

Adventitious Shoot Production from a *Vireya* Hybrid of *Rhododendron*

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Abstract. An efficient adventitious shoot production protocol has been developed for *Rhododendron laetum* × *aurigeranum*. Shoot tips taken from greenhouse-grown plants were cultured on Anderson's medium supplemented with 74 μM 2iP. Axillary shoots were excised and cultured on medium containing 23 μM IAA and 74 μM 2iP. After 6 months, brown callus developed at the cut surfaces of the shoot-tip explants. This callus produced many adventitious shoots (up to 70 per explant). Clusters of adventitious shoots were divided, subculture, and continued to proliferate shoots. An estimated 1600-fold increase in the number of shoots could be readily achieved in 6 months. In vitro rooting of adventitious shoots was accomplished in 4 weeks. Seventy-three percent of shoots rooted on 1/4 strength Anderson's medium supplemented with 28 μM IAA. Plantlet survival was 100% 3 weeks after transfer to soil. Chemical names used: 1-H-indole-3-acetic acid (IAA); N-(3-methyl-2-butenyl)-1H-purine-6 amine (2iP).

The genus *Rhododendron*, section *Vireya*, comprises ≈ 300 species endemic to Southeast Asia (Sleumer, 1966). Almost half of the 300 *Vireya* species, including the species *R. laetum* J.J. Smith and *R. aurigeranum* Sleumer, are native to New Guinea, which lies between lat. 2° and 12°S. According to the Royal Horticultural Society (Leslie, 1980), *Vireya* rhododendrons are classified as "normally requiring greenhouse protection during the winter months". Those species native to low altitudes in New Guinea are irreparably damaged if exposed, even for a short time, to temperatures below 0°C (Withers, 1984). Since *Vireya* rhododendrons do not require a resting period, they may flower at any time of the year. Therefore, they may have potential as greenhouse-grown ornamental plants. Moreover, they represent a source of breeding material for the improvement of hardy rhododendron flower quality. *Vireya* flowers range from < 1 cm to 10 cm in length and have a variety of colors, from white to yellow, from orange to deep red; the flower has diverse shape, from tubular

form to semi-open bells (Williams and Rouse, 1983). Within the section *Vireya*, the hybrid *R. laetum* × *aurigeranum* deserves particular attention because of its long-lasting golden yellow flowers that are not found in most common cultivated hardy rhododendrons.

Many species of *Rhododendron* are currently propagated by tissue culture (Chee, 1985). However, there are no reports concerning the micropropagation of *Vireya* species. The development of a micropropagation system would facilitate mass propagation of these species. The objective of this research was, therefore, to develop a protocol for the propagation of the *Vireya* hybrid *R. laetum* × *aurigeranum*.

Establishment of shoot-tip cultures. Stock plants of the *Vireya* hybrid *R. laetum* × *aurigeranum* were grown in 7.7-liter plastic pots containing a 1 perlite :1 peat :1 sandy soil (by volume) mixture. The greenhouse was at 26 to 32°C. Plants were watered as needed to keep the medium moist.

Actively growing apical shoot tips, 7 to 8 cm in length, were collected from the stock plants. All leaves, except those immature leaves enclosing the apical meristem and the leaf primordia, were removed. Shoot tips were rinsed under running tap water for 20 min. The explants were submersed for 2 min in 70% ethanol, followed by soaking for 40 min with gentle agitation in 1.05% NaOCl containing 20 drops/liter of Tween 20. After being rinsed three times in sterile distilled water, the basal ends were removed and shoot tips (≈ 5 cm long) were placed horizontally on

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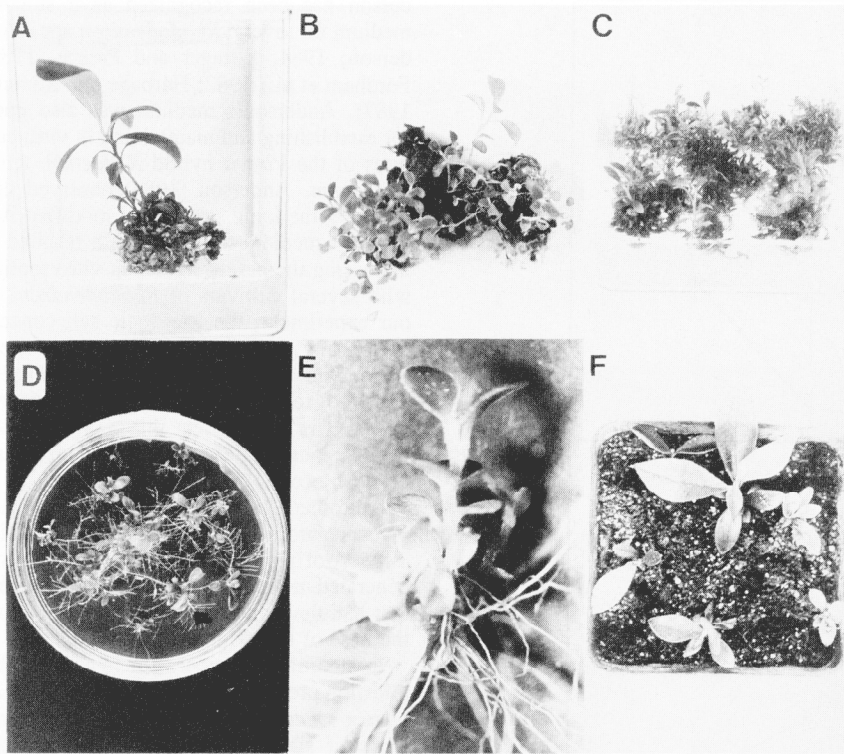


Fig. 1. Adventitious shoot formation from callus of *R. laetum* × *aurigeranum* and rootings. (A) Organogenic callus induced at the base of an axillary shoot cultured on Anderson's medium supplemented with IAA and 2iP. (B) Cluster of shoots with small amount of callus proliferated in fresh Anderson's medium with 23 μM IAA and 74 μM 2iP. (C) Proliferation of organogenic callus clumps consisting of adventitious shoots and callus tissues. (D) In vitro rooting of adventitious shoots on 1/4-strength Anderson's medium supplemented with 28 μM IAA, after 1 month in culture. (E) Plantlets with regenerated root system before transfer to soil. (F) Micropropagated plants 2 months after transplanting to soil.

the surface of various media. Explants were cultured on revised Anderson's medium (Anderson, 1984) containing 88 mM sucrose, 560 μM inositol, 200 μM adenine sulphate, and 8 g Difco Bacto agar/liter. A 5 × 4 factorial experiment, with all possible combinations of five concentrations of IAA (11 to 34 μM) and four concentrations of 2iP (49 to 172 μM), was used to determine the optimum growth regulator combination for the elongation of axillary shoots. Growth regulators were added to the media before autoclaving. After adjusting the pH to 4.5 and adding agar, media were autoclave at 121C for 20 min. Fifty milliliters of medium were dispensed into each sterilized GA7 vessel (Magenta Corp., Chicago).

Explants, one per vessel, were transferred into each of the various media tested. Vessels were sealed with parafilm and incubated at 25 ± 1C with a 16-h photoperiod and 50 μmol·s⁻¹·m⁻² provided by cool-white fluorescent lamps. A completely randomized block design was applied. Five vessels were used for each treatment combination. Treatments were replicated three times over time. Cultures were evaluated for the number of axillary shoots (> 2 mm in length) after 3 months of culture. The data were subjected to analysis of variance (ANOVA).

Axillary buds started to grow after 1 month

of culture. Although the most axillary shoots were induced on medium supplemented with 23 μM IAA and 74 μM 2iP (5.3 shoots per shoot-tip explant), there was not a significant interaction between IAA and 2iP. Furthermore, IAA was not required to induce axillary budbreak, as there was no difference between the media containing 0 to 23 μM IAA. Axillary bud proliferation was significantly induced by 74 μM 2iP, whereas lower 2iP concentrations only stimulated main shoot growth (data not shown). Similar results were observed by Anderson (1984) in the cultivar Scintillation, by Fordham et al. (1982) in 'Exbury' azaleas (*Rhododendron* spp.), and by Douglas (1984) in several cultivars of *Rhododendron* grown in Europe. Conversely, lower levels of 2iP were reported to be effective for some rhododendrons (Blazich et al., 1986; McCown and Lloyd, 1983). The function of 2iP and other cytokinins is thought to reduce apical shoot dominance and, consequently, induce axillary shoot proliferation (Economou and Read, 1984).

Anderson (1984) and McCown and Lloyd (1983) reported that IAA and 2iP concentrations above certain limits are not effective and/or can be detrimental in inducing *Rhododendron* multiple shoot formation. This is consistent with our results using *R. laetum* × *aurigeranum*. IAA and 2iP concentrations

>23 and 74 μM, respectively, did not increase the number of axillary shoots (data not shown). Axillary buds of *R. laetum* × *aurigeranum* are clustered at the apex of the shoot-tip explant. These buds remain inhibited unless an exogenous stimulus is provided.

Shoots developed from callus proliferated from axillary shoot-tip explants. Axillary shoots were excised and placed on medium supplied with 23 μM IAA and 74 μM 2iP. Brown, friable callus formed at the basal end of the axillary shoots. After ≈ 6 to 7 months without subculturing, many adventitious shoots were produced from the calli (up to 70 per explant) (Fig. 1A). Clusters of adventitious shoots with small amounts of callus (Fig. 1B) were divided and subculture every 2 months onto fresh medium supplied with 23 μM IAA and 74 μM 2iP. This procedure resulted in the greatest average shoot proliferation per callus (≈ 25 shoots per cluster) (Fig. 1C). Similar observations were made by McCown and Lloyd (1983) on *R. canadense*, *R.* × 'Boule de Neige', and *R.* × 'PJM'. We estimated that once organogenic callus is induced, a 1600-fold increase in shoot count in 6 months can be obtained. This protocol represents the most successful method of micropropagating the hybrid *R. laetum* × *aurigeranum*. However, uniformity and genetic stability of the plant material must be carefully evaluated before such a propagation method can be adopted.

Shoot proliferation from adventitious shoots. Individual adventitious shoots ranging from 2 to 10 mm were excised from callus clusters and placed into 10 × 60-mm petri dishes containing 10 ml of Anderson's medium supplemented with various combinations of IAA (11 to 34 μM) and 2iP (49 to 172 μM) concentrations. A completely randomized design was used, with five replicates per treatment. A replication was defined as a petri dish containing four single shoots. Explants were subculture every 2 months, and the number of newly formed shoots per individual shoot explant was recorded after 6 months of culture. ANOVA was performed to evaluate the effects of each main effect, as well as interactions.

A small amount of callus and green shoot primordia developed from the base of individual adventitious shoots after 2 months in culture. The primordia differentiated into small leaves. New shoots were distinguished as they emerged from the mass of leaves after 6 months of culture. There was no significant difference among 2iP treatments, nor was there any interaction between the growth regulators. However, 34 μM IAA with all concentrations of 2iP was most effective for inducing new shoot formation from the basal end of single shoots (7.4 shoots per single shoot), compared to lower levels of IAA (data not shown). The average number of shoots per single shoot obtained in this experiment (about seven shoots per cultured shoot) was considerably smaller than that obtained when clumps of shoots with small amounts of callus were routinely subculture (≈ 40 shoots per cultured shoot clump).

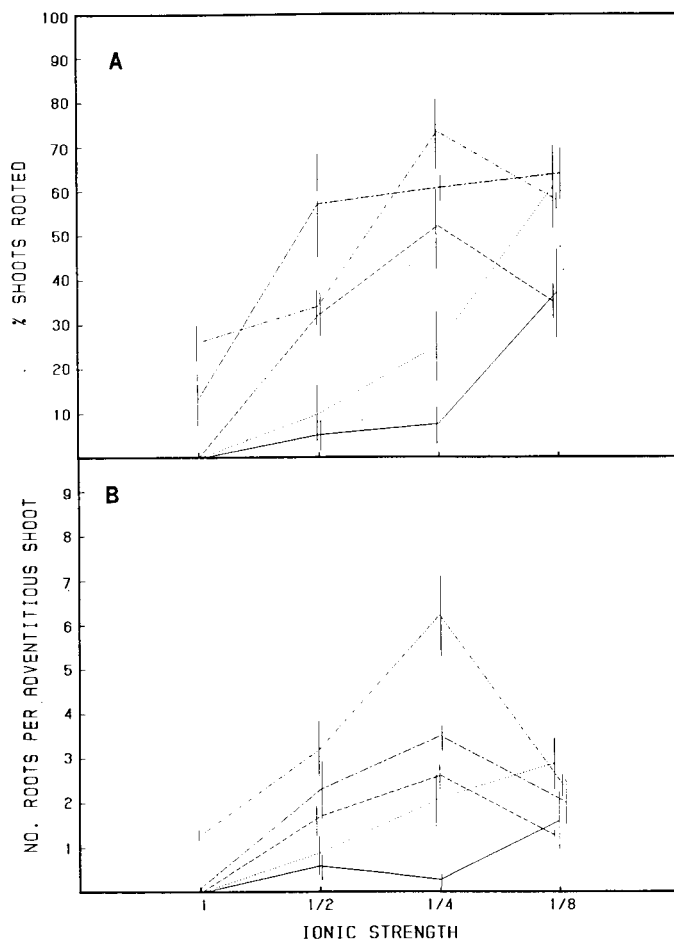


Fig. 2. Effect of ionic strength and IAA concentration on percentage of rooted adventitious shoots (A) and on the number of roots per adventitious shoot (B) of *R. laetum* \times *aurigeranum* after 1 month of culture. Vertical bars represent \pm S.E. (—) 0 μM IAA; ($\cdot\cdot\cdot\cdot$) 0.6 μM IAA; (- - - -) 6 μM IAA; (- - - -) 28 μM IAA; (- - - -) 57 μM IAA.

Rooting and establishment in the soil. To determine the optimum conditions for in vitro rooting of microshoots, four concentrations of the salts in Anderson's medium (1, 1/2, 1/4, and 1/8 strength) with five IAA concentrations (0, 0.6, 6, 28, and 57 μM) in a factorial experiment were used. A completely randomized design was used with five replicates. A replication consisted of a petri dish containing nine adventitious shoots. Data were recorded as percentage of shoots rooted after 4 weeks and number of roots per shoot. Percentage data were subjected to arcsin transformation before ANOVA analysis.

Rooted plantlets (Fig. 1D and E) were removed from culture medium, and agar was washed from the roots. Plantlets were then transplanted into compost (Jiffy Mix Plus; Jiffy Products of America, Bateria, Ill.) in seed trays and covered with clear plastic bags to maintain a high relative humidity. The compost was kept moist by frequent watering, and seed trays were kept in a greenhouse under the same growth conditions as the stock plants. Ventilation of the plantlets was increased with time by increasing the size of the holes made in the bags, and after 1 week, the plastic bags were removed. The percentage of plants that were successfully rooted was determined 3 weeks after transplanting.

In vitro rooting of excised shoots was accomplished in \approx 1 month. Ionic concentration of Anderson's medium in combination with concentrations of IAA influenced the rooting of shoots (Fig. 2A). Given the significant ionic strength \times IAA concentration interaction, valid comparisons among IAA concentrations can be made only within ionic strengths. Absent or low levels of IAA (< 28 μM) in combination with full-strength Anderson's medium resulted in no rooting. Using 1/4 ionic strength, the percentage of rooted plants ranged from 7.8% with 0 μM IAA to 73% with 28 μM IAA. About 60% rooting was obtained using 1/8 ionic strength and 0.6, 28, or 57 μM IAA. The percentage of rooted plants was significantly increased by increasing IAA concentrations and lowering the ionic concentrations. The highest value of in vitro-rooted shoots (73%) was obtained on medium with 1/4 strength supplemented with 28 μM of IAA.

The number of roots per shoot was also affected by the treatments tested, and again a significant interaction was found between IAA concentrations and ionic strength (Fig. 2B). The highest value, six roots per plant, was recorded using 1/4 ionic strength and 28 μM IAA.

The low-salt medium developed by An-

derson has been recognized as a suitable medium for several *Rhododendron* spp. (Anderson, 1984; Ettinger and Preece, 1985; Fordham et al., 1982; Harbage and Stimart, 1987). Anderson's medium was also good for establishing and maintaining in vitro cultures of the *Vireya* hybrid *R. laetum* \times *aurigeranum*. Anderson (1984) observed that lowering the ionic strength of modified Anderson's medium to 1/4 strength resulted in increasing the percentage of in vitro rooting with several cultivars of *Rhododendron*. In our experiment, the low ionic salt concentration of the medium and the IAA concentrations interacted significantly to induce in vitro root formation in *R. laetum* \times *aurigeranum*. It is also evident that a critical level of IAA must be reached before root induction can occur.

All rooted plantlets transferred to soil survived regardless of the medium used to induce rooting (Fig. 1F). Plants on soil generated new roots from the root system that had developed in vitro and, later, from the base of the plantlets.

In summary, a successful in vitro multiplication protocol has been developed for *R. laetum* \times *aurigeranum*. Among the three types of shoot multiplication, adventitious shoot multiplication from organogenic callus, induced at the cut surfaces of the axillary shoot-tip explants, gave the best results.

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