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Micropropagation of *Astragalus adscendens*: A Source of Gaz-angabin Manna in Iran (Persian Manna)

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ABSTRACT

The aim of this study was to investigate the *in vitro* proliferation of *Astragalus adscendens*. Explants were taken from hypocotyl and cotyledon and were cultured on the basic medium of Murashige and Skoog (MS) complemented with various plant growth regulators, (NAA, BAP, KIN, ZEA), to induce direct shoot regeneration. Callus induction was significantly affected by different concentrations of PGRs. Callus formation was observed from hypocotyl explants, but they were not induced to adventitious shoot regeneration and most of them were turned into brown. Therefore, rapid multiplication, performed using shoot apical buds, and obtained from 15-day old sterile seedlings. Apical buds were cultured on MS medium containing various levels of BAP, KIN and ZEA (0.5, 1.0, 2.0 and 4.0 mg L^{-1}) alone or in combination with 0.5 mg L^{-1} NAA. The highest number of shoot regenerants (8.5/explants) and leaves (22.4/explants) obtained on MS medium with 4 mg L^{-1} BAP. The highest root induction (100%) was obtained from MS media supplemented with 0.3 mg L^{-1} NAA. Rooted plantlets were successfully acclimatized in pots with 1:1:1 mixture of soil, peat, and perlite.

Keywords: Apical buds, Callus induction, Clonal propagation, Gavan-e-Gaz-angabin, Proliferation.

INTRODUCTION

Astragalus L. (*Fabaceae*) is a genus widely distributed throughout the temperate regions (Rios and Waterman, 1997). Iran is one of the main centers for various species of this genus and, based on the latest information, there are more than 804 species, with endemism rate of more than 60%, comprising more than 11% of the Iran's flora (Massoumi, 1998; Ghahremaninejad *et al*., 2012). Many species have been used in folk medicine for their hepatoprotective, antioxidative, immunostimulant, and antiviral properties (Erisen *et al*., 2010). The presence of three groups of chemical compounds including polysaccharides, saponins, and phenolics has been reported in this genus (Rios and Waterman, 1997).

Astragalus adscendens (Boiss. and Haussk) counts as an important plant in Iran, which is used for a special manna (called Gaz-angabin) production (Gerami, 1998). Gaz-angabin (Persian Manna) is a sweet exudate that is secreted by the puncture of an insect (*Psyllidae* class and *Cyamophila* genus) that feeds and is activated on a plant called Gavan-e-Gaz-angabin (*Astragalus adscendens*). Gavan-e-Gaz-angabin, a perennial shrub that was found in central indigenous of Iran and belongs to the *Tragacantha* section of the *Fabaceae* family (Farahnaky *et al*., 2009; Gerami, 1998). It is one of the oldest medicinal plants widely used in Iranian traditional medicine as antispasmodic, anti-headache and carminative. The main constituents of the

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manna collected from this plant is fructose (40%), which is useful for diabetics and has extraordinary importance in term of tragacanth production (Samsam Shariat and Moatar, 2003). In addition, this plant, especially the manna, has a socio-economic importance due to providing new job position which, because of harvesting this manna, was a source of income for the local people. This plant is also used for erosion control and consolidates the sand (Farahnaky *et al*., 2009; Azimi *et al*., 2005).

Recent studies have focused on the insect and its host (Gavan-e-Gaz-angabin), for instance Seifollahi and Ebadi (2002) studied different species of *Psyllids* and their distribution in Isfahan province. Mohammadi and Deni (2002) also studied about different manna sources, production mechanisms, and utilization in Iran. Also, Azimi *et al.* (2005) investigated the relationship between *Cyamophila dicora* Loginova population and vegetation parameters of *Astragalus adscendens* in Feridounshahr region (located in the middle of Iran). Farahnaky *et al.* (2009) studied physicochemical properties and rheological behavior of Gaz-angabin.

Manna production has decreased drastically during the past three decades in Iran. Furthermore, extinction of this plant by native habitats, extensive fires in grasslands, and other human activities led to destruction of native grasslands in recent years (Seifollahi and Ebadi, 2002). Particularly, these reasons are the cause of extinction risks and loss of genetic variability in this plant. Therefore, protection of the plant would be necessary. Several researches seem to be necessary to solve this problem on the effective factors such as host plant, insect, environmental conditions, and manna harvest method.

Unfortunately, because of the long juvenility period, low production of high-quality seeds and difficulty of winnowing, utilization of *Astragalus adscendens* seeds for reproduction is limited (Luo *et al*., 1999). Although, there are many reports on *in vitro* regeneration of *Astragalus* species such as *A. adsurgens* (Luo *et al*., 1999), *A. cariensis* Boiss (Erisen *et al*., 2009), *A. chrysochlorus* (Hasançebi *et al*., 2011), *A. cicer* L. (Basalma *et al*., 2008), *A. maximus* (Turgut Kara and Ari, 2006), *A. melilotoides* (Hou and Jia, 2004), *A. nezaketae* (Erisen *et al*., 2010) and *A. schizopterus* (Yorgancilar and Erisen, 2011), but there is no report on tissue culture of *Astragalus adscendens*. The original result of investigations on the establishment of micropropagation of *A. adscendens* is reported by the current study.

MATERIALS AND METHODS

Seed Germination and Explant Preparation

Seeds of *Astragalus adscendens* were collected from a wild population in Isfahan province, located in the middle of Iran (Figure 1-a). To increase the germination rate and dormancy breaking, the seeds were incubated in H_2SO_4 solution (98% v/v) for 15 minutes followed by three rinses with sterile distilled water. They were surface-sterilized in ethanol (70% v/v) for 1 minute followed by seed soaking in sodium hypochlorite (2.5% v/v) for 15 minutes, then, they were washed 3 times with sterile distilled water for 5 min. The seeds were germinated on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar, at 23-25°C under cool-white fluorescent lamps of 40 µmol m^{-2} s^{-1} photon density (Figures 1-b and -c). Cultures were placed in a growth chambers with a photoperiod of 16 hours light (24±2ºC) and 8 hours dark (20±2ºC). The medium pH was set to 5.7 with 1N KOH or 1N HCl prior to autoclaving at 121ºC, 1.4 kg cm^{-2} for 20 minutes. Hypocotyl (each approximately 5 mm in length) and cotyledon (approximately 0.5×0.5 mm) explants were excised from 15-day old seedlings for direct regeneration and callogenesis. Apical meristem (0.5-1 cm) was excised from 15-day-old seedlings for micropropagation.

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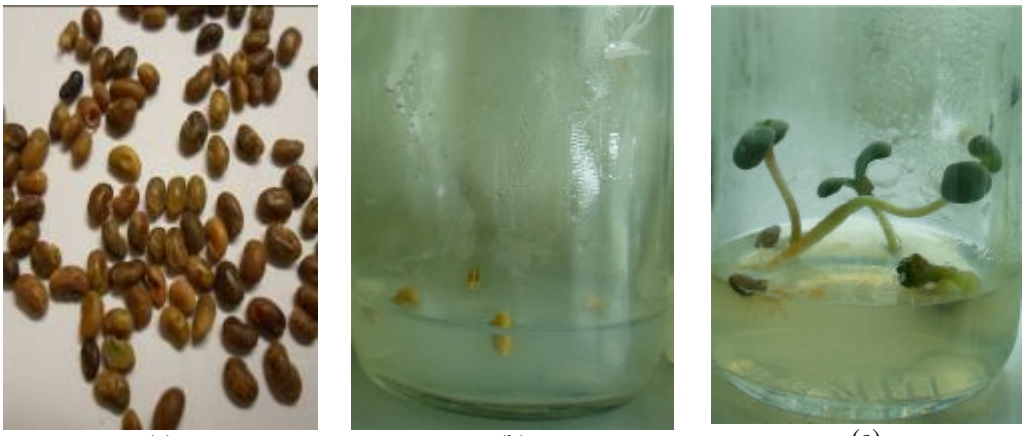

(a) (b) (c)

Figure 1. (a) The seeds of *Astragalus adscendens* were collected from a wild population; (b) The seeds cultured in half-strength MS medium, and (c) Seedling prepared in half-strength MS medium.

Direct Regeneration

To induce direct shoot regeneration, hypocotyl and cotyledon explants were cultured on MS media containing BAP, KIN and ZEA (0, 1, 2, 4 mg L^{-1}) alone or in combinations with 0.5 mg L^{-1} NAA. The rate of shoots and callus induction, as well as callus size was determined after 4 weeks. Each treatment had 4 replicates consisting of 50 mL sterile vials containing 4 explants for direct regeneration.

Micropropagation

For micropropagation, apical meristem was cultured on MS medium supplemented with different cytokinins including BAP, KIN and ZEA in various concentrations (0.5, 1, 2 and 4 mg L^{-1}) alone and in combination with 0.5 mg L^{-1} NAA. Each treatment had 6 replications and two explants were cultured in 50 mL sterile vials containing 15 mL medium. All cultures were placed in a growth chambers at 24±2°C with fluorescent light and 16 hours light and 8 hours dark photoperiod. After 4 weeks, all treatments were sub-cultured on the same medium. After 6 weeks by the beginning of the experiment, number of shoots per explant, shoots length, and number of leaves per shoot were recorded.

Rooting and Acclimatization

After 8 weeks, the regenerated shoots (2–3 cm) were excised from apical meristem and individually transferred to different basic media (MS and ½MS) PGR-free medium or in various levels (0.3, 0.6 and 0.9 mg L^{-1}) of NAA or IBA. The root induction rate, number of roots, and root lengths were determined 6 weeks after culture. Each treatment consisted of 6 replications and two explants in each culture vials. Rooted plantlets were transplanted to pots containing a mixture of sterilized soil, peat, and perlite (1:1:1).

Statistical Data Analysis

Analysis of variance was performed using a completely randomized block design and the comparison of means was performed by LSD test and JMP8 statistical program.

RESULTS AND DISCUSSION

Preliminary experiments on percent seed germination revealed that there was a

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significant difference between treatments consisting of scarification (mechanical and chemical), stratification and GA_3. The H_2SO_4 (98%v/v) treatment considerably softened hard seed coat of *A. adscendens* and considerably increased germination rate (data not shown). Recent studies stated that there were some successful treatments in seed scarification such as using sulfuric acid in *A. cicer* (Basalma *et al*., 2008) and excising of seed coats in *A. polemoniacus* (Mirici, 2004) and in *A. cariensis* Boiss (Erisen *et al*., 2009). Therefore, the results showed hard seed coat in *Astragalus* genus. The leguminous species seeds usually have a physical and mechanical dormancy. This different reaction clearly indicates hard seed coat in *Astragalus* genus (Rolston, 1978).

Direct Regeneration

Cotyledon explants on MS basal medium remained non-responsive and became gradually brown in all treatments (Figure 2-a). Similar results have been reported by Vidoz (2006) for *Lotononis bainesii (Fabaceae)*. The purpose of the experiment was direct shoot regeneration in explants, but most of the hypocotyl explants were enlarged and formed callus within 3-5 weeks after culture initiation. Therefore, we evaluated the callus production. Significant variations in frequency of callus induction and size were obtained depending on the cytokinin types and NAA concentration in culture media ($P< 0.05$). The callus induction frequency was the highest in the medium containing 1 mg L^{-1} BAP (91.6%) and the least (0%) was observed in the PGRs free medium (Figure 3). Medium containing 0.5 mg L^{-1} NAA combined with different concentrations of KIN and ZEA worked better in callus induction than the medium containing only cytokinin (Figure 3).

The effect of various PGRs on callus size

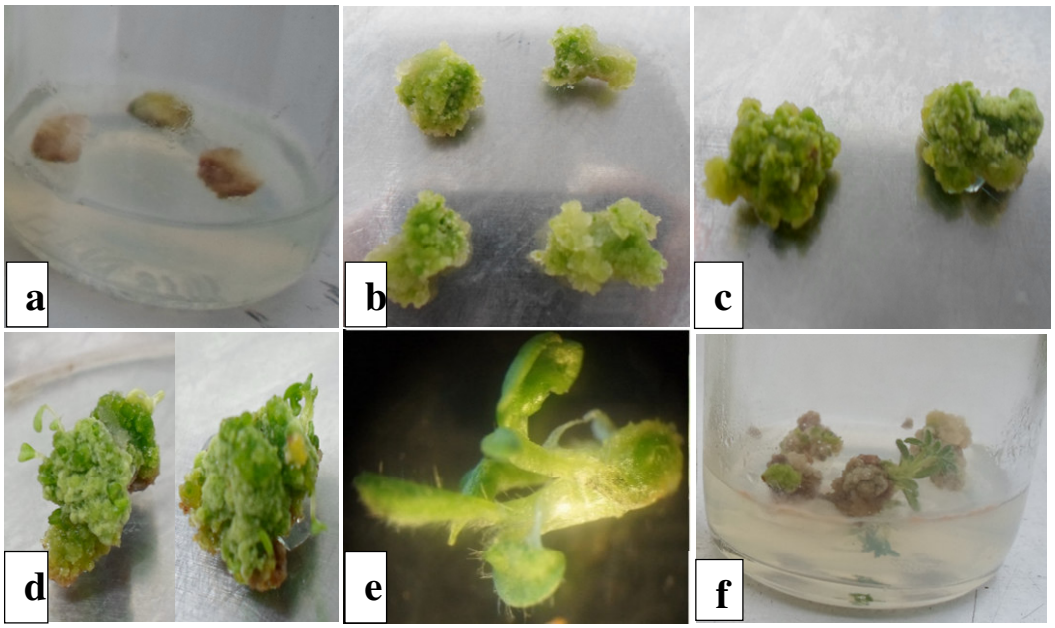

Figure 2. (a) Browning of cotyledon explants on MS medium; (b) Appearance of calli on medium supplemented with KIN and ZEA at different concentrations; (c) Appearance of calli on medium supplemented with BAP; (d) and (e). Shoot initiation on hypocotyl derived callusm after 6 weeks of culture on MS medium containing 4mgl/l BAP + 0.5 mg/l NAA and (f) Callus browning after sub-culturing onto the fresh culture medium. MS: Murashige and Skoog medium; NAA: α-NaphthaleneAcetic Acid; BAP: BenzylAminoPurine; ZEA: Zeatin, and KIN: Kinetin.

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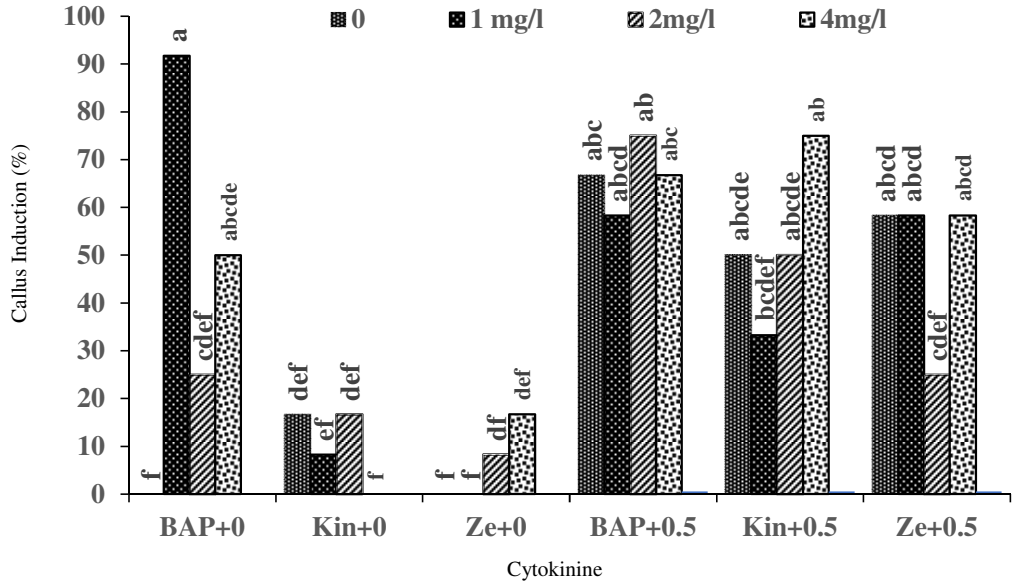

Figure 3. Interaction between cytokinin types (BAP, KIN, ZEA) and concentration of cytokinin (0.5, 1, 2 and 4 mg L^{-1}) and NAA (0 and 0.5 mg L^{-1}) on callus induction (%)**.** NAA: α-NaphthaleneAcetic Acid; BAP: BenzylAminoPurine; ZEA: Zeatin, and KIN: Kinetin.

has also been investigated. Four types of callus size were obtained: 0: No callus induction; 1: Small calli (with 0.5-1 mm diameter); 2: Medium calli (with 1-1.5 mm diameter), and 3: Long calli (with more than 1.5 mm diameter). Maximum calli size was obtained from a medium containing 2 mg L^{-1} BAP and 0.5 mg L^{-1}NAA, followed by the medium containing 4 mg L^{-1} ZEA and 0.5 mg L^{-1} NAA.

According to color and texture, there were two types of callus (I and II) that could be recognized after 6 weeks. Callus I was soft and yellow-brown, with a smooth texture without shoot induction (Figure 2-b). The type was obtained from MS medium containing KIN and ZEA in different concentration. All the calli turned into brown and were dead. Callus II was compact and green (Figure 2-c). The type was obtained from a medium containing BAP, especially in high concentrations. Some of the calli showed differentiation and produced shoot primordia on the surface (Figures 2-d and -e). But most of them were brown and died after sub-culturing in the fresh culture medium.

Erisen *et al*. (2009) reported the best callus induction rate with 100% frequency in MS medium containing 4 mg L^{-1} BAP and 0.5 mg L^{-1} NAA from leaf of *A. cariensis*. Similarly, MS medium containing 2 or 4 mg L^{-1} BAP and 0.5 mg L^{-1} NAA was the best medium for callus induction and shoot regeneration in the petiole of *A. nezaketae* (Erisen *et al*., 2010). Vidoz (2006) reported the formation of friable, light brown callus that died after sub-culturing into the fresh culture medium from leaf explant of *Trifolium polymorphum* (*Fabaceae*). Also, previous reports indicate that *Astragalus* species can be induced to regenerate by various combinations of NAA and BAP depending on the species and explants sources.

In the present study, callus formation occurred in some of the cultures, but could not regenerate, and most of them turned brown (Figure 2-f). Previous studies reported the problem in *A. schizopterus* (Yorgancilar and Erisen, 2011) and *A. cicer* (Basalma *et al*., 2008). Other researchers also reported that both types of callus and the medium played an important role in

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inducing of shoot formation in *Astragalus* spp (Luo and Jia, 1998a, b; Luo *et al*., 1999; Hou and Jia, 2004).

Micropropagation

Efficient proliferation was developed through apical meristem. Results of the experiment showed that there were significant differences in shoots number, shoots length, and leaf number in various treatments (P< 0.01) (Table 1). The highest number of shoots and leaves (8.5 shoot/explant and 22.8 leaf/shoot) was obtained from the medium containing 4 mg L^{-1} BAP; while the results for the media containing KIN in different concentrations had the lowest shoot proliferation (2 shoot/explant). The lowest leaf number (7 leaf/shoot) was achieved in media supplemented with 1 mg L^{-1} ZEA and 0.5 mg L^{-1} NAA.

Although the numbers of shoots were found the highest in the BAP-containing media, the reduction of shoot length was observed as a negative effect of BAP on shoots regeneration. Note that there was no proliferation in the medium without auxin and cytokinin.

After 4 weeks of callus culture, primordia initiation became apparent under the shoots (Figure 4-b). By sub-cultures, the number of shoots had increased (30 shoot/explant) and the callus became dark. Similar observations were reported by Sharma *et al*. (1993) in *Gentiana kurroo* (*Gentianaceae*) and by Rout *et al*. (1999) in *Plumbago zeylanica* (*Plumbaginaceae*). High concentrations of cytokinin had a negative effect on shoot formation and caused callus formation (Hasançebi *et al*., 2011). Combination of NAA with different cytokinins had also a negative effect on shoot multiplication and leaf number. It also increased the formation of callus. Shoot length was affected by cytokinin concentration as the raise in shoot length observed along with the increase in concentration of cytokinin in media. Rout (2005) reported the enhanced shoot multiplication by combination of BAP and NAA in *Clitoria ternatea* linn (*Fabaceae*).

Yorgancilar and Erisen (2011) suggested MS medium containing 1 mg L^{-1} BAP for micropropagation of *A. schizopterus*. Hasançebi *et al*. (2011) also reported that the

Table 1. Effects of different plant growth regulators on shoot proliferation of *Astragalus adscendens.*[a]

PGR	PGR-Con (mg L^{-1})	SHN	SHL (cm)	LN	PGR	Con (mg L^{-1})	SHN	SHL (cm)	LN
BAP	0.5	5.8^{b}	1.5^{e-g}	13.6^{c-f}	BAP + 0.5 mg L^{-1} NAA	0.5	3.0^{d-e}	2.8^{a-b}	12.5^{c-g}
	1	5.0^{b-c}	2.1^{b-e}	14.3^{b-e}		1	3.0^{d-e}	1.3^{g}	12.3^{d-g}
	2	5.5^{b}	2.1^{b-e}	18.0^{b}		2	3.5^{c-e}	1.5^{e-g}	14.8^{b-d}
	4	8.5^{a}	1.8^{d-g}	22.8^{a}		4	$4.3^{b.d}$	1.4^{f-g}	16.5^{b-c}
KIN	0.5	2.3^{e}	2.0^{c-f}	11.0^{d-h}	KIN + 0.5 mg L^{-1} NAA	0.5	2.0^{e}	2.6^{a-c}	9.4^{g-h}
	1	2.0^{e}	2.0^{c-f}	9.5^{f-h}		1	2.0^{e}	2.9^{a}	7.8^{h}
	2	2.0^{e}	2.3^{a-d}	10.4^{e-h}		2	2.0^{e}	2.9^{a}	10.5^{e-h}
	4	2.3^{e}	2.3^{a-d}	9.4^{g-h}		4	2.0^{e}	2.1^{b-e}	9.3^{g-h}
ZEA	0.5	2.3^{e}	2.1^{b-e}	10.0^{f-h}	ZEA + 0.5 mg L^{-1} NAA	0.5	2.0^{e}	2.4^{a-d}	10.1^{e-h}
	1	2.3^{e}	2.0^{c-f}	10.8^{d-h}		1	2.0^{e}	1.3^{g}	7.0^{h}
	2	2.5^{e}	2.5^{a-c}	9.5^{f-h}		2	2.3^{e}	1.5^{e-g}	10^{f-h}
	4	2.8^{d-e}	2.1^{b-e}	12.4^{c-g}		4	2.3^{e}	1.4^{f-g}	8.6^{g-h}

[a] Means followed by the same letters are not significantly different by the LSD test (P< 0.05). "a-d" means group abcd, with similar meaning for other letters separated by dash. PGRs-Con: Plant Growth Regulation Concentrations; SHN: Shoot Number; SHL: Shoots Length; LN: Leaves Number; NAA: α-NaphthaleneAcetic Acid; BAP: BenzylAminoPurine; ZEA: Zeatin, and KIN: Kinetin.

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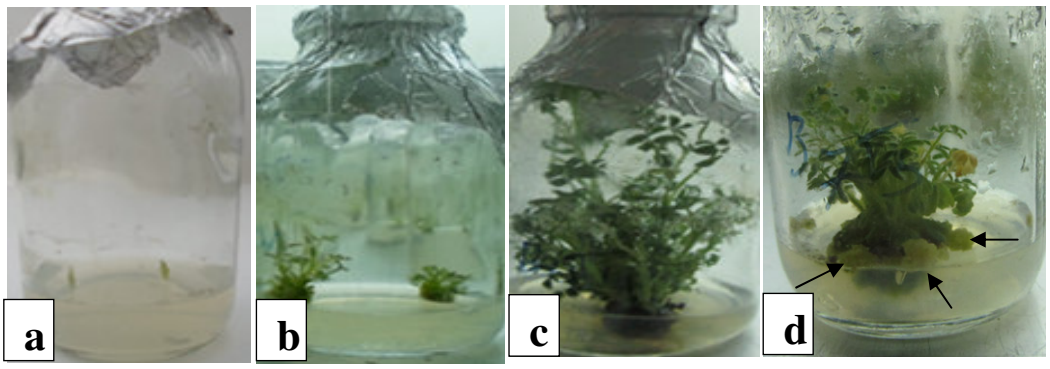

Figure 4. Different stages of proliferation (a, b and c). Induction of an apical meristem after one week, 2, and 4 weeks on MS medium containing 4 mg L^{-1} BAP; d. Initiation of callus was apparent under the shoot. BAP: BenzylAminoPurine . Initiation of callus showed with Arrows.

shoots formation was strongly induced in a medium containing 0.5 mg L^{-1} ZEA. Puspashree and Shiba (2012) mentioned that BAP and KIN were most effective on micropropagation of *Desmodium gangeticum* (*Fabaceae*) when used alone at 0.5 mg L^{-1} concentration. Many researchers reported that high concentrations of BAP and KIN could form small mass of callus and decrease shoots length (Rout, 2005; Puspashree and Shiba, 2012).

Rooting and Acclimatization

The proliferated shoots (after 8 weeks) were transferred into rooting medium (MS and ½ MS with 0, 0.3, 0.6, and 0.9 mg L^{-1} NAA or IBA). Rooting of shoots revealed significant differences in root induction percent in culture media ($P< 0.05$) depending on the medium types and concentrations of PGRs. The highest root induction (100%) was obtained from MS media supplemented with 0.3 mg L^{-1} NAA, but the highest root number (4/shoot), roots length (9.3 cm) and the best lateral rooting development was observed in ½ MS media containing 0.3 mg L^{-1} IBA.

After 6 weeks, none of the high concentration treatments (above 0.6 mg L^{-1}) induced rooting, but they formed callus in the end basal part of the shoots (Figure 5-b).

Astragalus chrysochlorus shoots were reported to root best in PGR-free medium

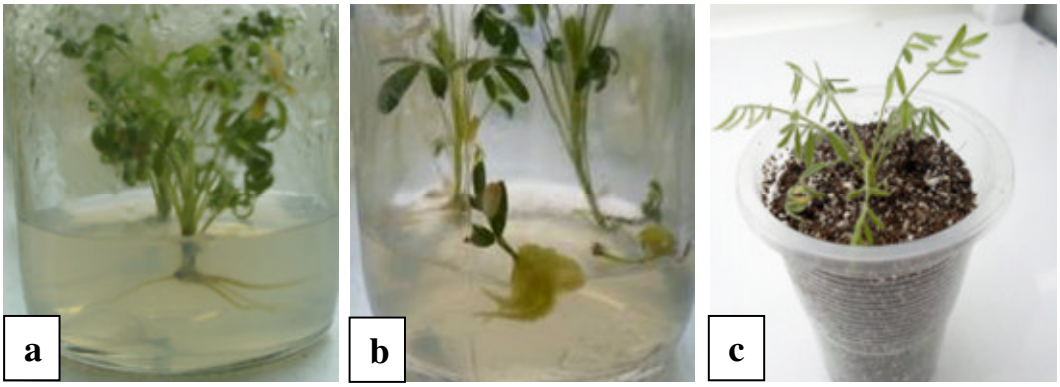

Figure 5. (a) Root development on regenerated shoots after 4 weeks on rooting medium; (b) Callus formation on a basal end of the shoots in high auxin concentration, (c) *In vitro* raised plantlet after a week of the transfer to a plastic pot containing 1:1:1 mixture of soil, peat, and perlite.

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(Hasançebi *et al*., 2011). Erisen *et al*. (2010) reported MS medium containing NAA stimulated root induction, while the MS medium with 0.5 mg L^{-1} IBA increased the mean number of roots per shoots and longer roots. Our results agreed with these researches.

In the current research, effects of IBA and NAA in 0.2, 0.3, 0.4 mg L^{-1} combinations were investigated on root induction. In addition, we examined the impact of $AgNO_3$ (0, 10 and 20 mg L^{-1}) and charcoal (0.5 mg L^{-1}) on the root formation. However, root induction was not observed (data has not shown).

Well-developed plantlets were transferred into the sterile soil by perlite and peat for acclimatization and covered with plastic bags to ensure high humidity around the plants (Figure 5-c). After 2 weeks, the plastic bags were removed and the plants were transferred to *ex-vitro* conditions.

In conclusions, in the study we reported a successful proliferation system for *Astragalus adscendens*. For future research, we recommend cytokinins in above concentration for proliferation and specially rooting of the plant. The results could be used in future studies on insects that have an important role is Gaz-angabin manna production at in situ conditions that need to be determined.

Abbreviations

NAA: α-NaphthaleneAcetic Acid, BAP: BenzylAminoPurine, KIN: Kinetin, ZEA: Zeatin, MS: Murashige and Skoog (1962) medium, PGRs: Plant Growth Regulators.

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ریزازدیادی *Astragalus adscendens*: یک تولیدکننده مان گزانگبین در ایران (مان ایرانی)

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چکیده

در این تحقیق، پرآوری *Astragalus adscendens* مورد بررسی قرار گرفت. ریزنمونه‌ها از هیپوکوتیل و کوتیلدون تهیه شدند و در محیط کشت پایه MS حاوی تنظیم کننده‌های رشد مختلف (NAA, BAP, KIN و ZEA) جهت باززایی شاخساره کشت شدند. غلظت‌های مختلف تنظیم کننده‌های رشد بصورت معنی‌داری القاء کالوس را تحت تاثیر قرار دادند. تولید کالوس در ریزنمونه‌های هیپوکوتیل مشاهده شد، اما هیچ گونه شاخساره ای از این کالوس‌ها القا نشد و اغلب کالوس‌ها قهوه ای شدند. بنابراین پرآوری سریع از طریق ریزنمونه جوانه انتهایی که از گیاهچه‌های ۱۵ روزه تهیه شده

بودند، انجام شد. جوانه های انتهایی در محیط کشت پایه MS حاوی غلظت‌های ۰/۵، ۱، ۲ و ۴ میلی‌گرم در لیتر ازKIN BAP , وZEA و تلفیق غلظت‌های فوق با ۰/۵ میلی‌گرم در لیتر NAA کشت شدند. بیشترین تعداد شاخساره به تعداد ۸/۵ شاخساره از هر ریزنمونه و برگ به تعداد ۲۲/۴ در هر ریزنمونه از محیط کشت MS حاوی ۴ میلی‌گرم در لیتر BAP بدست آمد. بیشترین مقدار القاء ریشه (۱۰۰٪) از محیط کشت MS حاوی ۰/۳ میلی‌گرم در لیتر NAA بدست آمد. گیاهچه‌های ریشه‌دار شده در محیط حاوی خاک، پیت و پرلیت به نسبت مساوی ۱:۱:۱ با موفقیت سازگار شدند.

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