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

Gh. Esmaeili¹, M. Azizi¹*, H. Aroei¹, and L. Samiei²

**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 H2SO4 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 23-25ºC 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.
**Micropropagation of Persian Manna**

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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**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.
There was a significant difference between treatments consisting of scarification (mechanical and chemical), stratification and GA₃. The H₂SO₄ (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 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).

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The effect of various PGRs on callus size.
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.

Figure 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
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

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<th>PGR</th>
<th>PGR-Con (mg L(^{-1}))</th>
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<th>SHL (cm)</th>
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<th>PGR Con (mg L(^{-1}))</th>
<th>SHN (cm)</th>
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\(^{a}\) 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.
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 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.

**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.
(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 adscurdens. 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**


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Astragalus adscendens: یک تولید کننده مان گزانتی در ایران (مان ایرانی)

چکیده

در این تحقیق، یک آزمایش برای بررسی رشد کشت و توسعه از MS هیپوکتول و کوتیدون تهیه شدند. در محیط کشت پایه (ZEA و NAA, BAP, KIN) ژنتیکی تغییرات شاخه‌کاری کشت شدند. غلظت‌های مختلف تغییراتی از کشت‌های شاخه‌کاری رشد مفرغ شدند. با توجه به نتایج، نتایج نشان دادند که کشت‌های مادری گونه‌شاخه‌کاری از این کالوسها تا به انتهای ۱۵ روز بهره‌برداری یافته‌اند.
بودن، انجام شد. جوانه‌های انها در محیط کشت پایه MS حاوی غلظت‌های 5/0 و 4/2 میلی‌گرم در لیتر از ZEA، KIN BAP و تلفیق غلظت‌های فوق یا 5/0 میلی‌گرم در لیتر A NAA کشت شدند. بیشترین تعداد شاخه‌های بکر را به تعداد 8/5 نشاختن از هر ریزیمیونه و بزگ به تعداد 2/4 در هر ریزیمیونه از محیط کشت کشت MS حاوی 4/2 میلی‌گرم در لیتر BAP بدست آمد. بیشترین مقدار افزایشی نسبی MS حاوی 3/0 میلی‌گرم در لیتر A NAA بدست آمد. گیاه‌های بهترین رشد در شده در محیط حاوی خاک، یتیم و پرلیت به نسبت مساوی 1:1:1 با موفقیت سازگار شدند.