

SHORT COMMUNICATION

Reversion to juvenility: the use of epicormics in the micropropagation of mature wild shrubs of *Vaccinium cylindraceum* Smith (Ericaceae)

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An efficient *in vitro* method was developed to propagate the shoots of mature wild shrubs of *Vaccinium cylindraceum* Sm. by axillary bud proliferation on epicormic stems. In the initial culture, 46.9% of the epicormic buds produced new shoots with juvenile morphological features. The number of nodes per shoot and the shoot length for explants which originated from epicormic buds was significantly higher when compared to that of winter dormant buds but similar when compared to that of seedling buds. The three most distal buds of epicormic twigs produced the best rates of shoot initiation: 87.5% (position 1) to 62.5% (position 3).

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INTRODUCTION

The use of micropropagation as a strategy for species conservation should include both mature shrubs (which represent established wild shrubs), and seedlings (since the current forces of natural selection are different from those that successfully selected the adult shrubs).

Seedlings grown in a greenhouse or in a growing chamber are appropriate for micropropagation. The risk of contamination is minimal and, in a suitable medium, these explants show vigorous vegetative growth and the new shoots exhibit juvenile morphologic characteristics (Pereira 2006; Guang-Jie et al. 2008).

However, when dealing with the conservation of endangered endemic species, especially with small populations or populations located in almost inaccessible habitats, it can be difficult to collect seeds or germinate them. Therefore, cloning should also be used to ensure the survival

of specific individuals (Edson et al. 1997). Micropropagation using explants from mature wild trees or shrubs has, however, two main problems: high rates of contamination (Brissette et al. 1990; Leifert et al. 1994; Pereira 1999) and, occasional reversion to morphological juvenility (Pereira 1999; McCown 2000).

The aim of this study was to establish an efficient method of *in vitro* shoot propagation of mature wild shrubs of *Vaccinium cylindraceum* Smith using axillary bud proliferation.

Vaccinium cylindraceum is an endemic shrub of the Azores Archipelago. Its *status* varies between islands, from its extinction on the island of Graciosa to its relative abundance on Pico Island. On all islands the populations at low altitudes are very rare due to human settlement. The breeding system of the species is that of facultative xenogamy while spontaneous autogamy has little importance in fruit development (Pereira 2008). The outcrossing

maximizes the percent of fruit set, the berry weight, the number of seeds per berry and the seed weight (Pereira 2008). Therefore, there is a special interest in recovering specific genotypes by micropropagation, of wild adult plants of *V. cylindraceum* from populations which are small, old, isolated and/or located at low altitudes or places with difficult access.

Although prior *in vitro* research into the micropropagation of wild shrubs of *V. cylindraceum*, describes initiation, multiplication and *ex vitro* rooting, high rates of contamination were present and reversion to juvenile characteristics (shoots with small and rounder leaves) was only occasionally observed (Pereira & Debergh 1998; Pereira 1999). The practical implication of these results was that only the large shrubs could be micropropagated.

In *V. cylindraceum*, early suppressed buds on old shrubs develop into epicormic shoots depending on the light available (Fig. 1C), whilst the apical and lateral winter dormant buds (Fig. 1A) are released each year in February. Based on the knowledge that epicormic twigs, i.e. basal branches, grow from dormant buds formed when the shrubs were younger, we tested the hypothesis that explants from epicormic twigs would produce more *in vitro* shoots with juvenile morphological characteristics than explants whose origin was winter dormant buds formed when the shrub was older, in the last growing season (Jain & Häggman 2007). The best bud position for shoot induction in epicormic twigs was also evaluated.

MATERIAL AND METHODS

CULTURE INITIATION AND PROPAGATION WITH EXPLANTS FROM MATURE WILD SHRUBS

In February, stems with winter dormant buds (Fig. 1A) and epicormic stems (Fig. 1C) were harvested from mature wild shrubs growing on the island of São Miguel. The shoots (≈ 10 cm in length) were washed in running tap water for 5 minutes, the surfaces disinfested for 30 seconds in 96% commercial ethanol and rinsed in sterile distilled water. The segments were then soaked for 5 minutes in 0.3% HgCl_2 (Sigma[®]) and rinsed for a few seconds in sterile distilled water. The

explants were soaked for a further 10 minutes in 10% commercial bleach (5% sodium hypochlorite) solution, rinsed 3 times in sterile distilled water and soaked again for 30 minutes in 12% benomyl (Benlate[®]) solution. Finally, the segments were rinsed 3 times, for 10 minutes each time, in sterile distilled water. A few drops of Tween 20 were added to the solutions of bleach and HgCl_2 prior to usage (Pereira & Debergh 1998). Both ends of the segments were re-cut to obtain about 8 cm segments. These segments were placed vertically in Pyrex[®] glass test tubes (125 X 25 mm) containing 10 ml of sterile tap water and after 24 hours the disinfection procedure of surfaces was repeated. The segments were re-cut to obtain single node cuttings or shoot tips; both explants were then vertically planted in tubes with 10 ml of Zimmerman and Broome's (1980) Z2-medium.

CULTURE INITIATION AND PROPAGATION WITH EXPLANTS FROM SEEDLINGS

Seedlings were obtained from the disinfested seeds of wild shrubs on São Miguel Island, as described by Pereira (2006). The explants used were single node cuttings with one leaf, planted vertically in tubes with 10 ml of Z2 medium. (Pereira 2006).

MEDIA COMPOSITION AND CULTURAL CONDITIONS

The Z2 medium was supplemented with sucrose (20g/l), N^6 [2-isopentenyl]adenine - 2iP (24.6 μM) and Bacto Agar Difco[®]. (10g/l), the pH adjusted to 5.0 and, finally, the medium was autoclaved at 121°C for 20 minutes (1.5 bar). The chemicals were from Sigma[®] or Merck[®]. All tubes were capped with Kaput[®] translucent closures and placed in a growing chamber at $20 \pm 1^\circ\text{C}$, with a light intensity of approximately 56 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ and a photoperiod of 16 hours per day.

DATA ANALYSIS

A χ^2 test was used to analyse the contingency table: the occurrence of juvenile shoots and the type of bud (winter dormant buds and epicormic buds). Three different types of responsive nodal explants were evaluated for shoot proliferation: nodal explants from winter dormant buds (n=30), from epicormic buds (n=30) and from seedlings (n=30); the following variables of these 90

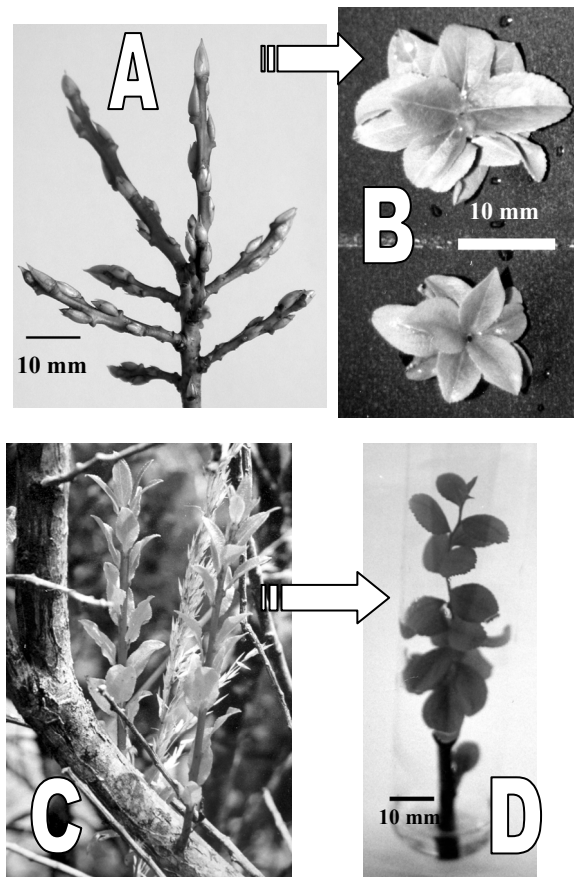


Fig. 1. *Vaccinium cylindraceum* Smith; A - Stems formed in the last growing season with apical and lateral winter dormant buds, B - Non juvenile shoots grown *in vitro* from apical and lateral winter dormant buds (*in vitro* initiation stage); C - Epicormic stems; D - Juvenile shoot developed *in vitro* from an epicormic bud (epicormic stem on tap water).

reactive explants were calculated: the number of shoots (>3 mm long) per explant, the mean number of nodes per shoot and the mean shoot length. The values used in the statistical analysis were taken after 8 weeks in culture. For measured quantitative data a single factor analysis of variance (ANOVA) was performed. The homogeneity of variances for each parameter was verified using the Levene's test. For a parametric multiple comparison of means, Tukey's test was used at the 5% significance level. The best bud position for shoot induction in epicormic twigs was also evaluated by calculating the proportion of responsive buds in positions one (the most

distal) to seven; and 16 buds were evaluated for each position.

RESULTS

THE EFFECT OF BUD TYPE ON SHOOT JUVENILITY

In the initiation period nearly half (46.9%) of the epicormic buds (n=113) produced new shoots with juvenile morphological features (Fig. 1D) while winter dormant buds (n=337) produced a significantly different ($P<0.01$) proportion of juvenile shoots (4.46%).

THE EFFECT OF BUD TYPE ON SHOOT PROLIFERATION

All the explants formed shoots on the Z2 medium with 24.6 μ M 2iP. Shoots grew directly from the nodes of the explants taken from mature shrubs whereas they grew both from the axillary buds and calluses of those taken from seedlings. Table 1 shows the effect of bud type on the number of shoots per explant, the number of nodes per shoot and shoot length; the analysis of variance demonstrates that the type of explant significantly ($P < 0.05$) affected the number of nodes per shoot and the shoot length. The number of nodes per shoot and shoot length for explants which originated from epicormic buds was higher when compared to that of winter dormant buds but similar when compared to that of the seedling buds.

Table 1. *Vaccinium cylindraceum* Sm. establishment on a Z2-medium with 24.6 μ M 2-iP: effect of bud type on shoot proliferation (30 explants per bud type). Data were collected after 8 weeks in culture. Means marked with the same letter within columns do not differ significantly (confidence level 95%).

Bud type	Shoots per Explant	Nodes per Shoots	Shoot Length (mm)
Winter dormant	1.0a	3.1a	3.8a
Epicormic	1.5b	4.5b	12.6b
Seedlings	1.5b	4.6b	13.0b

THE EFFECT OF BUD POSITION ON SHOOT INDUCTION

Table 2 shows that the proportion of reactive buds decreases from the distal position from 87.5% in position 1, to 6.3% in position 7. The three most distal buds of the epicormic twigs produced the best rates of shoot initiation: from 87.5% (position 1) to 62.5% (position 3).

Table 2. *Vaccinium cylindraceum* Sm. establishment on a Z2-medium with 24.6 μ M 2-iP: Effect of epicormic bud position on shoot induction (position 1 = distal bud).

Bud position	n	Responsive buds (%)
1	16	87.5
2	16	75.0
3	16	62.5
4	16	50.0
5	16	37.5
6	16	37.5
7	16	6.3

DISCUSSION

The *in vitro* plant production search for reactive explants producing new shoots with several and well spaced nodes to be subcultured, easy to root shoots, and successful acclimatization of the produced plants to the environment (George 1993); these traits have been related with tissue juvenility. Poor *in vitro* response or unsuccessful rooting of explants with origin in old trees or shrubs is attributed to the lack of juvenility factor in older tissues (George 1993).

Establishing a new clone of *V. cylindraceum* *in vitro* from mature, wild plants is known to be difficult: the response to the 2iP concentration of explants is not constant in the initial culture (Pereira 1999), contamination rates are usually high (Pereira & Debergh 1998) and, reversion to juvenility is only occasional or may need several subcultures to occur (Lyrene & Perry 1988; Pereira 1999). The latter means a longer period in culture, which results in more shoots arising from callus tissue, and an increased probability of somaclonal variation (George 1993; Pereira 1999).

Greenhouse or laboratory forcing of epicormic sprouts as a source of juvenile explants for *in vitro* culture has been successfully used with several trees: *Arbutus unedo* L. (Gomes & Canhoto 2009); *Acer saccharinum* L., *Fraxinus pennsylvanica* Marshall, *Carya illinoensis* K.Koch, *Tectona grandis* L.f. (Aftab et al. 2005; Aftab & Preece 2007; Akram & Aftab 2009); *Corylus avellana* L. (Sánchez-Olate et al. 2004); *Sorbus aucuparia* L. (Chalupa 2002); *Juglans*

nigra (Bailey et al. 1998), and *Eucalyptus grandis* W.Hill (Ikemori 1987). Still this technique and its potential for micropropagation have not been determined in many woody plants (Aftab & Preece 2007).

Although the majority of the studies have been done with trees, the use of epicormic buds as a source of juvenile explants can also be applied to shrubs as was demonstrated with *V. cylindraceum*. In fact the juvenility associated with the lower branches and epicormics is a characteristic of woody branching plants (Aftab & Preece 2007).

Gomes & Canhoto (2009) found that shoot apices from epicormic shoots of *Arbutus unedo*, which were developed in a growth chamber, showed higher rates of *in vitro* establishment. In the case of *S. aucuparia*, Chalupa (2002) found that explants from the epicormic shoots of mature trees exhibited high multiplication coefficients in cultured microshoots. He also showed that the height and diameter increments of micropropagated trees originating from the juvenile parts of mature trees were comparable with the dimensions of trees originating from seeds. The juvenile explants of *V. cylindraceum* obtained from epicormic buds in this study exhibited shoot proliferation traits similar to those of the juvenile explants from seedlings.

Gomes & Canhoto (2009) indicate that shoot apices of epicormics are more effective for *in vitro* plant establishment than nodal segments. The same scenario was observed for *V. cylindraceum*, wherein the shoot apices of epicormics presented the highest rate of shoot induction, and the rate of shoot induction decreased with the increasing distance of the buds from the shoot apex. A similar conclusion was achieved by Hruskoci & Read (1993), working with internodes stem segments instead of node stem segments.

It has been shown previously that explant juvenility is an essential condition to the successful *in vitro* culture of woody adult plants (George 1993). Our experiments with the *in vitro* cultivation of distal epicormic buds from the old shrubs of *V. cylindraceum* led to the successful rejuvenation and micropropagation of these shrubs; in addition, all the produced plants rooted and were successful acclimatized. Thus, these

results indicate the possibility of a new environmental conservation tool which can be used for the regeneration of old, small and fragmented *V. cylindraceum* populations. This technique could be applied to revive the weaker population of Santa Maria Island (Pereira 1999) and to the almost extinct population on Corvo (Pereira et al. 2007). In addition, combined with *in vitro* culture of seedlings, it could be used to return the *V. cylindraceum* populations to the surroundings of Lagoa do Fogo (the Natural Reserve on São Miguel Island) and Lagoa das Furnas (São Miguel).

The plants produced in this study were used by local authorities 'Direcção de Serviços de Agricultura e Pecuária' to establish an experimental field of blueberries production near Lagoa das Furnas (Hummer et al. 2009).

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