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Micropropagation and root culture of Turkish endemic *Astragalus chrysochlorus* (Leguminosae)

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Abstract: Efficient micropropagation and root culture protocols were developed for the endemic *Astragalus chrysochlorus* Boiss. & Kotschy. A high frequency of shoot formation (100%) and maximum multiplication (13 shoots per hypocotyl explant) were achieved on Murashige and Skoog (MS) media supplemented with 0.5 mg/L trans-Zeatin riboside (ZR). By using single regenerated hypocotyl explants, rooting was achieved at a rate of 93% on MS medium containing 2% sucrose without growth regulators. High frequency callus initiation and growth were achieved when single hypocotyl explants were inoculated on MS medium supplemented with 0.5 mg/L 2,4-Dichlorophenoxyacetic acid. Plant regeneration through indirect organogenesis was achieved on MS medium supplemented with 0.5 mg/L ZR. Root cultures were successfully established in liquid MS medium containing 0.5 mg/L α-Naphthaleneacetic acid. The optimised in vitro propagation, callus culture, and root culture protocols offer the possibility to use cell/root culture techniques for vegetative propagation and secondary metabolism studies on *Astragalus* L. species.

Key words: *Astragalus*, endemic, regeneration, organogenesis, plant conservation

Türkiye endemiklerinden *Astragalus chrysochlorus* (Leguminosae) türünün mikroçoğaltımı ve kök kültürleri

Özet: Endemik bir tür olan *Astragalus chrysochlorus* Boiss. & Kotschy için etkin bir mikroçoğaltım ve kök kültürü protokolü geliştirilmiştir. En yüksek gövde gelişim frekansı (%100) ve maksimum gövde sayısı (13 gövde/hipokotil eksplantı), 0.5 mg/L trans-Zeatin riboside içeren Murashige ve Skoog (MS) besiyerinde sağlanmıştır. Geliştirilen gövdecikler %2 sukroz içeren MS besiyerinde %93 oranında köklendirilmiştir. Hipokotil explantından yüksek frekansta kallus oluşumunun indüklenmesi ve gelişimi 0.5 mg/L 2,4-Dichlorophenoxyacetic acid içeren MS besiyerinde elde edilmiştir. İndirekt organogenez yoluyla kallustan bitki rejenerasyonu 0.5 mg/L ZR içeren MS besiyerinde gerçekleştirilmiştir. Kök kültürü gelişimi ise 0.5 mg/L α-Naphthaleneacetic acid içeren sıvı MS besiyerinde elde edilmiştir. Bu çalışma ile geliştirilen ve optimize edilen in vitro mikroçoğaltım, kallus ve kök kültürü protokolleri, *Astragalus* L. türlerinin vejetatif çoğaltımına ve sekonder metabolit çalışmaları için hücre/kök kültürlerinin kullanımına olanak sağlayacaktır.

Anahtar sözcükler: *Astragalus*, endemik, rejenerasyon, organogenez, bitki koruma

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Introduction

Plants are a major source of products used in the speciality chemicals industry (pharmaceuticals, flavours, fragrances, and pesticides). Unfortunately, many plant species are extinct and also a great number of species are becoming irreversibly lost due to both the destruction of their habitats by increasing human population as well as over-collection for their commercial phytochemicals (Flores et al., 1999). Therefore, in many countries, various strategies have been developed to conserve their plant resources, especially rare and endangered species. Plant tissue culture is one of the most important conservation techniques because it offers great potential for rapid cloning from a minimum of plant material. In addition, it plays a key role in the production of plant material required for different purposes such as breeding, genetic and biotechnological research, and the acquisition of industrial raw material (Endress, 1994; Sahai et al., 2010).

Astragalus L. species are very old and well-known curative plants with immunostimulant, hepatoprotective, antiperspirant, diuretic, and tonic properties (Tang & Eisenbrand, 1992). It is the largest genus of the family Leguminosae, with more than 2000 species, and also the largest genus, with 450 species, of flora in Turkey. About 48% of the Turkish species (218) are endemic (Akan et al., 2008, Pınar et al., 2009). In Turkey, *Astragalus* spp. are used primarily for the production of the economically important gum tragacanth (Çalış & Sticher, 1996) and also for curative purposes (Bedir et al., 2001). *Astragalus chrysochlorus* Boiss. & Kotschy (2n = 16) is one of the rare Turkish endemic species and it is listed in the Turkish red list (Ekim et al., 2000). This species is traditionally used for wound healing and also a crude ethanol extract of the roots has shown antioxidant, enhancement effects on the phagocytic activity of lymphocytes (unpublished data) and cytotoxic activity (Karagöz et al., 2007). In addition, a new cycloartane-type triterpenoic glycoside was isolated from *A. chrysochlorus* (unpublished data), and this plant takes up selenium as a secondary accumulator (Arı et al., 2010).

Successful application of plant biotechnology for plant improvement requires the development of efficient in vitro regeneration systems. However, very few plant regeneration systems have been reported for *Astragalus* spp. to date. *Astragalus* plants have been regenerated from hairy roots (Cho et al., 1998), axillary buds (Turgut-Kara & Arı, 2006), hypocotyls and cotyledons (Basalma et al., 2008), calli and protoplasts (Luo & Jia, 1998), leaves and leaf petioles (Uranbey et al., 2003; Mirici, 2004; Erisen et al., 2010), and somatic embryos (Luo et al., 1999; Hou & Jia, 2004; Turgut-Kara & Arı, 2008).

The aim of this study is the establishment of efficient tissue culture systems in order to ensure species conservation and to produce plant material for secondary metabolite studies. We report a protocol for direct and indirect regeneration through organogenesis and the establishment of a root culture of endemic *A. chrysochlorus*. In practical terms, these results could provide for clonal propagation of this medicinally important plant species for further biotechnological applications.

Materials and methods

Plant material and seed germination

Astragalus chrysochlorus seeds were collected from Sertavul, Karaman, Turkey, and classified by Prof. Dr. Zeki Aytaç (Gazi University, Ankara, Turkey), and a voucher specimen was deposited in the herbarium at İstanbul University, Faculty of Science Herbarium (ISTF). Seeds were surface-sterilised in 70% EtOH for 1 min, then in 5% commercial bleach (Domestos) for 15 min, followed by 3 rinses for 15 min each with sterile distilled water. Seeds were placed in groups of 10 in petri dishes containing 25 mL of plant growth regulator-free MS (Murashige & Skoog, 1962) medium supplemented with 3% (w/v) sucrose and solidified with 0.8% agar (w/v). This medium was defined as our basal medium. The pH was adjusted to 5.8 before sterilisation by autoclaving at 121 °C for 20 min. Seeds were incubated in a growth chamber (Hereaus, Vötsch, No: 440/0026/86) and illuminated with fluorescent light (1400 $Em^{-2}s^{-1}$) in 16 h/day photoperiods at 25 ± 2 °C for germination. This growth chamber condition was used for the micropropagation and indirect organogenesis experiments discussed below.

Micropropagation

Three types of cytokinin [trans-Zeatin riboside (ZR), Kinetin (K), and 6-Benzylaminopurine (BA)], consisting of basal medium combined with 2 different auxins [α-Naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA)], were tested for shoot multiplication. In the first set of shoot multiplication experiments, 5 different concentrations (0.1, 0.5, 1.0, 1.5, and 2.0 mg/L) of each cytokinin were used. In the second set, a 0.5 mg/L concentration of each cytokinin was combined with 5 different concentrations (0.1, 0.3, 0.5, 0.7, and 1.0 mg/L) of auxins. The basal medium without growth regulators was used in control experiments.

Detached hypocotyls (~1 cm length) from 15-day-old germinated seedlings served as the explant source for shoot cultures. They were placed on the surface of the media described above and incubated in the growth chamber. Cultures were transferred to fresh media at 3 week intervals. For each treatment, 10 hypocotyls were used and each treatment was replicated 3 times. Shoot multiplication ratio and multiplied shoot number per hypocotyl were determined on the 40th day of culture. Multiplied shoots were used for rooting experiments.

Indirect organogenesis

Divided hypocotyl explants (~0.5 cm) from 15-day-old in vitro germinated seedlings were used for callus induction. In experiments, four different concentrations (0.5, 1.0, 1.5, and 2.0 mg/L) of 2,4-Dichlorophenoxyacetic acid (2,4-D) was tested for callus initiation. Ten explants were oriented in a horizontal position on the surface of the petri dish media, and they were incubated in the dark at 25 °C. Five replicates were used per treatment. Callus formation frequency and callus growth index were calculated as [(final callus fresh weight - initial callus fresh weight) / initial callus fresh weight] after 30 days of culture.

Actively growing, yellow, and friable calli were divided into small pieces on the 30th day of culture and callus material was cultured on a MS basal medium containing 0.5 mg/L ZR, and the remaining callus material was cultured on plant growth regulator-free MS basal medium for shoot formation. All cultures were incubated in the growth chamber. Subcultures were done at 3-week intervals. For each treatment, 10 calli were used and 5 replications were performed. On the 40th day of culture, the number of adventitious shoots per callus was recorded. Subsequently, regenerated shoots were separated from calli and used for rooting experiments.

Rooting of the propagated shoots

Micropropagated shoots (minimum length of 1 cm) and adventitious shoots which originated from calli were used for the rooting experiment. Three types of auxin [NAA, IBA, and Indole-3-acetic acid (IAA)] at 3 different concentrations (0.1, 0.5, and 1.0 mg/L) and 3 sucrose concentrations (1%, 2%, and 3%) on plant growth regulator-free MS media were tested for their effects on rooting. The cultures were incubated in the growth chamber. For each rooting experiment, 10 micropropagated and adventitious shoots were used and each treatment was replicated 3 times. Data on root-formed shoot percentage, root number per shoot, and mean root length were recorded on the 40th day of culture.

Acclimatisation

For acclimatisation, 75 healthy plantlets which had well-developed root and shoot systems were chosen. They were removed from the culture media, washed in water to remove agar, and then transferred to plastic pots containing sterile soil with 15% perlite. The transplanted plants were covered with clear plastic bags to maintain humidity, placed in an acclimatisation chamber, and watered with quarter-strength MS mineral salt solution at 4 day intervals. After 2 weeks, the plastic bags were opened and the uncovered plants were maintained under natural daylight conditions at 19-23 °C in the laboratory. The survival frequency of the in vitro propagated plants was evaluated at the end of the 10th week of the acclimatisation process.

Establishment of root cultures

Root tips (1-1.5 cm) excised from 30-day-old in vitro germinated seedlings and cultured on MS basal medium were used for root culture studies. Root tips were inoculated in 50-mL Erlenmeyer flasks containing 15 mL of MS liquid basal medium with 0.5 or 1.0 mg/L of NAA, IBA, or IAA. The cultures were maintained on a rotary shaker at 120 rpm in the dark, and root culture fresh weight was measured at

15, 30, 45, and 60 days. There were 10 replicates per treatment.

Statistical analysis

Data were analysed by one-way analysis of variance. The statistical significance of differences between means was estimated at the 5% level by the Tukey HSD test.

Results and discussion

Shoot multiplication

Data shown in Table 1 indicate that the shoot formation was strongly induced in 0.5 mg/L ZR concentration. The percentage of proliferation was 100% and shoot number per explant was 13 without callus growth (Figure 1). Higher than 1 mg/L ZR concentration did not have any effect on propagation, but 1.5 mg/L concentration of ZR caused callus formation. Shoot induction was also observed at 0.5 and 1.0 mg/L concentrations of K and BA, but the induction ratio was very low (7%-36%, respectively) and accompanied by high callus formation. High concentrations (2.0 mg/L) of all cytokinins had negative effects on shoot formation; moreover, they inhibited hypocotyl survival.

For shoot propagation, all tested cytokinin/auxin combinations (0.5 mg/L ZR, K, and BA with 0.1-1.0 mg/L NAA and IBA) were found not to be as effective as 0.5 mg/L ZR alone and excessive callus growth

Table 1. Effects of plant growth regulators (PGRs) on shoot multiplication.

PGR	**Con (mg/L)**	**SP* (%)**	**SN / Hyp**	**CF**	**PGR**	**Con (mg/L)**	**SP* (%)**	**SN / Hyp.**	**CF**	**PGR**	**Con (mg/L)**	**SP* (%)**	**SN / Hyp.**	**CF**
ZR	0.1	42c	4	-	**0.5 (mg/L) ZR + NAA**	0.1	-	-	-	**0.5 (mg/L) ZR + IBA**	0.1	-	-	+
	0.5	100a	13	-		0.3	33e	3	+		0.3	-	-	+
	1.0	53b	8	+		0.5	29f	3	+		0.5	26g	4	+
	1.5	-	-	+		0.7	13i	2	+		0.7	-	-	+
	2.0	-	-	-		1.0	-	-	+		1.0	-	-	+
K	0.1	-	-	-	**0.5 (mg/L) K + NAA**	0.1	-	-	+	**0.5 (mg/L) K + IBA**	0.1	-	-	+
	0.5	11ij	2	+		0.3	11ij	2	+		0.3	11ij	2	+
	1.0	7k	3	+		0.5	13i	2	+		0.5	2 l	3	+
	1.5	-	-	+		0.7	-	-	+		0.7	-	-	+
	2.0	-	-	-		1.0	-	-	+		1.0	-	-	+
BA	0.1	-	-	-	**0.5 (mg/L) BA + NAA**	0.1	-	-	+	**0.5 (mg/L) BA + IBA**	0.1	-	-	+
	0.5	36d	3	+		0.3	-	-	+		0.3	36d	3	+
	1.0	22h	3	+		0.5	33e	4	+		0.5	23h	3	+
	1.5	-	-	+		0.7	-	-	+		0.7	13i	1	+
	2.0	-	-	-		1.0	-	-	+		1.0	-	-	+
Cont	-	-	-	-	**0**	-	0.1	-	-	**0**	-	-	-	-

*Means followed by same letters are not significantly different by the Tukey HSD test ($P < 0.05$)

Con = Concentration**, SP** = Shoot propagation, **SN/Hyp** = Shoot number per hypocotyl, **CF** = Callus formation, **Cont** = Control

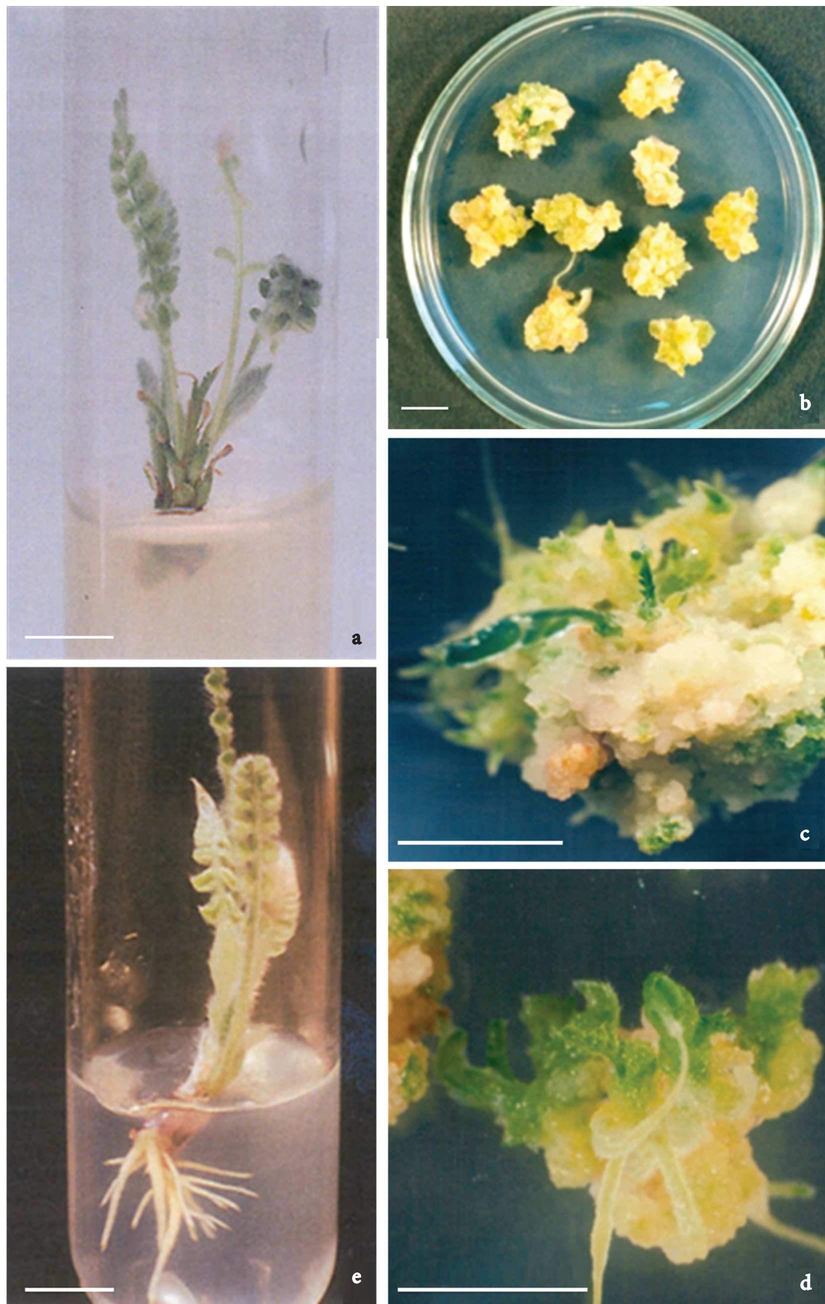

Figure 1. Micropropagation of *Astrgalus chrysochlorus* by direct and indirect organogenesis. a- Multiple shoot formation from a single node explant on MS medium with 0.5 mg/L ZR, b- Maximum callus induction (100%) on MS medium with 0.5 mg/L 2,4-D, c and d- Adventitious shoot regeneration from callus on MS medium with 0.5 mg/L ZR, e- Rooted shoots on plant growth regulator-free MS medium containing 2% sucrose. (Scale bars = 1 cm)

interfered with the propagation process. However, compared to the others, the combination of plant growth regulators 0.5 mg/L BA + 0.3 mg/L IBA gave better proliferation results (36% and 3 shoots per explant). There was no shoot propagation in the control medium.

In this work, it was observed that ZR was the most effective cytokinin on *Astragalus chrysochlorus* and a similar effect was recorded in *A. maximus* Willd (Turgut-Kara & Arı, 2006). However, BA is a commonly used cytokinin for in vitro culture of *Astragalus* species such as *A. adsurgens* Pall (Luo & Jia, 1998), *A. melilotoides* Pall (Hou & Jia, 2004), *A. aquilonius* Barneby, *A. amblytropis* Barneby, *A. columbianus* Barneby (Edson et al., 1994), and *A. cariensis* Boiss (Erisen et al., 2010). Many micropropagation studies produced similar results as ours, namely that different regeneration and propagation responses can be observed in different plants when the same growth regulators are used. It is clear that these differences originate from their genotypes.

Callus induction and shoot regeneration

The earliest sign of callus formation from cut ends of hypocotyl explants was visible within 3 days. Callus proliferation was fast and the whole explant was covered with calli 20 days after initiation. The frequency of hypocotyls which produced calli varied 30%-100% in all 2,4-D concentrations tested. The maximum callus formation was achieved in 100% of the explants cultured on MS medium with 0.5 mg/L 2,4-D in the dark (Figure 1). Moreover, 1.5 and 2.0 mg/L concentrations of 2,4-D were also effective for callus induction but less effective than 0.5 and 1.0 mg/L (Table 2). The mean callus fresh weight decreased with increasing 2,4-D concentrations. A 0.5 mg/L concentration of 2,4-D supported the highest callus fresh weight (growth index, 0.77 ± 0.02). Similarly, callus formation was achieved in 100% of cultures with 2,4-D in some *Phyllanthus* Webster species (Catapan et al., 2002), but, in contrast, the mean callus fresh weight was positively affected by increasing 2,4-D concentrations. However, in *A. melilotoides,* maximum callus formation (100%) from hypocotyl and stem explants resulted from 2 mg/L 2,4-D and 1 mg/L BA combinations (Hou & Jia, 2004).

Table 2. Effect of 2,4-D on callus induction (30th day).

2,4-D (mg/L)	*Callus induction (%)	Growth index
0	5 ± 0.57e	0.38 ± 0.07d
0.5	100 ± 0a	0.77 ± 0.02a
1.0	85 ± 1.05b	0.74 ± 0.06a
1.5	63 ± 2.5c	0.63 ± 0.07b
2.0	30 ± 1.52d	0.45 ± 0.12c

*Means followed by same letters are not significantly different by the Tukey HSD test ($P < 0.05$)

After 60 days of culture on 0.5 mg/L 2,4-D, proliferated, actively growing, yellow and friable calli were transferred to MS medium supplemented with 0.5 mg/L ZR and plant growth regulator-free MS medium to evaluate their potential for shoot formation. Within 10 days of transfer, small, dark-green patches emerged on the surface of the calli and 13 adventitious shoots regenerated per piece of callus (Figure 1) in the 0.5 ZR mg/L concentration MS medium. In addition, shoot formations were also observed in hormone-free MS medium but slower and fewer than in medium containing ZR (~4 shoots per piece of callus within 18 days).

Rooting

Micropropagated and adventitious shoots were used for rooting experiments. At the end of 6 weeks, none of the tested auxins (0.1-1.0 mg/L NAA, IBA, or IAA) induced rooting, but they strongly promoted callusing. However, root induction was strongly stimulated by plant growth regulator-free MS medium containing 2% sucrose (Figure 1). The rooting ratio of the multiplied shoots was 93% and the root number per explant was ~4. On the other hand, root formation was not observed in 3% sucrose plant growth regulator-free MS medium. Regenerated shoots, after being transferred to rooting medium lacking growth regulators, showed rapid root initiation. Similar results on the effect of plant growth regulator-free MS media on root induction were obtained for *A. maximus* (Turgut-Kara & Arı, 2006), *A. adsurgens* (Luo & Jia, 1998), *A. amblytropis, A. aquilonius* (Edson et al., 1994), and some *Phyllanthus* species (Catapan et al., 2002). However, it is known that high sucrose concentration (3%-4%) and auxins (especially NAA) have an inducing effect on rooting under tissue culture conditions (Werbruck & Debergh, 1994).

Root culture

Auxins (0.5 or 1.0 mg/L NAA, IAA and IBA) were tested for their effect on root induction in MS liquid medium containing 3% sucrose. Between

the 30th and 60th days, NAA and IAA strongly stimulated the root growth, and maximum root fresh weight was obtained in the 0.5 mg/L NAA culture after 60 days (Figure 2). Auxins tested in this study also affected the root morphology that is important for mass production. IAA at 0.5 and 1.0 mg/L concentrations supported thick and long roots but the number of newly branched lateral roots was very few. However, in NAA and IBA, plentiful short and thin roots regenerated from single cells in the culture and they had characteristic newly branched lateral roots. Similarly in another legume, *Pueraria lobata* Willd, induced roots with NAA were thick and short without branches (Theim, 2003).

Acclimatisation

Well-developed plantlets were transferred to sterile soil with perlite for acclimatisation and covered with plastic bags to ensure high humidity around the plants. After 2 weeks, the plastic bags were removed and the plants were transferred to ex vitro conditions. In the 10th week of acclimatisation, 77% of the plantlets survived and they showed normal growth.

Conclusion

Many rare and endemic plant species are propagated in vitro because they do not respond well to conventional methods of propagation. We report for the first time a procedure for the successful regeneration of *Astragalus chrysochlorus* plants through hypocotyls and callus explants. This is also a first report on root culture establishment for *A. chrysochlorus* and it is being used to assess the presence of the main biologically active compounds. The availability of protocols to establish callus and root culture systems will allow further studies on secondary metabolism aimed at increasing the production or the isolation of new compounds of interest.

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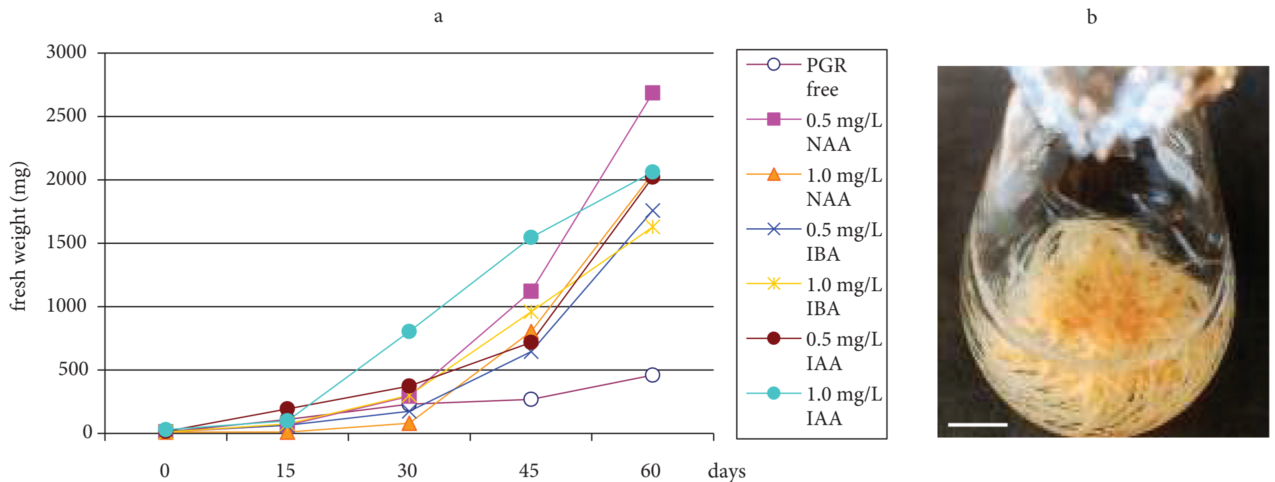

Figure 2. Establishment of the root culture in *Astragalus chrysochlorus*. a- Effects of different auxins and their different concentrations on cultured root development, b- Root culture in liquid MS medium with 0.5 mg/L NAA. (Scale bar = 1 cm)

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