African Journal of Biotechnology Vol. 10(83), pp. 19407-19414, 21 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1311
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Studies on seed germination and *in vitro* shoot multiplication of *Satureja khuzistanica* Jamzad, an important medicinal plant

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Accepted 12 October, 2011

Satureja khuzistanica **Jamzad is an important multipurpose medicinal plant in Iran. The essential oil of *S. khuzistanica* is characterized by high concentration of carvacrol (93%). The seeds of *S. khuzistanica* are dormant and have a very low germination under normal conditions. This is the first protocol for *in vitro* seed germination and plantlet regeneration of this plant. Experiments were done to determine the effects of cold stratification (two, four and six weeks), chemical treatments including various levels of gibberellic acid (GA_3) (50, 100, 200 and 500 ppm), potassium nitrate (0.2, 0.4 and 0.6%), sulfuric acid (50%) and also the synergistic effect between cold stratification and other factors on seed germination of *S. khuzistanica*. The most germination percentage was achieved when six weeks stratification was followed by 200 ppm GA_3. For shoot proliferation, the node explants were cultured on Linsmaier and Skoog (LS) medium supplemented with different concentrations of benzylaminopurine (BAP) and kinetin (Kn) within the range of 0.5 to 2 µM and combinations of indole-3-butyric acid (IBA: 2 and 5 µM) with BAP (2 and 5 µM). Multiple shoots were obtained from the nodal explants, the higher frequency (77%) of shoot formation was observed in the LS that contained BAP (5 µM) in combination with IBA (2 µM). The best condition for rooting were LS medium plus 2.5 µM of IBA. The rooted plants were successfully transferred to garden soil, exhibiting a normal development.**

Key words: Germination, gibberellic acid, growth regulators, node explants, *Satureja khuzistanica*, stratification.

INTRODUCTION

Satureja khuzistanica Jamzad is an endemic plant and of a dispersed distribution in the southern of Iran. *Satureja* belongs to the tribe mentheae of lamiaceae-nepetoideae (Jamzad, 1994). This plant has been used as analgesic and antiseptic among the inhabitants of southern parts of Iran (Abdollahi et al., 2003). In the classical Iranian medical books, a few varieties of herbs are described under the names ‘saatar’, ‘nadgh’ and ‘marzeh kouhi’ (a montane variety of savory). These herbs are described as stomachic, sedative and analgesic, especially in toothache (Avicenna, 1985; Amiri, 1974).

The main constituents of *S. khuzistanica* are mono-terpenoids such as carvacrol (93%), γ-terpinene (2.6%) and *p*-cymene (1.7%) (Majd et al. 2009). Carvacrol as the main component of the wild *S. khuzistanica* has been found to have significant antioxidant properties (Abdollahi et al., 2003). In recent years, antimicrobial (Amanlou et al., 2004), anti-inflammatory and anti nociceptive (Amanlou et al., 2005) as well as plasma glucose

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lowering (Shahsavari et al., 2009) activities were reported for the species. The seeds of *S. khuzistanica* are dormant and have a very low germination rate under the normal conditions. Hitherto, despite the potential commercial interest of this important multipurpose medicinal plant, there has been no report on the seed germination and propagation for this species.

In vitro culture techniques can be an alternative method for the continuous provision of plantlet stocks for the large scale field cultivation. More and more medicinal plant species of Lamiaceae are now propagated via *in vitro* culture techniques, just to mention a few, such as *Lavandula stoechas* (Nobre, 1996), *Cunila galioides* (Fracaro and Echeverrigaray, 2001), *Salvia fruticosa* (Arikat et al., 2004), *Salvia brachyodon* (Misic et al., 2006), *Ocimum gratissimum* (Gopi et al., 2006) and *Ocimum basilicum* (Begum et al., 2002).

Moreover, *in vitro* propagation of *S. khuzistanica* is considered to be important for chemical analysis of essential oils, physiological studies as well as pharmacological and genetic transformation programs. To the best of our knowledge, this paper is the first report on the seed germination and *in vitro* multiplication of *S. khuzestanica*.

MATERIALS AND METHODS

Plant material and seed germination

S. khuzistanica seeds were collected from Paalam region, located in the south-eastern part of Lorestan province in Iran. The mature plants were identified according to Jamzad, (1994) and a voucher specimen was deposited at the Tarbiat Modarse University Herbarium. Seed viability was determined by tetrazolium (TZ) test (Perry, 1987).

Seeds were placed in a sealed plastic box in a refrigerator at temperature of 4°C, for two, four and six weeks. The stratified (six weeks) and non-stratified seeds were surface sterilized by soaking in 1% sodium hypochlorite (NaOCl) for 5 min and subsequently rinsed thoroughly with sterilized water. After sterilization, the stratified (six weeks) and non-stratified seeds were soaked in four GA_3 (SIGMA) concentrations (0, 50, 100, 200, and 500 ppm) in dark at room temperature for 48 h. Stratified (six weeks) and non-stratified seeds were soaked in different concentrations [0, 0.2, 0.4 and 0.6 (w/v%)] of KNO_3 (MERCK) for 72 h in dark at room temperature.

Stratified and non-stratified seeds after sterilization, were soaked in H_2SO_4 (80% v/v) for 5 min then washed several times by distilled water. The seeds were then placed in 10 cm diameter Petri-dishes with 10 ml of water agar (7%). All dishes were sealed with a trip of parafilm to reduce water loss. The seeds were transferred to germinators with continuous darkness, constant temperature of 20°C and relative humidity between 70 and 75%. Germinated seeds were counted and removed every 24 h for 25 days. A seed was considered germinated when the tip of the radicle had grown free of the seed coat (Wiese and Binning, 1987).

The germination rate was calculated as follows (Wiese and Binning, 1987): Germination rate = ∑ (number of germinating since n-1) / n; n = the days of incubation. The seed vigor index was calculated as follows (Abdul-baki and Anderson, 1973): Vi = (Ls × Pg) / 100, where Vi is vigor index, Ls is the mean of seedling length and Pg is the germination percentage. The experiments were carried out in completely randomized design with four replications and 50 seed per each replication. Data were subjected to analysis of variance using SPSS version 18. Duncan's multiple range test (DNMRT) was used to detect significant differences among the treatments with a probability of 99% ($p \leq 0.01$). Germination percentage values were correlated using linear regressions ($p = 0.05$).

In vitro propagation

Establishment of cultures

Treated seeds (six weeks stratification + 200 ppm GA_3) were placed on MS medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.7 % (w/v) agar. The 3-week-old seedlings provided material for *in vitro* cultures used in this study. Four basal media were used: LS (Linsmaier and Skoog, 1965), B_5 (Gamborg et al., 1968), MS and half-strength MS. All the media were supplemented with 1 µM of BAP and except half-strength MS, other media contained 3% sucrose as the carbon source. The pH of the media was adjusted to 5.8 prior to the addition of 0.7% agar and autoclaving (1 atm, 127°c, 20 min). Glass tube cultures were incubated in a culture room at 25 ± 2°C with 16 h photoperiod under standard cool white fluorescent tubes (35 µmol s^{-1} m^{-2}).

Shoot multiplication

For shoot multiplication, single node segment, approximately 1 cm in length were transferred to LS medium supplemented with different concentrations of BAP and kinetin (Kn) within the range of 0.5 to 2 µM and combinations of indole-3-butyric acid (IBA: 2 and 5 µM) with BAP at two concentrations (2 and 5 µM). After six weeks, the efficacy of each treatment on shoot proliferation and growth was determined. Hormone-free medium was used as control.

Shoot rooting and acclimatization

To initiate rooting and elongation, the micro-shoots (around 3 cm height) were placed on LS medium containing 30 g/L sucrose and three different auxins, indoleacetic acid (IAA), naphthaleneacetic acid (NAA) and IBA, at three concentrations (2.5, 5 and 10 µM). The medium was solidified with 7 g L^{-1} agar and dispersed at 40 ml per glass tube cultures. Four shoots were cultured in each tube and were incubated in dark condition for three days at 25 ± 2°C in a growth chamber, afterwards the culture tubes were set under standard cool white fluorescent tubes (35 µmol s-1 m-2) with a 16 h photoperiod for 45 days. After 45 days, plantlets with well-grown roots were removed from the culture tubes; the roots were washed in running tap water and the plants were transferred to pots containing sterilized garden soil, covered with polythene bags to maintain humidity and kept at 25°C in a growth chamber for one week. The bags were removed gradually and acclimatized plants were transferred to the greenhouse.

All the experiments were conducted in randomized design with 25 explants per treatment and each was repeated twice. The data were submitted to statistical analysis by ANOVA and the means compared by the Tukey´s test.

RESULTS

Seed germination

Seed viability of *S. khuzistanica* was 77% according to TZ staining. As shown in Table 1, stratification had a

Table 1. Mean values (Duncan means test; p≤0.01) of seed germination and seedling characters for *S. khuzistanica* under stratification, chemical and combined stratification (6w) and chemical treatments.

Treatment	Germination percentage (%)	Germinationrates (seeds per day)	Shoot length (cm)	Root length (cm)	Vigor index
Stratification (week)					
2	14^{h}	0.63^{g}	1^{ab}	2^{ab}	0.423^{e}
4	18^{gh}	0.53^{gh}	1.1^{ab}	2.3^{ab}	0.621^{d}
6	21.5^{g}	0.38^{h}	1.08^{ab}	2.18^{ab}	0.699^{d}
GA_3 (ppm)					
50	30.5^{f}	0.93^{f}	1.18^{ab}	1.6^{b}	0.844^{c}
100	39.75^{e}	1.37^{d}	0.93^{ab}	2.35^{ab}	1.30^{bc}
200	38.5^{e}	1.215^{e}	0.9^{ab}	1.6^{b}	1.06^{bc}
500	44.5^{d}	1.198^{ef}	0.95^{ab}	1.95^{ab}	1.25^{bc}
KNO_3 (v/v%)					
0.2	22.25^{g}	0.645^{g}	0.83^{ab}	1.15^{de}	0.44^{e}
0.4	29.75^{f}	1.010^{ef}	0.85^{as}	1.5^{c}	0.69^{d}
0.6	33.75^{f}	0.937^{f}	1.13^{ab}	1.13^{e}	0.76^{d}
H_2SO_4 (50%)	4^{i}	0.107^{i}	0.98^{ab}	2.58^{ab}	0.14^{f}
Stratification for six weeks + GA_3 (ppm)					
50	53^{c}	1.697^{c}	1.025^{ab}	1.750^{ab}	1.485^{b}
100	56^{c}	1.805^{bc}	1.075^{ab}	2.725^{a}	2.147^{a}
200	72.5^{a}	2.313^{a}	1.025^{ab}	2.275^{ab}	2.398^{a}
500	66.75^{b}	2.065^{ab}	1.025^{ab}	2.200^{ab}	2.150^{a}
KNO_3 (v/v%)					
0.2	44.25^{d}	1.357^{de}	0.825^{b}	1.175^{d}	0.889^{c}
0.4	56.25^{c}	1.795^{bc}	0.800^{b}	1.475^{cd}	1.293^{bc}
0.6	62.5^{b}	1.995^{b}	1.050^{ab}	1.050^{f}	1.313^{bc}
H_2SO_4 (50%)	21.75^{g}	0.920^{f}	1.200^{a}	2.050^{ab}	0.706^{cd}
Control	2.5^{i}	0.055^{it}	0.77^{b}	1.88^{ab}	0.087^{f}

Means with different letters in each column differ significantly according to Duncan's multiple range tests at p≤ 0.01.

significant effect on seed germination of *S. khuzistanica* (p≤0.01).

Further improvement of germination through stratification was tried, but it was not a complete success. Seeds stratified for six weeks recorded a moderate increase in germination (21%) with high vigor index (0.699). Except root length, other traits such as germination rate and vigor index were also affected by stratification (Table1). Treatment with GA_3 significantly improved germination. Also, increasing the concentration of GA_3 resulted in an increase in germination percentage where, at 500 ppm, concentrations caused the highest germination percentage (44%) (Table1).

Regressions indicate that the germination percentage had significant positive correlations with the GA_3 concentrations (r = 0.67, p≤0.05). Seedling characters such as germination rate, root length, and vigor index were also affected by GA_3 and showed a significant difference at various concentration of GA_3.The combined treatment with GA_3 and stratification (6w) had a statistically significant effect on germination (Table1). Seeds treated with 200 ppm GA_3 without stratification, gave 38% germination, whereas seeds treated with 200 ppm GA_3 preceded by six weeks of stratification gave

Table 2. Influence of culture medium composition on *in vitro* response of *S. khozistanica*

Culture media	Number of shoots per explant	Shoot height (cm)	Number of leaf	Viability (%)
LS	6.8 ± 1[a]	6.9 ± 0.6[a]	25 ± 3[a]	86
MS	5.5 ± 0.8[a]	5.4 ± 1[b]	20 ± 2[a]	65
1/2MS	5.0 ± 0.6[a]	4 ± 0.5 b[c]	17 ± 2[b]	54
B5	2.8 ± 0.9[b]	3.2 ± 0.6[c]	8 ± 1[c]	19

Values represent the mean ± SD; means followed by same letters within a column are not significantly different ($p < 0.05$) using Tukey test. All media were supplemented with 1 µM BAP.

72% germination.

The results also show that potassium nitrate can effectively release seeds dormancy in *S. khuzistanica*. Soaking of seeds in 0.6% (w/v) KNO_3 for 72 h increased the germination to 33% (Table1). Although stratification produced much better results compared with separate application of these two factors, the combination of stratification and KNO_3 significantly enhanced germination percentage at 0.6% KNO_3 (62%) (Table 1).

Moreover, the results of the study reveal that germination rate, root length, and vigor index were also affected through various levels of potassium nitrate. Recorded experimental findings (Table1) show that scarification with sulphuric acid did not promote germination of *S. khuzistanica* seeds. The root length (2.58 and 2.05 cm) and shoot length (0.98 and 1.2 cm) were recorded to be in their maximum length in H_2SO_4 and a combination of six weeks of stratification and H_2SO_4 treatment.

In vitro regeneration

The effects of four salt formulations on the micropropagation of *S. khuzestanica* are shown in Table 2. The number of shoots were not significantly different among MS, 1/2MS, and LS media, but the high explants viability (86%) and shoot formation with the most number of nodes and leaves were observed on the LS media (Figure 1a). Nodal explants cultured on B_5 medium produced the shortest shoot with abnormal leaves.

The presence of both BAP and Kn on the LS media positively influenced shoot proliferation, where among cytokinins, BAP responded better when compared with Kn regarding shoot proliferation (Table 3). The synergistic effect of BAP with IBA for direct plant regeneration was evaluated and the best response and the maximum induction of multiple shoots (9.5 ± 0.5) were achieved from medium supplemented with 5 µM BAP and 2 µM IBA. Although the treatment of 5 µM BAP and 5 µM IBA resulted in lower shooting compared with 5 µM BAP and 2 µM IBA, it did not show any significant difference in terms of number of shoots, shoot height, and number of leaves.

Rooting and acclimatization

As can be observed in Table 4, three types of auxines (IAA, NAA and IBA, at three concentrations: 2.5, 5 and 10 µM) had different effects on rhyzogenesis. The most effective auxin for root number and root length was IBA (2.5 µM) (Figure 1b). Shoots exposed to higher concentrations of NAA and IAA (10 µM) became necrotic, lost leaves, and died gradually. For acclimatization, plantlets were removed from rooting medium after 45 days of incubation and transferred to plastic pots containing sterilized garden soil, covered with polythene bags, to maintain humidity and were kept under culture room conditions for one week (figure 1c).

After one week, polythene bags were removed and transferred to green house. The survival rate was 80% and the appearance of acclimatized plants was normal without any morphological abnormalities or variations (figure 1d).

DISCUSSION

Stratification was found to be effective in increasing germination and in breaking dormancy in several species of Labiatae, for example in *Stachys alpina* by Pinfield (1971); in *Isanthus brachiatus* by Baskin and Baskin (1975), and in *Salvia columbariae* by Hashemi and Estilai (1994). The reports and the results of this study show that stratification was successful in breaking seed dormancy, though the duration of treatment may vary with the species. At cold temperatures, more oxygen is soluble in water, so the oxygen requirements of the embryo are better satisfied. Cold-moist stratification imitates overwintering in a field seed bed (Young and Young, 1986). The actions of low temperatures in terminating dormancy may promote a fall in the level of inhibitors and it automatically increases the level of promotive hormones (Bewley and Black, 1982).

It has been reported that germination can be induced by gibberellic acid in *Salvia glutinosa*, *Lycopus europaeus*, and *Scutetlaria galericulata* (Thompson, 1969), *S. columbariae* (Hashemi and Estilai, 1994), and *Stachys alpine* (Pinfield, 1971). It has also been reported

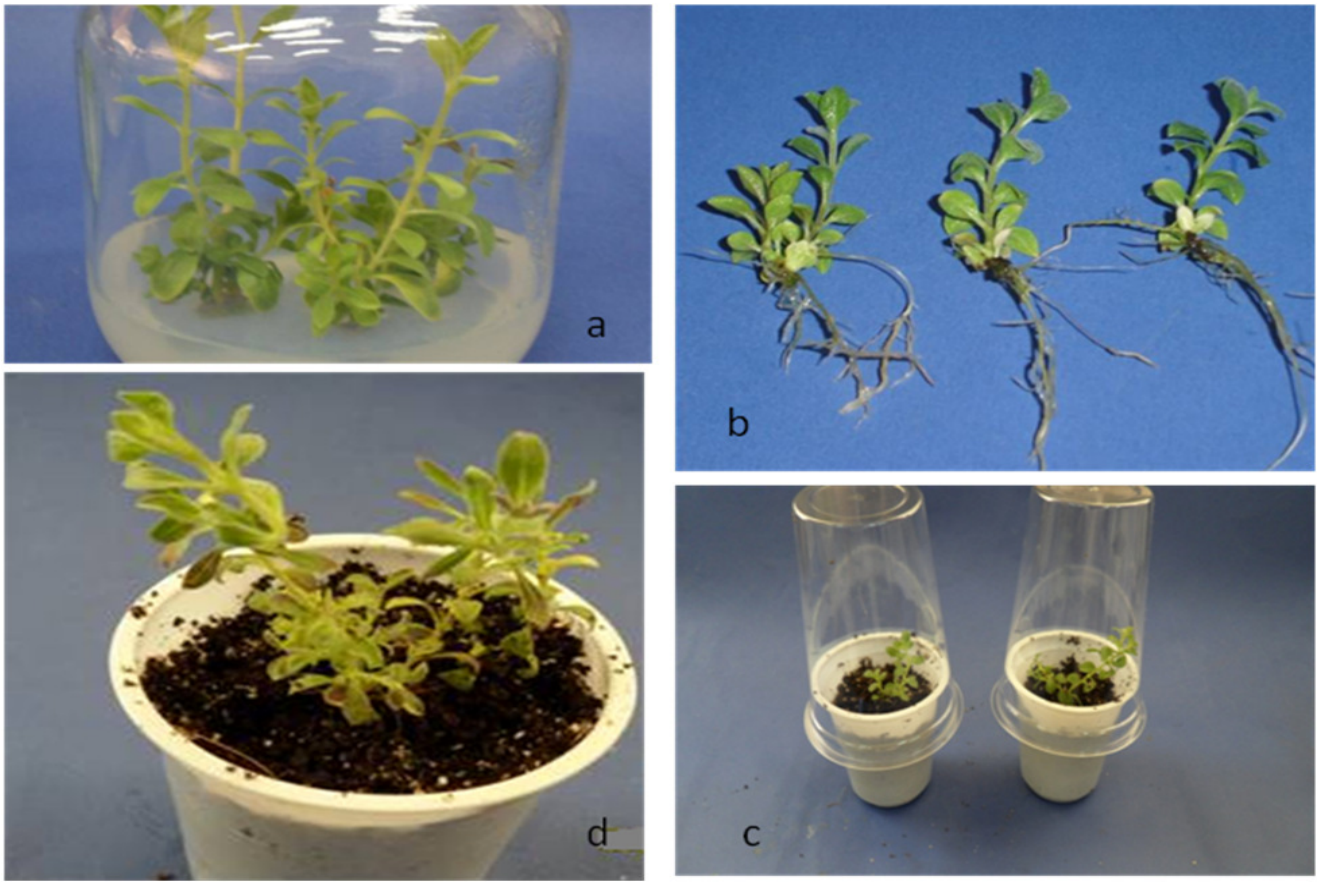

Figure 1. Regeneration of *S. khuzistanica* from the nodal explants. (a) Multiple shoots regeneration from the nodal explants; (b) rooted plantlets; (c) plantlets covered with polythene bags; (d) hardened plants.

Table 3. Effect of BA, Kn and combination of IBA with BAP on shoot induction and proliferation from the Nodal Segments of *S.khozistanica* in LS medium after 6 weeks in culture.

Growth regulator (µM)	Shooting (%)	Number of shoot	Shoot height (cm)	Number of leaf
BAP				
0.5	50	7.8 ± 1.3^{ab}	6.0 ± 1.8^{c}	24 ± 7^{c}
1	65	9.0 ± 0.8^{a}	7.3 ± 0.5^{ab}	29 ± 2^{abc}
2	25	2.8 ± 1.2^{de}	3.2 ± 0.6^{d}	13 ± 2^{d}
Kn				
0.5	37	3.0 ± 0.8^{cde}	4.8 ± 1.2^{cd}	19 ± 5^{cd}
1	40	4.0 ± 0.8^{cde}	3.3 ± 0.9^{d}	13 ± 3^{d}
2	27	2.8 ± 0.9^{de}	3.1 ± 1.3^{d}	12 ± 5^{d}
BAP + IBA				
2 2	65	5.5 ± 0.6^{bc}	6.8 ± 0.5^{bc}	27 ± 2^{bc}
2 5	58	5.3 ± 1^{bcd}	6.5 ± 1.9^{bc}	26 ± 8^{bc}
5 2	77	9.5 ± 0.5^{a}	9.5 ± 0.6^{a}	38 ± 2^{a}
5 5	52	7.3 ± 2^{ab}	9.0 ± 0.8^{ab}	36 ± 3^{ab}
Control	7.5	1.5 ± 0.6e	2.5 ± 0.5^{d}	10 ± 2^{d}

Means followed by same letters within a column are not significantly different ($p < 0.05$) using Tukey test.
Values represent the mean ± SD.

Table 4. Effect of different types and concentrations of auxin on root induction in LS media.

Auxin (µM)	Rooting (%)	Number of root	Root length (cm)
IBA			
2.5	91	7.5 ± 1.3^{a}	4.5 ± 1.7^{a}
5	62	4.8 ± 1.9^{a}	4.2 ± 1.5^{a}
10	29	3.3 ± 1.2^{b}	2.5 ± 0.6^{ab}
NAA			
2.5	25	3.2 ± 1^{b}	3.3 ± 0.5^{ab}
5	28	4.5 ± 1.2^{ab}	2.8 ± 0.6^{ab}
10	0.0	0.0^{c}	0.0^{c}
IAA			
2.5	26	4.3 ± 2^{a}	3.2 ± 0.6^{ab}
5	52	5.5 ± 1.2^{a}	3 ± 0.8^{ab}
10	0.0	0.0^{c}	0.0^{c}
Control	25	1.2 ± 1.5^{c}	0.9 ± 1^{c}

Means followed by same letters within a column are not significantly different ($p < 0.05$) using Tukey test. Values represent the mean ± SD.

that treatment with GA_3 can increase the formation of rough endoplasmic reticulum and polyribosomes (Evins, 1971). Moreover, it is found that GA_3 stimulates the synthesis of mRNA which is specific for α-amylase (Higgins et al., 1976). Effects of GA_3 preceded by stratification were found to be successful for *Prunus mahaleb* L. (Gercekcioglu and Cekic, 1999) and *Morus nigra* L. (Koyuncu, 2005). These reports are in consistent with our results showing that stratification followed by GA_3 enhance germination of *S. khuzistanica* seeds.

The results of this study are similar to other findings demonstrating that potassium nitrate treatment was an important factor in breaking seed dormancy and increasing seed germination (Brandel, 2005; Pupala and Fowler, 2003). Nitrogen containing compounds are considered to be effective in breaking seed dormancy and thus inducing seed germination (Tang et al., 2008). Nitrate plays the role of a co-factor in phytochrome action (Grubisic and Konjevic, 1990). Potassium nitrate may be helpful for reactivation of metabolic process of seeds. This compound may cause biosynthesis of auxin, which ultimately triggers the growth of the embryo (Khan et al., 1999).

Although *Satureja* species do not have thick seed coat, the strong inhibitory effect of seed coat on the seed germination may be caused by several possible mechanisms, including mechanical constraint, prevention of water and oxygen uptake, and retention or production of chemical inhibitors (Taiz and Zeiger, 2002). The integument breaking or softening is, therefore, necessary to remove dormancy imposed by seed coat hardness or impermeability.

Results in Table1 indicate that H_2SO_4 scarification did not increase the germination percentage of *S. khuzistanica* seeds; therefore, seed coat cannot be considered as a dormancy factor in *S.khuzistanica* . H_2SO_4 scarification has a positive effect on root and shoots length of *S.khuzistanica* seeds (Table1). Positive effect of H_2SO_4 on the length and height of the root and shoot was also reported in *Aframomum corrorima* (Eyob, 2009) and *Lupinus varius* (Karaguzel et al., 2004). According to the results found in this study, *S. khuzistanica* species probably exhibits a deep physiological dormancy. Seeds with intermediate and deep physiological dormancy are characterized by a requirement for a one to three month period (sometimes more) of chilling, while in an imbibed and aerated state (Crocker 1948). Seeds of this type ripen in the fall overwinter in the moist leaf litter, and germinate in the spring.

This requirement led to the horticultural practice of “stratification”, in which seeds are placed between layers of moist sand or soil in boxes (or in the ground) and exposed to chilling temperatures, either out-of-doors or in refrigerators. Temperature is the most important factor controlling stratification. The most effective temperature is next to freezing (1 to 10°C). The time required to stratify seeds results from the interaction of the genetic characteristics of the seed population, seed development environment, and the stratification environment (Wartidininghsih and Geneve 1994).

The results related to the effects of four salt formulations, which can be observed in Table 3, can be attributed to the NO^{3-}/NH^{4+} ratio on LS and MS media, both with 66 : 34, compared with the B_5 media (80 : 20). Nitrate/ammonium relation is essential for the nitrogen supply and pH control during *in vitro* culture, which drastically affects culture performance (George, 1993).

Based on these results, LS medium was used in shoot proliferation and rooting. The highest number of shoots per explants and the tallest plantlets were obtained on 1 µM of BAP, a concentration that is on the range (0.22 to 4.4 µM) recommended for most species of the Labiatae family (Furmanowa and Olszowska, 1992; Andrade et al., 1999; Agostini and Echeverrigaray, 2006). The number of shoots produced increased as the BAP concentration increased from 0.5 to 1 µM, but explants on 2 µM of BA produced callus and hyperhydric shoots, indicating an upper limit on BA concentration for the micropropagation of *S. khuzestanica.* High concentrations of cytokinins have been reported to be one of the most important factors involved in the induction of hyperhydricity during *in vitro* culture (Kadota and Niimi, 2003). This physiological disorder was previously observed in *C. galioides* by Fracaro and Echeverrigaray (2001) and in *Cunila incise* by Agostini and Echeverrigaray (2006). It has been reported that the combination of cytokinin and auxin stimulate the *in vitro* multiplication and the growth of shoots in several species of the Labiatae family (Dode et al., 2003; Agostini and Echeverrigaray, 2006 and Cristea et al., 2008).

Several researchers reported that IBA had positive effects on the rooting of various medicinal and aromatic plants, such as *Origanum vulgare* L., *Mentha piperita* L. and *Melissa officinalis* L. (Kuris et al., 1980) and *Origanum vulgare* var. *hirtum* (Sarihan et al., 2003). Although *in vitro* rooting of *Lavandula stoechas* shoots resulted in 100% rooting when basal medium contained 1.0 mg/L NAA (Nobre, 1996), Echeverrigaray et al. (2010) reported that the best condition for rooting of *Salvia guaranitica* Benth was the MS medium with 2.85 µM IAA.

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