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BRIEF COMMUNICATION

Micropropagation of *Salvia brachyodon* through nodal explants

D. MIŠIĆ*[1], D. GRUBIŠIĆ* and R. KONJEVIĆ**

Institute for Biological Research "Siniša Stanković",
*Bulevar Despota Stefana 142, 11060 Belgrade, Serbia and Montenegro**
*Institute for Botany, Faculty of Biology, Takovska 43, 11000 Belgrade, Serbia and Montenegro***

Abstract

A protocol for *in vitro* propagation of Balkan endemic plant *Salvia brachyodon* Vandas from nodal segments was developed. 6-benzylaminopurine was more effective in axillary buds promotion when compared to thidiazuron. The rooting of regenerated shoots was induced by transferring them to the media supplemented with auxins. All tested auxins (indole-3-acetic acid, indole-3-butyric acid, and α-naphthaleneacetic acid) stimulated the rooting of *S. brachyodon* shoots. The acclimatization of *in vitro* rooted shoots was successful.

Additional key words: Balkan endemic sage, shoot culture, axillary buds, acclimatization.

Salvia brachyodon Vandas is a Balkan endemic plant, growing wild in dry rocky habitats of Mt Orjen and Pelješac peninsula. It is a perennial herbaceous plant, 70 - 80 cm high, with large (35 - 40 mm long), light-purplish flowers, which are a real ornament of this sage (Fig. 1*A*). Medical properties of *S. brachyodon* are not well elucidated. Like in some other species of the genus *Salvia*, its essential oils are characterized by a high content of 1,8-cineole, bornyl acetate, camphene and β-pinene (Šavikin-Fodulović *et al*. 2002, Tzakou *et al.* 2002, Soković *et al*. 2005).

This study was primarily initiated to develop a protocol for *in vitro* propagation of this endemic and rare plant, with the aim in *ex situ* conservation, but also in commercial propagation. As an attractive flowering herb, this species could be clonally propagated and grown as garden plant. To our knowledge, there is no documented literature on protocols for micropropagation of wild *Salvia brachyodon* Vandas. Some other *Salvia* species have previously been micropropagated (Frett 1986, Olszowska and Furmanowa 1990, Hosoki and Tahara 1993, Cuenca and Amo-Marco 2000, Arikat *et al*. 2004).

Seeds of *S. brachyodon* were collected in August 2001 at the locality Vrbanj, the area of Mt Orjen, Bosnia and Herzegovina. Embryos were isolated by removing the testa mechanically. Explants were surface sterilized in 20 % solution of commercial bleach with two drops of liquid detergent for 10 min, and than rinsed 5 times with sterile distilled water. After the treatment with 1 mM GA_3 solution containing 500 mg dm^{-3} nystatin for 24 h, explants were rinsed 3 times with sterile distilled water and than aseptically transferred on half-strength Murashige and Skoog (1962; MS) medium supplemented with 100 mg dm^{-3} *myo*-inositol, 30 g dm^{-3} sucrose and 7 g dm^{-3} agar (*Torlak*, Belgrade, Serbia and Montenegro). The pH of the medium was adjusted to 5.8 before sterilizing by autoclaving at 114 °C for 25 min. Subculturing was performed once a month, on the same medium composition, until enough stock was available.

For shoot multiplication, one-node stem segments, approximately 10 mm in length, were taken from *in vitro*-derived shoots and transferred to half-strength MS medium supplemented with 6-benzylaminopurine (BAP) or thidiazurone (TDZ) within the range of 0.01 to 30.0 μM. In all treatments the media were supplemented with 0.57 μM indole-3-acetic acid (IAA). After four weeks in culture, the efficacy of each medium on shoot proliferation and growth was determined by recording: the percentage of primary explants developing shoots, the number of shoots per explant, and the length of the shoots. Treatments were carried out with 25 explants and each treatment was repeated twice.

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Abbreviations: BAP - 6-benzylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid; TDZ - thidiazurone.
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[1] Address for correspondence; fax: (+381) 2761 433, e-mail: dmisic@ibiss.bg.ac.yu

In order to induce rooting, one-month old shoots were transferred on media supplemented with indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or α-naphthaleneacetic acid (NAA) within the range of 0.5 to 1.7 μM. The rooting percentage was recorded four weeks after the onset of experiment. The experiment was repeated twice with 25 explants each.

For all treatments, cultures were grown in 100 cm^3 glass vessels closed with tent caps, with 50 cm^3 of culture medium each. All cultures were grown in a growth chamber under 16-h photoperiod, with photon flux density of 50 μmol $m^{-2}s^{-1}$ (white fluorescent *Tesla* tubes, Pančevo, Serbia and Montenegro, 60 W), temperature of 25 ± 2 °C, and a relative humidity of 60 - 70 %.

The rooted shoots were transferred to greenhouse for acclimatization. Before they were potted in soil, containing a mixture of 80 % forest peat and 20 % earthworm compost, plantlets were treated with 0.15 % solution of *Previcur* (*Aventis*, Berlin, Germany) to prevent fungal contamination. Pots were shielded with plastic covers to initially maintain the plants at high humidity, and the plantlets were acclimatized by gradually opening the covers. After two weeks they were completely uncovered and hardened to the greenhouse conditions (temperature of 25 ± 2 °C and relative humidity of 60 - 90 %). Observations on acclimatization percentage were recorded 6 weeks after the transfer of rooted plants to the soil.

Statistical analyses were performed using *StatGraphics* software, version 4.2 (*STSC Inc*., Rockville, Maryland, USA). Data were subjected to analysis of variance (*ANOVA*) and comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at the confidence level of $P \leq 0.05$.

Fig. 1. Micropropagation of *Salvia brachyodon* Vandas: *A* - ornamental, light-purplish flowers of *S. brachyodon*, pictured on species natural habitats of Mt Orjen (photo by Pedja Janjaćković); *B* - *in vitro* cultures of *S. brachyodon* grown on ½ MS culture medium; *C* - acclimatized plants grown in greenhouse.

Two weeks after the transfer of embryos on hormone-free ½ MS culture medium, seedlings developed *in vitro*. After four months of culturing on the same media composition (Fig. 1*B*), shoots were used in experiments to evaluate the effect of cytokinins on multiple shoot formation from nodal explants. Regardless of the cytokinin type, nodal explants showed axillary bud formation within two weeks. The percentage of explants showing shoot regeneration was high for both BAP and TDZ (Table 1). The explants that did not developed shoots became brownish and died. With increasing BAP and TDZ concentrations in culture medium, a slight increase in the multiplication rate was observed (Table 1). It was previously reported that low concentration of an auxin in combination with a cytokinin positively modifies the shoot induction frequency and their growth (Yuan *et al*. 1994, Vandemoortele *et al*. 1996, Singh and Sehgal 1999, Çöçü *et al*. 2004). Considering the percentage of explants producing shoots and the mean number of shoots per explant, the highest shoot regeneration capacity was achieved from explants on ½ MS medium supplemented with 30.0 μM BAP and 0.57 μM IAA. In our experiments, TDZ was less effective in axillary buds promotion than BAP. Furthermore, the addition of TDZ to the media reduced the shoot length significantly (Table 1). The inhibitory effect of TDZ on shoot elongation has also been reported for *S. fruticosa* (Arikat *et al*. 2004). BAP showed no inhibitory effect on *S. brachyodon* shoot elongation (Table 1). Contrary to our results, the presence of BAP in media inhibited shoot elongation in some other *Lamiaceae* species (Sánches-Gras and Calvo 1996, Singh and Sehgal 1999, Meszaros *et al*. 1999, Mišić *et al*. 2005).

The rooting of *S. brachyodon* shoots grown on hormone-free ½ MS culture medium was ~ 16 %. All the tested concentrations of BAP and TDZ completely inhibited the rooting of *S. brachyodon* shoots. In terms of rooting percentage, there were no differences between

Table 1. The effect of different BAP and TDZ concentrations, in combination with 0.57 M IAA, on axillary buds formation and growth of *S. brachyodon* shoots grown. Means ± SE, n = 50. Within each treatment, values with the same letter are not significantly different at the $P \leq 0.05$ level according to the LSD test.

IBA [μM]	TDZ [μM]	Explants producing shoots [%]	Number of axillary buds [explant^{-1}]	Shoot length [mm]
0		98 ± 2.0a	2.10 ± 0.069a	19 ± 0.9a
0.01		98 ± 2.0a	2.18 ± 0.284a	20 ± 0.9a
0.10		90 ± 4.2a	2.28 ± 0.115a	19 ± 1.2a
0.30		98 ± 2.0a	2.85 ± 0.199b	22 ± 1.5a
1.00		100 ± 0.0a	2.52 ± 0.169ab	19 ± 1.3a
3.00		100 ± 0.0a	3.00 ± 0.183b	20 ± 1.4a
10.00		98 ± 2.0a	3.47 ± 0.239bc	17 ± 1.2a
30.00		100 ± 0.0a	3.80 ± 0.162c	18 ± 1.1a
	0.01	66 ± 7.0a	1.83 ± 0.117b	16 ± 1.1a
	0.10	80 ± 5.7a	2.10 ± 0.129a	18 ± 1.1a
	0.30	82 ± 5.4a	1.83 ± 0.113ab	12 ± 1.1b
	1.00	82 ± 5.4a	1.90 ± 0.104ab	15 ± 1.5ab
	3.00	96 ± 0.0a	2.05 ± 0.085a	13 ± 1.6b
	10.00	94 ± 3.4a	2.35 ± 0.179ac	14 ± 1.5ab
	30.00	100 ± 0.0a	3.05 ± 0.203c	15 ± 2.0ab

shoots transferred to a hormone-free culture medium after one subculture on media with different concentrations of BAP and TDZ, and shoots without previous cytokinin tretment. Therefore, the additional experiments for rooting of shoots were performed with shoots treated with cytokinins during one subculture. All the tested concentrations of IBA, IAA and NAA, except 1.6 μM NAA, stimulated the rooting of *S. brachyodon* shoots (Table 2). It was previously reported that auxins play an essential role for root induction in shoots of *S. fruticosa* (Arikat *et al*. 2004). However, shoots of *S. bancoana*, *S. valentine* (Cuenca and Amo-Marco 2000), and *S. milthiorrhiza* (Morimoto *et al*. 1994) were able to form roots in the absence of auxins in the medium. As for rooting percentage, NAA was the least successful auxin

Table 2. Rooting [%] of *S. brachyodon* shoots after one month of culturing on ½ MS culture medium supplemented with different concentrations of IAA, IBA and NAA (means ± SE, n = 50). The value of the control group was 16 ± 6.4.

IAA [μM]	IBA [μM]	NAA [μM]	Rooting [%]
0.57			46 ± 10.0
1.10			54 ± 10.0
1.70			63 ± 9.9
	0.49		58 ± 10.1
	1.00		63 ± 9.9
	1.5		67 ± 9.6
		0.55	25 ± 7.7
		1.10	42 ± 10.0
		1.60	19 ± 6.9

in our experiments, which is in agreement with the results reported by Arikat *et al*. (2004) for *S. fruticosa* and for *S. valentine* (Cuenca and Amo-Marco 2000). Besides, Sánches-Gras and Calvo (1996) reported NAA inhibitory effect on rooting of *Lavandula latifolia*. We established that regardless of the auxin type and its concentration in the culture medium, roots developed after about twenty days of culturing.

The rooted shoots were transferred to pots and successfully acclimatized under greenhouse conditions. The survival of plants during acclimatization did not depend on the pretreatment with different types and concentrations of auxin. In all cases, the survival rate was ~75 %. The appearance of acclimatized plants was normal without any morphological abnormalities or variations (Fig. 1*C*).

In conclusion, the micropropagation protocol described makes rapid and large scale production of plantlets possible, starting with a small quantity of plant matherial. This should ease the prasure and diminish collection of this endemic species from the wild. That makes this method appropriate for *ex situ* conservation and for commercial production of *S. brachyodon* plantlets.

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