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. mulfordiae*) 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 N⁶-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.

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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
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
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% H₂SO₄ 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
121°C, 1.4 kg/cm² 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
condition 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

**RESULTS AND DISCUSSION**

H₂SO₄ treatment considerably softened hard seed coat of
*A. cicer* and considerably increased germination ratio. The
seeds which were not treated with H₂SO₄ showed no
germination (0%) in both half-strength MS medium
containing 3% sucrose and 0.8% agar and moistened sterile filter papers. However, after H₂SO₄ 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
initiation. 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
frequent-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 containing 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 callus-ling in both hypocotyl
and cotyledon explants. 0.50 mg/l and much more
concentrations of TDZ did not increase shoot regeneration
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

\[\text{arcsine } (\sqrt{x})\] transformation (Snedecor and Cochran, 1967) before statistical analysis.
establishing an efficient regeneration system as reported by Babaoglu and Yorgancilar (2000), Koroch et al. Başalma 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*.

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Adventitious shoot development</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDZ (mg/l)</td>
<td>Explant type</td>
</tr>
<tr>
<td>0.05</td>
<td>Hypocotyl</td>
</tr>
<tr>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>Cotyledon</td>
</tr>
<tr>
<td>0.10</td>
<td></td>
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<tr>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

*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 0.25 and 0.50 mg/l NAA also gave the highest number of 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.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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