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THE EFFECT OF THIDIAZURON (TDZ) ON SHOOT REGENERATION OF *ASTRAGALUS SCHIZOPTERUS*

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ABSTRACT

The effect of thidiazuron (TDZ) on shoot regeneration of the endemic *Astragalus schizopterus* was investigated in Turkish flora. Explants (leaf and petiole) were cultured on the basic medium Murashige and Skoog (MS) containing various plant growth regulators (PGRs) [0.0, 0.4, 0.5, 0.6 mg l^{-1} thidiazuron (TDZ); 0.4, 0.5, 0.6 mg l^{-1} TDZ x 0.1, 0.2 mg l^{-1} α-naphthaleneacetic acid (NAA)] to induce shoot bud induction. The combinations and concentrations of PGRs showed significant variations for the frequency of callus formation, appearance of callus and the potential of callus differentiation. Callus formation occurred in cultures, but it was not obtained in adventitious shoot regeneration. Therefore, rapid multiplication was made by using lateral meristem which were obtained from 4 weeks sterile seedling. Lateral meristems were cultured on basic media MS containing various PGRs 0.5, 1.0 mg l^{-1} zeatin (ZEA), benzyladenine (BA) and 0.22, 0.5 and 1 mg l^{-1} TDZ. The highest number of shoots (23.6/explants) was obtained on MS medium containing 1 mg l^{-1} TDZ followed by MS medium cointaining 1 mg l^{-1} BA which produced 15.80 shoots per explant. It was observed that shoots didn't have healthy development in all media containing TDZ. So it was suggested that BA is a useful PGR for rapid multiplication in *A. schizopterus*. The regenerated shoots were transferred to rooting medium [MS with 0.5-1 mg l^{-1} NAA and 0.5-1 mg l^{-1} indole-3-butyric acid (IBA)] where they successfully rooted (100%) and showed rapid elongation. Rooted shoots were transferred to the *ex vitro* and produced normal plants.

Key words: *Astragalus,* endemic, lateral meristem, micropropagation, callus induction.

INTRODUCTION

Astragalus species are important and are used in many different way and areas in medicine because of their secondary metabolites (Tang, 1992; Karagöz *et al*., 2007). Some species are important for animal nutrition. Deep top root system makes these species can be used for erosion control (Erisen *et al*., 2010a) and establishing the pasture in the non-irrigated areas such as Karapınar (Acar *et al*., 2011). At the same time the genus *Astragalus* contains annual or perennial species. Some of them can resist to abiotic stress such as cold, salinity and drought etc. and can grow in a wide range of conditions. However, slow seedling development, poor seed germination capacity and low number of seed set are drawbacks affecting their wider adaptation capabilities requiring the application of tissue culture techniques for their propagation.

There are studies indicating the obtaining of adventitious shoot regeneration via organogenesis and embryogenesis in species such as *Astragalus adsurgens* (Luo and Jia, 1998ab), *Astragalus cicer* (Uranbey *et al*., 2003; Başalma *et al*., 2008), *Astragalus melilotoides* (Hou and Jia, 2004), *Astragalus polemoniacus* (Mirici, 2004), *Astragalus chrysochlorus* (Turgut-Kara and Arı, 2008), *Astragalus cariensis* (Erisen *et al*., 2010a), *Astragalus nezaketae* (Erisen *et al*., 2010b). However, there are a small number of research *in vitro* multiplication of this genus such as *Astaragalus duranii* (Çöçü *et al*., 2005), *Astragalus maximus* (Turgut-Kara and Arı, 2006) and *Astragalus nitidiflorus* (Cano-Castillo *et al*., 2009).

Thidiazuron (TDZ), is one of the cytokinin like substances, has often been used in the shoot regeneration system in recent years. However when TDZ was using for shoot regeneration it can lead to negative effects viz. hyperhydricity and poor shoot devolopment (Huetteman and Preece, 1993).

Astragalus schizopterus L. that belongs to Proselius section is an endemic species and cluster-shaped, woody at the base, creeping users, consists of perennial herbaceous plants (Davis *et al*., 1988). This plant has the potential to become fodder for animal feed.

There has not been any report on tissue culture of *A. schizopterus.* This studies overall goals were to investigate the effect of TDZ on shoot regeneration and to develop a propagation procedure for the multiplication of *A. schizopterus.* In this paper, report the original results of investigations to establish micropropagation of *A. schizopterus* is reported.

MATERIALS AND METHODS

A. schizopterus seeds collected from a wild population (Burdur: Dirmil-Gölhisar, 7. km, 1175 m,

25.07.2006) in Turkey were classified by Dr. Ahmet Duran and Dr. Muhittin Dinç (Selçuk University, Ahmet Keleşoğlu Education Faculty, Department of Biology Education, Turkey).

Seed germination and explant preparation: The seeds were surface sterilized in 10% (v/v) sodium hypochloride solution (NaOCl) for 10 min, then rinsed 3 times with sterile distilled water. After sterilization seeds were excised with sterile scalpel in order to increase germination. Excised seeds were placed on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) solidified with 0.8% (w/v) agar. Explants were excised from 30-days old seedlings.

Callus induction: Leaf and petiole explants were cultured on basal media MS containing thidiazuron (TDZ) (0.0, 0.4, 0.5, 0.6 mg l^{-1}) or with 0.1, 0.2 mg l^{-1}α-naphthaleneacetic acid (NAA) to induce shoot bud induction. The frequency of explant producing calli and callus fresh weights (g) were determined 6 weeks after culture.

Micropropogation: Lateral meristem explants cultured on MS, containing 3% (w/v) sucrose and 0.8 % (w/v) agar, and different concentration of 6-benzylaminopurine (BA), Zeatin (ZEA) (0.5, 1.0 mg/l) and TDZ (0.22, 0.5, 1.0 mg/l) contained in Magenta vessels for propagation of *A. schizopterus*. The shoot numbers per explant were determinated after 4 weeks. The medium was adjusted to pH 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving at 121°C, 1.4 kg/cm^2 for 20 min. Cultures were incubated in a growth chamber (SANYO: MLR-351H) illuminated with fluorescent light (5LS) for 16 hours per day at 24 ± 2°C.

Rooting and acclimatization: The propagated shoots (2-3 cm) were transferred onto rooting mediun including MS with various levels (0.5, 1.0) of NAA or IBA. After 4 weeks, the percentage of rooted shoots were determined and well rooted shoots were transplanted to pots containing a mixture of sterilized peat and perlite (1:1) under greenhouse conditions (Totally 60 plants were transferred). Five explants were cultured in each culture vessel and each treatment was repeated three times. Analysis of variance was performed with using a two factor completely randomised block design and the differences between the means were compared by LSD test using the Mstat-C statistical program (MStat-C, Version 3 Michigan State University, USA) (Freed *et al*., 1989).

RESULTS AND DISCUSSION

Callus induction: Without any PGR in culture media, neither leaf nor petiole explants genarated callus (Table 1).

TDZ and TDZ x NAA interaction was significant on callus fresh weights and frequency of callus induction and there was a significant interaction between explant and media ($p<0.01$). Explant types were not significant for callus fresh weights and frequency of callus induction. Leaf and petiole explants produced green dense globular calli including brown area after 6 weeks (Fig. 1 a-b).

Table 1 Effects of various TDZ and NAA combinations on callus induction of leaf and leaf petiole explants of *A. schizopterus*

Growth regulators(mg/l)		Frequency of callus induction* (%)			Callus fresh weight (g)		
TDZ	**NAA**	**Leaf**	**Petiole**	**[1]Mean**	**Leaf**	**Petiole**	**[1]Mean**
0.0	-	0.00 b	0.00 b	0.00 c	0.00 g	0.00 g	0.00 e
0.4	-	100.00 a	13.33 b	56.67 b	0.07 fg	0.00 g	0.04 e
0.5	-	73.33 a	100.00 a	86.67 a	0.15 fg	0.05 g	0.10 e
0.6	-	33.33 b	33.33 b	33.33 b	0.02 g	0.05 g	0.03 e
0.4	0.1	100.00 a	100.00 a	100.00 a	0.51 de	0.36 ef	0.44 d
0.5	0.1	100.00 a	100.00 a	100.00 a	0.49 de	0.75 cd	0.62 cd
0.6	0.1	100.00 a	100.00 a	100.00 a	0.56 de	1.48 a	1.02 a
0.4	0.2	100.00 a	100.00 a	100.00 a	0.68 cd	1.10 b	0.89 ab
0.5	0.2	100.00 a	100.00 a	100.00 a	0.92 bc	1.10 b	1.01 a
0.6	0.2	100.00 a	100.00 a	100.00 a	1.19 ab	0.28 efg	0.74 bc
[2]Mean		80.67	74.67		0.46	0.52	
Frequency of callus induction		$LSD_{0.01}$: 39.91		[1]$LSD_{0.01}$: 28.22			
Callus fresh weight		$LSD_{0.01}$: 0.30		[1]$LSD_{0.01}$: 0.21			

*Responses of leaf and petiole were evaluated together, Numbers in a column with the same letters were not significantly different

Mostly TDZ, TDZ x NAA combinations, with slide responsed to other plant growth regulator combinations. Shoot regeneration did not occure when the calli were cultured in MS medium without PGR at the end of 6 weeks. As seen in Table 1, the callus induction frequencies were 100% and 33-100% for the media

containing NAAxTDZ and TDZ, respectively. When leaf (80.67%) and petiole (74.67%) explants were considered, similar results were observed. When medium and explant interaction observed, the highest callus induction frequencies on media with 0.4, 0.5, 0.6 mg/l TDZ x 0.1, 0.2 mg/l NAA (100 %) from petiole and leaf explants, the lowest callus induction frequencies on medium with 0.4 mg/l TDZ (13.33%) from petiole explant was obtained.

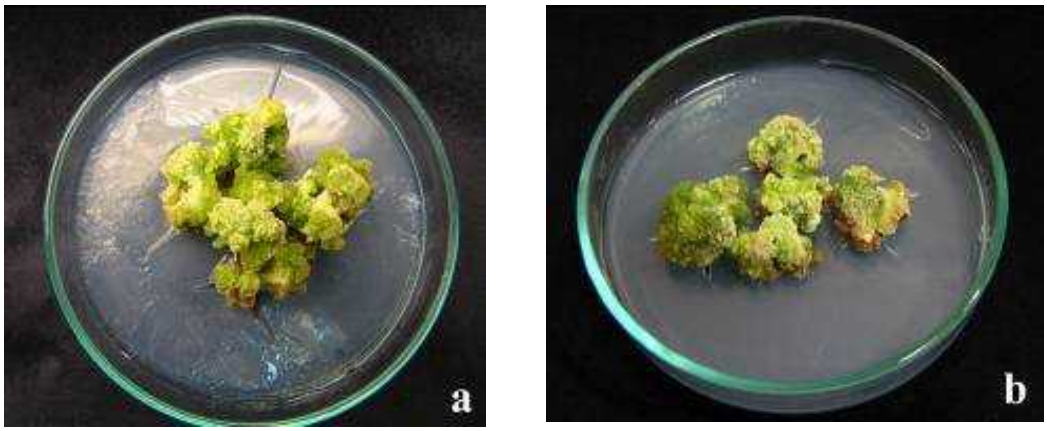

Figure 1. Callus formation from *A. schizopterus*
(a) Development of callus formation on petiole explants on medium supplemented with 0.6 mg/l TDZ and 0.1 mg/l NAA after 6 w of culture.
(b) Callus formation on leaf explants on medium supplemented with 0.5 mg/l TDZ and 0.2 mg/l NAA (petri dishes= 9 cm).

Mirici (2004) reported that the best result callus with 100 % frequency was obtained on MS containing 0.2 mg/l TDZ and 0.1 mg/l NAA from leaf and petiole of *A. polemoniacus*. Başalma *et al*. (2008) reported in *A. cicer* that callus frequency induction was 100 % on media containing 0.25-1 mg/l TDZ from hypocotyl explants, whereas the best result (50 %) from cotyledon explants were obtained on media containing 1 mg/l TDZ. Erisen *et al*. (2010b) reported that the highest frequency of callus induction (100 %) were obtained on media containing 0.2, 0.4 mg/l TDZ and 0.2 mg/l NAA from leaf and petiole of *A. nezaketae* and these results similar to our findings.

While among the media the highest callus fresh weight was obtained from 0.6 mg/l TDZ sup 0.1 mg/l NAA(1.02 g), the lowest callus fresh weight was obtained from 0.4 mg/l TDZ (0.00 g) indicating the importance of initial medium type cultured. When leaf (0.46 g) and petiole (0.52 g) explants were considered, similar results were observed.

The higest callus fresh weight was obtained on medium with 0.6 mg/l TDZ x 0.1mg/l NAA (1.48 g) from petiole explant, leaves had the highest callus fresh weigth on medium with 0.6 mg/l TDZ x 0.2 mg/l NAA (1.19 g). This result showed explant types and medium interaction is important in *A. schizopterus*. TDZ has been shown to induce callus formation in tissue culture studies of *Astragalus* ssp. But little information is available concerning the callus fresh weight induced by TDZ, however, the callus fresh weight from leaf and petiole explants was lower on medium with TDZ alone than adding NAA in *A. nezaketae* (Erisen *et al*., 2010b).

In this study, callus formation occurred in cultures, but it could not produce adventitious shoot regeneration. Although adventitious shoots regeneration have been obtained via organogenesis using TDZ in *Astragalus* ssp. such as *A. cicer* (Başalma *et al*., 2008) and *A. polemoniacus* (Mirici, 2004), in *A. nezaketae* was reported that petiole and leaf explants cultured on MS including TDZ showed no shoot regeneration (Erisen *et al*., 2010b). Similarly in *Tylophora indica*, leaf explants cultured on MS including low concentrations of TDZ (<0.55 mg/l) produced nonorganogenic and small calli (Sahai *et al*., 2010). In this study shoots regeneration can not be obtained. Other researchers, Luo and Jia (1998b); Luo *et al*. (1999); Hou and Jia (2004) also reported that both the type of callus and the medium played an important role in inducing shoot formation in *Astragalus* ssp.

Consequently, the highest callus induction frequencies were obtained on media with 0.4, 0.5, 0.6 mg/l TDZ x 0.1, 0.2 mg/l NAA (100 %) from petiole and leaf explants, the highest callus fresh weight was obtained on medium with 0.6 mg/l TDZ x 0.1mg/l NAA from petiole explant for *A. schizopterus*.

Micropropogation: According to rapid propagation applications, the lateral shoots were grown in whole media in 6-7 days from the beginning of the culture, then, the callus was apparent under the shoots. By this time, the number of the shoots had increased and the callus had become darker (Figure 2 e-f).

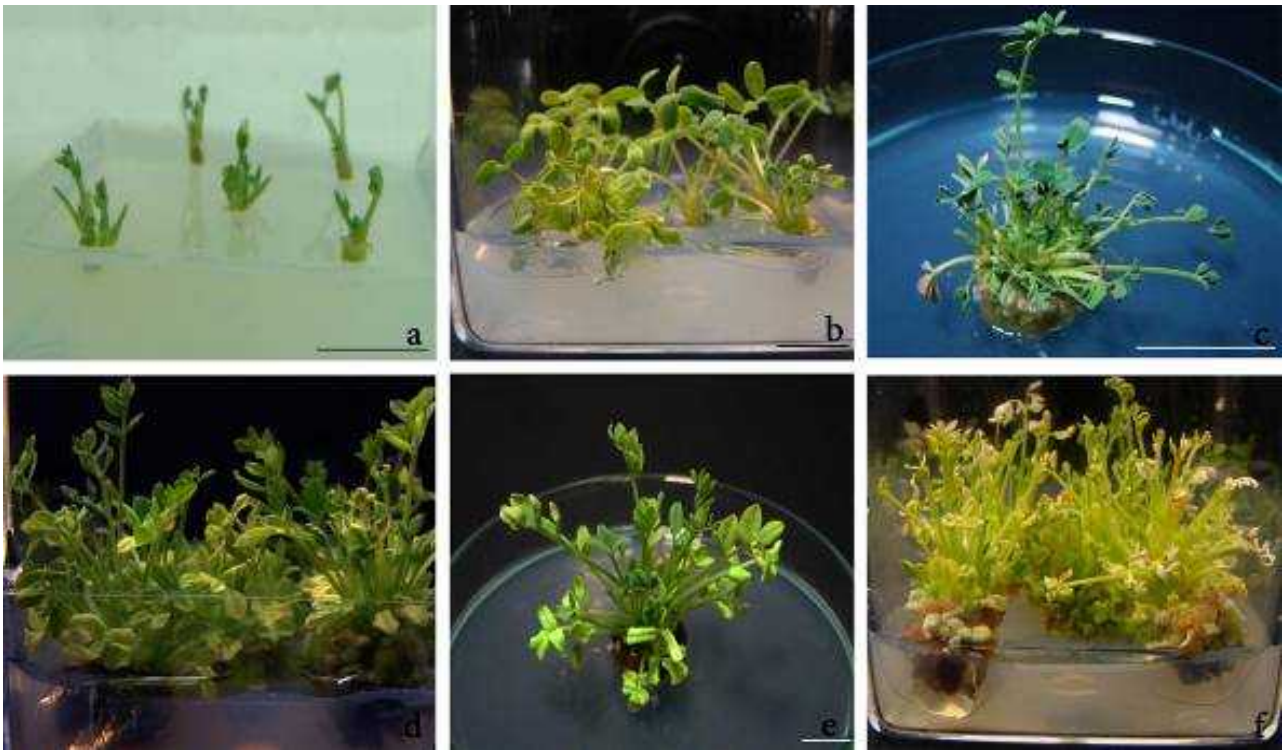

Figure 2. Micropropagation from lateral meristems explants of *A. schizopterus* (bar=1 cm)
(a) Induction of lateral shoots after a week from culture, **(b-c)** Developed shoots after 2-3 w, **(d-e)** Developing shoots after 4 w on medium supplemented with 1 mg/l BAP, **(f)** Developing shoots on medium supplemented with 1 mg/l TDZ.

The grown shoots were counted and taken to rooting media after the 4 weeks. The usage of different media had a significant effect ($p<0.01$) on the number of the shoots. While the highest value (23.60 shoots) for the shoot number per explants was obtained from the medium containing 1 mg/l TDZ, the results for the media containing ZEA were the lowest (2.4 and 3.13 shoots) (Table 2). Although the numbers of shoots were found higher in the media containing TDZ, the chlorosis of the leaves were observed as a negative effect of TDZ on shoots. Han and Park (2008) observed necrosis in *Philodendron cannifoliu,* when the shoots grown on MS media with TDZ. Kim *et al*. (1997) also reported that extended culture with TDZ resulted in abnormal leaf morphology, compact shoots and occasional necrosis of tissues in sweetgum which was similar to this study findings.

The highest shoot regeneration was obtained on medium containing 0.5 mg/l zeatin riboside (ZR) in *A. maximus willd.* (Turgut-Kara and Arı, 2006). Çöcü *et al.* (2005) reported that in *A. duranii* the maximum shoot formation was observed on media containing 0.2 mg/l TDZ. In plant *A. nitidiflorus*, the best proliferation was obtained with 0.1 mg/L BA (Cano-Castillo *et al.* 2009). These results indicated that cytokinins which produced the best results showed differences depending on species.

Table 2. The effects of different concentrations of TDZ, BA and ZEA on adventitious shoot regeneration from lateral meristem explants of *A. schizopterus*

Growth regulators (mg/l)	Mean number of shoots/explant
TDZ (0.22)	10.07 bc
TDZ (0.50)	15.60 ab
TDZ (1.00)	23.60 a
BA (0.50)	7.33 bc
BA (1.00)	15.80 ab
ZEA (0.50)	2.40 c
ZEA (1.00)	3.13 c

Numbers in a column with the same letters were not significantly different, $LSD_{0.01}$: 9.37

In this study, the best results for the shoot numbers were observed (15.60-23.60 shoots) for the TDZ containing media. The shoots were not healthy in the TDZ containing media. Because of that healthy shoots (15.80 shoots), for the micropropagation of *A. schizopterus,* medium containing 1 mg/l BA was offered.

Rooting and acclimatization: The regenerated shoots were transferred to rooting medium (MS with 0.5; 1 mg/l NAA or IBA) and rooted successfully (100%) with rapid elongation (Figure 3).

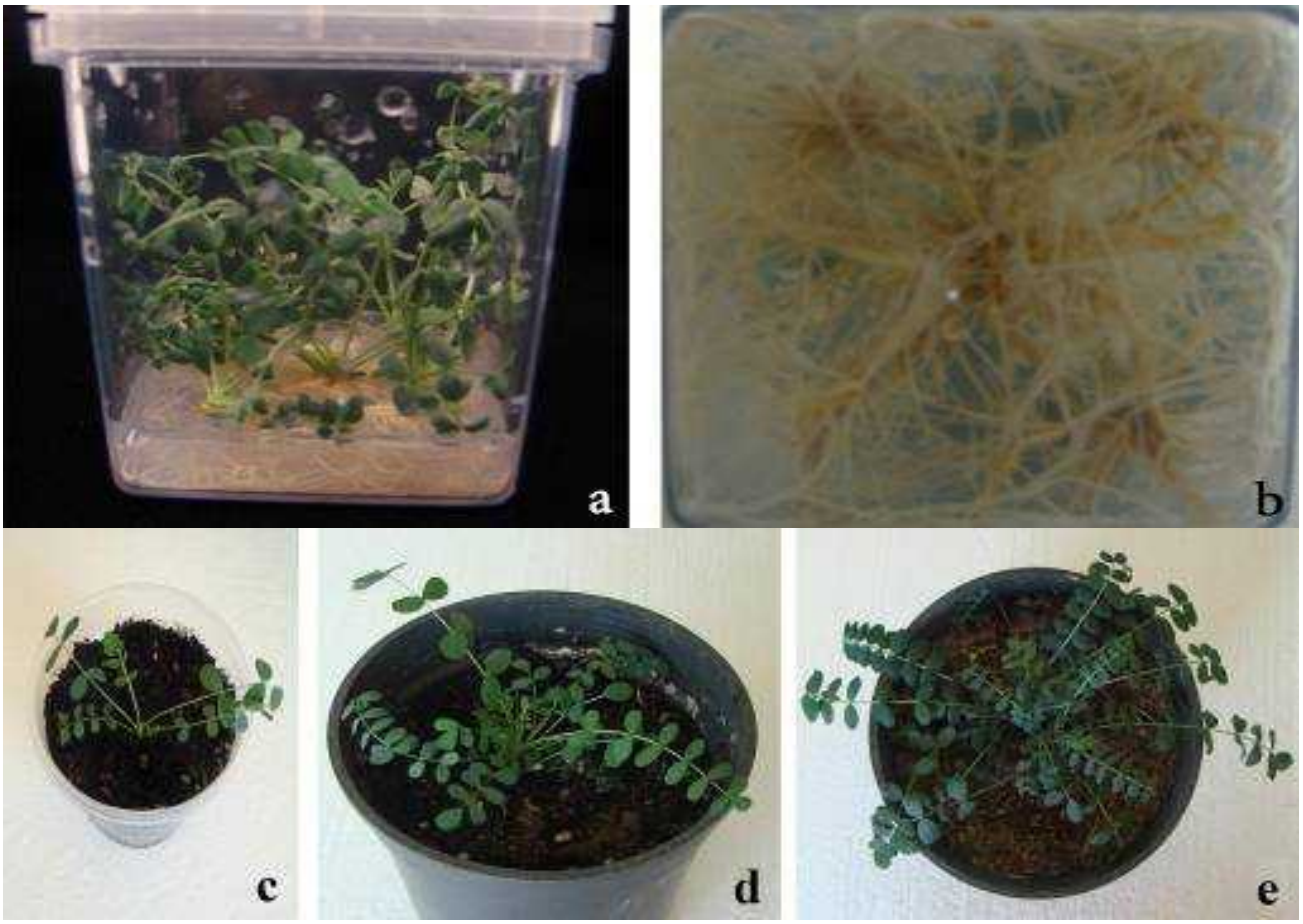

Figure 3. Rooting of regenerated shoots from *A. schizopterus* and acclimatization.
(a) Development of rooting formation on medium supplemented with 0.5 mg/l IBA after 4 w of culture, **(b)** Lateral root development on medium supplemented with 0.5 mg/l IBA of shoots, **(c-d-e)** Acclimatization and development of rooted shoots in *ex vitro* at *A. schizopterus* (at the end of 2, 5 and 8 w).

Rooting of shoots revealed significant variations ($p<0.05$) in number of root induction depending on the concentrations of PGR in culture media. The highest root number was obtained from media supplemented with 1 mg/l NAA (10.33) or 0.5 mg/l IBA (10.00) but the best lateral rooting development was observed in 0.5 mg/l IBA (Table 3).

Table 3. The number of roots in different rooting media of *A. schizopterus*

Rooting media (mg l^{-1})		The number of roots (paece)
NAA	IBA	
0.5	-	8.67 ab
1	-	10.33 a
-	0.5	10.00 a
-	1	8.00 b

Numbers in a column with the same letters were not significantly different, $LSD_{0.05}$: 1.80

All the plantlets of rooting were transferred to *ex vitro* conditions. The survival rate was 100% and tissue-culture derived plants were phenotypically normal (Figure 3).

In conclusions, this study presents the first report of micropropagation of endemic species *A. schizopterus*. Astragalus species are defined by a successful micropropagation system and applicable to other species of *Astragalus*.

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