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RESEARCH NOTE

Prolific shoot regeneration of *Astragalus cariensis* Boiss

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Abstract Prolific shoot regeneration via organogenesis was induced from leaf and leaf petiole explants of the endemic *Astragalus cariensis* species on Murashige and Skoog (MS) medium with α-naphthaleneacetic acid (NAA) and benzyladenine (BA) within 8 week. The highest number of shoots (23/explants) was obtained from leaf explants cultured on MS with 0.5 mg/l NAA and 4 mg/l BA. Elongated shoots were successfully rooted in MS medium with 0.5 mg/l indole-3-butyric acid. Rooted plantlets were acclimatized in pots containing 1:1 mixture of peat and perlite.

Keywords Astragalus · Endemic · Leaf · Organogenesis · Petiole

Abbreviations

BA	Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kin	Kinetin
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthaleneacetic acid
Zea	Zeatin

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Introduction

Astragalus L. (Leguminosae) is the richest genus of vascular plants in the world, represented by nearly 2,500 species (Maassoumi 1998). It is also the largest genus in Turkey where it is represented by approximately 455 taxa with 210 endemic species. *Astragalus* species are generally adapted to especially semiarid regions and steppe areas of Turkey with great importance in the supply of forage for grazing animals.

Astragalus species are used in traditional medicine as immunostimulants, hepatoprotectors, antiperspirants, diuretics etc., and for the treatment of nephritis, diabetes, leukemia, and cancer (Tang 1992; Karagöz et al. 2007; Martin et al. 2008a). Various species are used for gum production in medicine and textile industry. Some perennial *Astragalus* species are used for forage production. Forage quality of *Astragalus cicer* appears to be equal to that of alfalfa (Towsend 1970).

Astragalus species can tolerate adverse environmental conditions such as drought, cold and salt etc., and they can be used for erosion control because of their top root systems. Long growth periods, low seed germination rates and low percentage of seed set are drawbacks affecting their wider adaptation capabilities that may require development of regeneration systems in order to improve the genus through molecular techniques.

Hypocotils and protoplasts of *Astragalus adsurgens* (Lou and Jia 1998a, b), and *Astragalus melilotoides* (Hou and Jia 2004a, b), hypocotils and cotyledons of *Astragalus cicer* (Başalma et al. 2008) and suspension cells of *Astragalus chrysochlorus* (Turgut-Kara and Arı 2008) were reported to regenerate in vitro. However, only a few reports are available on plant regeneration from leaf and leaf petiole explants of *Astragalus* species (e.g. *A. cicer*, Uranbey et al. 2003; *A. polemoniacus*, Mirici 2004).

This study presents the first successful plant regeneration system using leaf and petiole explants of *A. cariensis* an endemic species of Turkey for which only some morphological (plant and chromosome number) data were reported up-to-date (Davis et al. 1988; Martin et al. 2008b).

Materials and methods

Seeds of *Astragalus cariensis* which were classified by Dr. Ahmet Duran (Selçuk University, Ahmet Keleşoğlu Education Faculty, Department of Biology Education, Turkey) were collected from a wild population (Kale Road, 1,360 m C2 Muğla) in Turkey. Surface sterilization of seeds was carried out with 20% (v/v) commercial bleach (HES, Turkey) for 10 min followed by three rinses with sterile distilled water. Some seeds were excised with a sterile scalpel prior to culture to increase germination rates. Control (unexcised) and excised seeds were then placed aseptically in Magenta vessels containing half-strenght Murashige and Skoog (MS) medium (Murashige and Skoog 1962) solidified with 0.8% agar (w/v) to obtain aseptic seedling as explant sources.

Seeds germinated after 4–5 days. Leaf and leaf petiole explants were removed from 30-day old in vitro germinated seedlings and were initially placed on MS, supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, and with various levels of α-naphthaleneacetic acid (NAA) (0.5 mg/l) × benzyladenine (BA) (1, 2, 4 mg/l), 2,4-dichlorophenoxyacetic acid (2,4-D) (2, 4, 8 mg/l) × kinetin (Kin) (0.5 mg/l), indole-3-acetic acid (IAA) (1, 2, 4 mg/l) × Zeatin (Zea) (0.1, 0.2, 0.5 mg/l) combinations contained in Petri plates for 6 weeks. Calli induced were transferred to growth regulator free MS medium 6 weeks after culture. These preliminary experiments showed that NAA × BA combinations resulted in better shoot regeneration response compared to other combinations tested (data not shown). Further callus induction and shoot regeneration optimization experiments were carried out by testing NAA (0.2–0.5 mg/l) and BA (1–4 mg/l) in combinations. Fresh weights (g) of calli were determined 6 weeks after culture followed by subculture on growth regulator free MS medium. Percentages of callus production and shoot number per callus were recorded 2 weeks after subculture. The pH of media was adjusted to 5.8 before autoclaving at 121°C for 20 min. All cultures were maintained at 24 ± 2°C in growth chamber (SANYO: MLR-351H, Japan) with fluorescent light (5LS) and 16 h light and 8 h dark photoperiod. All chemicals were provided from Sigma at analytical grade.

The regenerated shoots (2–3 cm) from leaf and leaf petiole derived calli, were excised and individually transferred to full strength MS medium without plant growth regulators or with various levels (0.5, 1.0, 3.0 mg/l) of NAA or indole-3-butyric acid (IBA) contained in 50 ml Magenta vessels to test rooting potentials. The number of roots per shoot and root lengths were determined 4 weeks after culture. Rooted plantlets were acclimatized in a growth chamber and transferred to 16 cm pots containing 1:1 mixture of peat and perlite and grown till maturity under greenhouse conditions.

Each treatment had three replicates containing five explants in each culture vessel. The experiments were repeated twice. The results were pooled. Significance level was determined by analysis of variance 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).

Results and discussion

Preliminary experiments revealed that control, unexcised seeds had low mean germination percentage (10%) compared to excised seeds in which a 100% germination rate was observed (data not shown) after 30-day of culture. This different behaviour clearly indicates that the hard seed coat had a negative effect in *A. cariensis* germination rates. All further germination studies were used excised seeds. In previous studies, sulfuric acid treatment of seeds in *A. cicer* (Başalma et al. 2008), excising seed coats in *A. polemoniacus* (Mirici 2004) produced successful germination rates similar to our study. Such results show that hard coat in *Astragalus* should be excised before culture for efficient germination in this genus.

Preliminary experiments testing callus and shoot regeneration potential of leaf and leaf petiole explants revealed that all explants types produced callus but shoot production rates from calli varied between leaf and leaf petiole explants. Callus produced on medium with 2,4-D and Kin did not produce shoots, and only media containing IAA × Zea or NAA × BA produced regenerative calli with the latter combination being superior to all other medium combinations (Table 1). Further studies were conducted aiming to determine optimum NAA × BA combination for best shoot regeneration response of the subcultured calli.

Both leaf and leaf petiole explants produced green and compact calli starting from first week (Fig. 1a). Significant variations ($P < 0.01$) in callus fresh weights were obtained depending on the concentrations of NAA and BA in culture media (Table 2). In general, leaves were more responsive than petioles to callusing. Leaf petioles yielded more callus fresh weights on medium with 0.2 mg/l NAA × 1 mg/l BA whereas leaves had the highest ($P < 0.01$) callus fresh

Table 1 Effects of various auxin and cytokinin combinations on shoot organogenesis in leaf and leaf petiole explants of *Astragalus cariensis*

Growth regulators (mg/l)		Frequency of organogenic explants (%)		
Auxin	Cytokinin	Leaf	Petiole	Mean
0.5 NAA	1 BA	80.0	66.7	73.3 abc
	2 BA	86.7	73.3	80.0 ab
	4 BA	100.0	93.3	96.7 a
1 IAA	0.1 Zea	0.0	0.0	0.0 e
	0.2 Zea	0.0	46.7	23.3 de
	0.5 Zea	13.3	60.0	36.7 cde
2 IAA	0.1 Zea	6.7	20.0	13.3 de
	0.2 Zea	33.3	20.0	26.7 de
	0.5 Zea	46.7	33.3	40.0 bcde
4 IAA	0.1 Zea	46.7	53.3	50.0 bcd
	0.2 Zea	66.7	33.3	50.0 bcd
	0.5 Zea	33.3	60.0	46.7 bcd
				$LSD_{0.01}$: 40.5

Numbers in a column with the same letters were not significantly different

weights on medium with 0.5 mg/l NAA × 4 mg/l BA indicating the importance of initial explants type cultured.

Calli from leaf explants were more responsive than leaf petiole explants derived calli for shoot regeneration. Shoot regeneration was observed within 2–3 weeks of culture (Fig. 1b). The highest number of shoots (23) was induced from leaf explants on medium with 0.5 mg/l NAA and 4 mg/l BA (Table 2).

Astragalus cariensis explants responded well to higher cytokinin/auxin rates to induce shoot regeneration. Mirici (2004), reported that leaf petiole explants of *Astragalus polemoniacus* had the highest number of shoots when cultured on MS medium with 4 mg/l BA and 0.1 mg/l NAA whereas leaf explants responded more to 1 mg/l BA and 0.1 mg/l NAA combination. Similarly, MS medium with 2.0 mg/l BA and 0.5 mg/l NAA was the optimum medium for the induction of organogenesis from hypocotyl explants of *Astragalus melilotoides* (Hou and Jia 2004a). Both reports indicate that *Astragalus* species can be induced to regenerate by various combinations of NAA and BA depending on the species and explants sources.

Fig. 1 Adventitious shoot regeneration via organogenesis from leaf explants of *Astragalus cariensis*. **a** Callus formation on leaf explants on medium supplemented with 0.5 mg/l NAA and 2 mg/l BA after 4 week of culture. **b** Development of shoot initials on leaf explants on a medium supplemented with 0.5 mg/l NAA and 4 mg/l BA after 3–4 week of culture. **c–d** Adventitious shoots on leaf explants on medium supplemented with 0.5 mg/l NAA and 3–4 mg/l BA after 8–10 week of culture. **e** Root development on regenerated shoots after 4 week on rooting medium. **f** In vitro plantlet after 10 week of the transfer to onto plastic pot (*bar* 1 cm)

Table 2 The effects of various concentrations of NAA and BA on adventitious shoot regeneration frequencies of leaf and leaf petiole explants of *Astragalus cariensis*

Growth regulators (mg/l)		Callus fresh weight (g)[a]		Frequency of organogenic explants (%)[a]		Mean number of shoots/explant[a]	
NAA	BA	Leaf	Petiole	Leaf	Petiole	Leaf	Petiole
0.2	1	1.2 a–e	1.8 a	100.0 a	60.0 abc	5.1 def	1.9 ef
0.2	2	1.6 abc	0.5 f	100.0 a	33.3 bc	6.9 def	5.1 def
0.2	3	0.8 def	0.6 ef	93.3 a	0.0 c	9.6 cd	0.0 f
0.2	4	1.6 abc	1.7 ab	100.0 a	93.3 a	7.1 def	4.3 def
0.5	1	0.8 def	1.0 c–f	93.3 a	73.3 ab	5.7 def	2.2 ef
0.5	2	1.3 a–d	1.2 a–e	93.3 a	86.7 a	15.1 bc	5.3 def
0.5	3	1.0 c–f	1.4 a–d	100.0 a	86.7 a	21.1 ab	8.4 cde
0.5	4	1.7 ab	1.1 b–f	100.0 a	80.0 a	23.2 a	7.6 de
	Mean	1.3	1.2	97.5	64.2	11.7	4.4
		$LSD_{0.01}$: 0.7		$LSD_{0.01}$: 42.8		$LSD_{0.01}$: 7.2	

[a] Responses of leaf and petiole were evaluated together

Numbers in columns with the same letter were not significantly different

Table 3 In vitro rooting of *Astragalus cariensis* shoots in full strength MS medium with various concentrations of NAA or IBA after 4 week of culture

Rooting media		Mean number of roots/shoot	Mean root length (cm)
NAA mg/l	IBA mg/l		
0.0	0.0	3.1 d	1.9 b
0.5	0.0	6.1 c	7.3 ab
1	0.0	4.5 cd	7.2 ab
3	0.0	3.7 d	8.8 a
0.0	0.5	10.7 a	12.7 a
0.0	1	8.5 b	11.5 a
0.0	3	8.4 b	11.9 a
		$LSD_{0.01}$: 1.9	$LSD_{0.01}$: 6.7

Numbers in a column with the same letters were not significantly different

Shoots elongated (2–3 cm) after 8–10 weeks (Fig. 1c–d) and were successfully rooted (100%) in 3 weeks in full strength MS medium with or without NAA or IBA. However, based on the mean number of roots per shoot and root lengths 0.5 mg/l IBA (Fig. 1e; Table 3) resulted in better values compared to both growth regulator free MS medium or media with various concentrations of NAA.

Rooted plants were transplanted into pots in greenhouse for further acclimatization. Plants grew successfully into normal mature plants (Fig. 1f) and set seed.

A. polemoniacus shoots were reported to root best in MS medium without plant growth regulators or with 2.0 mg/l NAA (Mirici 2004). On the other hand, *A. cicer* shoots rooted better in ½ MS containing 0.25–0.50 mg/l NAA (Başalma et al. 2008). *A. adsurgens* shoots were reported to induce roots (90%) in ½ MS with 0.2–1.0 mg/l NAA (Lou and Jia 1998a, b). Uranbey et al. (2003) stated that 40% of regenerated shoots of *A. cicer* were rooted in ½ MS medium containing 1.0 mg/l NAA. Although our results conform to previous reports on the positive effect of NAA in rooting frequencies of in vitro shoots of different *Astragalus* species, MS medium with 0.5 mg/l IBA resulted in higher mean number of roots per explants with mean longer roots than other growth regulators or growth regulator free media. This result shows that each *Astragalus* species may require a different auxin for best overall rooting performance.

Well rooted shoots were rinsed with water to remove residual rooting media and transferred to plastic pots containing 1:1 mixture of peat and perlite and kept in a growth chamber under a day/night temperature regime of 24°C, 16 h photoperiod at 90% humidity. Rooted plantlets collected from IBA containing medium had higher survival rate (55%) (data not shown) than plantlets from other media upon acclimatization further confirming superiority of IBA in rooting medium.

In conclusion, this study presents the first report of plant regeneration via organogenesis from leaf and leaf petiole explants of a Turkish endemic species; *Astragalus cariensis*. Both explants types produced calli similar in their morphologies but shoot regeneration potentials of calli derived from leaf explants were superior over calli from leaf petioles. Optimum shoot regeneration medium was MS with 0.5 mg/l NAA and 4 mg/l BA.

The regeneration system described here can be successfully used in studies dealing with in vitro preservation of *Astragalus* species and other genetic manipulation studies where appropriate.

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