation from culture of *Viburnum opulus* 'Nanum,' an economically important landscape plant.

Materials and Methods

Explants were removed from actively growing shoot tips of greenhouse-grown plants of *V. opulus* 'Nanum' and soaked successively in 0.1% sterile detergent, 0.5% sodium hypochlorite (10% commercial bleach) with 1 drop of 'Tween 20' per 50 ml, and twice in sterile distilled water. After disinfestation, shoots were trimmed to 3-4 cm (1.25-1.50 in) and Stage I cultures were ini-

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tiated on either solid or liquid media composed of 0.10, 0.25, 0.50 or full strength Murashige and Skoog (MS) salts (12), 3% sucrose and, per liter: 100 mg myo-inositol, 0.5 mg nicotinic acid, 0.5 mg pyridoxine.HCl, 0.5 mg thiamine.HCl, 0.1 mg indoleacetic acid (IAA) and 6 g K.C.Biological Inc., T.C. agar (for solid medium only). Another initiation medium contained 0.25 MS salts, the other addenda and 0, 50, 100, 150 or 200 mg/L $NaH_2PO_4.H_20$. Agar-based medium was dispensed in 10 ml aliquots to 25 x 150 mm test tubes, autoclaved at 1.4 hg/m² and 121 °C (250 °F) for 15 min, then cooled at room temperature for 24 hours before use. The liquid medium was dispensed in 5 ml aliquots and, because of the small volume of medium, the tissue responded favorably without agitation. Shoot tip cultures were incubated at 26-27 °C (79-81 °F) with a light intensity of 56 μE/m²/sec (4500 f.c.) for 18 hours/day unless noted otherwise.

Growing shoots initiated in Stage I cultures were transferred to full strength MS basal medium plus 2.5, 5.0, 10.0 and 20.0 mg/L 2-isopentenyladenine (2iP) for Stage II multiplication. A number of other compounds were used to increase shoot production. As 2.5 mg/L 2iP gave the best results, it was used in combination with 1.0, 2.5, 5.0, 10.0 and 15.0 mg/L 6-benzylaminopurine (BA) and with 0.025 and 0.05 mg/L gibberellic acid (GA_3). In addition, 1 mg/L BA and 2.5 mg/L were used in the medium to which (a) 50, 100, 150 and 200 mg/L $NaH_2PO_4.H_20$, (b) 40, 80, 120 and 160 mg/L adenine sulfate (AdS) and (c) 0.5, 1.0, 2.0 and 4.0 mg/L glycine were added individually. Transfers were made every 6 weeks and it was not until the 4th subculture that sufficient shoots were produced for analysis. With the exception of the effects of GA_3 (Table 1) all the data are from the 4th subculture.

Rooting was accomplished *in vitro* by culturing shoot tips on multiplication medium containing (a) 2.5 mg/L 2iP plus 0.25, 0.5, 1.0, 2.5 and 5.0 mg/L naphthalene acetic acid (NAA), (b) no cytokinin and 0.5, 1.0, 2.5 and 5.0 mg/L NAA, (c) no cytokinin and 0.5, 1.0, 2.0, 4.0 and 8.0 mg/L indolebutyric acid (IBA), (d) no cytokinin and 2.5, 5.0 and 10.0 mg/L IAA. Alternatively, the base of shoots of various sizes was treated with either 0.1 or 0.3% IBA talc and placed in sterilized moistened vermiculite under mist (8 sec every 2 min) during a 15 hour day with cheesecloth shading the shoots for the first 7 days. Other shoots went into sterilized vermiculite in flats covered with a plastic lid and placed in a growth room at 26 °C (79 °F), 56 μE/m²/sec (4500 f.c.) for 16 hours/day.

After 4 weeks rooted shoots were transplanted to a flat containing a mixture of peat, perlite and vermiculite (1:1:1 by vol) and placed next to the other shoots in the mist bed. After 3-4 weeks in the mist bed, shoots were moved to a shade house and gradually exposed to full sunlight.

An analysis of variance was used to analyze the data. If the F test indicated a significant difference in either

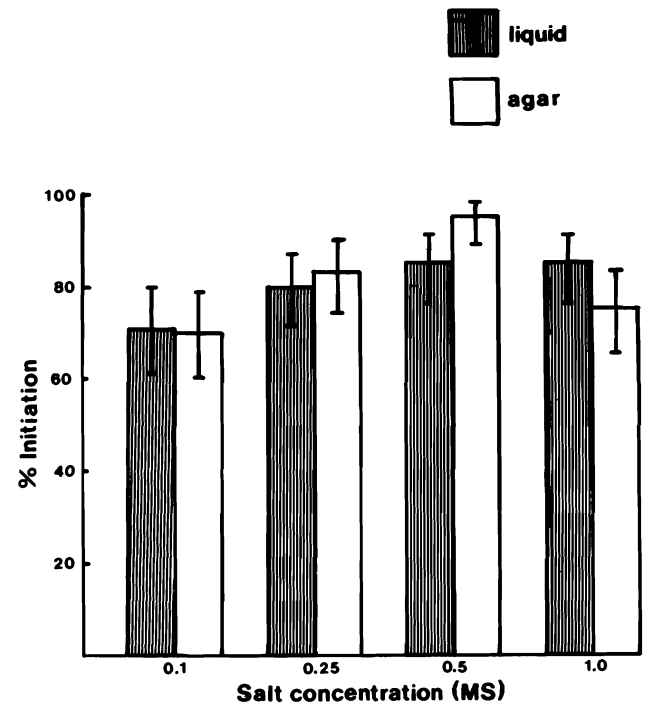

Fig. 1. Effect of varying salt concentrations in liquid and solid media on explant initiation in *Viburnum opulus* 'Nanum.' Vertical lines represent 95% confidence limits.

Table 1. Multiplication of *Viburnum opulus* 'Nanum' explants[z] from different nodes with 2.5 mg/L 2iP and varying concentrations of GA_3.

GA_3 (mg/L)	Nodes 1	2	3	4
	No. shoots/explant			
0**	2.6 (2.1-3.1)[y]	1.8 (1.4-2.3)	1.2 (0.8-1.7)	1.6 (1.1-2.1)
0.025	2.6 (2.2-3.1)	1.0 (0.5-1.5)*	0.8 (0.4-1.2)	1.0 (0.4-1.6)**
0.05	1.8 (1.4-2.2)	1.1 (0.7-1.6)*	0.8 (0.4-1.2)	1.0 (0.5-1.5)**
	Shoot length (mm)			
0**	34 (31-36)	32 (29-36)	30 (26-34)	30 (26-33)
0.025	29 (26-32)	32 (27-37)	24 (19-28)	25 (19-30)
0.05	28 (25-31)	24 (20-29)	24 (19-29)	24 (18-29)

[z]third passage in multiplication medium
[y]95% confidence interval
*significant at P = 0.05 (ANOVA)
**significant at P = 0.01 (ANOVA)

shoot number or shoot length, general or simple contrasts of the means were analyzed. Each treatment was replicated by obtaining data from at least 20 cultures. Every experiment was repeated at least once.

Results and Discussion

Shoot Initiation. After 4-5 weeks, 70-95% of the explants initiated shoots in all media (Fig. 1). Neither the salt concentration nor the physical condition of the medium had any effect on shoot initiation. This was contrary to findings of Anderson (1) that half-strength MS produced optimum shoot initiation in rhododendron and of Lloyd and McCown (8) that more shoots initiated in liquid than in solid medium. Similarly, there was no beneficial effect of $NaH_2PO_4.H_2O$ with 90% (82-95) initiation at all concentrations tested compared to 86% (78-92) for the control. Miller and Murashige (10) had found phosphate to increase shoot initiation in several tropical species.

Shoot Multiplication. Contamination and tissue necrosis often occurred in the first culture on multiplication media with few explants producing more than one shoot. With the second transfer half of the explants produced more than 2 shoots in media containing 1-20 mg/L 2iP or 1-15 mg/L BA. If the explants were inverted as Lane (7) did with pears there was no increase in shoot formation and the shoots that did form were twisted. Shoots incubated in a light intensity of 17 $\mu E/m^2/sec$ (1500 f.c.) were tall, spindly and etiolated whereas those at 57 $uE/m^2/sec$ (4500 f.c.) had shorter internodes and looked much healthier. By the 4th transfer, after 6-8 weeks in culture, the best results were obtained with 2.5 mg/L 2iP. The apparent lag between the initial culture and rapid proliferation in the 3rd or 4th subcultures has also been reported for lilac (4) and mountain laurel (8) cultures. Subculturing explants over a number of months increased the number of shoots harvested until a maximum level of 8 or 9 shoots was reached in 6-8 months. Since apical dominance was a problem, 1, 2, 3 or 4 node explants were separated from growing shoots and incubated on medium with or without GA_3 (Table 1). By general contrast, single node explants produced greater number of shoots than did 2, 3 or 4 node explants. Gibberellic acid had no effect on shoot production in single node explants and decreased shoot production in 2 and 4 node samples. Unexpectedly, shorter shoots were obtained in GA_3 medium than in medium without GA_3, which is contrary to its usual effect (11).

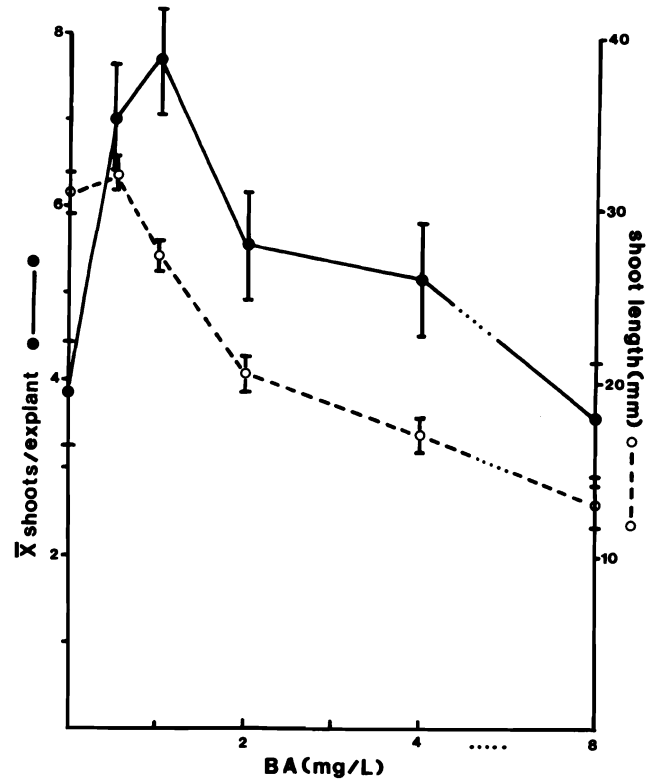

Fig. 2. Effect of varying concentrations of BA with 2.5 mg/L 2iP on shoot production and shoot length in *Viburnum opulus* 'Nanum.' Vertical lines represent 95% confidence limits.

The use of BA in combination with 2iP increased the number of shoots produced with the optimum concentrations being 0.5 and 1.0 mg/L BA (Fig. 2). The length of the shoots decreased significantly at concentrations of BA greater than 1.0 mg/L. An inverse relationship between shoot number and shoot length had been noted previously with other species including lilac (4) and mountain laurel (8).

The addition of $NaH_2PO_4.H_2O$ to the multiplication medium decreased shoot number, while glycine did not significantly affect multiplication (Table 2). The addition of AdS was toxic at the concentrations tested. The number of shoots produced was halved by the addition

Table 2. Effect of $NaH_2PO_4.H_2O$ or glycine concentration on shoot multiplication in *Viburnum opulus* 'Nanum.'[z]

	$NaH_2PO_4.H_2O$ (mg/L)				
	0	**50**	**100**	**150**	**200**
X shoots/explant	8.6* (6.4-10.7)[y]	4.6 (2.6-6.6)	7.0 (5.2-8.8)	4.4 (2.6-6.2)	4.8 (3.0-6.6)
	glycine (mg/L)				
	0	**0.5**	**1.0**	**2.0**	**4.0**
X shoots/explant	5.6 (3.9-7.3)	6.0 (4.4-7.7)	7.1 (5.5-8.7)	7.4 (5.7-9.1)	8.5 (6.8-10.2)

[z]2.5 mg/L 2iP, 1.0 mg/L BA

[y]95% confidence interval

*significant at P = 0.05

Table 3. Rooting of *Viburnum opulus* 'Nanum' shoots treated with 0.1% and 0.3% IBA in either a mist bed in the greenhouse or in a plastic covered flat in the growth room.

shoot length (mm)	Percent Rooting	
	In mist bed	In plastic covered flat
0.1% IBA talc		
20	75% (65-83)[z]	37% (28-47)
20-40	80% (71-87)	68% (58-77)
40	55% (45-65)	52% (42-62)
0.3% IBA talc		
20	52% (42-62)	57% (47-67)
20-40	80% (71-87)	32% (23-42)
40	83% (74-90)	28% (19-38)

[z]95% confidence limit

of 40 mg/L, and 120 and 160 mg/L AdS killed all explants.

Root Initiation. About half the shoots in medium containing 2.5 mg/L NAA and 2.5 mg/L 2iP rooted within 4 weeks. However, this number dropped when the NAA concentration was 5.0 mg/L (Fig. 3). Also, as the auxin concentration increased, more callus formed and more roots formed on the stem above the surface of the agar. Lane (5) had found a short incubation in a rooting medium with high NAA optimal for rooting apple shoots. After 4 weeks in a mist bed, roots formed on up to 100% of the shoots that had not rooted in the NAA, 2iP medium. Approximately 70-80% of the viburnum shoots rooted *in vitro* in a medium containing NAA and no cytokinin (Fig. 4). IBA and IAA were less effective for root initiation, as 40% was the highest rate of rooting with the former and 28% with the latter. In lilac cultures, healthier plants were produced in medium containing a small amount of BA as well as NAA (4).

Depending on the size of the shoots and the concentration of IBA in the talc dusted on them, approximately 80% rooted in the mist bed. Only about 50% of the shoots over 40 mm dipped in 0.1% IBA talc and those under 20 mm dipped in 0.3% IBA talc rooted (Table 3). Rooting of similar shoots in the covered flat was generally less successful due to a fungal infestation. It is possible, however, in the case of smaller shoots treated with a high IBA talc and the larger shoots treated with a low IBA talc that the problem may lie with the auxin concentrations. Too high a concentration

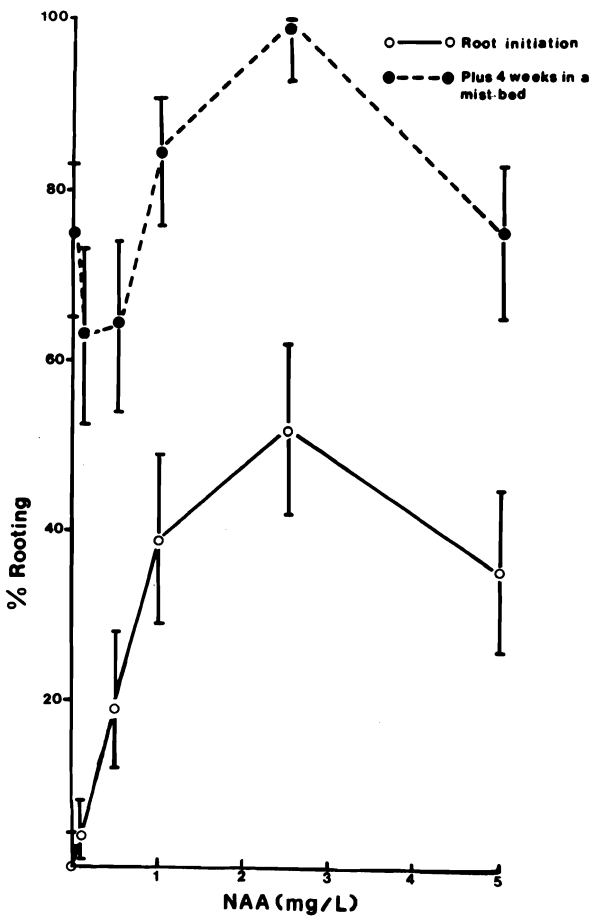

Fig. 3. Rooting of *Viburnum opulus* 'Nanum' shoots in media containing 2.5 mg/L 2iP and varying concentrations of NAA. Also, rooting in mist of shoots that had not rooted *in vitro*.

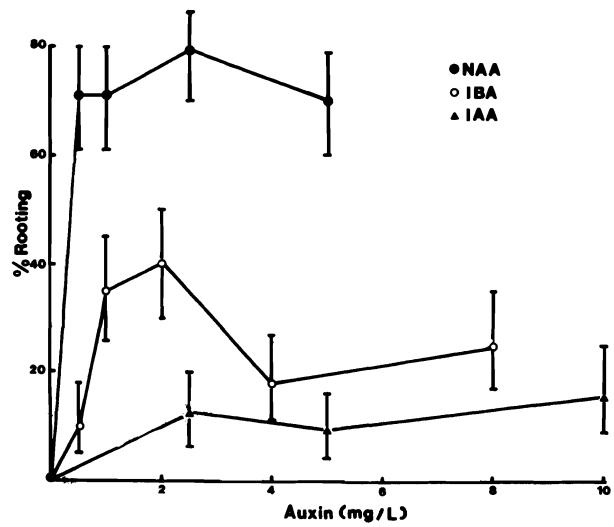

Fig. 4. Rooting of *Viburnum opulus* 'Nanum' shoots in media containing no cytokinins and varying concentrations of NAA, IBA or IAA.

may injure the succulent tissue of the smaller shoots whereas the lower concentration may not have been strong enough to induce roots on larger more mature shoots. In the case of the smaller shoots, at least, the fungal infestation may have been secondary. It is noteworthy that, although about the same proportion of shoots rooted *extra vitrum* as *in vitro,* more have rooted *extra vitrum* in a shorter time.

The following technique is recommended for successful micropropagation of *V. opulus* 'Nanum':

Stage I (initiation)—actively growing shoot tips from greenhouse grown plants onto modified MS plus 0.1 mg/L IAA, incubated at 26-27 °C (79-81 °F) with a light intensity of 57 μE/m^2/sec (4500 f.c.) for 18 hr/day.

Stage II (multiplication)—transfer to MS plus 1 mg/L BA and 2.5 mg/L 2iP, same light and temperature as Stage I.

Rooting—20-40 mm long shoots in vermiculite in mist bed in greenhouse. Dust base of shoot with 0.3% IBA in talc. Mist on 8 sec every 2 min.

Significance to the Nursery Industry

Although most explants initiated shoots in 4 to 5 weeks only one shoot was produced per explant. It was not until the 4th subculture, after 6-8 months, that approximately 7 explants were produced per shoot. However, once this lag was overcome, the multiplication rate was high with the possibility of producing 2400 plants from one shoot in a year.

There are a number of advantages of *in vitro* culture for woody ornamentals, in spite of the aforementioned lag in the production of large numbers of shoots in some species. These include propagation at any time of the year and the rapid proliferation of plants that may be difficult to multiply vegetatively by more traditional methods. In addition, techniques not described in this paper, including heat treatment, meristem culture and indexing, lead to the production of disease-indexed plants.

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