THE EFFECT OF CYTOKININ TYPE AND CONCENTRATION ON *in vitro* MULTIPLICATION OF *Salvia fruticosa*

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Abstract

Salvia fruticosa Mill. (Lamiaceae), Greek sage, is a perennial sage species endemic of the Mediterranean region, part of the macchia vegetation, with a wider distribution from Sicily to Israel. Aiming to impove and promote the species for ornamental and medicinal use, in the present work the effect of cytokinin type and concentration on its *in vitro* propagation was studied. 6-benzyladenine (BA), zeatin (ZEAT) or meta-Topolin (mT) at 0.0, 0.4, 0.8, 1.6 or 3.2 mg L^{-1} were combined with 0.01 mg L^{-1} 1-naphthaleneacetic acid (NAA) in the culture medium (solid MS). Shoot tip or single-node explants from microshoots originated from explants excised from *in vitro* grown seedlings were used. The response for shoot production was very high in shoot tip explants (over 90%) in most media, while in nodal explants was slightly lower (75-85%) when the medium was supplemented with ZEAT or BA. The presence of cytokin compared to the hormone-free medium promoted slightly the number of shoots produced decreasing simultaneously their length. However, cytokinin in high concentrations, regardless of cytokinin type, resulted in the formation of hyperhydrated shoots, which reached up to 62% in the substrate with 3.2 mg L^{-1} ZEAT. There was an indication that most normal shoots (2.4-2.5 shoots explant) were produced on 0.8 or 1.6 mg L^{-1} BA medium from nodal explants, while the highest multiplication rate was observed on 0.8 mg L^{-1} mT medium. The increase in concentration of all three types of cytokins tested resulted in an increase in the number of shoots produced, but this increase was mainly reflected in hyperhydrated shoots.

Keywords: *6-benzyladenine, hyperhydricity, Mediterranean sage, meta-Topolin, zeatin*

Introduction

S. fruticosa Mill. (Lamiaceae), Greek sage, is a strongly aromatic, perennial evergreen shrub, up to 1.20 m high, growing mainly in bushy rocky areas, often on coastal cliffs, at altitudes 1-700 m (Blamey and Grey-Wilson, 1993; Thanos and Doussi, 1995). It is endemic to the eastern Mediterranean, including southern Italy, North Africa and the Canary Islands, while in Greece, it is found in the Central country, the Peloponnese and the Aegean islands (Thanos and Doussi, 1995). In Greece, it has been traditionally used as a medicinal, culinary and melliferous plant since the antiquity (Clebsch and Barner, 2003). Nowadays, it is widely used for the preparation of an herbal tea (faskomilo) (Hanson, 2004).

The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants, while plant tissue culture techniques offer an integrated approach for the production of active compounds and standardized quality phytopharmaceutical for herbal and pharmaceutical industries (Debnath *et al.*, 2006; Sidhu, 2011). There are a few reports about the use of tissue culture techniques in *S. fruticosa* aiming to its micropropagation and the accumulation of essential oils or other compounds (Karam et al., 2003; Arikat et al., 2004). Meta-topolin (mT) is a natural aromatic cytokinin that is shown to be more effective in morphogenesis than other cytokinins, regarding regeneration capacity,

reduced hyperhydricity and root inhibition (Krishna Vrundha *et al.*, 2021), which would worth being tested on *in vitro* shoot multiplication of *S. fruticosa*. It has been used on *in vitro* propagation of *Salvia sclarea* and was found superior to other cytokinins (Erişen *et al.*, 2020)
So, in the present study the effect of cytokinin type and concentration on *in vitro* propagation of *S. fruticosa* was studied, aiming to impove and promote the species for ornamental and medicinal use.

Materials and Methods

Shoot tip or single-node explants excised from microshoots of *in vitro* cultures of *S. fruticosa* initiated from *in vitro* grown seedlings were used. The explants were cultured on MS medium (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose either without plant growth regulators (control) or supplemented with three different cytokinins, i.e. 6-benzyladenine (BA), zeatin (ZEAT) or meta-Topolin (mT), at four concentrations, i.e., 0.4, 0.8, 1.6, and 3.2 mg·L^{-1} in combination with 0.01 mg L^{-1} 1-naphthaleneacetic acid (NAA).
All media were solidified with 8 g L^{-1} agar and their pH was adjusted to 5.7 before agar addition and autoclaving (121 ^{o}C for 20 min). The cultures were maintained at 25 ^{o}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 days of culture.
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- or three-way analysis of variance (ANOVA) and the means of the treatments were compared by Student's *t* test at $P < 0.05$ (JMP 13.0 software, SAS Institute Inc., Cary, NC, 2013, USA).

Results and Discussion

The 3-way ANOVA in most parameters measured revealed significant interaction of the three nain factors of the experiment. The type and concentration of cytokinin had a significant effect on the length of normal shoots, while the explant type had an effect on the number of normal shoots and their node number, as well as on the multiplication index. Nodal explants resulted in higher number of normal shoots produced per responded explant, while tip explants in higher node number per shoot and higher multiplication index.
The response for shoot production was very high in tip explants (over 90%) in most media, while in nodal explants was slightly lower (73-85%) when the medium was supplemented with ZEAT or BA (Table 1). Cytokinin in high concentrations, regardless of cytokinin type, resulted in the formation of hyperhydrated shoots, which reached up to 62% when nodal explants were cultured on a medium with 3.2 mg L^{-1} ZEAT or mT (Table 1).
The presence of cytokinin compared to the hormone free medium promoted slightly the number of shoots produced per explant decreasing simultaneously their length. There was an indication that the highest number of normal shoots (2.4-2.5 shoots per responded explant) was produced on 0.8 or 1.6 mg L^{-1} BA medium when nodal explants were used, while the longest shoots (2.2 cm) with a higher number of nodes (3.3 nodes /shoot) were produced when tip explants were cultured on hormone-free medium, but with no significant difference from many other media (Table 1, Figure 1). The increase in concentration of all three types of cytokinins tested resulted in an increase in the number of produced shoots, but this increase was mainly reflected in hyperhydrated shoots. The highest number of hyperhydrated shoots

was observed on the 3.2 mg L^{-1} ZEAT medium for both explant types (2.3-2.4 hyperhydrated shoots per responded explant) (Table 1).

Taking into consideration the highest percentage of explants that responded to form normal shoots, hormone-free medium and media with 0.4 mg L^{-1} ZEAT or BA and 0.4 or 0.8 mg L^{-1} mT were distinguished (Table 1). In previous studies on micropropagation of *S. fruticosa* (Arikat *et al.*, 2004) and *S. officinalis* (Petrova *et al.*, 2015) was also shown that shoot proliferation was favored by low concentrations of BA (0.2-0.5 mg L^{-1}). In the present study, the medium with 0.8 mg L^{-1} mT was superior to others, since it resulted to the highest multiplication rate and good tissue quality (Figure 1), followed by that with 0.4 mg L^{-1} mT (Table 1). This result verifies other studies on mT, which have shown its superiority in shoot regeneration capacity, along with increased plant tissue quality (Kőszeghi *et al.*, 2014; Erişen *et al.*, 2020; Krishna Vrundha *et al.*, 2021).

Table 1. Effect of explant type and cytokinin type and concentration on shoot multiplication of explants excised from *in vitro* seedlings of *S. fruticosa* and cultured in a medium with marked cytokinin type and concentration in combination with 0.01 mg L^{-1} NAA.

Cytokinin concn (mg L^{-1})	Shoot production[1] (%)	Shoot production[2] (%)	Mean NSh$^{\overline{T}}$ number	Mean NSh length$^{\overline{T}}$ (cm)	Mean NSh node number$^{\overline{T}}$	Mean HSh$^{\overline{TT}}$ number	Multiplication index for NSh
Shoot tip explant							
0.0 (Hf$^{\overline{TTT}}$)	84 a[z]	8 m	1.0 f	2.2 a	3.3 a	0.1 l	2.8 bcd
0.4 ZEAT	83 a	13 l	1.1 f	1.6 cdef	2.5 bcd	0.2 k	2.3 def
0.8 ZEAT	67 def	21 j	1.4 def	1.3 cdef	2.5 bcd	0.3 jk	2.3 def
1.6 ZEAT	59 gh	29 h	1.3 ef	1.1 efg	2.3 cde	0.6 hi	1.8 fgh
3.2 ZEAT	21 lm	58 a	2.2 ab	0.7 hi	1.2 f	2.3 a	0.6 i
0.4 BA	80 ab	20 j	1.6 cde	0.8 ghi	1.2 f	0.3 jk	1.5 ghi
0.8 BA	67 def	27 h	1.6 cde	0.8 ghi	1.6 ef	0.5 i	1.7 fgh
1.6 BA	67 def	33 fg	1.9 abcd	0.7 hi	1.7 def	0.8 fg	2.2 def
3.2 BA	67 def	33 fg	2.0 abc	0.7 hi	1.6 ef	0.8 fg	2.1 efg
0.4 mT	73 c	21 j	1.7 bcd	2.0 ab	2.9 ab	0.6 hi	3.6 b
0.8 mT	77 b	23 i	2.2 a	1.8 abcd	2.7 bc	0.6 hi	4.6 a
1.6 mT	65 ef	35 f	1.8 bcd	1.6 cde	2.4 cde	0.9 def	2.8 cde
3.2 mT	62 fg	38 de	2.1 abc	1.4 cdef	1.7 def	1.0 de	2.2 def
Nodal explant							
0.0 (Hf$^{\overline{TTT}}$)	71 cd	17 k	1.7 bcd	1.4 cdef	2.3 cde	0.3 jk	2.8 cde
0.4 ZEAT	50 ij	29 h	2.0 abc	1.2 def	1.7 def	0.7 gh	1.7 fgh
0.8 ZEAT	46 jk	33 fg	1.9 abcd	1.1 efg	1.7 def	1.0 de	1.5 ghi
1.6 ZEAT	46 jk	37e	2.0 abc	1.0 fgh	1.7 def	1.0 de	1.6 gh
3.2 ZEAT	13 m	62 a	2.0 abc	0.8 ghi	1.3 f	2.4 a	0.3 i
0.4 BA	67 def	20 j	2.0 abc	0.7 hi	1.5 ef	0.6 hi	2.0 efg
0.8 BA	50 ij	27 h	2.5 a	0.6 i	1.2 f	1.0 de	1.5 ghi
1.6 BA	40 k	40 cd	2.4 a	0.6 i	1.2 f	1.0 de	1.2 hi
3.2 BA	23 l	50 b	2.2 ab	0.6 i	1.1 f	1.2 c	0.5 i

0.4 mT	56 hi	27 h	1.7 bcd	1.9 abc	2.7 bc	0.6 hi	2.6 def
0.8 mT	67 def	29 h	2.2 ab	1.4 cdef	2.4 cde	0.9 def	3.5 bc
1.6 mT	58 gh	42 c	1.7 bcd	1.2 def	1.6 ef	1.4 b	1.6 gh
3.2 mT	38 k	62 a	1.5 def	1.0 fgh	2.0 def	1.6 b	1.1 hi
$F_{\text{cytokinin}}$	-	-	-	***	-	-	-
$F_{\text{concentration}}$	-	-	-	*	-	-	-
F_{explant}	-	-	*	NS	**	-	***
$F_{\text{cytok x concn}}$	***	***	**	NS	*	***	**
$F_{\text{cytok x explant}}$	***	***	NS	NS	NS	NS	NS
$F_{\text{concn x explant}}$	**	***	NS	NS	NS	***	NS
$F_{\text{cyt x exp x concn}}$	***	***	NS	NS	NS	***	NS
$F_{\text{one-way}}$	***	***	***	***	***	***	***

[z]Mean separation in columns by Student's *t*, $P \leq 0.05$.
NS: not significant or *, **, ***: significant at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$, respectively, n=30.
Multiplication Index = Shooting (%) x mean shoot number[Ŧ] x Mean node number[Ŧ]
[1]The explants produced normal and hyperhydrated shoots
[2]The explants produced hyperhydrated shoots only
[Ŧ]NSh = normal shoot
[ŦŦ]HSh = hyperhydrated shoot
[ŦŦŦ]Hf = hormone free

Conclusions

The increase in concentration of all three types of cytokinins tested (ZEAT, BA, mT) resulted in an increase in the number of shoots produced, but this increase was mainly reflected in hyperhydrated shoots. Low concentration (0.4 or 0.8 mg L^{-1}) was preferable for all tested cytokinins, but meta-Topolin at 0.8 mg L^{-1} was superior to others, due to high shoot multiplication rate simultaneously with good plant tissue quality.

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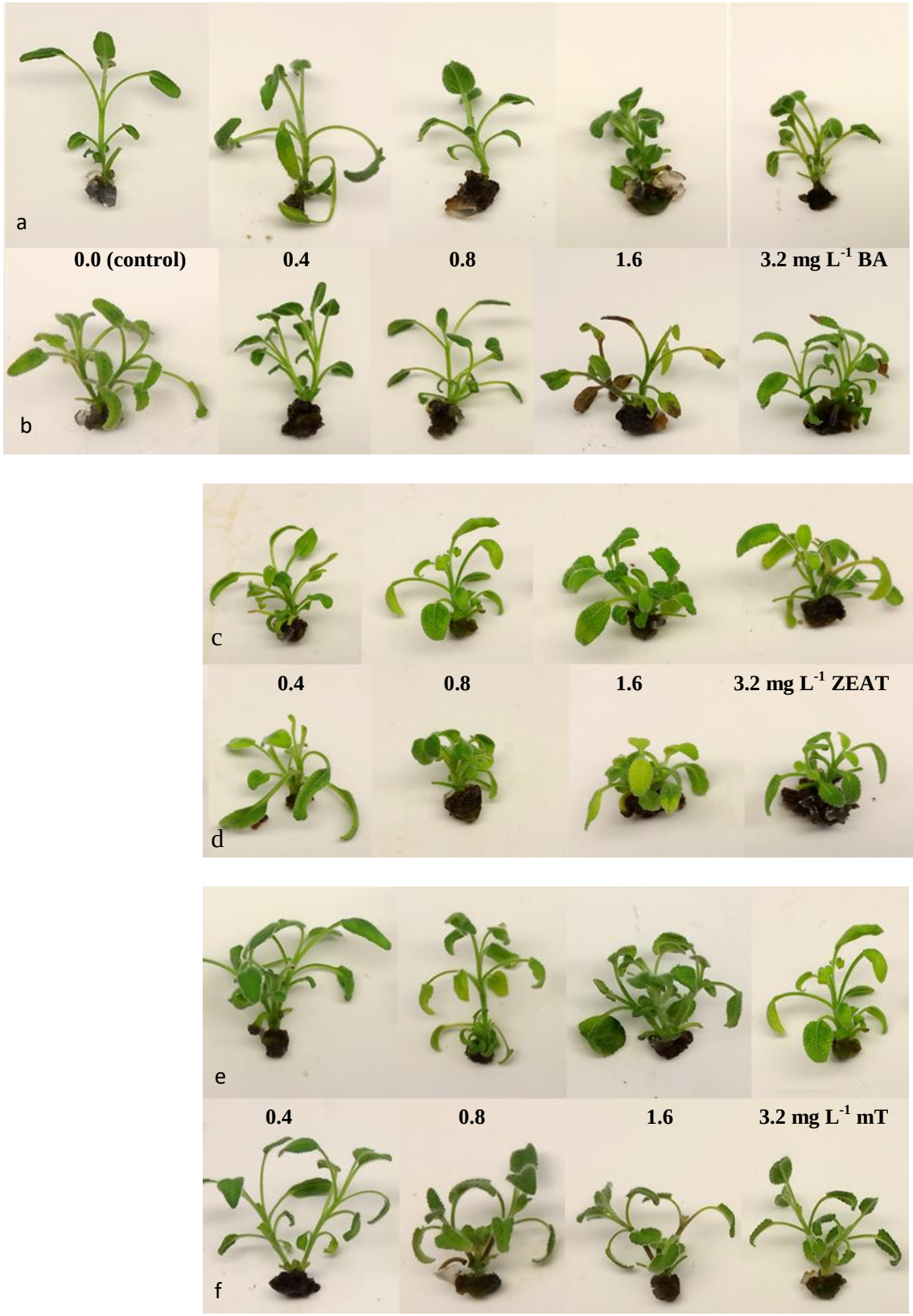

Figure 1. Typical response of shoot tip (a, c and e) and nodal (b, d and f) explants of *S. fruticosa*, after 4-week culture *in vitro* on solid MS medium without plant growth regulators

(control) or supplemented with marked concentration (mg L^{-1}) of BA or ZEAT or mT, respectively, in combination with 0.01 mg L^{-1} NAA.

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INTRODUCTION

S. fruticosa Mill. (Lamiaceae), Greek sage, is a strongly aromatic, perennial evergreen shrub, up to 1.20 m high, growing mainly in bushy rocky areas, often on coastal cliffs, at altitudes 1-700 m (Blamey and Grey-Wilson, 1993; Thanos and Doussi, 1995). It is endemic to the eastern Mediterranean, including southern Italy, North Africa and the Canary Islands, while in Greece, it is found in the Central country, the Peloponnese and the Aegean islands (Thanos and Doussi, 1995). In Greece, it has been traditionally used as a medicinal, culinary and melliferous plant since the antiquity (Clebsch and Barner, 2003). Nowadays, it is widely used for the preparation of an herbal tea (faskomilo) (Hanson, 2004).

MATERIALS AND METHODS

Shoot tip or single-node explants excised from microshoots of *in vitro* cultures of *S. fruticosa* initiated from *in vitro* grown seedlings were used. The explants were cultured on MS medium (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose either without plant growth regulators (control) or supplemented with three different cytokinins, i.e. 6-benzyladenine (BA), zeatin (ZEAT) or meta-Topolin (mT), at four concentrations, i.e., 0.4, 0.8, 1.6, and 3.2 mg L^{-1} in combination with 0.01 mg L^{-1} 1-naphthaleneacetic acid (NAA).

RESULTS

The response for shoot production was very high in tip explants (over 90%) in most media, while in nodal explants was slightly lower (73-85%) when the medium was supplemented with ZEAT or BA (Table 1).

Nodal explants resulted in higher number of normal shoots produced per responded explant, while tip explants in higher node number per shoot and higher multiplication index (Table 1).

The presence of cytokinin compared to the hormone free medium promoted slightly the number of shoots produced per explant decreasing simultaneously their length (Table 1).

Cytokinin in high concentrations, regardless of cytokinin type, resulted in the formation of hyperhydrated shoots, which reached up to 62% when nodal explants were cultured on a medium with 3.2 mg L^{-1} ZEAT or mT (Table 1).

There was an indication that the highest number of normal shoots (2.4-2.5 shoots per responded explant) was produced on 0.8 or 1.6 mg L^{-1} BA medium when nodal explants were used, while the longest shoots (2.2 cm) with a higher number of nodes (3.3 nodes /shoot) were produced when tip explants were cultured on hormone-free medium, but with no significant difference from many other media (Table 1, Figure 1). The highest number of hyperhydrated shoots was observed on the 3.2 mg L^{-1} ZEAT medium for both explant types (2.3-2.4 hyperhydrated shoots per responded explant) (Table 1).

CONCLUSIONS

The increase in concentration of all three types of cytokinins tested (ZEAT, BA, mT) resulted in an increase in the number of shoots produced, but this increase was mainly reflected in hyperhydrated shoots. Low concentration (0.4 or 0.8 mg L^{-1}) was preferable for all tested cytokinins, but meta-Topolin at 0.8 mg L^{-1} was superior to others, due to high shoot multiplication rate simultaneously with good plant tissue quality.

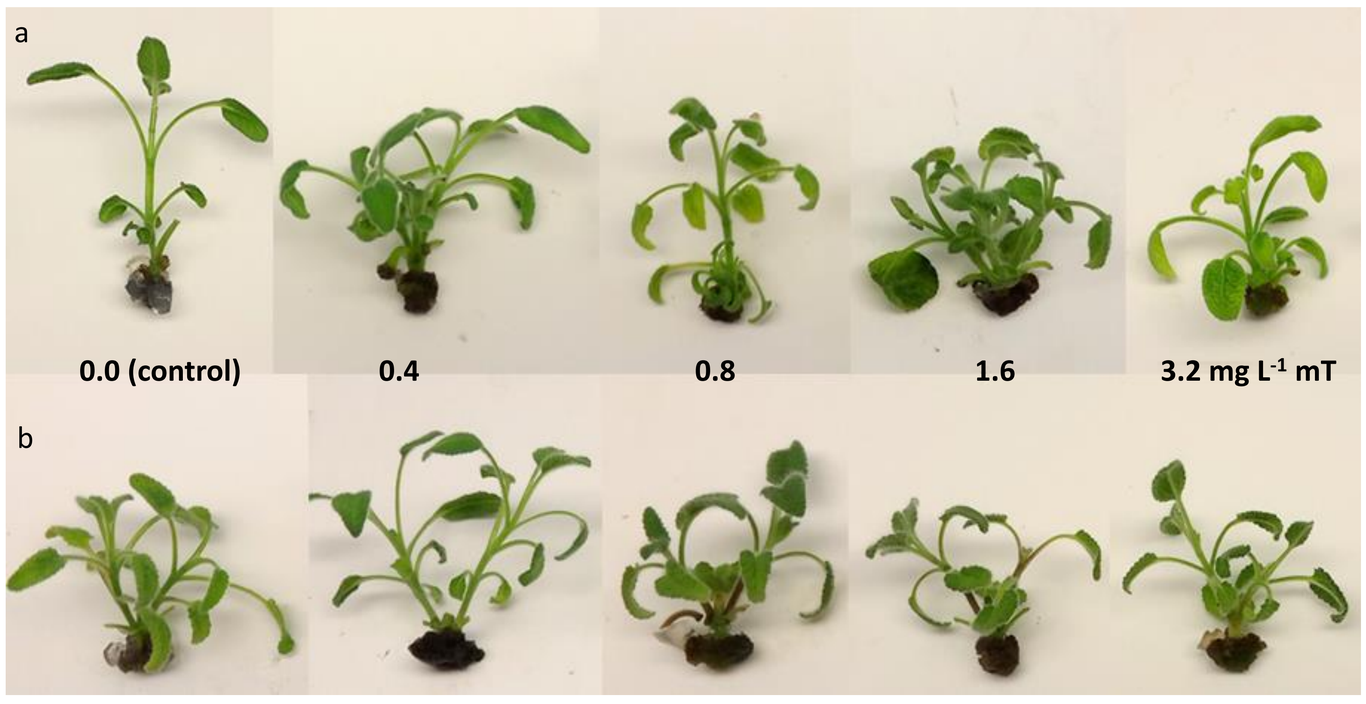

Figure 1. Typical response of shoot tip (a) and nodal (b) explants of S. fruticosa, after 4-week culture in vitro on solid MS medium without plant growth regulators (control) or supplemented with marked concentration (mg L^{-1}) of mT, respectively, in combination with 0.01 mg L^{-1} NAA.

Table 1. Effect of explant type and cytokinin type and concentration on shoot multiplication of explants excised from in vitro seedlings of S. fruticosa and cultured in a medium with marked cytokinin type and concentration in combination with 0.01 mg L^{-1} NAA.

Cytokinin concn (mg L^{-1})	Shoot production[1] (%)	Shoot production[2] (%)	Mean NSh$^{\overline{T}}$ number	Mean NSh length$^{\overline{T}}$ (cm)	Mean NSh node number$^{\overline{T}}$	Mean HSh$^{\overline{TT}}$ number	Multiplication index for NSh
Shoot tip explant							
0.0 (Hf$^{\overline{TTT}}$)	84 a[z]	8 m	1.0 f	2.2 a	3.3 a	0.1 l	2.8 bcd
0.4 ZEAT	83 a	13 l	1.1 f	1.6 cdef	2.5 bcd	0.2 k	2.3 def
0.8 ZEAT	67 def	21 j	1.4 def	1.3 cdef	2.5 bcd	0.3 jk	2.3 def
1.6 ZEAT	59 gh	29 h	1.3 ef	1.1 efg	2.3 cde	0.6 hi	1.8 fgh
3.2 ZEAT	21 lm	58 a	2.2 ab	0.7 hi	1.2 f	2.3 a	0.6 i
0.4 BA	80 ab	20 j	1.6 cde	0.8 ghi	1.2 f	0.3 jk	1.5 ghi
0.8 BA	67 def	27 h	1.6 cde	0.8 ghi	1.6 ef	0.5 i	1.7 fgh
1.6 BA	67 def	33 fg	1.9 abcd	0.7 hi	1.7 def	0.8 fg	2.2 def
3.2 BA	67 def	33 fg	2.0 abc	0.7 hi	1.6 ef	0.8 fg	2.1 efg
0.4 mT	73 c	21 j	1.7 bcd	2.0 ab	2.9 ab	0.6 hi	3.6 b
0.8 mT	77 b	23 i	2.2 a	1.8 abcd	2.7 bc	0.6 hi	4.6 a
1.6 mT	65 ef	35 f	1.8 bcd	1.6 cde	2.4 cde	0.9 def	2.8 cde
3.2 mT	62 fg	38 de	2.1 abc	1.4 cdef	1.7 def	1.0 de	2.2 def
Nodal explant							
0.0 (Hf$^{\overline{TTT}}$)	71 cd	17 k	1.7 bcd	1.4 cdef	2.3 cde	0.3 jk	2.8 cde
0.4 ZEAT	50 ij	29 h	2.0 abc	1.2 def	1.7 def	0.7 gh	1.7 fgh
0.8 ZEAT	46 jk	33 fg	1.9 abcd	1.1 efg	1.7 def	1.0 de	1.5 ghi
1.6 ZEAT	46 jk	37e	2.0 abc	1.0 fgh	1.7 def	1.0 de	1.6 gh
3.2 ZEAT	13 m	62 a	2.0 abc	0.8 ghi	1.3 f	2.4 a	0.3 i
0.4 BA	67 def	20 j	2.0 abc	0.7 hi	1.5 ef	0.6 hi	2.0 efg
0.8 BA	50 ij	27 h	2.5 a	0.6 i	1.2 f	1.0 de	1.5 ghi
1.6 BA	40 k	40 cd	2.4 a	0.6 i	1.2 f	1.0 de	1.2 hi
3.2 BA	23 l	50 b	2.2 ab	0.6 i	1.1 f	1.2 c	0.5 i
0.4 mT	56 hi	27 h	1.7 bcd	1.9 abc	2.7 bc	0.6 hi	2.6 def
0.8 mT	67 def	29 h	2.2 ab	1.4 cdef	2.4 cde	0.9 def	3.5 bc
1.6 mT	58 gh	42 c	1.7 bcd	1.2 def	1.6 ef	1.4 b	1.6 gh
3.2 mT	38 k	62 a	1.5 def	1.0 fgh	2.0 def	1.6 b	1.1 hi
$F_{\text{one-way ANOVA}}$	***	***	***	***	***	***	***

[z]Mean separation in columns by Student's t, $P \le 0.05$.
NS: not significant or *, **, ***: significant at $P \le 0.05$, $P \le 0.01$, $P \le 0.001$, respectively, n=30.
Multiplication Index = Shooting (%) x mean shoot number$^{\overline{T}}$ x Mean node number$^{\overline{T}}$
[1]The explants produced normal and hyperhydrated shoots
[2]The explants produced hyperhydrated shoots only
$^{\overline{T}}$NSh = normal shoot
$^{\overline{TT}}$HSh = hyperhydrated shoot
$^{\overline{TTT}}$Hf = hormone free

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