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HIGH-FREQUENCY *IN VITRO* DIRECT SHOOTS REGENERATION FROM AXILLARY NODAL AND SHOOT TIP EXPLANTS OF CLARY SAGE (*SALVIA SCLAREA* L.)

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Abstract

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In vitro bud induction and shoot regeneration of *Salvia sclarea* L. was investigated. *In vitro* regeneration was achieved on MS media supplemented with various combinations of BAP, TDZ and IAA. In the first phase of the experiment, the interaction between explant type (shoot tip and axillary node) and plant growth regulators (TDZ and BAP) (0, 2.2, 4.4 and 8.8 μM) on bud induction and plant regeneration was investigated. According to the results, the interaction of explants and growth regulators on bud induction and regeneration of shoots on the 5% level was not significant. Maximum average of bud induction and shoot regeneration was achieved in axillary nodal explants. Compare data obtained from the effect of TDZ and BAP on the induction of buds and regenerated shoots showed that in culture media supplemented with different concentrations of the TDZ in compared with the same concentration of BAP, bud induction and shoot regeneration was better. In the second experiment, the effect of axillary bud explants in culture media containing different concentrations of TDZ (0, 2.2, 4.4 and 8.8 μM) in combined with 1.1 μM IAA were studied. Results showed that the maximum average of bud induction and shoot regeneration were in media containing 2.2 μM TDZ in combined with IAA. Root induction also was evaluated on ½ MS and MS media with two concentrations (0.5 and 1 mgl^{-1}) of IAA or IBA. MS media supplemented with IAA (0.5 mgl^{-1}) was the best medium for rooting.

Key words: bud induction, rooting, *Salvia sclarea*, shoot proliferation

Abbreviations: MS - Murashige and Skoog, BAP - 6-Benzylaminopurine, TDZ - Thidiazuron, IAA - Indole-3 Acetic Acid; IBA - Indole-3 Butyric Acid

Introduction

The use of medicinal plants for health reasons started thousands of years ago and is still an apart of medicinal practice in all countries of the world (Aftab and Sial, 1999). The genus *Salvia* consists of many species, which have wide applications in folk medicine and also many commercial uses, especially in the production of essential oils and flavoring agents. *Salvia sclarea* L. (Clary sage) is grown in the Mediterranean basin and Iran (Dweck, 2000). The essential oils or extracts of the aerial part of the *S. sclarea* plant have a broad spectrum of effects: analgesic, anti-inflammatory (Moretti et al*.,* 1997), antioxidant, antifungal (Pitarokili et al*.,* 2002) and antibacterial (Peana et al., 1999; Gülçin, 2004). Apart from the various medicinal uses, essential oils of Clary sage are widely applied in the food and cosmetic industries (Kuźma et al., 2009).

Cultivation of medicinal plants for the purpose of extraction of active constituents may face certain limitations such as climate, season, water availability, diseases, pests and

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scarcity of naturally growing plants. Such limitations have led to the use of tissue culture techniques for production of the active constituents (Arikat et al., 2004). In vitro culture techniques offer an option for the study and conservation of rare, threatened or endangered medicinal plants and a tool for efficient and rapid multiplication of species when high uniformity of progeny is required (Saha et al., 2010).

In vitro tissue culture of several species of the *Salvia* genus was carried out. Callus cultures of *Salvia miltiorrhiza* have been successfully induced from in vitro grown seedlings (Miyasaka et al., 1989; Waldemar, 1996) and shoot tips (Morimoto et al., 1994). Makunga and Van staden (2008) developed for *S. africana-lutea* an in vitro cultivation protocol. Irina (2008) reported the highest regeneration of adventitious shoots and micropropagation in *Salvia officinalis* which was achieved on MS media supplemented with BAP (2.22 μM), Kin (4.65 μM) and NAA (2.68 μM). Misic et al., (2006) developed a protocol for in vitro propagation of *Salvia brachyodon* (Bulkan endemic plant). They reported effective role of BAP in axillary buds promotion.

Approximately 80% of the plants inhabiting in north-west of Iran declined due to habitat loss and human interferences (Heidary and Shanki, 2011). Because of these, west Azerbaijan agricultural and natural resource research center recommended special attention to the new propagation methods and conservation of these medicinal plants. The use of tissue culture techniques has played a major role in the growth and regeneration, propagation and conservation of important medicinal plants. Due to the lack of proper studies on adventitious shoot development from shoot tip and node segments of Clary sage, this work describes successful regeneration of *Salvia sclarea* L. under in vitro conditions using two different explants. In present article we have reported the effect of some plant growth regulators including cytokinins (BAP and TDZ) and auxins (IAA and IBA) with various concentrations and combination on micropropagation of this species in order to obtain high shoot regeneration rate and high rooting frequency.

Materials and Methods

Seed germination and explant preparation

Seeds of *Salvia sclarea* L. were obtained from medicinal plants garden of the Horticultural Sciences Department of Urmia University, West Azerbaijan, Orumieh, Iran. The seeds were surface sterilized by soaking in a solution of 70% (v/v) ethanol for 1 min., then by soaking in 1% (v/v) sodium hypochlorite for 5 min followed by rinsing three times with sterile distilled water for 5 min. These surface-sterilized seeds germinated on MS (Murashige and Skoog, 1962) medium containing 3% (v/v) sucrose supplemented with 40 mg l^{-1}Chitosan. Shoot tip and single nodes were prepared from 16-day-old in vitro growing plants and used as the explant (Figure 1 A and B).

Basal medium and Culture conditions

In all of the experiments, MS medium containing 3% (w/v) sucrose and 0.7% agar (Duchefa, Netherlands) was used as basal medium. The pH of the media was adjusted to 5.8 before adding agar. Media were autoclaved for 20 min. at 121°C. IAA and IBA were added to the medium after autoclaving by filter sterilization (0.22 μm) (Millipore). All the cultures were kept in growth chambers at 25 ± 2°C under a 16/8 h (light/dark) photoperiod at a photon flux rate of 60 μmol m^{-2} s^{-1} provided by cool daylight fluorescent lamps.

Effect of explant types on bud induction and shoot regeneration

For evaluation the effects of explant type on bud induction and shoot regeneration, shoot tips (7 mm in length) and nodal explants were isolated from 16-day-old plants and used as initial explants for the experiments. In this part of experiments, different concentrations of BAP and TDZ (0, 2.2, 4.4, 8.8 μM) were used. BAP was added to the medium before autoclaving and TDZ was added to the medium after autoclaving by filter sterilization (0.22 μM Millipore). All explants were placed vertically on the shoot regeneration medium surface and were subcultured on the same shoot induction media every three weeks interval. After nine weeks, the number of induced buds and regenerated shoots per explants were calculated for each treatment.

Effect of plant growth regulators (PGRs) on bud induction and shoot regeneration

Different concentrations of TDZ (0, 2.2, 4.4, 8.8 μM) in combination with IAA (1.1μM) were used to find the best combination and concentrations of them for bud induction and shoot regeneration of axillary nodal explants.

Rooting and acclimatization

Regenerated shoots (5 cm length) were carefully excised and put on MS medium containing 4.4 μM BAP for one week, then they were transferred on MS or ½MS medium supplemented with various concentrations (0, 0.5, 1 mg l^{-1}) of IAA and IBA. After four weeks well rooted shoots were excided from the culture and rinsed with sterile distilled water to remove agar (Figure 2A). These plantlets were transferred to plastic pots containing soil mixture composed of peat and perlite (3:1) (v: v) moistened with half strength of MS basal liquid medium (Figure 2B) and covered with transparent

polythene bags to maintain high humidity (Figure 2C). They were kept in growth chambers at 24 ±2°C under a 16 h light and 8 h dark photoperiod at a photon flux rate of 60 µmol m^{-2} s^{-1} provided by cool daylight fluorescent lamps. After ten days the polythene bags were removed gradually from the pots and the acclimatized plantlets were finally transferred to the greenhouse (Figure 2D).

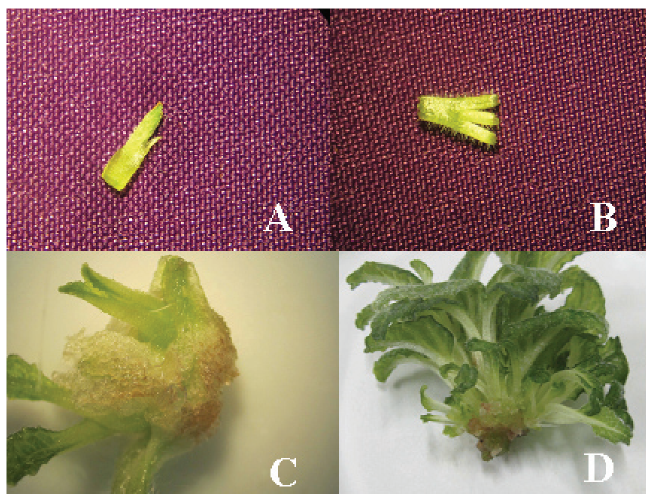

Fig. 1. A: Shoot tip explant. B: Nodal explants. (Explants were prepared from 16-day-old in vitro growing plants). C: Induced adventitious bud observed after two weeks of culture. D: Initiated multiple shoots after four weeks of culture

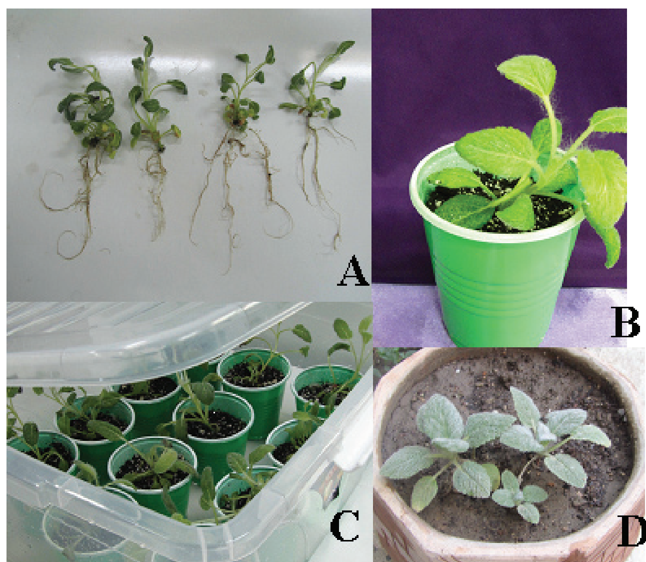

Fig. 2. A: Rooted shoots on MS or ½ MS medium. B: Transferred plantlets to plastic pots containing soil mixture composed of peat and perlite. C: Pots covered with transparent polythene bags. D: Acclimatized plantlets to the greenhouse

Experimental design and data analysis

The experiments were based on a completely randomized design with 4 replications per treatment. Analysis of variance (ANOVA) was performed for the data with the General Linear Model procedure using SAS 9.1 software and the means were compared using Duncan's multiple range test (DMRT) at the 5% probability level. Graphs were plotted with Excel program.

Results and Discussion

Effect of explants type on bud induction and shoot regeneration

Regeneration frequency depends on the type of the explant, its degree of differentiation, and how it is implanted on the medium. Adventitious bud induction was observed after two weeks of culture (Figure 1C). Multiple shoots were initiat-

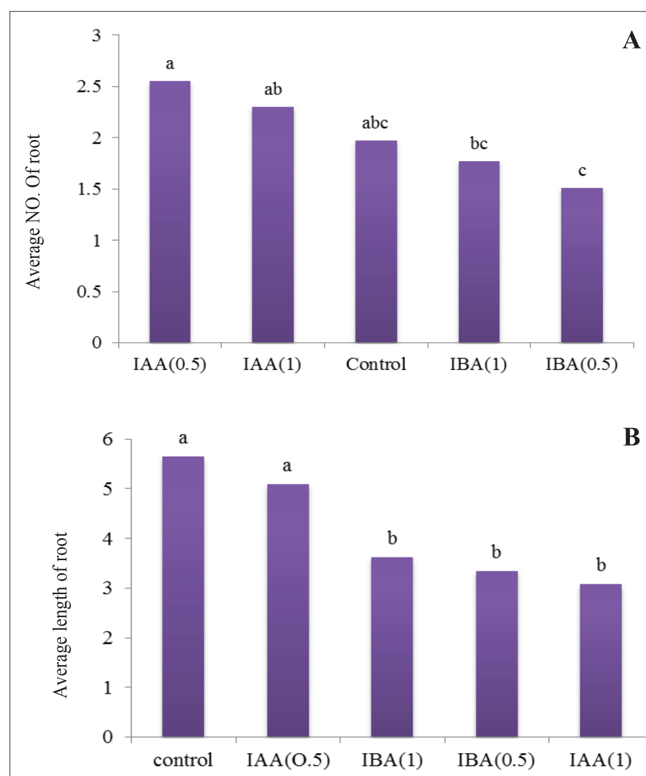

Fig. 3. Effect of different concentrations of auxins in root formation. A: Average num. of root. B: Average length of root

ed from all of the explants after four weeks of culture (Figure 1D). No plant regeneration was observed on explants planted on free PGRs MS medium (as control). Statistical analysis showed that the interaction between the explants sources (shoot tip and axillary node) and PGRs types (BAP, TDZ) was not significant in bud induction and shoot regeneration. Maximum average of bud induction (20.644) and shoot regeneration (14.862) achieved in axillary nodal explants (Table 1). Compare data obtained from the effect of TDZ and BAP on the induction of buds and regenerated shoots showed that in culture media supplemented with different concentrations of TDZ in compared with the same concentration of BAP, bud induction and shoot regeneration was better. Between different concentration of TDZ (0, 2.2, 4.4, 8.8µM), medium with 8.8 µM TDZ had highest mean of induced bud (33.83) and regenerated shoot (23.74). The lowest mean of bud induction and shoot regeneration was observed in medium without PGRs. The table indicates that with increasing concentration of TDZ, induced bud and regenerated shoot means, were increased (Table 2). Between different concentration of BAP (0, 2.2, 4.4, 8.8µM), medium with 8.8 µM BAP, had highest mean of induced bud (25.43) and regenerated shoot (18.85). With to be increased concentration of BAP, induced bud and regenerated shoot means, increased (Table 2).

The ability of *S. sclarea* explants to form new shoots varied with explants type and cytokinin concentrations. Explant type is one of the factors affecting *in vitro* regeneration. Our study results indicated that the axillary nodal explants are the best source of explants used for shoot regeneration. This has been suggested earlier in the case of other medicinal plants such as *Rauwolfia serpentian* (Roy et al., 1995), *Emblica officinalis* (Rahaman et al., 1999), *Holarrhena antidysenterica* (Ahmed et al., 2001). Begum et al. (2002) stated that "the proliferation efficiency of nodal explants was significantly higher than that of shoot tip explants, when evaluated five-six weeks after proliferation". Xim and Zhang (1987) obtained better *in vitro* plantlet regeneration from nodal explants.

Table 1
Effect of explant types on bud induction and shoot regeneration in media with different concentration of BAP and TDZ

Explant types	Bud induction	Shoot regeneration
Axillary node	20.644 a	14.862 a
Shoot tipe	16.121 b	11.456 b

*In each column followed by the same small letters are not significantly different at $p \leq 0.05$ by DMRT.

Table 2
Effect of different concentration of BAP and TDZ on bud induction and shoot regeneration from nodal and shoot tip explants of Clary sage

PGRs type	Concentration of PGRs, µM	Bud induction	Shoot regeneration
BAP	0	1.429 e	1.429 e
	2.2	8.00 d	6.250 d
	4.4	18.325 c	13.220 c
	8.8	25.425 b	18.850 b
TDZ	0	1.343 e	1.208 e
	2.2	26.111 b	18.805 b
	4.4	31.843 a	21.208 ab
	8.8	33.831 a	23.736 a

*In each column followed by the same small letters are not significantly different at $p \leq 0.05$ by DMRT.

Effect of plant growth regulators (PGRs) types on bud induction and shoot regeneration

The number of induced buds and shoots regeneration per explant were directly proportional to the concentration of TDZ in combination with IAA.

All tested TDZ concentration stimulated the fast development of new shoots from the control. Experiments showed that the major treatment was significantly different from the control. There were significant effects between TDZ and IAA in bud induction and shoot regeneration from nodal explants. The highest means of bud induction and shoot regeneration in axillary nodal explants were obtained in MS medium containing 2.2µM TDZ in combination with IAA (Table 3).

It is well known that cytokinins stimulate plant cell division and participate in the release of lateral bud dormancy, in the induction of adventitious bud formation, in the growth of lateral buds and in the cell cycle control (Gaspar et al., 2003). Our results showed that in control medium (without hormones), regeneration has not taken place or too little regeneration has taken place and this proves the importance of

Table 3
Effects of TDZ and IAA combination on bud induction and shoot regeneration from axillary nodal explants

TDZ, µM	IAA, µM	Bud induction	Shoot regeneration
0	1.1	1.645 c	1.583 c
2.2	1.1	30.165 a	21.498 a
4.4	1.1	23.498 b	18.185 ab
8.8	1.1	23.500 b	16.580 b

*In each column followed by the same small letters are not significantly different at $p \leq 0.05$ by DMRT

cytokinin in stimulating of proliferation and cell division in cultured explants and organ formation in tissue treated by cytokinin. Echeverrigaray et al. (2010) showed that "the highest multiplication rate from nodal segments from *Salvia guaranitica* was obtained on MS medium supplemented with 2.22 µM of BAP". Singh and Sehgal (1999) in their experiments on *Ocimum sanctum* from young inflorescence explants, used MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IAA, to shoot induction and proliferation. The role of BAP and IAA in shoot formation has been recorded in other medicinal plants (Begum et al., 2002; Dode et al., 2003; Valdez Melara and Gatica Arias, 2009; Taware et al., 2010; Daniel et al., 2010). We have found out that between two hormones, TDZ was better than BAP. TDZ is among the most active cytokinin-like substances and it induces greater *in vitro* shoot proliferation than many other cytokinins in many plant species (Khawar et al., 2004). The highest shoot multiplication of *Nothapodytes foetida* achieved on medium containing TDZ at a concentration of 2.2 µM (Rai, 2002).

The number of shoots per explant depends on concentrations of the growth regulators and the particular genotypes (Faria and Iiig, 1995). Our experiments showed that by increasing cytokinin concentration, a greater amount of regenerated shoots was obtained. This fact can be explained so that cytokinins especially at high concentrations overcome apical dominance and promote shoot formation (Echeverrigary and Fracaro, 2001). Our finding was in agreement with Tiwari et al., (2000) that they reported; BAP in high concentrations used in regeneration media caused high *in vitro* shoot regeneration of *Centella asiatica.*

Rooting of shoots and acclimatization of plantlets

The objective of the rooting experiment was to test the effect of IAA and IBA on rooting of Clary sage. Data analysis showed that simple effect of salt concentrations in root number and length had no significant differences at ($p \leq 0.05$). Simple effects of PGRs in root number and length was significant. Interaction effects between salt concentrations and PGRs were no significant ($p \leq 0.05$). Our results indicated that IAA (0.5 mgl^{-1}) established highest number of root (2.55 mean of root) and lowest mean of root number (1.52) was in IBA (0.5 mgl^{-1}) (Figure 3, A). Average root length was highest in control medium (5.65 cm) while in medium containing IAA (1 mg l^{-1}) the lowest average root length (3.08 cm) was obtained (Figure 3, B).

Shoot regeneration is most affected by cytokinin. Results showed that without the presence of cytokinin, shoot regeneration had not performed and root formation was caused. For most species, auxin is needed to stimulate rooting. Auxin to cytokinin ratio is important for root induction and growth. Generally by increasing auxin and reducing cytokinin, roots are formed. Auxin plays a major role in root induction through its effect on the first cell division which forms root initials (Farooq et al., 2008) In a study on *Hyssopus officinalis* rooting, it was observed that the highest percentage of rooting (90%) was in 0.1 mg l^{-1} IAA treatment (Nanova et al., 2007). Echeverrigaray et al. (2010) demonstrated that "the best condition for rooting of *Salvia guaranitica* plantlets was MS medium with 2.85 µM IAA".

The acclimatization protocol used for *in vitro* produced plantlets was successful and a total of 90% survival rate was obtained. Acclimatized plants appeared normal and did not exhibit any morphological abnormalities or variations.

Plant cultured *in vitro* grows usually under very different conditions as compared to those grown *in vivo*. Low irradiance and high relative humidity strongly alter their structure (Majada et al., 2000). Most species grown *in vitro* require an acclimatization process to support plant survival and growth when transferred to soil (Ebrahim et al., 2007). In our study after acclimatization, 90% survival rate was obtained.

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