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Research Note

Effect of Thidiazuron on Shoot Regeneration from Different Explants of Lentil (*Lens culinaris* Medik.) via Organogenesis

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Abstract: Thidiazuron (TDZ) is among the most active cytokinin like substances and induces greater in vitro shoot proliferation than many other cytokinins in many plant species. Leaf, stem, stem node and cotyledonary node explants of 2 extensively cultivated Turkish lentil cultivars, Ali Dayı and Kayı 91, were cultured on Murashige and Skoog (MS) media supplemented with various concentrations of TDZ. The present study was conducted to develop a rapid and efficient shoot regeneration system suitable for the transformation of lentil (Lens culinaris Medik.) using TDZ. Cotyledonary nodes and stem nodes after the initial callus stage regenerated prolific adventitious shoots via organogenesis. Shoot or callus formation was not achieved from leaf or stem explants. DMSO as a solvent for TDZ was necrotic on plant tissues and therefore TDZ was dissolved in 50% ethanol to carry out the studies. Cotyledonary nodes showed a higher shoot formation capacity than stem nodes. MS medium supplemented with 0.25 mg/l TDZ produced the highest frequency of shoot formation from cotyledonary nodes in both genotypes. Regenerated shoots (10-20 mm long) rooted in MS medium containing 0.25 mg/l indole-3-butyric acid (IBA). Rooted plantlets were finally transferred to sand in pots.

Abbreviations: TDZ - thidiazuron [1 Phenyl 3-(1,2,3-thiadiazol -5YL) urea], IBA – indole-3-butyric acid, MS - Murashige and Skoog, DMSO - dimethylsulphoxide

Key Words: Thidiazuron, in vitro, shoot regeneration, cotyledonary node.

Organogenesis Aracılığıyla Farklı Mercimek (Lens culinaris Medik.) Eksplantlarından Sürgün Rejenerasyonuna TDZ'nin Etkisi

Abstract: Thidiazuron (TDZ) en aktif sitokinin benzeri bileşiklerden olup, birçok bitki türünde sitokininlerden daha yüksek oranlarda sürgün rejenerasyonu sağlamaktadır. Yaygın olarak üretimi yapılan mercimek çeşitlerinden Ali Dayı ve Kayı 91 çeşitlerine ait yaprak, gövde, gövde bouğumu ve kotiledon boğum eksplantları farklı oranlarda TDZ içeren Murashige ve Skoog (MS) besin ortamlarında kültüre alınmıştır. Bu çalışma Mercimekte (*Lens Culinaris* Medik.) gen aktarımına uygun hızlı ve etkili bir sürgün rejenerasyon sistemi geliştirmek için yürütülmüştür. Kotiledon ve gövde boğumlarından, başlangıç kallus gelişimini takiben organogenesis aracılığıyla prolifik adventif sürgün rejenerasyonu elde edilmiştir. Yaprak ve gövde eksplantlarında ise kallus ve sürgün gelişimi gözlenmemiştir. DMSO, TDZ çözücüsü olarak kullanıldığında bitki dokularına ölümcül etki yapmış ve çalışmalarda TDZ %50 ethanolda çözülmüştür. Kotiledon boğumları gövde boğumlarından daha yüksek sürgün rejenerasyon kabiliyetine sahip olmuştur. Her iki çeşitte de en yüksek sürgün rejenerasyonu 0.25 mg/l TDZ içeren MS besin ortamlarında kotiledon boğumlarından elde edilmiştir. Elde edilen sürgünler (10-20 mm uzunlukta) 0.25 mg/l indol-3-butrik asid (IBA) içeren MS ortamında köklendirilmiştir. Köklenen sürgünler son olarak kum içeren saksılara aktarılmıştır.

Anahtar Sözcükler: Thidiazuron, *in vitro*, sürgün rejenerasyonu, kotiledon boğumu.

Introduction

The lentil (*Lens culinaris* Medik.) is an important seed legume widely cultivated in the Middle East, Southern Asia and throughout the tropical and subtropical regions, where it provides a large proportion of the dietary protein requirements. It also improves soil fertility by fixing atmospheric nitrogen, thereby providing an excellent break crop, profitable in its own right to the intensive cereal farmer. However, lentil production is threatened by many insects, diseases and weeds.

Because of its potential usefulness for human consumption in Turkey, we are interested in biotechnological methods to improve this important plant. Development of an efficient regeneration system

would substantially assist breeding of this crop for improvement.

Shoot regeneration has been previously reported by Williams et al. (1986) from the shoot meristem and epicotyl, by Polanco & Ruiz (1997) from the shoot tips, first nodes and bractlets of immature seeds with rooting frequency of 4.6 and 39% on 2 mg/l IAA, by Polanco et al. (1988) and Polanco (2001) from first stem nodes with rooting on NAA or IAA, and by Mallick & Rashid (1989) from cotyledonary seedlings of lentil. Similarly, Cambecedes et al. (1991) and Malik & Saxena (1992a) induced shoot regeneration from axenic seedlings established from mature lentil, chickpea and pea seeds on a medium supplemented with thidiazuron after 1 week of culture. However, previous studies generally involve extensive manipulation of culture conditions to induce shoot regeneration and the frequency of shoot regeneration is not high.

This study includes rapid and simple shoot regeneration from cotyledonary nodes and stem nodes of 2 popular Turkish lentil cultivars on MS media containing TDZ.

Materials and Methods

Plant regeneration

Uniform and uncracked seeds of the lentil genotypes Ali Dayı and Kayı 91 were obtained from the Central Field Crops Research Institute, Ankara, Turkey. The seeds were surface-sterilised in 100% commercial bleach (Axion) for 20 min under continuous stirring, followed by 3 rinses in sterile distilled water. Thereafter, they were cultured in petri dishes (100 x 10 mm diameter) containing regeneration medium. The seed germination medium consisted of mineral salts and vitamins of Murashige and Skoog (1962), 3% sucrose and 0.7% agar (Sigma agar type A). Leaf, stem, stem node and cotyledon node explants were isolated from 2-3 cm long 1 week-old seedlings and cultured on MS, 3% sucrose, 0.7% agar and 0.25, 0.5, 1.0 and 2.0 mg/l TDZ (Table 1). Stock solution of 1 mg/ml TDZ was prepared either by using dimethyl sulphoxide (DMSO; Sigma Technical Information bulletin 1996) or 50% ethanol as solvent. Ethanol diluted TDZ was incorporated into the medium and retained its high activity even after autoclaving. The number of explants producing shoots and the number of shoots per explant were scored after 8 weeks of culture. Regenerated shoots (20 to 30 mm in length) were excised and rooted in sealed Magenta GA-7™ vessels containing rooting media consisting of MS medium, 3% sucrose, 0.7% agar and 0.25 mg/l IBA, and root formation was scored after 4 weeks of culture. The pH of each medium was adjusted to 5.6-5.8 with 1M NaOH or 1M HCl before the addition of agar and autoclaving. All cultures were incubated at 24 ºC under cool white fluorescent light (42 mmol photons m^{-2} s^{-1}) with a 16/8-h light/dark photoperiod.

Rooted plantlets were acclimatised in growth cabinets under relative humidity of 90% during the first 7 days, which was decreased gradually thereafter to 40%, until they were established in a greenhouse. A control was planted without treatment both for shoot regeneration and rooting.

Each treatment was replicated 4 times and contained 5 explants in both regeneration and rooting experiments and was repeated twice. Shoot development was recorded weekly until the end of the experiment. Significance was determined by analysis of variance (ANOVA) using SPSS for Windows (v. 9. SPSS Inc. USA) based on a randomised complete block design. Differences between the means were compared by Duncan's multiple range test using the MSTAT-C computer program (Michigan State University). Data given in percentages were subjected to arcsine ($\sqrt{X}$) transformation (Snedecor and Cochran, 1967) before statistical analysis.

Results and Discussion

TDZ is among the most active cytokinin - like substances and it induces greater in vitro shoot proliferation than many other cytokinins in many plant species. It is very soluble in DMSO with slight solubility in water (NORAM Technical Bulletin, 1987). Although most published reports describe the use of DMSO as a solvent for TDZ, we found that the use of DMSO as a solvent resulted in necrosis on explants, with no callus initiation or shoot regeneration. Endress (1994) points out that DMSO penetrates rapidly into the cells and results in inhibitive dislocation of membrane, proteins and their aggregates in lipid areas. Perbal (1988) also reported that DMSO may have a detachment or killing effect on sensitive cells.

We found that cotyledonary node explants were more responsive than stem nodes on all TDZ concentrations (Tables 1 and 2; $p < 0.01$). Stem nodes did not produce shoots on media containing 1.0 or 2.0 mg/l TDZ. Ahmad et al. (1996-1997) produced optimal shoot regeneration without a callogenic stage on medium containing 2.89 µM GA3 in combination with 1.11 µM BA in MS medium lacking sucrose with rooting on MS medium supplemented with 5.37 µM NAA. The regeneration from cotyledonary nodes was proceeded by 7 to 10 days of callusing followed by green shoot initials on callus within 14 to 18 days. These subsequently developed into normal adventitious shoots by organogenesis after 8 weeks of culture in both genotypes (Figures 1a and b). Mallick & Rashid (1989) also obtained multiple shoot regeneration from cotyledonary nodes of lentil on a medium containing BAP (data not given). Polanco (2001) achieved 5-20 shoots per immature seed of 4 lentil genotypes on media supplemented with BAP. We have obtained a similar frequency of shoot regeneration from cotyledonary node explants using TDZ, which has not been reported previously. Immature seeds are only available during the growth period of the crop, whereas our protocol is not time barred and can be used at any time of the year and is therefore more practical. Fratini & Ruiz (2002) concluded that it is best to regenerate shoots on media containing kinetin or zeatin at low concentrations in order to be able to regenerate roots subsequently. They also found that TDZ inhibits rooting. Ahmad et al. (1996) stresses the use of gibberellic acid in combination with BA in MS medium lacking sucrose for optimal shoot regeneration and rooting in half strength MS medium.

Table 1. Shoot regeneration from different explants of lentil genotype *Ali Dayı* after 8 weeks in culture on MS medium supplemented with various concentrations of TDZ.

Explants /TDZ (mg/l)	Explants producing shoots (%)				Mean number of shoots per explant[1]			
	0.25	0.5	1.0	2.0	0.25	0.5	1.0	2.0
Leaves	0.0[c]	0.0[c]	0.0[b]	0.0[b]	0.0[c]	0.0[c]	0.0[b]	0.0[b]
Stems	0.0[c]	0.0[c]	0.0[b]	0.0[b]	0.0[c]	0.0[c]	0.0[b]	0.0[b]
Stem nodes	43.3[b]	50.0[b]	0.0[b]	0.0[b]	2.1[b]	1.1[b]	0.0[b]	0.0[b]
Cotyl. nodes	86.7[a]	73.3[a]	53.3[a]	86.7[a]	15.6[a]	6.4[a]	4.9[a]	4.1[a]

Each value is the mean of 3 replications with 5 explants each.
Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test ($p < 0.01$).
[1]From explants which regenerated shoots.

Table 2. Shoot regeneration from different explants of lentil genotype *Kayı 91* after 8 weeks in culture on MS medium supplemented with various concentrations of TDZ.

Explants /TDZ (mg/l)	Explants producing shoots (%)				Mean number of shoots per explant[1]			
	0.25	0.5	1.0	2.0	0.25	0.5	1.0	2.0
Leaves	0.0[b]	0.0[c]	0.0[b]	0.0[b]	0.0[c]	0.0[c]	0.0[b]	0.0[b]
Stems	0.0[b]	0.0[c]	0.0[b]	0.0[b]	0.0[c]	0.0[c]	0.0[b]	0.0[b]
Stem nodes	53.3[a]	30.0[b]	0.0[b]	0.0[b]	2.1[b]	0.7[b]	0.0[b]	0.0[b]
Cotyl. nodes	46.7[a]	46.7[a]	36.9[a]	26.8[a]	12.7[a]	5.8[a]	2.7[a]	1.3[a]

Each value is the mean of 3 replications with 5 explants each.
Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test ($p < 0.01$).
[1]From explants which regenerated shoots.

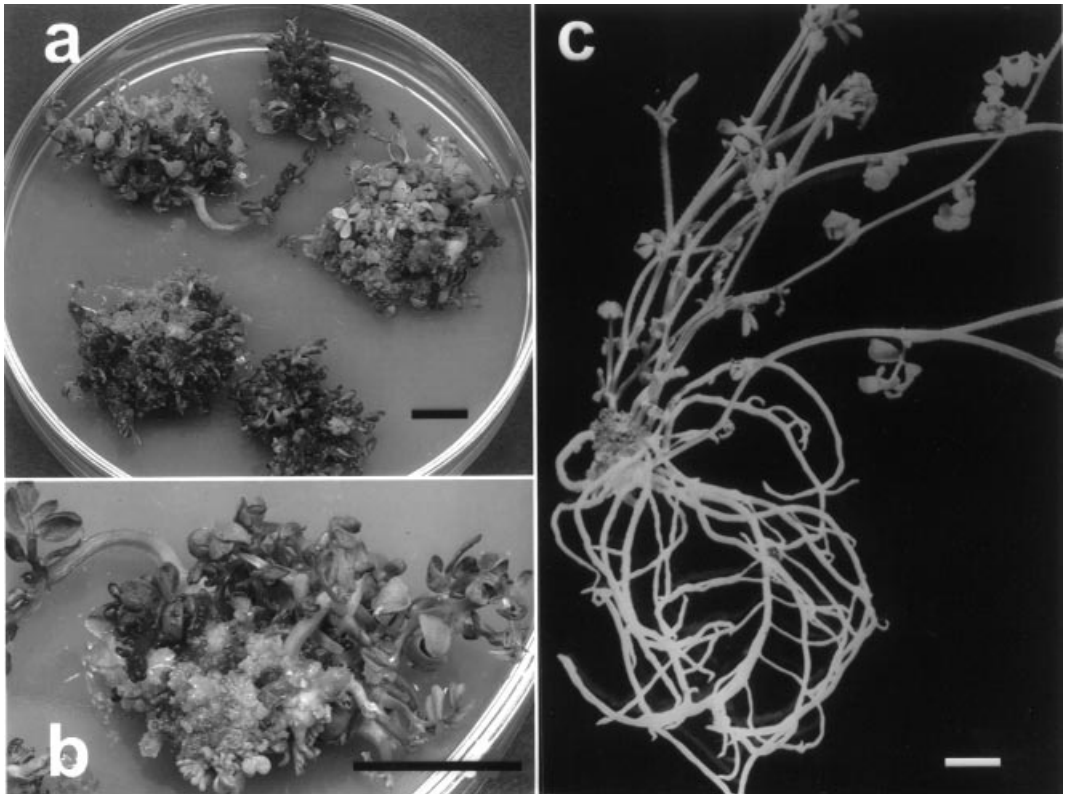

Figure 1. In vitro shoot regeneration from cotyledonary node explants of lentil on MS medium containing TDZ and root formation on regenerated shoots. (*a-b*) Well-developed shoots after 8 weeks in culture. (c) Root development on regenerated shoots on MS medium supplemented with 0.25 mg/l IBA after 4 weeks in culture. Bar = 1 cm.

Similar results were also obtained in *Cercis canadensis* L. var. *alba* (Rehder) Bean. (Yusnita et al., 1990), *Hibiscus rosa-sinensis* L. (Preece et al., 1987) and in muscadine grape (Gray & Benton, 1991) using TDZ. Similarly growth regulator free media failed to induce shoot regeneration on any explant (Tables 1 and 2). Higher TDZ concentrations reduced shoot regeneration and resulted in stunted shoots in both genotypes, as has been reported for pea (Malik & Saxena, 1992a). The highest shoot regeneration capacity was achieved on a MS medium supplemented with 0.25 mg/l TDZ in both genotypes (Tables 1 and 2; $p < 0.01$). These results underline the importance of TDZ and suggest that a lower dose of TDZ induces high frequency of shoot regeneration from cotyledonary nodes. Similarly, Malik & Saxena (1992b) obtained the highest shoot regeneration from nodal and basal regions of primary shoots developed from seed cultures of lentil on media supplemented with relatively low concentrations of TDZ.

Although the reason for the high activity of a low concentration of TDZ has not been investigated in lentil at the molecular level, we assume that TDZ is persistent in the plant tissue and is presumably metabolised in a manner similar to that reported for *Phaseolus lunatus* L. by Mok & Mok (1985). They found that even when bean callus was cultured on a medium supplemented with [^{14}C]-thidiazuron for 33 days, most of the label remained in the TDZ molecule. A portion of TDZ was glycolsylated by the bean tissue, possibly to inactivate the compound for storage. Gill & Saxena (1992) described organogenesis and somatic embryogenesis in intact seedlings of several *Phaseolus* L. species and explant cultures of peanut by using TDZ or BAP. They suggested a crucial role of TDZ in the interaction with endogenous hormones in reprogramming the mode of morphogenesis from organogenesis to somatic embryogenesis possibly by releasing, synthesising, protecting or even inhibiting auxins in situ in combination with other sub-cellular

metabolic changes, particularly in key regulatory enzyme and related proteins. Vinocur (1998), while investigating various levels of BAP and TDZ on shoot regeneration in root explants of aspen, found that TDZ had a marked effect on bud development as compared to BAP, inducing a 10-fold increase in the number of regenerated shoots.

Rooting of excised shoots may be difficult due to the "carry over" effect of TDZ in the rooting medium (Huetteman & Preece, 1993). However, in most species TDZ did not seem to inhibit root formation once shoots were excised (Fasolo et al., 1989; Yusnita et al., 1990; Preece et al., 1991). We found that the MS medium containing 0.25 mg/l IBA resulted in root formation in 25% of excised shoots preceded by an initial callus stage after 4 weeks of culture (Figure 1c; Table 3). Reduced root formation with initial callus growth may be attributed to the high cytokinin activity and "carry over" effect of TDZ. Higher IBA concentrations failed to induce roots even after 2 months of culture. It seems that root formation and elongation are controlled by interactions between multiple factors. It is suspected that higher levels of IBA resulted in dormancy, impeding signals for root development from developed callus. Rooted plantlets were transferred to pots containing sand in a growth chamber with 90% humidity gradually reduced to 60% at 24 °C for acclimatisation. After acclimatisation, they were transferred into a commercial soil mixture. The majority of regenerated plants were established without signs of water stress under greenhouse conditions.

Table 3. Effect of different IBA concentrations on in vitro rooting of lentil.

IBA (mg/l)	Rooting (%)	Mean number of roots/shoot	Mean root length (cm)
0.5	0.0	0.0	0.0
1	0.0	0.0	0.0
2	0.0	0.0	0.0
0.25	25.0	9.1	6.3

Each value is the mean of 4 replications with 4 explants

Previous studies (Polanco et al., 1988) and our results confirmed that rooting of regenerated shoots is a major limiting factor in obtaining whole plants in lentil. To overcome this problem, we also micrografted the regenerated shoots on root stocks from 3-4-day-old in vitro seedlings and achieved 100% graft setting. Micrografted plantlets were then acclimatised to ambient conditions and later transferred to a greenhouse.

In conclusion, the present study underlines the importance of lower concentrations of TDZ for high shoot regeneration from stem and cotyledonary nodes of lentil by organgenesis. The results also indicate partial cell specificity in the regeneration of lentil under in vitro conditions. We are now successful in obtaining transgenic shoots from cotyledonary node explants of various lentil genotypes using disarmed GV 2260, EHA 105 and LBA 4404 *Agrobacterium tumefaciens* strains that will potentially lead to the production of transgenic lentil plants.

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