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The effect of thidiazuron on the in vitro shoot development of endemic *Astragalus cariensis* in Turkey

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Abstract: Thidiazuron (TDZ) is a cytokinin-like substance that has often been used for shoot regeneration in recent years. We observed the effect of TDZ on callus formation and shoot regeneration from leaf and petiole explants of the endemic species *Astragalus cariensis* Boiss. Explants were cultured on Murashige and Skoog (MS) media including TDZ (in concentrations of 0.2, 0.4, or 0.6 mg/L) or in combination with α -naphthaleneacetic acid (NAA) (in concentrations of 0.2 or 1.0 mg/L). Significant variations in the frequency of callus formation and the morphogenesis of the callus were obtained depending on the explant type. Petiole explant was determined to be the best for callus formation, but calli from leaf explants were more responsive in terms of shoot formation. MS media including TDZ and NAA showed 100% callus formation, but shoot regeneration was enhanced by TDZ combined with a low concentration of NAA. The highest number of shoots, (15 per explant) was induced from leaf explants on MS medium with 0.4 mg/L TDZ and 0.2 mg/L NAA. Regenerated shoots were rooted in MS medium containing 0.5 mg/L indole-3-butyric acid (IBA). The rooted plantlets were acclimatised and produced normal plants.

Key words: *Astragalus cariensis*, endemic, callus, organogenesis, thidiazuron

Endemik *Astragalus cariensis* türünün in vitro sürgün gelişimine thidiazuron'un etkisi

Özet: Thidiazuron (TDZ) sitokinin benzeri bileşiklerden biridir ve son yıllarda sürgün rejenerasyon sistemlerinde sıklıkla kullanılmaktadır. Bu çalışmada endemik *Astragalus cariensis* Boiss. bitkisine ait yaprak ve yaprak sapı eksplantlarında TDZ'nin kallus oluşumu ve sürgün rejenerasyonuna etkisi araştırılmıştır. Eksplantlar 0,2, 0,4, 0,6 mg/L TDZ ile bunların 0,2 ve 1,0 mg/L α -naphthaleneacetic acid (NAA) ile kombinasyonlarını içeren Murashige ve Skoog (MS) besin ortamlarında kültüre alınmıştır. Kallus oluşum yüzdesi ve kallus morfolojisi bakımından eksplantlar arasında farklılık gözlenmiştir. Kallus oluşumunda, yaprak sapı eksplantı, sürgün oluşumunda ise yaprak eksplantı daha iyi sonuç vermiştir. TDZ ve NAA içeren ortamlarda kallus oluşumu % 100 olmasına rağmen, sürgün rejenerasyonunda TDZ ve düşük oranda NAA içeren ortamlar daha iyi sonuç vermiştir. En fazla sürgün rejenerasyonu (15 adet/eksplant) 0,4 mg/L TDZ ve 0,2 mg/L NAA içeren ortamda kültüre alınan yaprak eksplantlarından elde edilmiştir. Rejenere sürgünler 0,5 mg/L indole-3-butyric acid (IBA) içeren MS ortamında köklendirilmiş ve köklenen sürgünler dış ortama başarılı bir şekilde aktarılmıştır.

Anahtar sözcükler: *Astragalus cariensis*, endemik, kallus, organogenesis, thidiazuron

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Introduction

Astragalus L. is the largest genus of flowering plants, containing up to 3000 species. It is also the largest genus in Turkey, where it is represented by nearly 455 species in 61 sections (Chamberlain & Matthews, 1970; Davis et al., 1988; Duran & Aytaç, 2005). Turkey is a main centre of diversity of the genus (Ghahremaninejad & Behçet, 2003), containing 210 endemic taxa with a rate of endemism of about 47% (Duman & Akan, 2003; Martin et al., 2008). *Astragalus* species are used for medicine and in the textile industry. Some perennial *Astragalus* species are also employed for forage production, and they can be used for erosion control because of their top root systems.

Astragalus cariensis Boiss. is endemic to Honaz Mountain (Denizli) and Yılanlı Mountain (Muğla) in southwestern Anatolia. The species is known from 3 populations and from an area of approximately 1500 km² (criterion B). The populations are not severely fragmented and there are no extreme fluctuations; it should therefore be regarded as Near Threatened (NT) (IUCN, 2001). Nowadays, the conservation of wild plant genetic resources is very important for preventing a decrease in genetic variability resulting from culture-grown plants. *A. cariensis* has deep top roots and is tolerant to drought, but it has poor seed germination capacity, long growth periods, and a low seed set. For this reason, classical breeding and propagation methods are of limited effect. Biotechnological approaches, such as somatic hybridisation and genetic transformation, may be used not only for improving this species but also for transferring its favourable stress resistance traits to other legumes. To attain these goals, the establishment of a reliable regeneration system is one of the main prerequisites (Hou & Jia, 2004).

There have been several plant regeneration studies in *Astragalus adsurgens* Pall. (Luo & Jia, 1998a, 1998b), *A. sinicus* L. (Cho & Widholm, 2002), *A. cicer* L. (Uranbey et al., 2003; Başalma et al., 2008), *A. melilotoides* Pall. (Hou & Jia 2004), *A. polemoniicus* Bunge (Mirici, 2004), *A. chrysochlorus* Boiss. & Kotschy (Turgut-Kara & Arı, 2008), and *A. cariensis* (Erişen et al., 2010). Although a report is available on the in vitro plant regeneration of *A. cariensis*, there is no information on plant regeneration using

thidiazuron (TDZ) as an alternative cytokinin source for this species. In general, few reports are available on TDZ-aided plant regeneration from leaf and leaf petiole explants of *Astragalus* subspecies (Mirici, 2004). In this paper, the effects of TDZ on callus formation and shoot regeneration from leaf and petiole explants of *A. cariensis* are described.

Materials and methods

Mature seeds of *Astragalus cariensis*, which were classified by Dr. Ahmet Duran (Selçuk University, Turkey), were collected from a wild population (C2 Muğla, Kale road, 1360 m) in Turkey. Surface sterilisation of the seeds was carried out with 20% (v/v) commercial bleach (HES, Turkey) for 10 min followed by 3 rinses with sterile distilled water. Seeds were incised with a sterile scalpel prior to culture in order to increase the germination rates. Incised seeds were germinated on Magenta vessels containing half-strength Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) solidified with 0.8% agar. The medium was adjusted to a pH of 5.8 with 1 N NaOH or 1 N HCl prior to being autoclaved at 121 °C, 1.4 kg/cm², for 20 min.

Leaf and petiole explants were removed from 30-day-old seedlings germinated in vitro and were initially placed on MS media supplemented with 3% sucrose and 0.8% agar (Sigma) and various levels of TDZ (0.2, 0.4, or 0.6 mg/L) alone or in combination with α -naphthaleneacetic acid (NAA) (0.2 or 1.0 mg/L) contained in petri dishes. After 4 weeks, some calli showed proliferation and shoot formation. These calli were then transferred to MS medium without growth regulators for shoot elongation. The percentage of explants producing calli was scored after 4 weeks of culture. The percentage of explants producing shoots and the number of shoots per explant were recorded after 4 weeks of subculture. Regenerated shoots (2-3 cm) were transferred to MS medium with 0.5 mg/L indole-3-butyric acid (IBA) for rooting. All cultures were maintained at 24 \pm 2 °C in a growth chamber (SANYO MLR-351H, Japan) with fluorescent light (5LS) and a photoperiod of 16L:8D.

Each treatment contained 5 explants with 3 replicates; all experiments were repeated twice. The

significance level was determined by analysis of variance using a 2-factor, completely randomised block design. 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

Within a week, callus induction was observed on the cut surfaces of leaf and petiole explants from *Astragalus cariensis*. The frequency of callus formation varied between leaf and petiole explants (Table). Petiole explants showed 100% callus formation in all media tested, whereas leaf explants showed 40%-100% callus formation. In the leaf explants, callus formation was suppressed in the medium containing TDZ alone (<0.6 mg/L). Similarly, a lower concentration of TDZ was found to suppress callus formation in *A. cicer* hypocotyl and cotyledon explants (Başalma et al., 2008). In *A. polemoniicus*,

callus formation from leaf explants was lower in a medium with TDZ alone than in those with NAA added (Mirici, 2004).

TDZ has been shown to induce callus formation in many tissue culture studies, but little information is available concerning the type of callus induced by TDZ. However, lower concentrations of TDZ generally tended to promote the formation of compact, green, nodular calli (Murthy et al., 1998). In *Tylophora indica*, yellowish-green, slightly loose calli were produced from leaf explants on TDZ at 2.5 µM, whereas compact and nodular calli were produced at higher concentrations (Sahai et al., 2010). In this study, petiole explants produced slightly loose, yellowish-green calli, but leaf explants produced greener and more compact calli (Figure).

We observed that petiole explant was the best explant for callus formation, but the calli from leaf explants were more responsive in terms of shoot regeneration. Shoot regeneration was observed within

Table. The effect of various concentrations of TDZ and NAA on callus formation and adventitious shoot regeneration from leaf and petiole explants of *Astragalus cariensis*.

Growth regulators (mg/L)		Callus induction* (%)			Explants producing shoots (%)			Number of shoots per explant		
TDZ	NAA	Leaf	Petiole	Mean	Leaf	Petiole	Mean	Leaf	Petiole	Mean
0.2	-	40 b	100 a	70	33	20	26	2.60	1.34	1.97 c
0.4	-	73 a	100 a	86	60	73	66	6.20	3.07	4.64 bc
0.6	-	100 a	100 a	100	66	40	53	5.07	2.07	3.57 bc
0.2	0.2	100 a	100 a	100	80	20	50	4.07	2.20	3.14 bc
0.4	0.2	100 a	100 a	100	93	66	80	15.47	6.07	10.77 a
0.6	0.2	100 a	100 a	100	33	33	33	3.34	0.94	2.14 c
0.2	1.0	100 a	100 a	100	93	53	73	11.27	2.73	7 ab
0.4	1.0	100 a	100 a	100	-	-	-	-	-	-
0.6	1.0	100 a	100 a	100	13	-	7	2.60	-	1.31 c
Mean		90 b	100 a		43.4 a	33.8 b		5.62 a	2.04 b	

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

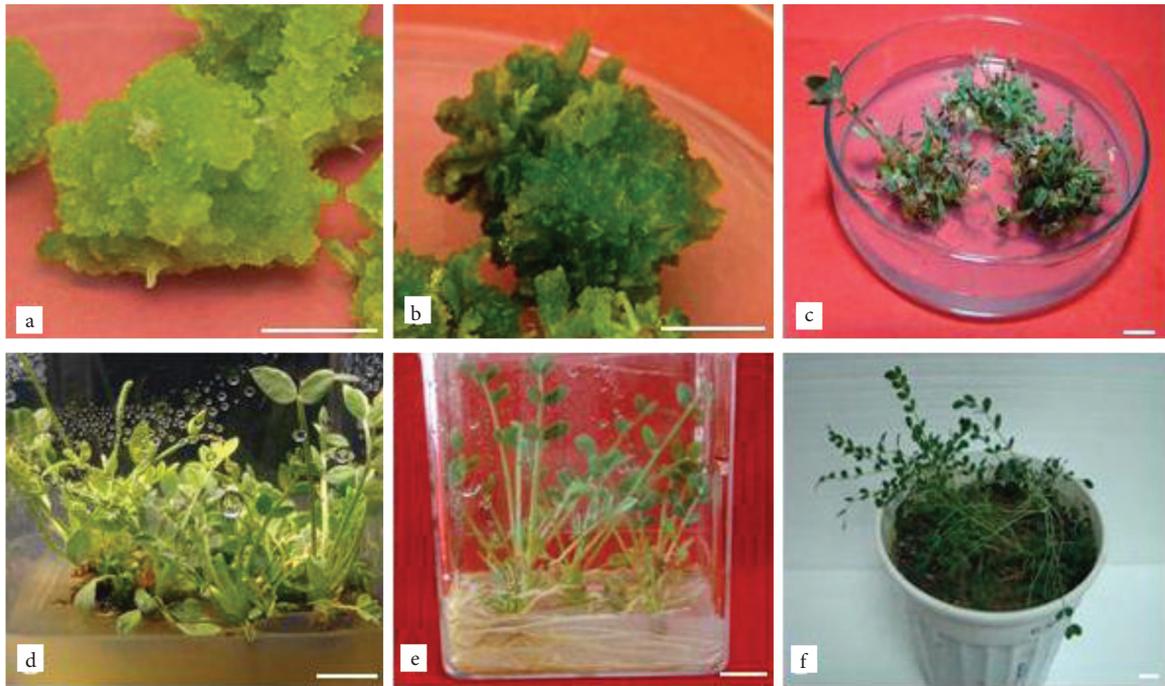


Figure. Callus induction and shoot regeneration in *Astragalus cariensis* using TDZ. In all photographs, the scale of the bar is equal to 1 cm: a and b- appearance of calli on medium supplemented with 0.4 mg/L TDZ and 0.2 mg/L NAA from petiole explant and leaf explant, c and d- adventitious shoots of leaf explants on medium supplemented with 0.4 mg/L TDZ and 0.2 mg/L NAA after 6 weeks and 8 weeks of culture, e- root development of the regenerated shoots after 4 weeks on rooting medium, f- in vitro plantlet 10 weeks after transfer into a plastic pot.

4-6 weeks of culture (Figure). Significant variations were obtained in the frequency of organogenic calli and the number of shoots per explant depending on the tested media and explant types (Table). Shoot regeneration was enhanced by the combination of TDZ and a low concentration of NAA compared with the use of TDZ alone. The highest number of shoots (15 per explant) was induced from leaf explants on MS medium with 0.4 mg/L TDZ and 0.2 mg/L NAA (Table and Figure). These results agree with the reports of Huettelman and Preece (1993) and Lincy and Sasikumar (2010), which suggested that combinations of TDZ and other plant growth regulators can be more effective than TDZ used alone. Mirici (2004), on the other hand, reported that TDZ alone prompted reasonable shoot multiplication in *Astragalus polemoniicus*. These variations may be due to species and genetic differences.

Adventitious shoot regeneration from leaf and petiole explants of *Astragalus cariensis* was reported by Erişen et al. (2010) using different concentrations

and combinations of BA and NAA. Generally, explants cultured on media supplemented with relatively high levels of cytokinins were positive for adventitious shoot regeneration. TDZ has been shown to stimulate shoot regeneration at much lower concentrations and was used mainly in combination with other plant growth regulators, primarily auxins (Murthy et al., 1998). In the present study, a cytokinin source (TDZ) of 0.2, 0.4, or 0.6 mg/L was combined with low and high concentrations of auxin (0.2 or 1.0 mg/L NAA) and tested for ability to induce shoot regeneration in *A. cariensis*. Neither TDZ alone nor combinations of TDZ and NAA produced reasonable shoot multiplication when compared to our previous study (Erişen et al., 2010). Mirici (2004) reported that BA or TDZ alone, or either used with combinations of NAA, showed adventitious shoot regeneration from leaf and petiole explants of *A. polemoniicus*. However, BA and NAA were found to be more effective than TDZ or TDZ combined with NAA, a result similar to our own findings. In other studies,

TDZ encouraged better shoot multiplication in *A. sinicus* root explants (Cho & Widholm, 2002) and *A. cicer* hypocotyl explants (Başalma et al., 2008) than BA or BA combined with NAA. This variation may be due to differences in the genotype and/or explant type. Different genotypes or explants having different culture responses under similar induction conditions have been documented before, a result that could be caused by different endogenous hormone levels in different genotypes or explants (Hung & Xie, 2008). Babaoglu and Yorgancilar (2000) also reported that explant type is highly important in the induction of adventitious shoot regeneration by organogenesis.

After being transferred to a rooting medium (MS with 0.5 mg/L IBA), the regenerated shoots were successfully rooted (100%) and showed rapid elongation (Figure). Well-rooted shoots were rinsed with sterile water to remove residual rooting media, transferred to 8-cm pots containing a 1:1 mixture of peat and perlite, and kept in a growth chamber under a day/night temperature regime of 24 °C with a 16-h photoperiod and 90% humidity. After 4 weeks, the

plantlets were transferred into pots containing soil and grown until maturity under environmental conditions. All such experimental plants grew successfully into normal mature plants (Figure).

In summary, this study presents the first report of shoot regeneration using TDZ from leaf and petiole explants of *A. cariensis*. The 2 explant types used in this study produced calli with different morphologies; petiole explant was the best explant for callus formation, but the shoot regeneration potential of leaf explant-derived calli was superior to that of calli from petiole explants. The highest number of regenerated shoots was obtained on MS medium with 0.4 mg/L TDZ and 0.2 mg/L NAA. These results could be useful for the genetic manipulation and propagation of this endemic species.

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