

# Vegetative propagation of the Azorean endemic shrub *Viburnum treleasei* Gand.

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*Viburnum treleasei* Gand. is a threatened hermaphroditic shrub or small tree endemic to the Azores islands. In this study we aimed at defining a fast, simple and cost-efficient propagation methodology that could be used by non-skilled workers in conservation action plans. Our objective was also to produce cleaner material for initiation of *in vitro* cultures and to determine the effects of season, placement of cuttings in the branch, placement of vegetative buds in cuttings and forcing solutions in shoot development. It was possible to produce clean shoots from cuttings using a forcing solution with 8-hydroxyquinoline sulphate (8-HQS), 2% sucrose and no growth regulators addition. Shoot development results obtained with apical and sub-apical cuttings indicate that *V. treleasei* possesses apical dominance and deep endodormancy. Apical semihardwood cuttings in autumn or air-layered branches in autumn and winter with 2 or 5% (w/w) of IBA produced excellent rooting results which will allow reinforcing depleted populations of *V. treleasei* efficiently and at reduced costs.

Key words: contamination, growth regulators, propagation, rooting, season

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## INTRODUCTION

*Viburnum treleasei* Gand., in Adoxaceae (Backlund 1996), is a hermaphroditic shrub or small tree endemic to the Azores islands. Although more common in some islands it is rare in others and in general populations do not have a large number of individuals. It has been recently included in the ninety indigenous Azorean vascular plants considered as priorities for conservation (Silva et al. 2009). Due to its threatened status and high potential as an ornamental plant, the development of conservation strategies for this endemic species was deemed a priority.

Forcing solutions have been indicated as a way to supply growth regulators to cuttings, promoting bud dormancy break and the development of new shoots, which is a material appropriate to

reduce contamination in the initiation stage of *in vitro* cultures (Read & Qiguang 1987; Read & Yang 1991; Yang & Read 1991; Ercisli et al. 2001). Previous micropropagation studies with *V. treleasei* (Moura et al. 2009) demonstrated that field material is very prone to fungal contamination hence a source of cleaner material will be highly beneficial to the micropropagation process. Additionally, this technique can bring information on type of bud dormancy and was recently applied with this purpose on a study of several species of *Vitis* (Gu & Read 2004).

There are a large variety of methodologies to promote rooting of *Viburnum* species, including softwood, hardwood cuttings and layering (Dirr & Heuser 1987; Hartmann et al. 1990). While *V. carlesii* is considered difficult to root, *V. burkwoodii*, *V. rhytidophylloides*, *V. lantana*, *V. sargentii* and *V. plicatum* easily develop an

adventitious root system (Hartmann et al. 1990), and several *Viburnum* species from Florida respond favourably to simple layering (Ingram & Yeager 1991). For most species, softwood cuttings planted in sand or perlite and mist irrigated produce good results, but hardwood cuttings can also be used to propagate some species, e.g. *V. opulus*, *V. dentatum* and *V. trilobum* (Hartmann et al. 1990). Younger cuttings (at the end of spring) may root more quickly, but those harder (at mid summer) are more likely to resist winter, and treatments with indole-3-butyric acid (IBA) and Tirame are recommended (Hartmann et al. 1990). IBA effect on rooting of *V. nudum* and *V. obovatum* varied with season and was only effective during flowering and/or at the end of the growing season (Dehgan et al. 1989).

Woody leafy cuttings in autumn or at the beginning of winter are indicated for *V. tinus* (Boutherin & Bron 1989), but Coccozza Talia et al. (2004) found a favourable effect of IBA and that apical cuttings of this species rooted more quickly and efficiently in autumn than in winter.

It was the aim of this study to: i) find a source of cleaner material to be used in the initiation stage of the existing micropropagation protocol for *V. treleasei*; ii) understand the effects of season, placement of cuttings in the branch, placement of vegetative buds in cuttings and forcing solutions in shoot development; and iii) find a fast, simple and cost-efficient propagation methodology for *V. treleasei* to be used by non-skilled workers in conservation action plans.

## MATERIAL AND METHODS

### PLANT MATERIAL

The specimens selected as stock plants pertained to two medium altitude natural populations of *V. treleasei* located in São Miguel Island at the localities of Lombadas and Lagoinha do Areiro. They were all flowering individuals with an average height of 1 m.

### SHOOT DEVELOPMENT

Softwood branches with approx. 15 cm were collected, cut in two sections (apical and sub-

apical) with approx. 4-9 cm each, and all leaves removed. Surface sterilization used immersion in 5% (w/v) HgCl<sub>2</sub> with 2 drops per 100 ml of Polysorbate 20 for 20 min, agitation, and three rinses with autoclaved distilled water. Cuttings were maintained for 4 weeks in 5 ml forcing solutions inside 125 x 25 mm Pyrex test tubes closed with translucent autoclavable plastic caps. The effect of 8-hydroxyquinoline sulfate (8-HQS) in the reduction of cuttings contamination was evaluated by comparing two forcing solutions, one composed by 200 mg/L 8-HQS and 2% sucrose in distilled water, and the other composed by 2% sucrose in distilled water. Contamination was registered when the occurrence of fungus growing on cuttings and/or a cloudy appearance of the forcing solution was visually observed. The effect of growth regulators in shoot development was tested using a 8-HQS solution supplemented with 3 levels of NAA (54, 269, 537 µM), IBA (49, 246, 492 µM), BAP (44, 222, 444 µM) or GA<sub>3</sub> (29, 144, 289 µM), plus two control solutions with 2% sucrose and no growth regulators added (8-HQS and water), in a total of 14 treatments. Each forcing treatment was carried out one time with 10 replications within each treatment for both apical and sub-apical cuttings, and repeated for each season of the year. Test tubes were maintained in a growing chamber at 19 ± 1 °C, with artificial illumination by daylight fluorescent lamps with a photosynthetic photon flux density (PPFD) of 33 µmol m<sup>-2</sup>.s<sup>-1</sup> and 16 hours of light. Percentage of contaminated cuttings and percentage of shoots developed per cutting (shoot development) were recorded after 4 weeks.

### ROOTING

To induce rooting several methods were tested in laboratory and in the field: A) Cuttings obtained by forcing were dipped in 0, 0.5, 2 or 4% (w/w) IBA in talc, single-planted in a perlite and peat mix (1:1), and kept inside 330 ml transparent colourless plastic containers with adjustable aeration. B) Semihardwood cuttings with approx. 15 cm and all leaves removed, except for the apical pair, were dipped in 0, 0.1, 0.5, 1, 1.5, 2 or 25% (w/w) IBA in talc, planted in fresh *Sphagnum* spp. or in a perlite and peat mix (1:1),

and kept in open plastic containers. All laboratory rooting experiments were conducted in a nursery room under natural illumination and photoperiod with an average temperature of 19 °C. Watering of the plants was done manually. C) Semi-hardwood branches with approximately 20 cm were selected from three locations in Lombadas. All leaves were removed except for the 2 or 3 apical pairs. Each set of cuttings was interchanged between the three locations and planted without any growth regulator treatment in autumn. D) Sixty adult plants from Lagoinha do Areiro were selected and air layering was performed on each plant in spring and autumn, in a vigorously looking branch with 12-15 mm diameter. A ring of bark with approximately 1 cm was removed from the branch at a distance from the apex of 25-30 cm. The appropriate IBA concentration was applied to the wound which was covered with a ball of wet *Sphagnum* spp. with approximately 8 cm diameter, and wrapped in a black polyethylene sheet fixed with two plastic bands. To promote rooting 0, 0.5, 1, 1.5, 2 or 5% (w/w) IBA in talc was applied. In all rooting experiments each treatment was carried out 3 times with 8-10 replications each, except for method D consisting of a single repetition of each treatment with 10 replications. After 6 months the average percentage of rooting and the average percentage of survival in rooted plants were determined.

#### STATISTICAL ANALYSIS

Nominal data were analysed using contingency tables and the Pearson ( $\chi^2$ ) test was calculated. Data normality was determined with the Kolmogorov-Smirnov test. Non-normal absolute data were log transformed and percentage data were arcsine transformed prior to analysis. Whenever data structure allowed, an analysis of variance (ANOVA) was used. A Tukey (honestly significant difference) test was used as the multiple comparison procedure. For stubborn non-normal data non-parametric statistics were used, namely the Kruskal-Wallis test and Dunn's multiple comparison test. When only two sets of data were compared the Mann-Whitney test was used. Statistical analyses were performed using SPSS 15.0 and Microsoft Office Excel 2003.

## RESULTS

#### SHOOT DEVELOPMENT

Season did not have a significant effect on the contamination percentage for apical (0-10%;  $\chi^2=3.077$ ;  $p > 0.05$ ) and sub-apical cuttings (0-20%;  $\chi^2=3.984$ ;  $p > 0.05$ ) forced in the 8-HQS solution, but there was a significant effect in the water treatment for both apical (0-100% contamination;  $\chi^2=30.595$ ;  $p < 0.001$ ) and sub-apical cuttings (60-100% contamination;  $\chi^2=12.926$ ;  $p < 0.001$ ), with the lowest contamination percentages occurring in summer with apical cuttings and in spring with sub-apical cuttings. The type of cuttings had a significant effect on contamination in summer, in the water treatment, with 100% contamination obtained with sub-apical cuttings and no contamination with apical cuttings ( $\chi^2=0$ ;  $p < 0.001$ ). The 8-HQS treatment significantly reduced contamination percentage compared with the water control, except in spring and when sub-apical cuttings were used (Table 1).

Table 1. Effects of 8-hydroxyquinoline sulfate (8-HQS) on contamination of apical and sub-apical cuttings from *Viburnum treleasei* for all seasons of the year. Comparisons of 8-HQS and water in rows: results of Pearson  $\chi^2$  tests (\*  $p < 0.01$ ; \*\*  $p < 0.001$ ).

Season	Cuttings	Contam. (%)		$\chi^2$
		8-HQS	Water	
Spring	Apical	10	80	9.899 *
	Sub-apical	20	60	3.333
Sum.	Apical	0	0	-
	Sub-apical	0	100	19.000 **
Aut.	Apical	0	100	19.000 **
	Sub-apical	11	100	15.354 **
Winter	Apical	0	100	19.000 **
	Sub-apical	0	100	20.000 **

Regarding the percentage of shoot development, only in spring the type of cuttings had a significant effect, with the highest values occurring with sub-apical cuttings (Table 2).

As for the effect of season in shoot development, apical cuttings produced a significantly higher number of shoots in summer than in autumn and winter, with intermediate values obtained in spring ( $\chi^2=18.932$ ;  $p < 0.001$ ; Table 2).

Table 2. Effects of type of cuttings and season on shoot development of *Viburnum treleasei*, in 8-hydroxyquinoline sulphate forcing solution. Results of the comparison of type of cuttings using Mann-Whitney test (U) in rows (\*  $p < 0.01$ ). Results of the comparison of seasons using Dunn test (applied after Kruskal-Wallis test) in columns (percentages followed by the same letters are not significantly different,  $p > 0.05$ ).

Season	Shoot development (%)		
	Apical	Sub-apical	U
Spring	17.71 ab	40.75 ab	19.500 *
Sum.	23.87 a	53.75 a	30.000
Autumn	1.43 b	14.26 abc	27.500
Winter	2.50 b	0.00 c	45.000

With sub-apical cuttings, the highest percentage of shoot development occurred in summer but this was only significantly different from the results obtained in winter ( $\chi^2=19.171$ ;  $p < 0.001$ ; Table 2). Considering the results obtained in autumn and winter, subsequent analysis was only conducted with values obtained in spring and summer.

Regarding the effect of the location of vegetative buds (apical versus axillary buds) in shoot development obtained from apical cuttings, there were significant differences for both spring ( $U=16.500$ ;  $p < 0.01$ ) and summer ( $U=12.000$ ;  $p < 0.001$ ), with highest shoot development occurring in apical buds (70% versus 5% in spring, and 80% versus 10% in summer).

Treatments using different forcing solutions with apical cuttings had a significant effect on shoot development in summer ( $\chi^2=45.258$ ;  $p < 0.001$ ) but not in spring ( $\chi^2=17.020$ ;  $p > 0.05$ ). With sub-apical cuttings, the type of forcing solution had a significant effect on shoot development in spring ( $\chi^2=48.676$ ;  $p > 0.001$ ) and in summer ( $\chi^2=63.214$ ;  $p > 0.001$ ). In fact, altogether, the addition of growth regulators to the forcing solution didn't produce any significant increase in the percentage of shoot development, since new shoots developed equally well from cuttings on 8-HQS solution or in water only (Fig. 1).

#### ROOTING

The percentage of rooting after six months obtained in modalities that produced positive results can be observed on Figure 2. The effect of season was not significant on air-layering ( $t=0.132$ ;  $p > 0.05$ ) and the results were henceforth analysed altogether. The percentage of rooting was significantly different between treatments ( $F=44.424$ ;  $p > 0.001$ ), with cuttings collected and planted in situ and air-layering producing the highest results. All treatments had a survival rate of 100% except for those using cuttings from forcing.

#### DISCUSSION

The addition of 8-HQS was very effective on contamination control. The anti-microbial properties of 8-HQS are well known, being used to preserve cut flowers (Gast 1997) and in micropropagation (Machado 1993).

Sub-apical cuttings only produced a significantly higher number of shoots in spring. The preferential development of the apical bud in apical cuttings indicates the occurrence of an apical dominance of the apical bud over the axillary buds (Brenner et al. 1987; Powell 1987; Tamas 1987; Cline 2000). The results obtained throughout the year with apical and sub-apical cuttings suggest that the buds enter dormancy during fall, and are deep dormant in winter (Powell 1987). This indicates a physiological dormancy connected to winter rest, i.e. an endodormancy, or an ecodormancy related to environmental stress (Lang 1987, 1994; Lang et al. 1987). As reviewed by Cesaraccio et al. (2004), buds with an ecodormancy i.e. quiescent, require forcing temperatures to become active. Since forcing of cuttings during autumn and winter in the growing chamber under spring temperatures did not promote shoot development, the occurrence of an ecodormancy can be ruled out.

Regarding the effect of the addition of growth regulators to the forcing solution, the treatments with 8-HQS alone generally originated the highest percentages of shoot development, particularly on axillary buds. It is known that

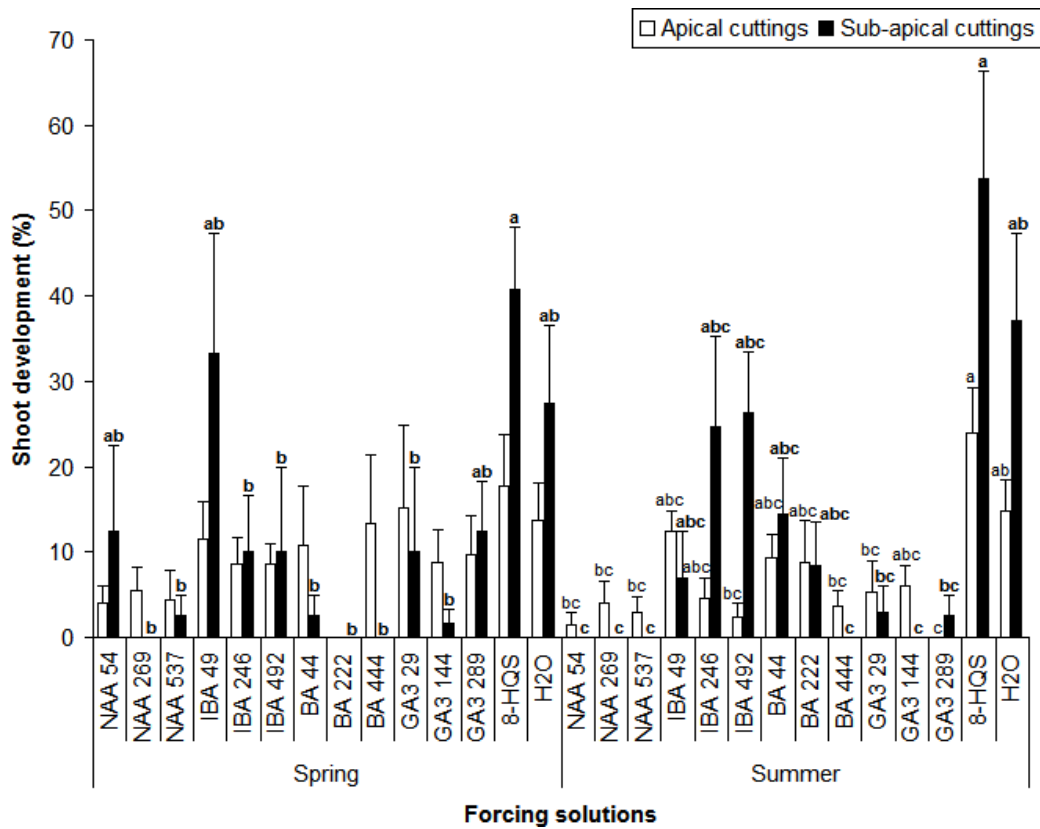


Fig. 1. Effect of forcing solutions with different growth regulators supplements (given in  $\mu\text{M}$ ) on shoot development of *Viburnum treleasei*, using apical or sub-apical cuttings in spring or summer. For additional information on growth regulators concentrations see text. Two controls with 8-hydroxyquinoline sulfate (8-HQS) or water (H2O) were also used. Results of Dunn tests (applied after Kruskal-Wallis test): percentages (+ SE) from the same season and type of cuttings followed by the same letters are not significantly different ( $p > 0.05$ ).

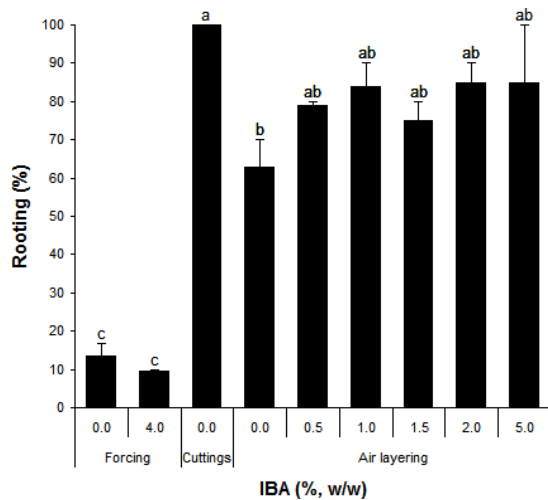


Fig. 2. Percentages of rooting for *Viburnum treleasei* cuttings, after six months, obtained from forcing in the laboratory (Forcing), from apical cuttings in situ (Cuttings), and from air layering in situ with different IBA concentrations (0-5%, w/w in talc). Results of Dunn test (applied after Kruskal-Wallis test): percentages (+ SE) followed by the same letters are not significantly different ( $p > 0.05$ ).

8-HQS and sucrose inhibit the effect of localized ethylene accumulation and its repressive action in axillary bud growth (Parups 1975; Su et al. 2001). The absence of a promoting effect on shoot development of all growth regulator treatments during winter, further sustains the hypothesis of a deep endodormancy requiring cold temperatures to achieve bud dormancy break.

Only treatments conducted in situ resulted in good rooting (>60%) and survival (100%) percentages. The poor rooting results obtained ex situ might be explained by the lack of appropriate acclimatisation in the nursery room, as mist is generally indicated for *Viburnum* species (Hartmann et al. 1990). Similarly to the recommendations of Coccozza Talia et al. (2004) and Boutherin & Bron (1989) for *V. tinus*, apical cuttings taken in autumn were appropriate for rooting of *V. treleasei*. Considering the good results obtained with cuttings without any growth regulator treatment it will be interesting to verify the occurrence of mycorrhizae symbiosis. In air layering, 2 or 5% (w/w) of IBA effectively promoted rooting in both spring and autumn.

#### CONCLUDING REMARKS

It was possible to produce clean shoots from cuttings using a forcing solution with 8-HQS and no growth regulator addition, which can be used with the already defined micropropagation protocol for *V. treleasei* as an alternative source of explant material, thus avoiding the use of plant material originating directly from natural populations which are more prone to contamination. The occurrence of apical dominance was confirmed for *V. treleasei* as well as an endodormancy.

Apical semiwoody cuttings without growth regulators in autumn or air-layered branches in autumn and winter dusted with 2 or 5% (w/w) IBA produced excellent rooting after six months. Our findings suggest that in situ vegetative propagation of *V. treleasei* by apical cuttings is a fast, efficient and cost-effective conservation strategy to be used in the reinforcement of depleted natural populations. Air-layering, although more time-consuming is also an effect-

tive technique and particularly advantageous from a conservation perspective as no damage is done to the mother plant if rooting fails. These results complement other research devoted to the propagation of *V. treleasei* by seed germination and by micropropagation.

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