Effect of plant growth regulators and explant type on in vitro shoot multiplication of *Salvia officinalis*

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Abstract

Salvia officinalis*, sage, is a strongly aromatic, perennial grayish shrub, up to 60 cm, usually with lilac flowers (May-July), which is found in garrigue, stony pastures, scrub, rocky places. It is one of the most important species of the genus *Salvia* worldwide, as it is cultivated in many varieties as a medicinal and ornamental plant. In the present study, the effect of plant growth regulators and explant type on in vitro shoot multiplication of this species was investigated, aiming to the development of a micropropagation protocol that could facilitate breeding and promotion of it for ornamental use. Shoot-tip and nodal explants excised from in vitro grown seedlings were initially cultured on MS medium without plant growth regulators (control) or supplemented with 0.1 mg L^{-1} 6-benzyladenine (BA) and 0.0 or 0.01 mg L^{-1} 1-naphthaleneacetic acid (NAA). In the following subculture, an additional medium supplemented with 0.1 mg L^{-1} BA and 0.02 mg L^{-1} NAA was also tested. Both during initial culture and the subculture, explants responded at high percentages (84-100%) producing shoots in all media. However, regarding the percentage of explants that produced shoots without hyperhydricity, this was higher in shoot-tip explants (79-98%) than in nodal ones (35-83%), as well as in the control (75-93%) and media containing BA and NAA (80-98%) compared to medium containing only BA (35-79%). More normal shoots per explant (almost double) were produced by nodal explants (1.7-2.1) in comparison to shoot-tip explants (1.0-1.1). During initial culture, the longest shoots (3.0-3.6 cm) were produced in the control, while during subculture, shoot-tip explants cultured on the control or on a medium containing BA and NAA produced longer shoots (2.1-2.4 cm). In conclusion, the use of shoot-tip explants and the addition of NAA in the medium limited shoot hyperhydricity, while the use of nodal explants resulted in higher shoot production.

Keywords: auxin, cytokinin, hyperhydricity, medicinal and aromatic plant, shoot tip or nodal explant

INTRODUCTION

Genus *Salvia* is the largest in the plant family of *Lamiaceae* (Labiatae) (Kamatou et al., 2010). Many species of this genus are used as ornamental plants, in cooking, and in herbal medicine due to the essential oil found in the leaves. In Greece, there are 30 taxa (species and subspecies) of the genus *Salvia* (Dimopoulos et al., 2013). The most representative species within the genus *Salvia* is *Salvia officinalis* L., the common sage. The name *Salvia* comes from the Latin verb "*salvare*" (= save lives) (https://bit.ly/2Swt2eK) and the name *officinalis* refers to medicinal use of plants *officina* being the warehouse of a monastery to store herbs and medicines (Stearn, 2004). The common name "sage" is attributed to different species of the genus *Salvia*, which are widely used as ornamental or medicinal plants (Vidic et al., 2010).

Salvia officinalis is a strongly aromatic, perennial grayish shrub, up to 60 cm, which is found in garrigue, rocky pastures, scrub, rocky places. It is cultivated worldwide in many varieties as medicinal and ornamental. It blooms from late spring (May) to summer (July). Flowers are lilac in color (most common), though they can also be white, purple or pink

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(Clebsch and Carol, 2003). The leaves are oblong, gray-green, rugose on the upper side, and nearly white underneath due to the many short soft hairs (Clebsch and Carol, 2003). The aboveground part of the plant contains essential oil, rich in camphor, cineole, borneol and thujone (Said-Al Ahl et al., 2015; Khedher et al., 2017). Many properties have been mentioned for this herb, such as anti-seizure and anti-cough, anti-inflammatory, antiviral, antibacterial and anti-inflammatory (Baricevic et al., 2001; Bozin et al., 2007; Šmidling et al., 2008; Qnais et al., 2010; Tosun et al., 2014; Fournomiti et al., 2015). The species is quite appropriate for ornamental use, particularly as landscape plant in xeriscaping, green roofs and landscape restoration mainly in the Mediterranean region.

The propagation of *S. officinalis* is presenting difficulties. There are many reports on seed propagation of the species where various treatments have been tested to improve the rather low germination ability (Vlachou et al., 2020a; Dastanpoor et al., 2013). Clonal propagation by stem cuttings is quite satisfactory resulting in rooting percentages over 70% with certain pretreatments (Martini et al., 2000; Paradiković et al., 2013; Kaçar et al., 2009), and is the method employed for commercial propagation of the species.

Micropropagation is appropriate for species that cannot be efficiently propagated by conventional horticultural techniques and an important tool to select, multiply and conserve the critical genotypes of medicinal plants in particular (Tripathi and Tripathi, 2003; Debnath et al., 2006). There are several studies concerning micropropagation of *S. officinalis*, but none of them present a complete micropropagation protocol of the species, indicating difficulties such as stress symptoms (hyperhydration, browning, reduced growth etc) in produced micro-shoots (Olszowska and Furmanowa, 1990; Avato et al., 2005) or unsatisfactory rooting of micro-shoots (Gostin, 2008; Petrova et al., 2015). BAP at concentration 0.5 or 1.0 mg L^{-1}, alone or in combination with 0.1 mg L^{-1} NAA, was tested for shoot multiplication in several studies (Avato et al., 2005; Gostin, 2008; Petrova et al., 2015) and it was found superior to kinetin (Avato et al., 2005) or thidiazuron, zeatin and 2iP (Petrova et al., 2015),

In the above mentioned studies, shoot tip or nodal explants or both types from seedlings or adult plants were used, but the two explant types were not compared regarding their response. Explant type is a factor that may affect shoot proliferation, since nodal explants have been found to produce more shoots than shoot tips in other species of the genus *Salvia*, such as *S. fruticosa* (Arikat et al., 2004), *S. splendens* (Sharma et al., 2014), *S. valentina* and *S. blancoana* subsp. *mariolensis* (Cuenca and Amo-Marco, 2000) and *S. sclarea* L. (Ghanbar et al., 2016). On the other hand, shoot tips were superior to nodal explants in shoot multiplication in other Labiatae species (Vlachou et al., 2016).

In the present study the effect of plant growth regulators and explant type on in vitro shoot multiplication of *S. officinalis* were investigated, aiming to the development of an efficient micropropagation protocol starting from in vitro seedlings that could facilitate breeding programs of the species and promotion of Greek sage clones for ornamental or/and medicinal use.

MATERIALS AND METHODS

The initial in vitro culture was established from shoot tip and nodal explants excised from one-month old seedlings *S. officinalis* grown in vitro, on MS medium (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose, either without plant growth regulators (control) or supplemented with 0.1 mg L^{-1} BA and 0.0 or 0.01 mg L^{-1} NAA. In shoot tip and nodal explants, 30 and 40 explants per treatment were used, respectively. In the following subculture, the explants (shoot tip or nodal) were cultured on solid MS medium with 30 g L^{-1} sucrose without plant growth regulators (control) or supplemented with 0.1 mg L^{-1} BA and 0.0 or 0.01 or 0.02 mg L^{-1} NAA. Thirty explants per treatment were used.

All media were solidified with 8 g L^{-1} agar and their pH was adjusted to 5.7-5.8 before agar addition and autoclaving (121°C for 20 min). The cultures were maintained for 5 weeks at 25°C with a 16 h photoperiod at 37.5 μmol m^{-2} s^{-1} fluorescent light, provided by cool-white fluorescent lamps. Data were collected after 30 d of culturing.

The "multiplication index" of each culture was calculated by multiplying the percentage of explants that produced shoots by the mean number of shoots per responding explant, and

by the mean node number per shoot.

The completely randomized design was used. The significance of the results was tested by either one- or two-way analysis of variance (ANOVA) and the means of the treatments were compared by Student's t test at $P \leq 0.05$ (JMP 11.0software, SAS Institute Inc., Cary, NC, 2013, USA).

RESULTS AND DISCUSSION

In the initial culture, explant response for shoot production was very high in both types of explant (87-100%) in all media. However, on the medium with 0.1 mg L^{-1} BA without NAA nodal explant response was slightly reduced, while many explants, particularly the nodal one, formed hyperhydrated shoots only (27 and 52% for tip and nodal explants, respectively) (Table 1). The possibility of high percentage of hyperhydrated or necrosed explants during establishment, reported also by Avato et al. (2005), led us use low concentration of BA. The highest number (almost double) of normal shoots per explant was produced by nodal explants, especially on the control medium, while shoot length and node number were highest in shoot tip explants on the control medium (Table 1). The multiplication index was highest in nodal explant on the control medium (Table 1).

Table 1. Effect of BA and NAA concentration on shoot multiplication from shoot tip or nodal explants excised from *S. officinalis* in vitro grown seedlings, at the establishment stage.

BA/NAA conc. (mgL^{-1})	Shooting[a] (%)	Shooting[b] (%)	Mean NSh[c] number	Mean NSh[c] length (cm)	Mean node number[c]	Mean HSh[d] number	Multiplication index[e]
			Shoot tip explant				
-/- (Hf[f])	93 a[g]	7 c	1.1 c	3.6 a	4.7 a	0.1 c	4.8 b
0.1/0.0	70 d	27 b	1.0 c	2.4 bc	3.7 b	0.1 c	2.6 d
0.1/0.01	97 a	0 d	1.1 c	2.5 bc	3.5 b	0.0 d	3.7 c
			Nodal explant				
-/- (Hf[f])	75 c	23 b	2.1 a	3.0 b	3.7 b	0.5 b	5.8 a
0.1/0.0	35 e	52 a	1.8 b	1.6 d	2.4 c	1.1 a	1.5 e
0.1/0.01	80 b	18 b	1.9 b	2.3 cd	3.3 b	0.6 b	5.0 b
$F_{\text{one-way ANOVA}}$	***	**	***	***	***	**	***

, *: significant at $P \leq 0.01$, $P \leq 0.001$, respectively, $n=30$.

[a]The explants produced normal and hyperhydrated shoots.

[b]The explants produced hyperhydrated shoots only.

[c]NSh=normal shoot.

[d]HSh=hyperhydrated shoot.

[e]Multiplication Index=Shooting (%) x mean shoot number[c] x Mean node number[c]

[f]Hf=hormone free.

[g]Mean separation in columns by Student's *t*, $P \leq 0.05$.

In the subculture explants responded in a similar way to the initial culture (Table 2; Figure 1), producing shoots at high percentage, but only 1-2 shoots per explant, either without or at low (0.1 mg L^{-1}) BA concentration. In other studies for the same species, optimum shoot induction from nodal explants was reported on MS medium supplemented with higher concentration of cytokinin, 0.5 or 1.0 mg L^{-1} BAP (4.4-4.6 shoots explant^{-1}) Avato et al. (2005) or 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA (5.3 shoots explant^{-1}) Petrova et al. (2015), while Santos-Gomes et al. (2002) found a higher shoot number (3.2) on medium with 1.5 mg L^{-1} BA and 0.05 mg L^{-1} 2,4-D, however stress problems of the shoots was often an issue (Avato et al., 2005). For other *Salvia* species, optimum shoot induction has been reported only for *S. fruticosa* on medium enriched with low BA (0.2 mg L^{-1}) in combination with, 0.02 mg L^{-1} NAA and 0.04 mg L^{-1} GA_3 (Arikat et al., 2004), while higher concentration of BA (1.0 mg L^{-1}) was

used for *S. splendens* (Sharma et al.,2014), *S. valentina* and *S. blancoana* subsp. *mariolensis* (Cuenca and Amo-Marco, 2000). The addition of NAA into the BA medium reduced hyperhydricity (independently of the explant origin), as in the initial culture, similarly to results reported for other Mediterranean species, *Clinopodium nepeta* (Vlachou et al., 2019) and *Anthyllis barba-jovis* (Vlachou et al., 2020b). Both tested concentrations of NAA gave equal explant response.

Table 2. Effect of BA and NAA concentration on shoot multiplication from shoot tip or nodal explants in the first subculture.

BA/NAA conc. (mgL^{-1})	Shooting[a] (%)	Shooting[b] (%)	Mean NSh[c] number	Mean NSh[c] length (cm)	Mean node number[c]	Mean HSh[d] number	Multiplication index[e]
Shoot tip explant							
-/- (Hf[f])	93 b[g]	7 c	1.0 d	2.4 a	3.3 a	0.1 d	3.1 b
0.1/0.0	79 c	17 b	1.0 d	1.8 bc	2.4 c	0.2 d	1.9 c
0.1/0.01	98 a	2 e	1.0 d	2.2 ab	3.4 a	0.1 d	3.3 ab
0.1/0.02	96 ab	4 d	1.0 d	2.1 abc	3.1 ab	0.1 d	3.0 b
Nodal explant							
-/- (Hf[f])	78 c	18 b	1.7 c	2.0 bc	2.4 c	0.4 b	3.2 b
0.1/0.0	38 d	46 a	2.0 a	1.5 c	2.0 c	1.1 a	1.5 c
0.1/0.01	80 c	18 b	1.8 ab	1.8 bc	2.5 bc	0.3 c	3.6 a
0.1/0.02	83 c	14 b	1.7bc	1.6 c	2.3 c	0.3 c	3.2 ab
F_{NAA}	-	-	NS	NS	**	-	***
$F_{explant}$	-	-	***	*	***	-	NS
$F_{NAA \times explant}$	***	***	NS	NS	NS	***	NS
$F_{one\text{-}way\ ANOVA}$	***	***	***	**	***	***	***

NS: not significant or *, **, ***: significant at $P \leq 0{,}05$, $P \leq 0{,}01$, $P \leq 0{,}001$, respectively, $n=24$.

[a]The explants produced normal and hyperhydrated shoots.

[b]The explants were produced only hyperhydrated shoots.

[c]NSh=normal shoot.

[d]HSh=hyperhydrated shoot.

[e]Multiplication Index=Shooting (%) x mean shoot number[c] x Mean node number[c]

[f]Hf=hormone free.

[g]Mean separation in columns by Student's *t*, $P \leq 0.05$.

The two-way ANOVA showed no significant interaction between explant type and NAA concentration concerning the mean number and length of produced shoots, mean number of nodes, and multiplication index (data for two-way ANOVA not shown). Shoot number and length was affected only by explant type, the number of nodes by both factors, and the multiplication index by NAA concentration.

More normal shoots (almost double) were produced by nodal explants (1.8-2.1 shoots $explant^{-1}$) in comparison to shoot-tip explants (1.0-1.1 shoots $explant^{-1}$), while shoot length and node number were highest on the medium without plant growth regulators for both explant types (Table 2; Figure 1). The use of nodal explants resulted in higher shoot production compared to shoot-tip explants, like in a number of other species of the genus *Salvia*, such as *S. fruticosa* (Arikat et al., 2004), *S. splendens* (Sharma et al., 2014), *S. valentina* and *S. blancoana* subsp. *mariolensis* (Cuenca and Amo-Marco, 2000) and *S. sclarea* L. (Ghanbar et al., 2016), although shoot-tip explants also responded satisfactorily. In the medium without plant growth regulators, spontaneous rooting of more than 80% was observed in both types of explants, both in the initial culture and in the first subculture (Figure 1). Conclusively, the use of shoot-tip explants and the addition of low NAA concentration in a BA-medium limited

shoot hyperhydricity, while the use of nodal explants resulted in higher shoot production. Experimentation is in progress for improving the micropropagation protocol, testing higher concentrations of BA and other cytokinins, in combination with auxin, aiming to higher productivity without hyperhydricity problems, as well as examining rooting of microshoots in relation to the multiplication media used for their production.

Figure 1. Variation in the response of *Salvia officinalis* cultured on Murashige and Skoog (1962) growth medium (MS) either hormone free (a), or supplemented with 0.1 mg L^{-1} BA (b), or 0.1 mg L^{-1} BA/0.01 mg L^{-1} NAA (c) or 0.1 mg L^{-1} BA/0.02 mg L^{-1} NAA (d) (Right: shoot tip explants and Left: nodal explants). Nodal explant at the first subculture on MS medium supplemented with 0.1 mg L^{-1} BA that produced either hyperhydrated shoots only (e) or normal shoots (NSh) along with hyperhydrated shoots (HSh) (f).

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Figure 1. Characteristic adult plant of *Salvia officinalis* during the flowering period

INTRODUCTION

Salvia officinalis is a strongly aromatic, perennial grayish shrub, up to 60 cm, which is found in garrigue, rocky pastures, scrub, rocky places (Figure 1). It is cultivated worldwide in many varieties as medicinal and ornamental. Many properties have been mentioned for this herb, such as anti-seizure and anti-cough, anti-inflammatory, antiviral, antibacterial and anti-inflammatory.

In the present study the effect of plant growth regulators and explant type on in vitro shoot multiplication of this species were investigated, aiming to the development of a micropropagation protocol from in vitro seedlings that could facilitate breeding and promotion of Greek sage species for ornamental use.

MATERIALS AND METHODS

In vitro culture was established from shoot tip and nodal explants, excised from one-month old seedlings of *S. officinalis* grown in vitro, on MS medium with 30 g L^{-1} sucrose and either without plant growth regulators (control) or supplemented with 0.1 mg L^{-1} BA and 0.0 or 0.01 mg L^{-1} NAA. In the following subculture, explants, shoot tip and nodal, were cultured on control or MS medium supplemented with 0.1 mg L^{-1} BA and 0.0 or 0.01 or 0.02 mg L^{-1} NAA. All media were solidified with 8 g L^{-1} agar and their pH was adjusted to 5.7 – 5.8 before addition of the agar and autoclaving.

RESULTS AND DISCUSSION

At the establishment, shoots were produced at high percentages (87-100%) by both types of explants in all media. However, on the medium with 0.1 mg L^{-1} BA without NAA nodal explant response was slightly reduced, while many explants, particularly the nodal ones, formed only hyperhydrated shoots at high percentage (Table 1). The highest number (almost double) of normal shoots per explant was produced by nodal explants, while shoot length and node number were highest in shoot tip explants on the control medium (Table 1).

In the subculture, explants responded in a similar way to the establishment (Table 2, Figure 2), producing shoots at high percentage, but only 1-2 shoots per explant, either without or at low (0.01 mg L^{-1}) BA concentration. The addition of NAA into BA medium reduced hyperhydricity (independently of the explant origin). Both tested concentrations of NAA gave equal explant response. More normal shoots (almost double) were produced by nodal explants (1.8-2.1 shoots per explant) in comparison to shoot-tip explants (1.0-1.1 shoots per explant), while shoot length and node number were highest on the medium without plant growth regulators for both explant types (Table 2, Figure 2). In addition, in the control, spontaneous rooting of more than 80% was observed in both types of explants, both in the initial culture and in the first subculture (Figure 2).

Table 1. Effect of BA and NAA on shoot multiplication of shoot tip and nodal explants excised from *S. officinalis* in vitro seedling, at the establishment stage.

BA / NAA concn (mg L^{-1})	Shooting[1] (%)	Shooting[2] (%)	Mean NSh^{T} number	Mean NSh length^{T} (cm)	Mean node number^{T}	Mean HSh^{TT} number
Shoot tip explant						
-/- (Hf^{TTT})	93 a[z]	7 c	1.1 c	3.6 a	4.7 a	0.1 c
0.1 / 0.0	70 d	27 b	1.0 c	2.4 bc	3.7 b	0.1 c
0.1 / 0.01	97 a	0 d	1.1 c	2.5 bc	3.5 b	0.0 d
Nodal explant						
-/- (Hf^{TTT})	75 c	23 b	2.1 a	3.0 b	3.7 b	0.5 b
0.1 / 0.0	35 e	52 a	1.8 b	1.6 d	2.4 c	1.1 a
0.1 / 0.01	80 b	18 b	1.9 b	2.3 cd	3.3 b	0.6 b
$F_{one\text{-}way\ ANOVA}$	***	**	***	***	***	**

[z]Mean separation in columns by Student's t, P ≤0.05.
, *: significant at P ≤0,01, P ≤0,001, respectively, n=30.
[1] referring to explants that produced normal shoots
[2] referring to explants that produced only hyperhydrated shoots
^{T}NSh = normal shoot, ^{TT}HSh = hyperhydrated shoot, ^{TTT}Hf = hormone free

Table 2. Effect of BA and NAA on shoot multiplication of *S. officinalis* shoot tip and nodal explants , at the first subculture.

BA / NAA concn (mg L^{-1})	Shooting[1] (%)	Shooting[2] (%)	Mean NSh^{T} number	Mean NSh length^{T} (cm)	Mean node number^{T}	Mean HSh^{TT} number
Shoot tip explant						
-/- (Hf^{TTT})	93 b[z]	7 c	1.0 d	2.4 a	3.3 a	0.1 d
0.1 / 0.0	79 c	17 b	1.0 d	1.8 bc	2.4 c	0.2 d
0.1 / 0.01	98 a	2 e	1.0 d	2.2 ab	3.4 a	0.1 d
0.1 / 0.02	96 ab	4 d	1.0 d	2.1 abc	3.1 ab	0.1 d
Nodal explant						
-/- (Hf^{TTT})	78 c	18 b	1.7 c	2.0 bc	2.4 c	0.4 b
0.1 / 0.0	38 d	46 a	2.0 a	1.5 c	2.0 c	1.1 a
0.1 / 0.01	80 c	18 b	1.8 ab	1.8 bc	2.5 bc	0.3 c
0.1 / 0.02	83 c	14 b	1.7 bc	1.6 c	2.3 c	0.3 c
F_{NAA}	-	-	NS	NS	**	-
$F_{explant}$	-	-	***	*	***	-
$F_{NAA\ x\ explant}$	***	***	NS	NS	NS	***
$F_{one\text{-}way\ ANOVA}$	***	***	***	**	***	***

[z]Mean separation in columns by Student's t, P ≤0.05.
NS: not significant or *, **, ***: significant at P ≤ 0,05, P ≤ 0,01, P ≤ 0,001, respectively, n=24.
Multiplication Index = Shooting (%) x mean shoot number^{T} x Mean node number^{T}
[1] referring to explants that produced normal shoots
[2] referring to explants that produced only hyperhydrated shoots
^{T}NSh = normal shoot, ^{TT}HSh = hyperhydrated shoot, ^{TTT}Hf = hormone free

Figure 2. Variation in the response of *Salvia officinalis* explants cultured on MS either hormone free (a), or supplemented with 0.1 mg L^{-1} BA (b), or 0.1 mg L^{-1} BA / 0.01 mg L^{-1} NAA (c) or 0.1 mg L^{-1} BA / 0.02 mg L^{-1} NAA (d) (Right: shoot tip explants and Left: nodal explants). Nodal explant at the first subculture on MS medium supplemented with 0.1 mg L^{-1} BA that produced either hyperhydrated shoots only (e) or normal shoots (NSh) along with hyperhydrated shoots (HSh) (f).

CONCLUSIONS

Use of shoot-tip explants and addition of low NAA concentration in a BA-medium limited shoot hyperhydricity, while use of nodal explants resulted in higher shoot production.

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