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Micropropagation of *Salvia broussonetii* Benth. - A Medicinal Plant Species

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Abstract

The purpose of this study was to establish culture medium requirements for micropropagation of *Salvia broussonetii* Benth., an important medicinal plant. Cultures were initiated from axillary shoots collected from mature plants. Most satisfactory results were achieved using a MS.2 medium supplemented with 1 mM ascorbic acid, 1.44 µM GA_3 and 1.11 µM BAP. Axillary nodes were used for *in vitro* regeneration of multiple shoots and best results were achieved with MS.2 medium plus 1.44 µM GA_3, 2.66 µM BAP and 1.14 µM IAA. Shoots rooted without symptoms of chlorosis or necrosis in half-strength MS.2 medium plus 1.44 µM GA_3 and 2.28 µM IAA.

Introduction

Published research on *in vitro* culture of *Salvia* species endemic to the Canary Islands, Spain is limited (Mederos-Molina 1991, 2004b; Mederos-Molina et al. 1994, 1997). So far, there are no reports on *in vitro* culture and plantlet regeneration in *Salvia broussonetii* Benth., an endemic shrub species from the Canary Islands. This species is a woody medicinal plant of the family Lamiaceae and it is found across Anaga region and locally abundant, Teno region, Tenerife, Canary Island. This medicinal plant shows a good deal of variation in seed-derived population. It also possesses antioxidant and antifungal chemicals (Mederos-Molina et al. unpublished data). This paper reports for the first time a protocol for *in vitro* propagation of *S. broussonetii* Benth.

Materials and Methods

The micropropagation of *Salvia broussonetii* Benth. has been carried out by axillary shoot culture from five-month-old juvenile branches collected from 20-year-old mature plants. Juvenile branches were washed with 1% benomyl

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solution for 10 min. The branches were then shaken in 96 % v/v ethanol for 3 min. Next, they were placed for 15 min in a 0.1 % v/v mercury chloride ($HgCl_2$)) solutions plus 0.3 % of Tween 20 surfactant. Finally, the branches were washed four times with sterile distilled water. After dissection, explants were placed into 24 × 16 mm test tubes containing 15 ml culture medium.

The explants were isolated and placed on several media supplemented with different plant growth regulators. For *in vitro* establishment of shoots, MS medium was tested. Also, this culture medium was used with various modifications involving the addition of 5 mM NH_4NO_3 [MS.1]; 10 mM NH_4NO_3 [MS.2], 15 mM NH_4NO_3 [MS.3], (Table 1). These media were supplemented with 1 mg/l of thiamine-HCl and pyridoxine-HCl, respectively 100 mg/l myo-inositol, 30 g/l sucrose, 1 mM ascorbic acid, 1.44 µM GA_3 and 5 g/l Difco Bacto agar. During the *in vitro* proliferation of shoots, culture media were supplemented with 1 mM ascorbic acid, 1.44 µM GA_3 plus BAP alone or combined with IAA (Table 2).

For *in vitro* rooting 23-day-old shoots obtained from proliferation stage were used. The shoots were cultured in half-strength MS.2 medium plus 1 mM ascorbic acid, 1.44 µM GA_3 and containing IAA, NAA or IBA (Table 3).

In all the culture media pH was adjusted to 5.6 which were sterilized under 0.5 atmosphere at 115 ^{o}C for 15 min. The cultures were incubated in a growth chamber at 25 ± 1.3 ^{o}C under 16 h photoperiod regimes and provided by Sylvania Gro-Lux fluorescent lights (between 15 and 35 µM/m^2s) and 75 % relative humidity. Each treatment had 24 replicates and all experiments were repeated twice.

To study the growth, development and proliferation of shoots in each of the above culture media, the following parameters such as axillary shoots with developed shoot and shoot proliferation; the elongation of shoots (Length, mm) and their quality (3 = vigour stemsx, 2 = slightly vigour stems and 1 = not vigour stem) were evaluated.

Data were analyzed using a one-way analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by the least significant difference test at the 5 % level of probability. Results were obtained from 23-day-old shoots or 23-day-old *in vitro* plantlets and each value represents the mean ± S.E. (Tables 1, 2.

Results and Discussion

In preliminary experiments, the effect of various concentrations of BAP or Kn was investigated in axillary shoots explants of *S. broussonetii* and maximum percentage of initial culture occurred on 1.11 µM BAP (data not shown). The NH_4NO_3 concentration has proved to be a vital factor in the *in vitro*

organogenesis and best results were obtained in the MS.2 and MS.1 media where the shoot development was 90 and 87 % respectively (Table 1). These modified media showed significant difference in terms of shoot lengths and quality; however, the longest and good quality shoots developed in MS.2 medium (Table 1) and in normal MS and MS.3 media shoots had malformed leaves (data not

Table 1. *In vitro* establishment of axillary shoots of *Salvia broussonetii* on different MS media plus 1.11 µM BAP. Cultures were incubated under 16 h photoperiod and 25 µE/m^{2}s.

Macroelement solutions	% shoot developed	Mean length of shoots (mm)	Quality of shoots
Control	0	0[a]	0[a]
MS	58	20[b] (0.3)	20[b] (0.3)
MS.1	87	25[d,c] (0.2)	25[c] (0.1)
MS.2	90	37[e] (0.4)	30[d] (0.0)
MS.3	71	23[c] (0.3)	25[c] (0.2)

Table 2. Effect of hormones supplemented into the MS.2 on shoots proliferation, length and quality of shoots of *Salvia broussonetii* Cultures were incubated under 16 h photoperiod and 35 µE/m^{2}s.

BAP (µM)	IAA	% shoot developed	Mean No. of shoot proliferate explant	Length of shoots (mm)	Quality of shoots
0	0	33	1.0[a] (0.0)	11.3[a] (0.2)	1.5[a] (0.2)
1.33	0	46	2.3[b] (0.5)	13.5[b,a] (0.3)	2.0[b] (0.3)
2.66	0	71	3.1[d] (0.7)	17.2[c] (0.6)	2.0[b] (0.3)
1.33	1.14	80	2.7[c,b] (0.3)	19.9[d,e] (0.5)	2.5[c] (0.3)
2.66	1.14	96	4.8[g] (0.5)	33.8[g] (0.6)	3.0[d] (0.0)
1.33	1.71	93	4.3[f,e] (0.3)	29.7[f] (0.5)	3.0[d] (0.0)
2.66	1.71	88	3.8[e] (0.5)	22.3[e] (0.6)	2.5[c] (0.2)

shown). The above study has shown how important it is to test a range of media when attempting to optimize the *in vitro* growth and development of shoots. These results confirmed that optimal composition of macronutrients strongly depended on explant's type and genotype of *Salvia* species (Mederos-Molina 1991, 2004b; Mederos-Molina et al. 1994, 1997). On the other hand, the effect of hormones on growth, development and proliferation of shoots were also observed. The highest number of multiple shoots regenerated from decapitated shoot-tips and maximal shoot proliferation occurred in MS.2 medium supplemented with 1.44 µM GA_3, 2.66 µM BAP and 1.14 µM IAA (Table 2). These results have shown that low levels of BAP were necessary for maximal shoot proliferation; this observation was different from those obtained with other medicinal species (Mederos-Molina 2002a,b; Dutta Gupta and Ibaraki 2006). In

contrast, at concentrations higher than 8.87 μM BAP the explants tended to develop adventitious shoots instead of axillary shoots without the intervention of callus, while in other medicinal species the behaviour was different (George 1993). Moreover, MS.2 medium had effect on shoot proliferation on shoot tips cultures (33 %).

Table 3. Effect of various auxins added into the half-strength MS.2 on roots proliferation of *Salvia broussonetii* cultures were incubated under 16 h photoperiod and 15 μE/m^{2}s.

IAA (μM)	NAA	IBA	% of shoots rooted	No of roots[(1)] per shoot	Mean length of roots (mm)	Quality of shoots
0	0	0	16	1.2^a (0.3)	13^a (0.5)	0^a
1.71	0	0	87	2.5^c (0.3)	38^d (0.6)	2.5^b (0.3)
2.28	0	0	90	4.7^f (0.4)	45^e (0.8)	3.0^c (0.0)
0	1.61	0	87	3.3^d (0.4)	34^c (0.7)	2.5^b (0.2)
0	2.15	0	85	3.7^{e,d} (0.5)	38^d (0.6)	3.0^c (0.0)
0	0	1.48	75	2.5^c (0.4)	26^b (0.7)	3.0^c (0.0)
0	0	1.97	71	2.3^b (0.3)	26^b (0.6)	3.0^c (0.0)

Individual shoots achieved from MS.2 + 1.44 μM GA_3, 2.66 μM BAP and 1.14 μM IAA exhibited differences in rooting ability on half-strength MS.2 + IAA, NAA and IBA (Table 3). There were no visible root development until two weeks after transfer to the rooting media. The percentage of rooting was markedly different ($p < 0.05$) in IBA treatment (75 and 71 % plantlets) (Table 3) were obtained giving an average of rooting between 2.5 and 2.3 respectively), and similar behaviour was observed in other woody plant species (Mederos-Molina 2002a,b, 2003). Moreover, inhibition of shoot multiplication and callus proliferation were apparent in week two for high doses of auxins (2.85 - 3.99 μM IAA; 2.69 - 3.76 μM NAA; 2.46 - 3.44 μM IBA), (data not shown in Table 3). More than 83 % of rooted shoots were obtained in the medium supplemented with BAP combined with IAA and NAA at different concentrations and these figures were significantly higher than all other treatments ($p < 0.05$) (Table 3). NAA and IAA were more effective for root initiation and elongation compared to IBA. These results are in agreement with other studies (Reddy et al. 2001; Sudha et al. 2005). Working with some other medicinal species, a different set of authors reported that the capacity of cultured shoots to develop roots was affected by the cultural conditions before, during and after shoot production.

These observations suggest that selection of the correct multiplication medium and the right cytokinin concentration in the low range is important for rooting (George 1993; Dutta Gupta and Ibaraki 2006). Work on trying combinations with other chemical supplements for micropropagation of shoot tips from *in vitro* germinated seeds that developed into healthy *Salvia broussonetii* is in progress.

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