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Original Article

High-Frequency *in Vitro* Direct Shoot Regeneration from Nodal Explants of Hyssop Plant (*Hyssopus officinalis* L.)

Bahman Hosseini[1], Morteza Alizadeh[1], Abbas Hassani[1], Morad Jafari[2] and Amir Rahimi[3*]

[1]*Horticulture Department, Agriculture Faculty, Urmia University, Urmia, Iran*
[2]*Plant Breeding and Biotechnology Department, Agriculture Faculty, Urmia University, Urmia, Iran*
[3]*Field Crops Department, Agriculture Faculty, Urmia University, Urmia, Iran*

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Abstract

Considering great medicinal value of *Hyssopus officinalis* L. and possibility of its mass production through *in vitro* culture, two individual experiments was conducted. Effect of various concentrations (0, 2.2, 4.4 and 11 µmol) of TDZ and BAP in combination with 1 µmol of IAA on direct regeneration from nodal explants were assessed. Significant difference between treatments was observed ($P \leq 0.01$). In BAP treatments, the maximum shoot-buds induction (9 shoot-buds per explant) and shoot regeneration percentage (96.66%) were observed on MS medium fortified with 2.2 and 4.4 µmol BAP in combination with 1 µmol of IAA. In TDZ treatments, the highest regeneration percentage was achieved in MS medium supplemented with TDZ (2.2 µmol) and IAA (1 µmol), and the maximum shoot-buds induction (19.83 shoot-buds per explant) was observed in medium containing 4.4 µmol of TDZ in combination with 1 µmol of IAA. The highest root production frequency (89.5%) was achieved in medium contained 9.84 µmol of IBA. Rooted plants were acclimatized successfully in greenhouse conditions with 100% survival. The protocol described here could be applicable for mass *in vitro* production of the valuable medicinal plant *Hyssopus officinalis* L. for its genetic resource conservation as well as pharmaceutical purpose**.**

Keyword: Hormonal combination, Medicinal plant, Root induction, Shoot-bud induction

Introduction

Hyssop (*Hyssopus officinalis* L.) belongs to the Lamiaceae family and is a perennial herbaceous plant, which is widely distributed in Asia Minor covering a large area from Caspian to Black sea. Essential oils of Hyssop have been reported to possess antifungal [1] and antimicrobial [2] activities, and are used in canning, beverage, toilet and medical industry [3]. With an ever-increasing global inclination towards herbal medicine, there is not only an obligatory demand for a huge raw material of medicinal plants, but also of right stage when the active principles are available in optimum quantities at the requisite time for standardization of herbal preparations. Commensurate with this the intervention of biotechnology or to be precise, plant tissue culture for accelerating clonal multiplication of desired clones and strains (High-yielding) of medicinal plants through micro propagation and their conservation through establishing tissue banks or gene banks are warranted in the right earnest. Ideally, the herbal plants should be grown under uniform environmental conditions and the planting material must have the same genetic make-up as of the selected high-yielding clones which are possible when they are cloned through an *in vitro* strategy [4].

Micropropagation has many advantages over traditional propagation methods. In this method, propagation rate is considerably increased and

*Corresponding author: Field Crops Department, Agriculture Faculty, Urmia University, Urmia, Iran
Email Address: emir10357@gmail.com

pathogen-free plants can be obtained. Various factors including the type of genotype and explant, different combination of growth regulators can be influence successful *in vitro* propagation of a plant [5]. *In vitro* direct regeneration has been reported for many of medicinally important plants using multifarious explants. For instance, in *Rosmarinus officinalis* L. [6]. *Artemisia dracunculus* L. [7] and *Datura insignis* Barb.Rodr. [2] stem nodes, as an efficient explant, were used for micropropagation. The use of nodal explants offers several advantages over other explants as they can be easily isolated and manipulated. Also, much number of this explant can be prepared from a single plant. So far, tissue culture methods such as organogenesis and direct regeneration were not widely established in *H. officinalis*. Micropropagation of Hyssop has been recently reported by Nanova *et al*. [8] using shoot tip explants. Guo *et al.* [9] reported optimized *in vitro* propagation method of *Saussurea involucrata* (Kar. & Kir.) Sch.Bip. in MS medium supplemented with BAP and NAA. In *Hydrastis canadensis* L. 2.5 µmol/l of TDZ and 0.5 µmol/L of NAA were reported as the best factors for regeneration [10].

To our knowledge, no reports are available on shoot regeneration of *H. officinalis* using nodal explants. Therefore, the aim of the present study was to evaluate the effects of different concentrations of BAP and TDZ with combination of IAA on micro propagation features of this plant.

Material and Methods

In vitro Sseed Germination

The experiment was carried out at the Plant Tissue Culture laboratory of the Horticultural Sciences Department of Urmia University, Urmia in Northwest Iran during 2011. The seeds of Hyssop Plant were collected from medicinal plants collection of Urmia University and a voucher specimen (No. 7471) has been deposited at the herbarium of the Agricultural and Natural Resources Research Center of West Azerbaijan, Uremia, Iran. The seeds were surface sterilized with 70% (v/v) ethanol for 1 min and 2.5% (v/v) sodium hypochlorite for 7 min followed by rinsing in sterile distilled water three times. They were then germinated on MS [11] basal medium without plant growth regulators (PGRs) with 3% sucrose and 0.7% agar. Voucher specimens deposited at the herbarium of the Agricultural and Natural Resources Research Center of West Azerbaijan, Urmia, Iran.

Preparing Explants and Culture Condition

To investigate the effects of plant growth regulators (PGRs) in shoot-bud induction and direct shoot regeneration, two independent experiments experiment was carried out based on a completely randomized design (CRD) with different concentrations of cytokinins, 6-benzylaminopurine (BAP) (0, 2.2, 4.4 and 11 µM) and Thidiazuron (TDZ) (0, 2.2, 4.4 and 11 µM) in combination with 1 µM indole-3-acetic acid (IAA) and 3 replicates (each contains 10 explants) (Table 1). Influence of. Nodal explants (Fig. 1A) from 28-day-old *in vitro*-growing plants were isolated and cultured on MS basal medium and B5 [12] vitamins supplemented with above mentioned PGRs, 100 mg l^{-1} Myo-inositol, 3% sucrose, and 0.7% plant agar. Some explants were cultured on a hormone-free MS medium considered as control. TDZ and IAA was filter sterilized by Millipore filtration (0.22 µm pore size) and added to autoclaved media. In both experiments, explants were subcultured on the same shoot induction media at 3 weeks interval. The pH of the media was adjusted to 5.8 before autoclaving at 103 kPa for 15 min at 121°C. All cultures were kept in a plant growth chamber at 25 ± 2°C under a 16/8 h (light/dark) photoperiod with a light intensity of 33 μm^{-2} s^{-1} provided by cool white fluorescent lamps.

Table 1 Hormonal combination used for shoot-bud induction and regeneration in hyssop plant

IAA (µm)	TDZ (µm)	BAP (µm)	Culture media
-	-	-	SIM_0
1	-	2/2	SIM_1
1	-	4/4	SIM_2
1	-	11	SIM_3
1	2/2	-	SIM_4
1	4/4	-	SIM_5
1	11	-	SIM_6

Root Induction and Acclimatization

After 3 weeks, the new regenerated shoots (4-5 cm) were excised by cutting at the basal end and transferred individually on root induction medium contains MS basal medium fortified various concentrations of 3-Indole butyric acid (IBA; 0, 1, 2.56, 4.92 and 9.84 µM). The percentage of rooted

shoots and length of roots were recorded for each treatment. For acclimatization, plantlets with well-developed root were gently rinsed with tap water to remove the remnants of agar and then transferred to They kept in a plant growth chamber with a high relative humidity at 24±2 °C under a 16 h day/night photoperiod for two weeks. The acclimatized plantlets were finally transferred into greenhouse conditions.

Data Recording and Statistical Analysis

The number of shoot-bud per explant and the percentage of shoot regeneration were calculated and the percentage of rooted shoots and length of roots were also recorded at the end of rooting experiment.

Statistical Analysis

Experiments were set up in a completely randomized design (CRD) with 3 replicates (each contains 10 explants) per treatment. The analysis of variance (ANOVA, one-way analysis) was performed using SAS 9.1 (SAS Institute, Cary, North Carolina, USA) to detect the significance of differences among the treatment means and the means were compared using Fisher's least significant test (FLST) at the 1% probability level.

Results and Discussion

Effect of BAP on Adventitious Shoot-bud Induction and Regeneration

The analysis of variance results revealed that different hormonal levels were significantly different from point view of shoot bud induction and regeneration ($P \leq 0.01$) (Table 2). There was no significant difference among treatments SIM_1, SIM_2 and SIM_3 based on shoot regeneration percentage (Fig. 2). The results suggest that there is an optimum concentration (2.2 µmol) of BAP for achieving high level of regeneration and increasing of BAP level did not improve regeneration rate (Fig .1C, D, E, F). The highest number of shoot-buds induction (9 shoot-buds per explant) was obtained in 2.2 µmol BAP combined with 1 µmol IAA (Fig. 3). The lowest number of shoot-buds induction was observed in control medium, and no significant difference was observed between 1.1 and 4.4 µmol levels of BAP. The highest rate of regeneration was achieved in SIM_1 and SIM_2 (MS media supplemented with 2.2 and 4.4 µmol BAP in combination with 1 µmol IAA, whereas no plastic pots containing sterile soil: perlite (1:1) mixture in the plastic containers and moistened with liquid ½MS basal medium without vitamins, phytohormones and sucrose.
regeneration was observed in SIM_0 (hormone-free media).

Table 2 Effect of different concentrations of BAP on shoot regeneration percentage and number

		Mean of Squares	
S.O.V	df	Shoot regeneration percentage	Shoot regeneration
BAP	3	1767.24**	142.72**
Error	8	1593.64	14.37
CV (%)		37.34	36.69

** significantly different (P<0.01)

Table 3 Effect of different concentrations of TDZ on shoot regeneration percentage and number

		Mean of Squares	
S.O.V	df	Shoot regeneration percentage	Shoot regeneration
TDZ	3	3796.32**	2.97**
Error	8	185.37	0.07
CV (%)		24.07	13.03

** significantly different (P≤0.01)

Effect of TDZ on Adventitious Shoot-bud Induction and Regeneration

The results of ANOVA showed that including of TDZ in the basal media cause to significant differences ($P \leq 0.01$) in comparison with hormone free media (Table 3). The highest rate of regeneration was achieved in SIM_4 containing MS basal medium supplemented with 2.2 µmol TDZ in combination with 1 µmol IAA, whereas no regeneration was observed in medium containing 11 µmol TDZ (SIM_6) (Fig. 3). Moreover, there was no significant difference between treatments SIM_4 and SIM_5. The results revealed variation in plantlet regeneration rate between TDZ levels, since SIM_4 and SIM_5 media led to achieve the highest number of regenerated shoots (19.83 and 16.16 plantlet per explants), respectively and no regeneration was

observed in the treatment of SIM_6 the same as control media.

Root Induction

The ANOVA results showed significant ($P \leq 0.01$) effect of IBA Levels on root induction. The highest rooting percentage (89.5%) was achieved in MS medium supplemented with 9.84 µmol of IBA and the lowest rooting percentage (20%) was observed in hormone-free medium (Fig. 4). Meantime, increasing of IBA level promotes rooting response. Well rooted plants were acclimatized successfully in greenhouse conditions with 100% survival and no visible morphological alterations was observed in them with compared to control plants grown in the greenhouse (Fig .1G-H). Successful *in vitro* propagation of medicinal plants is determined by a wide range of factors and one of the most important factors, is the growth regulating compounds such as auxins and cytokinins that influencing shoot induction in different plants.

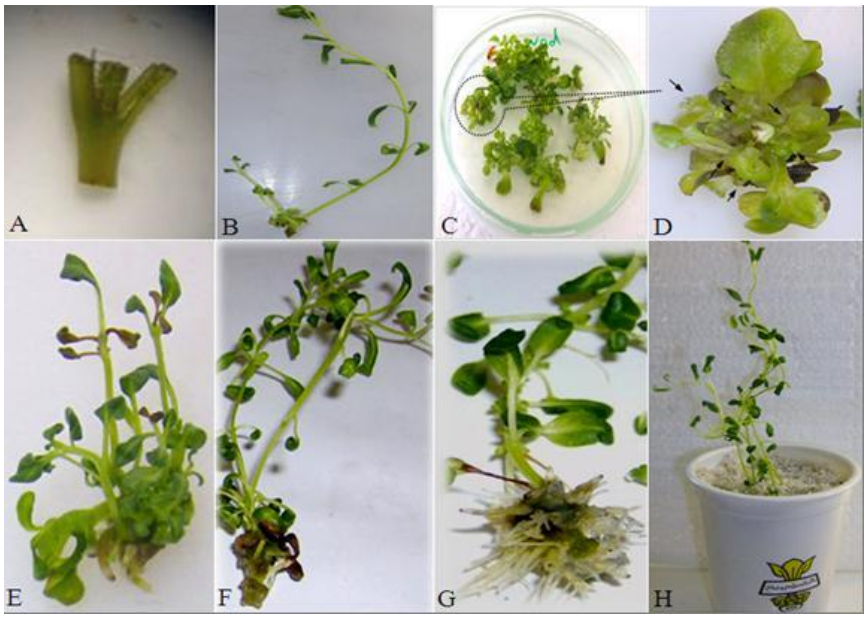

Fig. 1 *In vitro* organogenesis and regeneration of *Hyssopus officinalis* L. using Nodal explants. A: Nodal explant isolated from *in vitro* growing seedling, B: A nodal explant cultured on hormone-free MS medium as control that did not show any shoot-dud induction, C: multiple shoot development from stem node explants cultured on MS basal medium supplemented with a Cytokinin (TDZ or BAP) in combination with IAA, D: close-up view of adventitious shoot (Black arrows) induction, E: regenerated Plantlets on the same medium, F: Elongated plantlets, G: Rooted plantlet on MS basal medium containing IBA, H: Acclimated plantlet growing in a pot containing sterilized bed soil and perlite mixture.

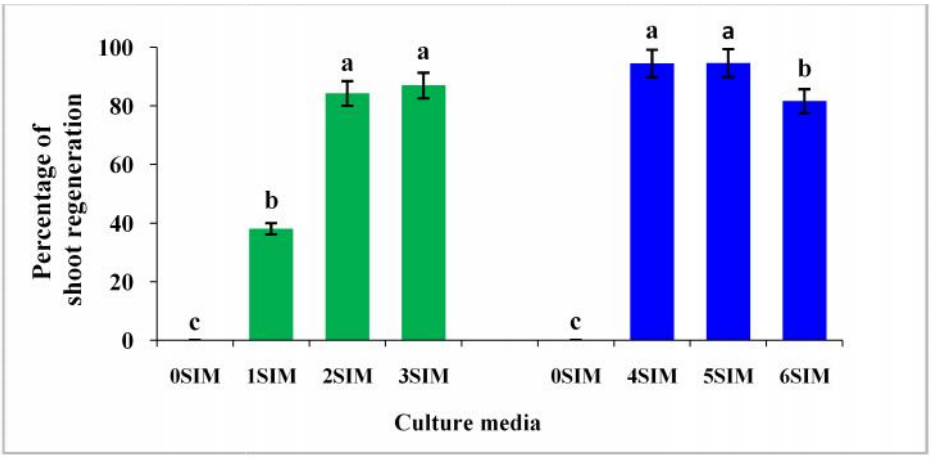

Fig. 2 Comparative effect of different concentrations of 6-benzylaminopurine (BAP) and thidiazuron (TDZ) in combination with 1 µM Indole-3-acetic acid (IAA) on potential regeneration of induced shoots from nodal explants of *Hyssopus officinalis* L,. The results are expressed as percentage of regeneration of three replicates (totally 30 explants) per treatment. Bars followed by different letters are significantly different ($P<0.05$) according to the Fisher's least significant difference (FLDS) test.

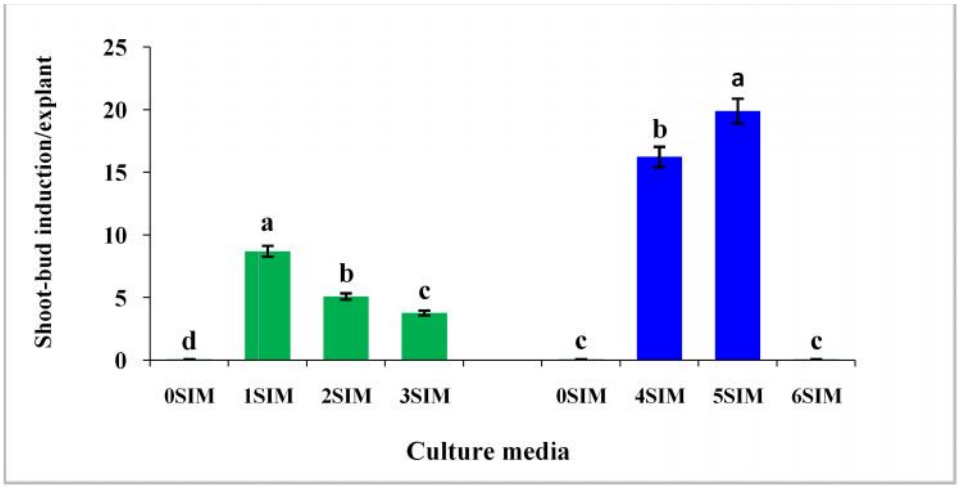

Fig. 3 Effect of shoot induced media (SIM) supplemented with different concentrations of BAP or TDZ with IAA on shoot-bud induction of *Hyssopus officinalis* L. The bars represent means±SE. Bars (separately for each phytohormone) followed by different letter are significantly different (P<0.05) according

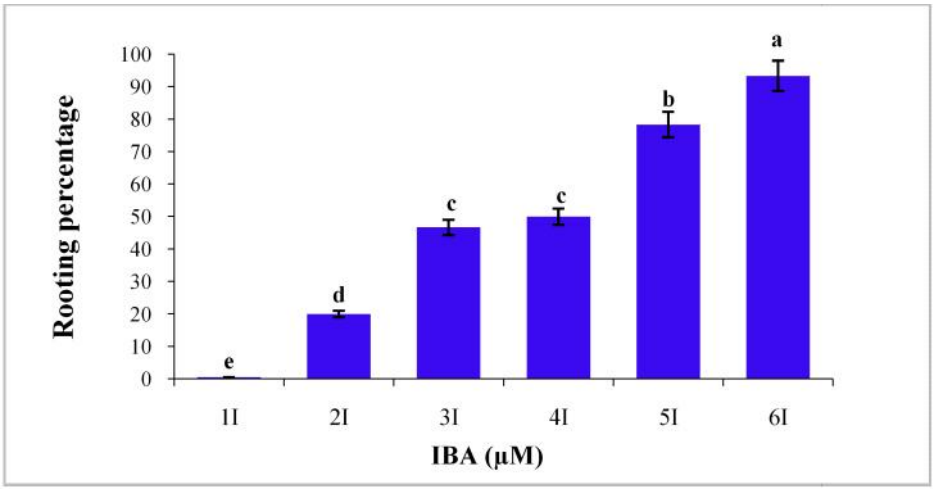

Fig. 4 Effect of different concentrations of IBA on root induction of *in vitro* obtained *Hyssopus officinalis* L. plantlets. Data represent rooting percentage of three replicates (totally 30 explants) per treatment. Vertical bars indicate the SE of three replications. Bars followed by different letters are significantly different (P<0.05) according to the Fisher's least significant difference (FLDS) test.

In the natural status, the axillary buds of the higher plants are dormant due to apical dominance and the mechanism of apical dominance has been demonstrated to be under the control of various growth regulators specially auxin [13]. Cutting the stem into segments and culturing them on medium supplemented with suitable PGRs can break the dormancy of the bud [14,15]. In general, bud induction and development of multiple shoots from stem node explants is a function of cytokinin activity [16].

Many authors had previously reported that a suitable concentration of cytokinins, alone or in combination with auxin, is necessary for shoot induction and regeneration of many medicinal plants [17]. When cytokinins are accompanied by a very low concentration of auxin, shooting percentage is considerably increased (Rout, 1999). It has been reported that various kinds of cytokinins have been used for shoot induction in *Ocimum basilicum* L, among which, the best results obtained with BAP [18]. Therefore, in this experiment, we used an optimized level of IAA (1 µmol), and a high level of regeneration (more than 90%) was obtained. Our result showed that the highest regeneration percentage was observed in BAP treatments. The same result was obtained in *O. basilicum*, where the highest level of regeneration was observed in MS medium containing 2.2 µmol of BAP and 1.4 µmol of IAA [19]. Nanova *et al.* [8] also reported the effectiveness of BAP at lower concentrations (0.2 or 0.5 mgl^{-1}) on microclonal Propagation of Hyssop plants. But in *Mentha piperita* L., the highest level

of shooting was obtained via nodal explants cultured on MS medium supplemented with 4.4 µmol of BAP [2].

Combinations of cytokinins such as BAP and Kin with low level of auxin (IAA or NAA) have been used to induce shoot formation in some plants [20-22]. (Chen, 2001; Sivanesan and Jeong, 2007; Sunil, 2009). Amutha *et al*., [23] have been reported the highest number of shoot induction in *Ocimum basilicum*, when they used a medium containing 8.88 µM BAP and 9.28 µM Kin. In *Artemisia absinthium*, 2.2 µM BAP and 0.5 µM NAA were reported as the most appropriate combinations of plant growth regulators [24]. 4.43 µM BAP was also reported as the best treatment for propagation of *Allium sativum* L. [25]. Roberson *et al.* [26] were found 4.4 µM BAP in combination with 2.7 µM NAA as effective levels in *in vitro* propagation of *Eucalyptus globulus* Labill. Combination of BAP+IAA was used for shoot culture in *Adhatoda vasica* Nees [27], *Centella asiatica* (L.) Urb. [28], *Hypericum perforatum* L. [29], and *Salvia officinalis* L. [30].

In the present study, the largest number of regenerated seedlings was achieved in medium containing 4.4 µmol of TDZ; while the optimum concentration for BAP was 2.2 µmol. Significant influence of TDZ on shoot multiplication of medicinal plants has been reported by other reports [31-32]. In *Hydrastis canadensis* L., combination of 2.5 µmol of TDZ and 0.5 µmol of IAA was used for plant regeneration and 25 seedlings per explant were obtained [10]. Whereas, in the present study the highest number of shoot regeneration (19.83 shoot per explant) was obtained in a high level of TDZ (4.4 µmol).

The most common growth regulators used for root induction include IAA (0.1-10 mgl^{-1}), IBA (0.5-3 mgl^{-1}) and NAA (0.05-1 mgl^{-1}). These auxins have similar effect on root induction and can be used on behalf of each other, but a special kind of auxin depends on plant species leads to better result [33]. In this study, IBA led to efficient root development. Different concentrations of IBA were used for multiplication of *H. officinalis* and the highest level of root extension was obtained in ½MS medium supplemented with 1 to 2 µmol of IBA [8]. The maximum rooting percentage (63.5%) with 3.8 roots per explant and average root length of 8.4 mm in *H. camadensis* was obtained in media containing 2 µmol of IBA [10]. In *Origanum vulgare* plant, root induction recorded on medium supplemented with 0.5 mgl^{-1} IBA [34]. In the present study, the highest rooting percentage (93.33%) was observed in 9.82 µmol of IBA and there was no significant difference between 4.92 and 2.56 µmol of IBA.

Conclusion

The results of the study showed that the highest level of shoot regeneration as 96.66% was achieved by using 2.2 and 4.4 µmol of BAP in combination with 1 µmol of IAA. The largest number of seedling was obtained when 4.4 µmol of TDZ was used in combination with 1 µmol of IAA. Comparing the two phytohormones, BAP seems to be better concerning the time and the percentage of direct regeneration of Hyssop plant.

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