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Full Length Research Paper

TDZ-induced plant regeneration in *Astragalus cicer* L.

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We developed a regeneration protocol using thidiazuron (TDZ) with a high frequency *in vitro* root induction in *Astragalus cicer*. High *in vitro* germination ratio (75%) for hard-seeds of *A. cicer* was also achieved. For this, hypocotyl and cotyledon explants were cultured on Murashige and Skoog medium supplemented with different concentrations of TDZ. The highest frequency of shoot regeneration (53.3%) was achieved from hypocotyl segments through an initial callus growth stage on MS medium containing 0.25 mg/l TDZ. The shoots were cultured on the different strength (1/1, 3/4, 1/2 and 1/4) of basal Murashige and Skoog medium containing different concentrations of NAA. High rooting (100%) and survival (100%) were achieved using half strength MS medium supplemented with 0.25 and 0.50 mg/l NAA.

Key words: *Astragalus cicer,* regeneration, hypocotyl, cotyledon, rooting.

INTRODUCTION

Astragalus cicer L. (Cicer milkwetch*)* is a perennial forage legume with good grazing potential. *A. cicer* is thought to be one of the most important forage crops in arid and semi arid countries such as Turkey (Adıgüzel et al., 2006), Spain, Afghanistan, Portugal, Greece, Iran and Iraq. When harvested frequently under simulated grazing conditions, forage yields are comparable with those of other pasture legumes in mixed stands (Acharya et al., 2006). Forage quality of *A. cicer* appears to be equal to that of alfalfa (Towsend, 1970; Johnson et al., 1975). It is also a relatively new forage legume suitable for rangeland and in establishment of artificial meadows. It is adaptable to a wide range of conditions, from irrigated land to dry lands receiving less than 400 mm of annual precipitation. However, *A. cicer* has poor seed germination capacity, due to hard-seed coat resulting in slow seedling development and poor competition with weeds particularly during the initial years. Therefore, the development of herbicide resistant *A. cicer* by plant genetic engineering methods may help to become widespread of its use. Successful application of plant biotechnology for plant improvement requires the development of efficient shoot regeneration systems from cultured cells or tissues. There are limited reports related to *in vitro* regeneration of *Astragalus* spp. Edson et al. (1998) reported micropropagation of four threatened North American species of *Astragalus* (*A. columbianus, A. amblytropis, A. aquilonius* and *A. mulfordea*) by *in vitro* shoot culture. Plant regeneration was achieved from callus derived protoplasts (Luo and Jia 1998a) and hypocotyl (Luo and Jia 1998b) explants of *A. adsurgens* Pall. Efficient plant regeneration was also obtained through somatic embryogenesis in *A. adsurgens* (Luo et al., 1999). Plant regeneration from hypocotyls, cotyledon, stem and petiole explants explants of *A. cicer* was achieved by Uranbey et al. (2003) using different concentrations and combinations of N^6-benzylamino-purine (BAP) and α-naphthaleneacetic acid (NAA). Although one report is available on the regeneration of *A. cicer,* there are still no data concerning plant regeneration using thidiazuron (TDZ) as a potent and alternative cytokinin source for this species. Also, *in vitro* rooting manipulations were, for the first time, tested in this study. Adventitious root formation is very important for the vegetative propagation and a key step in micropropagated systems. Low frequency of rooting has been reported for some forage legumes such as sainfoin (Pupilli et al., 1989) and alfalfa (Özgen et al., 1998) which appears to be a problem in regenerated shoots. There-

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Abbreviations: TDZ, thidiazuron; MS, Murashige and Skoog medium; NAA, α-naphthaleneacetic acid.

fore, improvement of rooting ability of *A. cicer* shoots is necessity in order to mass plant propagation and genetic manipulation of this species which may offer alternative for dry lands receiving limited rainfall.

The present study focuses on a) increasing of germination ratio of hard-seed coat, b) improvement of regeneration potential using TDZ and c) obtaining of high frequency of rooted plants.

MATERIAL AND METHODS

Mature seeds of *Astragalus cicer* L. were obtained from Department of Field Crops, Faculty of Agriculture, and University of Ankara, Turkey. In order to increase germination ratio and break dormancy, the seeds of *A. cicer* were incubated in 50% H_2SO_4 solution for two min. They were then rinsed twice with sterile water for two min and surface sterilized for 2 min in 70% (v/v) ethanol before soaking in 50% commercial bleach (Axion) for 30 min. The seeds were then rinsed 3 times with sterile deionised water for 2 min. Sterilized seeds were placed on half-strength MS (Murashige and Skoog 1962) medium containing 3% (w/v) sucrose and 0.8% (w/v) agar. The seeds were also put between two filter papers (Type MN 751) placed in petri dishes (100 x 10 mm) moistened with 7.5 ml sterile water and were cultured at 24±2°C under fluorescent light (35 µmol m^{-2} s^{-1}) in a 16 h photoperiod for testing germination ratio. In order to obtain explant sources for regeneration studies, sterilized seeds were germinated on MS (Murashige and Skoog 1962) medium containing 3% (w/v) sucrose and 0.8% (w/v) agar. The medium was adjusted to pH 5.7 with 1 N NaOH or 1 N HCl prior to autoclaving at 121ºC, 1.4 kg/cm^2 for 20 min.

Basal MS medium was supplemented with different concentrations (0.05, 0.10, 0.25, 0.50 and 1.0 mg/l) of TDZ. TDZ was filter-sterilized using a Milipore filter (0.22 µm pore size) and added to hot autoclaved medium before dispensed into culture tubes. Hypocotyl explants were excised from 15 days old seedlings. Hypocotyl segments were dissected by discarding axillary meristems and cut into pieces approximately 0.3 cm long. Cotyledon explants were cut across discarding the petiole. Edges of cotyledons were also trimmed off. The number of explants producing shoots and the number of shoots per explant were scored after seven weeks of culture. The shoots (2 - 3 cm) regenerated from explants were excised and individually transferred to 1/1, 3/4, 1/2, 1/4 strength MS medium supplemented with various concentrations (0.25, 0.50 and 1.0 mg/l) of NAA in Magenta vessels for rooting. After four weeks, the number of rooted shoots and the number of roots per shoot were recorded. Rooted plantlets were acclimatized in a growth chamber at 90% humidity and transferred to 16 cm pots containing 1:1 mixture of soil and vermiculite and grown till maturity under greenhouse conditions. All cultures were subcultured to fresh regeneration and rooting media after 24 days of culture initiation.

Each treatment had 5 replicates consisting of Petri dishes containing 6 explants for regeneration study. Each treatment had 6 replicates consisting of Magenta vessels each containing 5 shoots for rooting study. The experiments were repeated two times. The results were pooled. Total 60 explants were used for each treatment in both regeneration and *in vitro* rooting studies. Significance was determined by analysis of variance using a two factor completely randomised block design method and the differences between the means were compared by Duncan's multiple range test using a MSTAT-C computer program (Michigan State University). Data given in percentages were subjected to arcsine (√X) transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS AND DISCUSSION

H_2SO_4 treatment considerably softened hard seed coat of *A. cicer* and considerably increased germination ratio. The seeds which were not treated with H_2SO_4 showed no germination (0%) in both half-strength MS medium containing 3% sucrose and 0.8% agar and moistened sterile filter papers. However, after H_2SO_4 treatment, mean germination ratio was 25% on half-strength MS medium whereas, and it increased up to 75% within the sterile filter papers. MS medium and other compounds may change osmotic pressure of seeds and negatively affect germination.

Most of cotyledon and hypocotyl enlarged and formed greenish coloured compact callus within 3 - 5 weeks after culture initiation. In general, hypocotyl explants were more responsive than cotyledon explants in response to media containing TDZ. Calli covered whole hypocotyls with frequencies ranged from 73.3 to 100%, which were followed by the emergence of shoot primordia within 3 weeks (Figure 1A). These shoot primordia developed into normal shoots after 7 - 8 weeks (Figure 1B). Hypocotyl explants showed positive morphogenetic response and readily developed multiple shoots, whereas cotyledon explants produced only callus 4-5 weeks after culture initation. Compact calli were formed on the cut surfaces of cotyledon explants. High TDZ concentrations resulted in formation of somatic embryos at low frequency in both explant types.

There was a significant interaction between explant type and plant growth regulator concentrations on the frequen-cy of shoot regeneration ($p < 0.05$) (Table 1). Considering both percentage of explants producing shoots and the number of shoots per explant, the highest adventitious shoot regeneration frequency was achieved on a medium supplemented with 0.25 mg/l TDZ in hypocotyl explants. Whereas, the highest percentage of shoots regenerated from cotyledon explants was obtained on a medium con-taining 1.0 mg/l TDZ. The media supplemented with TDZ considerably promoted shoot regeneration from hypocotyl explants. At higher and lower concentrations of TDZ from 0.25 mg/l, number of shoots as well as frequency of shoot regeneration was TDZ significantly enhanced callu-sing in both hypocotyl and cotyledon explants. 0.50 mg/l and much more concentrations of TDZ did not increase shoot regenera-tion in hypocotyl explants. All TDZ treated explants yielded healthy shoots in both explants. The results emphasize the importance of TDZ and suggest that a reasonable TDZ concentration induces shoot regene-ration. Recent reports are available on the high frequency shoot regeneration of some crops and other legumes using TDZ (Malik and Saxena, 1992; Gill and Saxena 1992; Luo, 1993; Kim et al., 1997; Hosseini and Rashid, 2003; Thomas, 2003; Uranbey, 2005). These results indicate that the type of explant is highly important in establishing an efficient regeneration system as report-ted by Babaoglu and Yorgancılar (2000), Koroch et al.

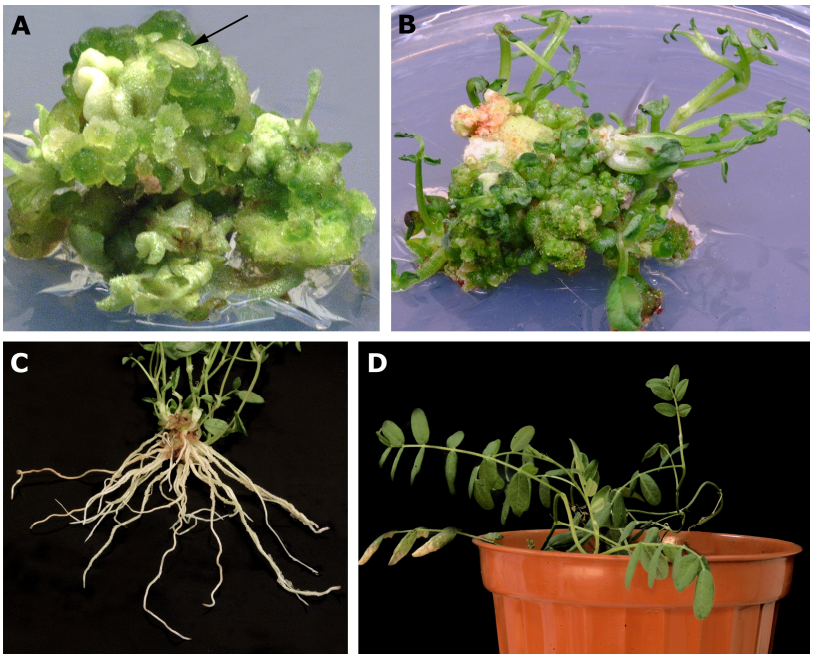

Figure 1. Adventitious shoot regeneration from hypocotyl explants of *Atragalus cicer* and root formation. *A*) Callus formation and development of shoot initials on hypocotyl explants on a medium supplemented with 0.25 mg/l TDZ after 4-5 weeks of culture. *B*) Adventitious shoots on hypocotyl explants after 7 weeks of culture. *C)* Root development on regenerated shoots after 3 weeks on rooting medium. D) *In vitro* raised plantlet after 4-week of the transfer to plastic pot containing 1:1 mixture of soil and vermiculite.

Table 1. Effect of various concentrations of TDZ on adventitious shoot regeneration from hypocotyl and cotyledon explants of *A. cicer*.

Growth regulator		Adventitious shoot development		
TDZ (mg/l)	Explant type	Callus formation (%)	Explants producing shoots (%)	Number of shoots per explant
0.05	Hypocotyl	93.3a*	26.6b*	2.03bc*
0.10		73.3b	16.6bc	2.13bc
0.25		100.0a	53.3a	5.60a
0.50		100.0a	20.0bc	2.93b
1.00		100.0a	20.0bc	3.13b
0.05	Cotyledon	33.3d	0.00d	0.00d
0.10		40.0cd	0.00d	0.00d
0.25		30.0d	0.00d	0.00d
0.50		46.6c	10.0c	1.36c
1.00		50.0c	13.3c	2.43bc

*Values within a column followed by different letters are significantly different at the 0.05 probability level, analyzed by Duncan's multiple range test.

(2002) and Uranbey et al. (2005). We also observed that adventitious shoots frequency of hypocotyl was faster than that of cotyledon explants. High adventitious shoot regeneration capacity of hypocotyl revealed the morphogenic potential of hypocotyl in legumes (Gu et al., 1987; Fakhrai and Evans, 1989; Özgen et al., 1998; Uranbey et al., 2003). Adventitious shoot regeneration from hypocotyls, cotyledon, stem and petiole explants of *A. cicer* was reported by Uranbey et al. (2003) using different concentrations and combinations of BAP and NAA. Whereas, different concentrations of TDZ as a drastically decreased. However, 0.25, 0.5 and 1.0 mg/l cytokinin source for

Table 2. *In vitro* rooting of *A. cicer* at various strengths of MS medium containing different concentrations of NAA after 4 weeks of culture.

Rooting media		Rooting (%)	Number of root/shoot
MS	NAA (mg/L^{-1})		
1/1	0.25	46.6d*	8.6gh*
1/1	0.50	40.0d	5.16i
1/1	1.00	70.0c	10.66fg
3/4	0.25	76.6c	8.33gh
3/4	0.50	80.0bc	7.33hi
3/4	1.00	90.0ab	14.46de
1/2	0.25	100.0a	21.20a
1/2	0.50	100.0a	19.63ab
1/2	1.00	93.3ab	16.56cd
1/4	0.25	80.0bc	12.13ef
1/4	0.50	100.0a	17.33bc
1/4	1.00	76.6c	16.00cd

*Values within a column followed by different letters are significantly different at the 0.05 probability level, analyzed by Duncan's multiple range test.

the first time were tested to induce shoot regeneration of *A. cicer*. In this study and TDZ gave reasonable shoot multiplication when compared to our previous study (Uranbey et al., 2003). Moreover, all tested concentrations of TDZ were more callogenic than combinations of BAP and NAA when compared to our previous study. Elongated shoots (>10 mm) were cultured on various strengths of MS basal media supplemented with different concentrations of NAA. Uranbey et al. (2003) stated that 40% of regenerated shoots of *A. cicer* were rooted in half-strength MS medium containing 5.4 µM NAA. Therefore, different concentrations of NAA were tested to induce the development of roots and to reduce duration of root induction in this study. Rooting started within 30 days of culture on all media. Different strengths of MS medium showed a noticeable effect on rooting rates. Higher strengths of MS (1/1 and ¾ MS) resulted in increased callus production. The highest percentage of rooting and the number of roots per shoot changed significantly with different concentrations of MS media and concentrations of NAA. Different strengths of MS x NAA interaction was significant ($p < 0.05$) (Table 2) on the rooting. It was seen that 1/2 MS medium containing all the concentrations of NAA showed the promoting effect on the root formation (Figure 1C). The highest rooting ratio (100%) was achieved on ½ MS media contaning 0.25 and 0.50 mg/l NAA and 1/4 MS supplemented with 0.50 mg/l NAA. When considering both rooting ratio and the number of roots per shoot, the best rooting was achieved on 1/2 MS medium containing 0.25 and 0.50 mg/l NAA. Similarly, 1/2 MS medium containing 0.25 and 0.50 mg/l NAA also gave the highest number of root per shoot (21.2 and 19.63, respectively). While mean rooting ratio was 40% for *A. cicer* in our previous study, rooting ratio was increased up to 100% with high number of root per shoot. Reduced MS and high concentrations of NAA strongly stimulated root formation; however, callus formation was strongly stimulated by high concentrations of NAA. Also, a drastic inhibitory effect on root formation was observed in the 1/1 and 3/4 strengths of MS medium. Komalavalli and Rao (2000) also reported that the best rooting frequency was achieved on the 1/2 strength MS strength medium, the lowest root formation and elongation were observed on the 1/4 strength MS medium in *Gymnema sylvestre*. Well rooted shoots were rinsed with sterile water to remove residual rooting media and transferred to plastic pots containing 1:1 mixture of soil and vermiculite and kept in a growth chamber under a day/night temperature regime of 24°C, 16 h photoperiod at 90% humidity. The survival rate of regenerated plantlets transferred to soil was the highest (100%) following root initiation on 1/2 strength MS medium (Figure 1D). All tissue culture-derived plants grew well had no morphological variations when compared with seed derived plants.

The present study emphasizes the importance of TDZ and suggests that a suitable TDZ concentration induces shoot regeneration in *A. cicer*. *In vitro* high frequency rooting of *A. cicer* may help adventitious shoot regeneration and *in vitro* micropropagation. Combinations of reduced MS and NAA strongly encouraged root formation in *A. cicer*. These results will be useful for both genetic transformation studies and for micropropagation of this important forage crop.

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