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*Full Length Research Paper*

# *In vitro* regeneration of Turkish dwarf chickling (*Lathyrus cicera* L) using immature zygotic embryo explant

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**Dwarf chickling (*Lathyrus cicera* L) is an important forage legume crop. However, due to the presence of neurotoxin amino acid (b-N-oxalyl- L-a,b-diaminopropionic acid) in the dwarf chickling, its cultivation is limited. There is need to carry out studies for the development of varieties with reduced or none of this neurotoxin for beneficial and prolong consumption by animals. The study reports tissue culture approaches for multiplication of the plant using thidiazuron (TDZ), 6-benzylaminopurine (BAP), and - naphthalene acetic acid (NAA) from immature zygotic embryos of the plant. The highest number (16.25) of shoots per explant was recorded on MS medium supplemented with 0.45 mg/l TDZ and 0.4 mg/l ascorbic acid. Much reduced shoot regeneration was recorded on MS medium containing different combinations BAP+NAA+ascorbic acid. The TDZ-regenerated shoots were rooted by pulse treatment with 50 mg/l IBA for 7 min. The plantlets were successfully acclimatized under the greenhouse conditions.**

**Key words:** Immature zygotic embryos, dwarf chickling, *Lathyrus cicera* L., axillary shoot regeneration, rooting.

## INTRODUCTION

Dwarf chickling (*Lathyrus cicera* L.) family Fabaceae is a self-pollinated, annual, herbaceous legume plant that grows widely in Greece, Crimea, the Middle East, and Central Asia (Kislev, 1989). It grows in range of conditions from fertile to adverse agricultural conditions such as flooding, drought, salinity and low fertility soils, at altitude of 5 - 2000 m above sea level, in diffused bush and pine forests, rocky slopes and fields (Tubives, 2008). It flowers during April and May each year (Zohary and Hofp, 1988). It fixes atmospheric nitrogen and makes it suitable as green manure crop on nutrient poor soils. The plants belonging to *Lathyrus* species are also used as vegetable in some parts of the world. *Lathyrus* species are widely used as animal feed in green and dry form or as grains. However, their prolong use cause a disease, lathyrism, both in animals and human beings due to the presence of a neurotoxic amino acid, b-N-oxalyl-L-a,b-diaminopropionic acid (ODAP/BOAA). Lathyrism result in paralysis in humans and the lower limbs of animals by affecting the central nervous system. Traditional breeding practices explored to date have not been successful in substantially reducing the toxin (Sachdev et al., 1995; Barik et al., 2004; Basaran et al., 2007). ODAP was identified in the 1960s and since that time plant breeding has produced lines with low toxin levels (Hanbury et al., 2000). Due to the presence of ODAP, *Lathyrus* species have almost disappeared from many regions including Europe, where they were once cultivated extensively (Hanbury and Hughes, 2003). There is need to explore ways to improve the quality of the plant by reducing or eliminating the neurotoxins in the plants for future plant breeding work, thereby improving commercially valuable species along with *Pisum*, *Lens* and *Vicia*, (McCutchan, 2003).

Since one of the goals of establishing *Lathyrus* cultivation in Turkey is to make efficient use of the plant

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**Abbreviations:** BAP, 6-benzylaminopurine; TDZ, thidiazuron, NAA, -naphthlene acetic acid.

**Table 1.** Effects of various concentrations of BAP+NAA on axillary shoot regeneration from immature zygotic embryos of dwarf chickling.

| Treatment | | Frequency (%) of shoot regeneration | Mean number of shoots per explant | Mean shoot length (cm) |
|---|---|---|---|---|
| BAP (mg/L) | NAA (mg/L) | | | |
| 1 | 0.5 | 100 a | 1.58 b | 5.35 b |
| 2 | 0.5 | 93.33 a | 2.67 a | 7.86 a |
| 4 | 0.5 | 100 a | 3.20 a | 8.26 a |
| 6 | 0.5 | 93.33 a | 1.58 b | 3.43 c |
| MS (control) | | 0.00 b | 0.00 c | 0.00 c |

Mean values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test.

in the developing animal feed market, it is necessary to investigate different approaches for rapid multiplication of the plant for future use in the breeding programmes. *In vitro* plant regeneration has been reported in *L. cicera* through cotyledonary node (Malik et al., 1992), *L. sativus* through cotyledonary node (Barik et al., 2004), cotyledon, hypocotyl, epicotyl, internode and leaf explants (Barik et al., 2005) along with plant regeneration from protoplasts of *L. sativus* (McCutchan et al., 1999; Durieu and Ochatt, 2000) and *L. odoratus* (Razdan et al., 1980).

To date, there is no report on *in vitro* plant regeneration of *L. cicera* using immature zygotic embryos. In this paper, we describe an efficient and reproducible protocol for rapid and large-scale propagation of *L. cicera* by shoot proliferation from immature zygotic embryo explants obtained from field grown plants.

## MATERIALS AND METHODS

### Plant regeneration

The seed pods containing immature seeds belonging to Line No. LC 870 (of Osman Tosun Gene Bank of the Faculty of Agriculture, University of Ankara, Turkey) was obtained from the experimental fields of the Department of Field Crops, University of Ankara, Turkey during the last weeks of June 2007 after 18 - 20 days of anthesis. These were surface sterilized with 50% commercial bleach (5 - 6% NaOCl, Ace –Turkey) and magnetic stirrer in laminar flow hood for 20 min. It was followed by 3 x 5 min rinsing of the pods in sterile distilled water. The pods were cut open with forceps and the testa was removed from the seeds to get immature embryos by squeezing hard the seeds with forceps (1 - 2 mm in length).

These were cultured on MS medium (Murashige and Skoog, 1962) containing 1 - 6 mg/l BAP – 0.5 mg/l NAA (Table 1) or 0.05 – 0.65 mg/l TDZ (Table 2) with or without 0.4 mg/l ascorbic acid contained in 100 x 10 mm Petri dishes™ and incubated at 24 ± 2$^{O}$C in 16 h day length photoperiod.

The developing shoots on MS medium containing different concentrations of TDZ were pulse treated with 50 mg/l IBA for 7 min followed by culture in ½ strength of the regular MS mineral salts and vitamins for rooting.

The pH of all cultures was adjusted to 5.6 - 5.8 before adding 0.65% agar (Duchefa, Germany) and autoclaving at 121$^{O}$C, 118 kPa pressure for 20 min.

### *Ex vitro* culture

The rooted plantlets were transferred to potting mixture containing peat moss vermiculite and perlite (1:1:1). Potted plants were maintained in the greenhouse at 24 ± 2$^{O}$C under natural light and watered every 2 days for 2 weeks or as and when needed.

### Statistical analysis

Each treatment had 4 replicates (Petri dishes) containing 5 explants and all experiments were repeated twice (4 x 5 x 2 = 40 explants). Data was analyzed with SPSS 15.0 using one- way ANOVA and the post hoc tests were performed using Duncans Multiple Range test. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran 1967) before statistical analysis.

## RESULTS

Axiliary shoot regeneration on MS medium containing different concentrations of BAP- NAA or TDZ was accompanied with blackening of the explant with no or diminished shoots (data not shown). However, this problem could not be detected when 0.4 mg/l ascorbic acid was added to the culture medium.

### Axiliary shoot regeneration

No shoot regeneration was observed on MS medium (control) without plant growth regulators (Table 1). High frequency of shoot regeneration was recorded on MS medium containing different concentrations of BAP+NAA with a range of 93.33 to 100%. It was found that MS medium containing 4 mg/l BAP + 0.5 mg/l NAA induced the longest (8.26 cm) and maximum number (3.20) of shoots on all explants within 8 weeks of culture (Table 1). This was closely followed by 2.67 shoots per explant with shoot length of 7.86 cm on MS medium containing 2 mg/l BAP + 0.5 mg/l NAA. Compared to the above mentioned concentrations, dramatic decrease in the number of shoots per explant was very evident on 1 or 6 mg/l BAP + 0.5 mg/l NAA. However, 1 mg/l BAP + 0.5 mg/l NAA was

**Table 2.** Effects of various concentrations of TDZ on axillary shoot regeneration from immature zygotic embryos of dwarf chickling.

| TDZ (mg/l) | Frequency (%) of shoot regeneration | Mean number of shoots per explant | Mean shoot length (cm) |
|---|---|---|---|
| 0.05 | 100 a | 6.60 c | 1.04 b |
| 0.15 | 100 a | 11.40 b | 1.44 b |
| 0.30 | 100 a | 12.33 ab | 1.51 b |
| 0.45 | 100 a | 16.25 a | 2.14 a |
| 0.65 | 100 a | 6.60 c | 0.97 b |
| Control (MS) | 0.00 b | 0.00 b | 0.00 b |

Mean values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test.

less inhibitory in terms of shoot length compared to 6 mg/l BAP + 0.5 mg/l NAA with shoot length of 5.35 and 3.43 cm, respectively.

### Axiliary shoot regeneration

Shoot regeneration was recorded on all concentrations of TDZ on all immature zygotic embryos of *L. cicera* (Table 2) . Development of shoot meristems was recorded with in 2 - 3 days of culture. These meristems grew into well developed shoots after 8 weeks of culture. It was observed that MS medium containing 0.45 mg/l TDZ induced the longest (2.14 cm) and maximum number (16.20) of shoots on all explants (Table 2). It was closely followed by 12.33 shoots per explant with mean shoot length of 1.51 cm on MS medium supplemented with 0.30 mg/l TDZ. All other concentrations of TDZ were inhibitory or less stimulatory and resulted in significantly lower number of shoots per explant and reduced shoot length. Mean number of shoots per explant and shoot length increased gradually in ascending order on MS medium containing 0.05 to 0.45 mg/l TDZ.

### Rooting

Irrespective of the concentration of TDZ used for shoot regeneration, all pulse treated shoots from all culture media rooted on ½ strength regular MS mineral salts and vitamins.

### *Ex vitro* culture

The rooted plantlets were easily acclimatized in pots in the greenhouse at 24 ± 2$^{o}$C under natural light. No plant showed signs of abnormality, physical stress or weakness. Once established, they were transferred to fields for further studies.

## DISCUSSION

The success of tissue culture largely relies on the selection of a suitable explant for use as the starting material for the experiment. Recently, there has been an increased interest in using immature zygotic embryos as an experimental plant material to reinforce the technique of micropropagation in a number of plant species that are difficult to regenerate including pigeon pea (Gosal and Bajaj, 1979), Hungarian vetch (Sancak et al., 2000), *Vigna unguiculata* (Choi et al., 2003) and lesser burnet (Cocu et al., 2003).

The immature zygotic embryo explants failed to regenerate shoots on plant growth regulator free MS basal medium, but addition of thidiazuron or BAP+NAA to the medium was essential to induce axiliary shoot proliferation. Of the all combinations of plant growth regulators used, TDZ at 0.45 mg l $^{-1}$ was the most effective (Table 2). The concentration and type of cytokinin used significantly affected the percentage shoot regeneration, shoot number, and shoot length.

The results showed that both BAP and TDZ were suitable for induction of shoot regeneration on immature zygotic embryos only if they contained 0.4 mg/l ascorbic acid in the regeneration medium. However, TDZ was more effective to regenerate shoots compared to BAP. Similar results have also been reported in grasspea (Barik et al., 2004). Successful shoot regeneration through cotyledon node (Barik et al., 2004), cotyledon, hypocotyl, epicotyl, internode, leaf explants (Barik et al., 2005) and protoplasts of *L. sativus* (McCutchan et al. 1999, Durieu and Ochatt, 2000), *L. odoratus* (Razdan et al., 1980) and cotyledon nodes of *L. cicera* (Malik et al., 1992) has already been reported.

The results signified that the concentration of BAP+NAA or TDZ in the culture medium significantly affected the frequency, mean number of shoot and mean shoot length. Comparing the two plant growth regulators, it was observed that the TDZ was more potent compared to BAP+NAA. This is in contrast to the results of Barik et al. (2004) who found that BAP was more favorable for shoot regeneration compared to TDZ. This might be due to the effect of different explant or due to different dose of the plant growth regulators used by Barik et al. (2004). Again it was not necessary to repeatedly subculture the

explants in contrast to Barik et al. (2004, 2005) who regularly subcultured the original cotyledonary nodes on a fresh multiplication medium for 3 to 4 times after each harvest of newly formed shoots to produce an average of eight to nine shoots per explant. Sinha et al. (1982) observed callus cultures from stem explants of six *L. sativus* L. cultivars tested for their morphogenic capacity, and they found that shoot-buds were formed in calli of only one cultivar. Maximum response was observed in the medium containing $10^{-8}$ M picloram and $10^{-6}$ M benzylaminopurine. Supplementation with adenine sulphate was required for shoot-bud formation. Roy et al. (1992) developed a protocol for the *in vitro* production of plants from callus derived root explants of *L. sativus* cv. P-24. They found that callus and shoot regeneration were achieved only in MS medium supplemented with 10.7 µM NAA and an increased concentration of kinetin (0.9 µM for 14 days to 1.4 µM for 18 days) during callusing. Similarly, Malik et al. (1992) recorded multiple shoots from cotyledon node explant of *L. cicera*

All pulse treated shoots with 50 mg/l IBA rooted easily. Barik et al. (2004, 2005) found it necessary to root the regenerating shoots on in half strength MS supplemented with IAA, IBA, or IPA. They were able to elongate the roots only after the rooted shoots were transferred to half strength MS lacking the auxin. Roy et al. (1992) also rooted the shoots of *L. sativus* in 1/2 MS supplemented with 0.5 µM indole butyric acid. No root formation was observed when shoots were cultured in the medium devoid of auxins in agreement with Barik et al. (2004, 2005). Gulati and Jaiwal (1994) and Polisetty et al. (1997) emphasized that auxins promote root induction, but inhibit subsequent root growth if allowed to persist in the culture medium.

The *in vitro* grown plantlets were easily acclimatized in soil compost contained in transparent plastic pots under greenhouse conditions. All plantlets survived in the greenhouse. This protocol has high potential for improvement of the plant through *Agrobacterium*-mediated genetic transformation or through *in vitro* mutation breeding for reducing or eliminating the neurotoxic amino acid, b-N-oxalyl-L-a,b-diaminopropionic acid.

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