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RESEARCH ARTICLE

Effect of plant growth regulators and explant types on in vitro direct plant regeneration of *Hyoscyamus reticulatus* L.

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Abstract

Hyoscyamus reticulatus L. is an important medicinal plant, belonging to Solanaceae family. This research was performed to study the effects of different explants (cotyledons, hypocotyls, shoot tips, and nodal segments) and various concentrations of 6-benzyladenine (BA) in combination with Indole-3-acetic acid (IAA) on shoot regeneration of *Hyoscyamus reticulatus* L. in vitro culture conditions. Regenerated shoots were transferred to half- or full-strength MS medium with various growth regulators for root regeneration. The experiment was done in completely randomized designs in four replications. Data was registered after nine weeks (three subcultures). Statistical analysis showed the highest average of regenerated shoots (162.25 shoot per treatment) was obtained on shoot tip explant in MS medium supplemented with BA (5mg.l⁻¹) and IAA (0.1 mg.l⁻¹) the lowest average of regenerated shoots (2.52, 1.75 shoot per treatment) respectively on cotyledon and hypocotyl explants in MS medium containing IAA (0.1 mg.l⁻¹) and without BA and control treatment (0). Regenerated shoots have been rooted on full-strength MS medium containing 1.1 and 2.2 μM IBA and in other treatments no rooting induction was observed. Rooted plantlets derived from in vitro culture were transferred to plastic pots to acclimatization, with 90% survival. After 10 days they were transferred to greenhouse conditions.

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INTRODUCTION

Hyoscyamus reticulatus L. is an herbaceous, annual or biennial, belonging to Solanaceae family (Al-Qura, 2008). *Hyoscyamus reticulatus* L. recently is known as a medicinal plant for the treatment of neurological diseases of postpartum. In this herb, Tropane alkaloids have antispasmodic, antichlorogenic and analgesic characteristics. This plant is also commonly used to treat asthma, ulcers and Parkinson. The alkaloids are used in ophthalmology, and treatment of diseases of the cardiovascular and gastrointestinal function (Akramian et al., 2008; Bahmanzadegan et al., 2009; Oto et al., 2013). This genus contains tropane alkaloids Hyoscyamine and scopolamine which are found mainly in the leaves. Hyoscyamine is the major alkaloids in *H. reticulatus* L. and its rate is higher than scopolamin at all the organs and all stages of development of different parts of the plant; the highest amount of scopolamine is recorded at the time of flowering and fruit formation. *H. reticulatus* seeds have a higher amount of Hyoscyamine. Commercial sources of tropane alkaloids in Solanaceae family include, Mandragora, Atropa, Datura, Duboisia,

Plant tissue culture techniques offer a valuable tool for mass propagation, biomass production, plant pathology, plant tissue preservation and scientific research (Nejadhabibvash et al., 2012; Oto et al., 2013; Kaur et al., 2013). Pharmaceuticals supplied directly from human nature, such as geographical and native varieties of seeds and fruits genetic and biochemical models are costly to society as protectors of drugs in the nature of the bank kept. Replicating this model only is used for mass production of pharmaceutical substances in Agro-industrial. So putting yourself at risk for public consumption model is Non-normative (Tasheva and Kosturkova, 2011; Omidbaigi, 2007). Therefore, to prevent the extinction of the national capital, different methods such as in vitro culture for mass production of these plants are used to maintain basic properties. In vitro regeneration has been accomplished in many species of the medicinal plant, for example: The maximum number of stem on *Hyoscyamus niger* in MS medium supplemented with 5.0 mg l⁻¹ NAA and 1 mg.l⁻¹ BA (Ghorbanpour et al., 2013), the maximum shoot induction of the *Solanum trilobatum* L. was observed in nodal and inter nod explants (Arockiasamy et al., 2002), The best combination of the *Silybum marianum* was reported to be MS medium containing 0.3 mg.l⁻¹ zeatine and BA for leaf explant regeneration (Samandari et al., 2012). Due to the growing importance of *H. reticulatus* L. and considering the fact that no studies on the in vitro culture of this plant have been reported to date this study aims to identify the best, fast and most economical protocol of multiplication, and access to culture medium for plant regeneration and proliferation in the plant as well as the development of in vitro technology.

In this study, effects of various plant growth regulators including BA and auxin (IAA) with different concentrations were studied on shoot regeneration of *H. reticulatus* L.

Materials and Methods

Plant material, explants preparation, and culture conditions. Seeds of *H. reticulatus* collected from the Botanical garden of Urmia University in 2013, were used for obtaining in vitro explants. Seeds were surface sterilized and for uniform germination, seeds were submerged in sulfuric acid solution (10%) for 7 min and then transferred to laminar flow cabinet disinfection by 70% ethanol (v/v) for 1 min and then 5% sodium hypochlorite (v/v) for 10 min. Afterwards, the seeds were rinsed three times. The sterilized seeds were cultured in growth regulator-free MS (Murashige and Skoog 1962) medium. Hypocotyls and cotyledons segments were obtained 15 days after seedlings and Shoot tips and nodal segments were obtained 20 days after culture. In all experiments, MS medium containing 3% sucrose and 0.7% plant agar (Duchefa, Haarlem, Netherlands), were used as the basal medium. All cultures were kept in growth chambers at 25±2°C under a 16/8 h (light/dark) of photoperiod at a photon flux rate of 60 μmol/m²/s provided by cool daylight fluorescent lamps.

Shoot bud induction and regeneration. All explants were cut into 1- to 1.5- cm pieces and cultured on shoot regeneration medium (SRM) supplement with 0, 1, 3, 5 mg.l⁻¹ 6-benzyladenine (BA) in combination with 0, 0.1, 0.5 mg.l⁻¹ indole-3-acetic acid (IAA); the medium without PGR served as the control (Table1). Each PGR treatment had four replicates, each replicate contained four explants of the same type in 7-cm×9-cm vials. Explants were subcultured onto similar fresh media at 3-wks and 9-wks intervals. The average number of adventitious shoots formed per explant was determined after 12 -wks of culture.

Rooting and acclimatization of plantlets: Well-developed shoots (5 cm long) were excised from all regenerated explants and maintained in medium without PGR for 1-wk before transferring to rooting media containing half- or full-strength MS salts and 0, 1.1, or 2.2 μM indole-3-butyric acid (IBA) and IAA. Four shoots were cultured in each 7-cm×9-cm vial. After 4-wks rooted plantlets and the average induction of root per plantlet were recorded. For acclimatization, plantlets with well-136 developed roots were gently removed from culture vials to avoid any damage and washed with tap water to remove traces of medium adhered to the roots. The plantlets were then transplanted to 6-cm plastic pots filled with perlite and pit (3:1) nourished with half-strength MS basal salts. Plantlets were kept in a chamber with 80–90% relative humidity under a 16/8 h (light/dark) of photoperiod at a photon flux rate of 60 μmol m⁻²s⁻¹ provided by cool daylight fluorescent lamps for 2 wks. Finally, plantlets were transferred to greenhouse conditions after 2-wks.

Statistical analysis. The shooting experiments consisted of factorial arrangements with a 4×4 of shoot regeneration medium with different combinations of BA and IAA and explant type and rooting experiments consisted of factorial arrangements of 2×2×3 of MS salt strength, auxin type and auxin concentration. Each treatment consisted of at least 16 cultures (four replications with four explants per replicate). The experiments were set up in a completely randomized designs and each treatment had four replicates of four explants each. Analysis of

variance (ANOVA) was performed on 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 a 95% confidence level.

Results

Effect of explant type and PGR treatment on shoot regeneration: Shoot regeneration was observed in all treatments, and all tested explant types after 25–30 days of culture, and shoots continued to proliferate for 9-wks (Table1). The interaction among explant type and SRM type were statistically significant ($p < 0.01$) (table1). The highest shoot regeneration frequency was recorded for shoot tip explants, followed by nodal, cotyledons and hypocotyls respectively. For shoot tip explants, the highest shoot regeneration frequency (162.25 shoots in each treatment) was obtained using MS medium supplemented with BA (5 mg.l^{-1}) and IAA (0.1 mg.l^{-1}) (Table1, Fig. 1A-C). For nodal explants the highest shoot regeneration frequency (137 shoots in each treatment) was observed on the MS medium including BA (1 mg.l^{-1}) with IAA (0.1 mg.l^{-1}). Cotyledon and hypocotyl explants inoculated on BA-free medium yielded no shoots, and the highest shoot regeneration frequency (96, 84 respectively) was provide on the MS medium supplemented with BA (5 mg.l^{-1}) and IAA (0.5 mg.l^{-1}) (Table1).

Effect of growth regulators on root induction: Roots were not induced during shoot multiplication in the cytokinins treatments. Individual shoots when implanted in half or full strength MA medium free from growth regulators, poor numbers of roots were elicited with low frequency. Addition of auxin, IAA and IBA on MS medium enhanced the rate of frequency as well as number of roots. Among these two auxin tests, 1.1 and 2.2 μM IBA on full MS medium induced maximum number (87.50) of root per shoot (Fig1C) compared to other concentrations of IAA tested (Table 2). Similar results were also reported by Al-Bakhit et al., (2007), Samandari., et al (2012) and Babaei .,et al (2014) but Hossain., et al (2007) reported that there was no root formation on MS medium containing any concentration of IBA. This report does not correspond with our results.

The rooted shoots were kept for 4- weeks in the rooting medium and then transferred to MS medium free PGRs for 2 wks. About 80-90% of the transplanted *H. reticulatus* survived, if the plants in the culture tubes were kept under normal room temperature for 7-8 days before transplantation in pots and reared under semi-controlled temperature ($30 \pm 2^\circ\text{C}$) in a chamber with 80% humidity. During this period of acclimation the shoots elongated, leaves expanded and were healthier. After 3 wks, plants were transferred to an open place and gradually acclimated to outdoor condition, where 80% of plants survived.

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Table 1. In vitro shoot regeneration from various explants of *Hyoscyamus reticulatus* MS medium supplemented with different concentrations of BA in combination with IAA.

Medium	Plant growth regulator		Explant type			
	IAA	BA	Cotyledon	Hypocotyl	Shoot tip	Nodal segment
SRM0	0	0	0 ^{op}	0 ^{op}	8 ^{mn}	11 ^m

SRM1	0	1	1.75 ^o	24.25 ^{ki}	60.5 ^g	127.75 ^{bc}
SRM2	0	3	6.5 ⁿ	29 ^k	81 ^e	79 ^{ef}
SRM3	0	5	60 ^g	20.75 ^{ki}	123.5 ^c	89.5 ^{de}
SRM4	0.1	0	2.25 ^o	1.75 ^o	29.5 ^k	34.75 ^j
SRM5	0.1	1	64 ^{fg}	27 ^{ki}	71.25 ^f	137 ^b
SRM6	0.1	3	30.5 ^k	78.5 ^{ef}	78.5 ^{ef}	82.75 ^c
SRM7	0.1	5	54.5 ^{gh}	48.75 ^{hi}	162.25 ^a	82.25 ^e
SRM8	0.5	0	35.5 ^j	6.5 ⁿ	55.25 ^{gh}	52 ^h
SRM9	0.5	1	42.75 ⁱ	35.5 ^j	17 ⁱ	52.25 ^h
SRM10	0.5	3	25.5 ^{ki}	46.5 ^{hi}	70 ^f	57 ^{gh}
SRM11	0.5	5	96 ^d	84 ^e	108 ^{cd}	64.25 ^{fg}

Table 2. Effects of MS salt strength and auxin treatment on adventitious root induction in regenerated shoots of *Hyoscyamus reticulatus* after 4 wks of culture on rooting media.

	MS					1/2MS				
	IAA(μ M)		IBA(μ M)		control	IAA(μ M)		IBA(μ M)		Control
	1.1	2.2	1.1	2.2	-	1.1	2.2	1.1	2.2	-
Root Induction	0 ^c	0 ^c	87.5 ^a	87.5 ^a	0 ^c	0 ^c	0 ^c	50 ^b	0 ^c	0 ^c

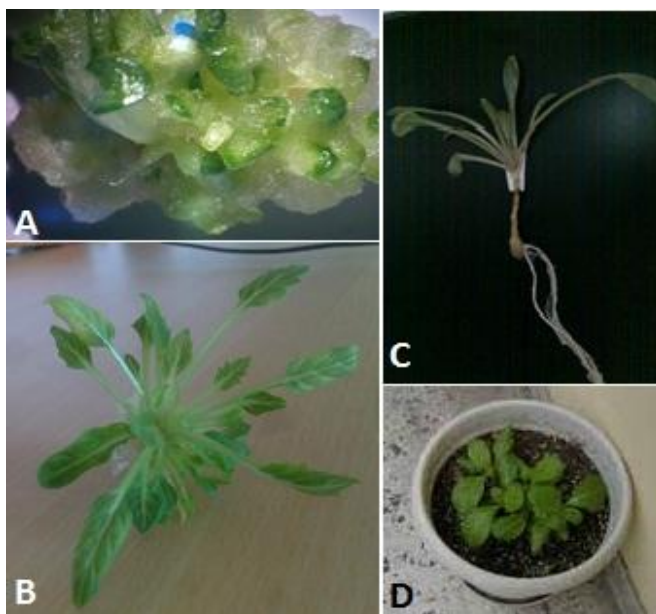


Figure1. Shoot and root regeneration in *Hyoscyamus reticulatus* L. (A-C). Shoot regeneration from shoot tip on MS medium containing 0.5 mg.l⁻¹ BA+ 0.1 mg.l⁻¹ IAA after 21d (A), 63 d (B) of culture. Rooting of an *in vitro*-regenerated shoot on MS medium containing 2.2 μ M IBA after 36d of (D) of culture. An acclimatized plantlet growing in a pot containing sterilized perlite (E).

Discussion

The plant regeneration system via direct shoot regeneration was established in this study. Type of explant, growth medium and internal hormones levels have been reported to play an important role in shoot induction in plant tissue culture

(Kesari et al., 2012). In this research, application of cytokinin (BA) and auxin (IAA) resulted in high-frequency shoot regeneration in *H. reticulatus* L. Combined effects of cytokinin and auxin proved to be useful in shoot organogenesis in *Cinnamomum tamala* (Sharma and Nautiyal, 2009), *H. niger* (Ghorbanpour et al., 2010) and *Ocimum tenuiflorum* (Gogoi and Kumaria, 2011). MS medium supplemented with 22 μ M BA was effective for shoot multiplication in shoot tips of *Melissa officinalis* (Jangjuo, 2010). Survey done in this study indicated regeneration was little in the control medium (free PGR), this indicates the importance of cytokinins in stimulating proliferation and cell division in cultured explants, forming organs and tissues treated with cytokinin (Arteca, 1996). Explant type is another factor influencing in vitro shoot regeneration. According to the study the highest and the lowest shoot regeneration frequencies were observed in shoot tip and hypocotyl explants, respectively. Similar results, showing variation among shoot regeneration capacity of different explant types, have been reported in other plants such as *Solanum trilobatum* L (Arockiasamy et al., 2002), *Scopolia carniolica* (Deliu et al., 2002), *Cassia auriculata* L. (Singh et al., 2011) and *Ocimum basilicum* L. (Asghari et al., 2012).

Root induction. There was a clear difference in rooting response of PGR-treated and untreated regenerated shoots of *H. reticulatus* L. In the presence of auxin regenerated shoots rooted earlier and had a much higher rooting rate than untreated shoots. Liu et al (2002) reported that auxin induced the complicated process of lateral root formation through repetitive cell division.

The best rooting response (100%) was obtained on MS medium containing 1.1 and 2.2 μ M IBA. Our results corresponded with the results of studies on plants *Lavandula angustifolia* (Al-Bakhit et al., 2007), *Silybum marianum* (Samandari et al., 2012) and *Curculigo latifolia* (Babaei et al., 2014). Complex process lateral root formation through repeats induces cell division by auxin (Liu et al., 2002). Finely shoots were placed in medium without auxin, led to no roots. Similar results was obtained for in vitro cultures of *Melissa officinalis* (Jangjuo, 2012).

The success of the protocol tissue culture adaptation of successful seedlings obtained from in vitro culture in the greenhouse and field conditions (Nalawade and Tsay, 2004).

The research plants produced from tissue culture, with 90% survival after acclimation were transferred to greenhouse conditions. In conclusion, a rapid and efficient shoot and root regeneration and acclimatization protocol for *H. reticulatus* L. was reported in this study. This protocol could be used for large-scale clonal propagation. It could also be used to obtain competent target tissue for genetic modification in order to generate plants with the desired characteristics

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