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Full Length Research Paper

In vitro shoot regeneration of fenugreek (*Trigonella foenum-graecum* L.) using different cytokinins

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Fenugreek (*Trigonella foenum-graecum* L.) is an important vegetable, spice and medicinal legume plant used as fresh and dried leaves and seeds in many parts of the world. Fenugreek seeds contain polysaccharide galactomannan and different saponins and have anti-microbial activity. The study presents an efficient shoot regeneration protocols from 8 - 10 days old *in vitro* grown cotyledon node explants cultured on Murashige and Skoog (MS) medium supplemented with 0.05 - 0.80 mg/l kinetin, 0.25 - 1.0 mg/l 6 benzyladenine (BA) with 0 and 0.20 mg/l α naphtalene acetic acid (NAA) and 0.05 - 0.80 mg/l thidiazuran (TDZ) with 0 and 0.10 mg/l indole 3 butyric acid (IBA). All culture mediums were solidified with 0.22% gelrite. Maximum shoot regeneration and number of shoots per explant were recorded on MS medium supplemented with TDZ with or without IBA. Maximum of 22.21 shoots per explant were recorded on MS medium containing 0.40 mg/l TDZ. Presence of auxins in the culture medium positively increased the mean shoot length. Regenerated shoots were transferred to rooting media containing 0.1 - 1.0 mg/l IBA or NAA.

Key words: Fenugreek , cotyledon node, hypocotyl, shoot regeneration.

INTRODUCTİON

Fenugreek (*Trigonella foenum-graecum* L.) is an important legume used as vegetable, spice and medicinal plant in India, Pakistan, Argentina, Egypt, France, Spain, Turkey, Morocco and China. The young leaves and sprouts are good source of protein, mineral and vitamin C (Khan et al., 2005; Chhibba et al., 2007) and are used as green vegetable in Pakistan and India alone or with potatoes, spinach and meat. The dried leaves have a bitter taste and a strong characteristic smell.

Fenugreek seeds are a rich source of polysaccharide, galactomannan and different saponins such as diosgenin, yamogenin, gitogenin, tigogenin and neotigogens. Other bioactive constituents of fenugreek include mucilage, volatile oils and alkaloids such as choline and trigonelline (Pribacl and Ardelean, 2008). Fenugreek seeds mixed with yogurt are used as a conditioner for hair. Seeds are used for making oily pickles in South Asia. Galactagogue in fenugreek seeds are used to increase milk supply in lactating women (Chantry et al., 2004). Fenugreek seeds also contain high level of iron and phosphorus and are used as insect and pest repellent in grain storage (Billaud

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Abbreviations: NAA, α-Naphtalene acetic acid; **MS,** Murashige and Skoog; **BA,** 6-benzyladenine; **IBA,** indole-3-butyric; **TDZ,** thidiazuran.

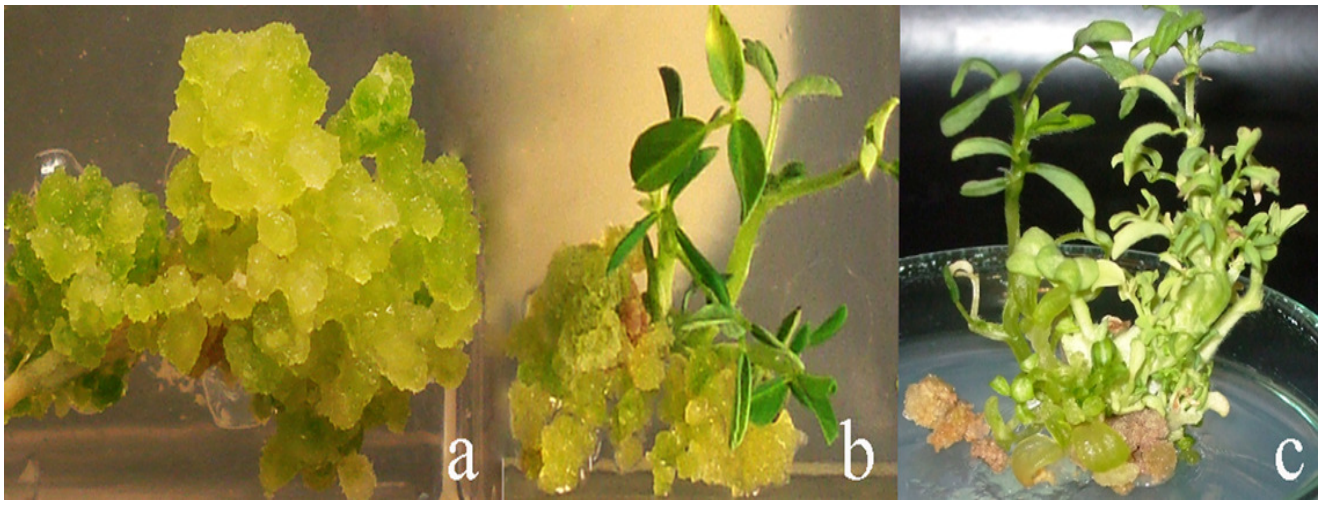

Figure 1. Callus induction and shoot regeneration from hypocotyl and cotyledon node explant. (a) Callus induction on hypocotyl explant; (b) shoot regeneration on cotyledon node explant on MS medium supplemented with BAP and NAA; (c) shoot regeneration on cotyledon node explant on MS medium supplemented with kinetin.

and Adrian, 2001).

Research on the medicinal aspects of fenugreek by-products has taken great importance in the past few years. However, it lacks the efficient and reliable *in vitro* micropropagation protocols. The present study was designed to develop reliable *in vitro* shoot regeneration protocol of fenugreek using different explants and cytokinins-auxin combinations.

MATERİALS AND METHODS

The seeds of cv. Qusoori of fenugreek were obtained from Faisalabad, Pakistan. Healthy and uniform seeds free of injuries were selected and surface sterilized with 70% commercial bleach (Ace, Turkey) for ten minutes followed by 5 min each 3 times rinsing with autoclaved bidistilled water. Sterilized seeds were germinated on MS medium solidified with 0.22% gelrite. Cotyledonary node and hypocotyl explants were isolated from 8 - 10 days old germinated seedlings and cultured under *in vitro* conditions on MS medium (Murashige and Skoog, 1962) containing 0.05 - 0.80 mg/l kinetin; 0.25 - 2.00 mg/l 6 benzyladenine (BA) with 0 and 0.20 mg/l α naphthalene acetic acid (NAA) and 0.05 - 0.80 mg/l TDZ (Thidiazuran) with 0 and 0.1 mg/l indole 3 butyric acid (IBA). After 8 weeks of culture, more than 1 cm long regenerated shoots were taken and cultured on rooting medium containing 0.1, 0.5 and 1 mg/l IBA or NAA.

All culture media was solidified with 0.22% gelrite and pH of all media was adjusted to 5.6 - 5.8 using 1 N NaOH or 1 N HCl before autoclaving at 121°C, 118 kPa for 21 min. All germination, regeneration and rooting cultures were maintained at 24 +2°C at 20 000 lux with 16 h light photoperiod.

Each treatment had 3 replicates containing 8 explants. Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple range test using the Statistical Package for the Social Sciences (SPSS) for Windows 16" computer program. Data given in percentages were subjected to arcsine (√X) transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS AND DISCUSSION

The study presents the effect of cytokinin singly or in combination with auxins on shoot regeneration of fenugreek using cotyledonary node and hypocotyl explant. Explants were cultured on MS medium containing 0.05 - 0.80 mg/l kinetin, 0.25 - 1.00 mg/l BA with or without 0.20 mg/l NAA and 0.05 - 0.80 mg/l TDZ with or without 0.1 mg/l IBA. Results showed that hypocotyl explant was very recalcitrant when compared to cotyledonary node explant and induced callus only on MS medium containing different concentrations of BA with 0.20 mg/l NAA (Figure 1a), whereas, the explants totally failed to give any response to other cytokinins-auxins combinations. Cotyledonary node explant was more responsive and induced callus irrespective of hormone type and concentration.

Results showed that hormone types and concentration in the culture medium significantly affected the frequency (%) of shoot regeneration, mean number of shoots per explant and shoot length of cotyledon node explant.

Shoot regeneration frequency of cotyledonary node explant ranged 66.67 - 91.67% (Table 1), 0 - 66.67% (Table 2) and 54.17 - 100% (Table 3) on MS medium containing kinetin, BA-NAA and TDZ-IBA, respectively. Cotyledon node explants failed to generate shoots on MS medium containing BA without NAA, but inclusion of NAA in the culture medium promoted shoot regeneration and ranged 50.00 - 66.67% (Table 2). Contrarily, 100% shoot regeneration was recorded on TDZ medium devoid of IBA (Table 3) and presence of 0.10 mg/l IBA resulted in decreased shoot regeneration frequency and ranged 54.17 - 87.50% (Table 3).

Number of shoots per explants ranged 4.50 - 8.64 on

Table 1. Effects of various concentrations of kinetin on shoot regeneration from cotyledon node explant of fenugreek.

Kinetin (mg/l)	Frequency (%) of shoot regeneration	Number of shoots per explant	Shoot length (cm)
0.05	66.67^{b}	4.44^{b}	1.57^{ns}
0.10	75.00^{ab}	4.67^{b}	1.50
0.20	66.67^{b}	4.50^{b}	1.47
0.40	91.67^{a}	8.64^{a}	1.43
0.80	75.00^{ab}	5.00^{b}	1.37

Values within a column followed by different letters are significantly different at the 0.05 probability level by Duncan test.

Table 2. Effects of various concentrations of BAP-NAA on shoot regeneration from cotyledon node explant of fenugreek

BAP (mg/l)	NAA (mg/l)	Frequency (%) of shoot regeneration	Number of shoots per explant	Shoot length (cm)
0.25	-	0.00^{b}	0.00^{b}	0.00^{c}
0.50	-	0.00^{b}	0.00^{b}	0.00^{c}
0.75	-	0.00^{b}	0.00^{b}	0.00^{c}
1.00	-	0.00^{b}	0.00^{b}	0.00^{c}
0.25	0.20	58.33^{a}	3.67^{ab}	1.63^{b}
0.50	0.20	50.00^{a}	4.33^{ab}	1.70^{b}
0.75	0.20	58.33^{a}	8.72^{a}	1.83^{ab}
1.00	0.20	66.67^{a}	4.94^{ab}	2.17^{a}

Values within a column followed by different letters are significantly different at the 0.05 level by Duncan test ($p < 0.05$).

Table 3. Effects of various concentrations of TDZ-IBA on shoot regeneration from cotyledon node explant of fenugreek.

TDZ (mg/l)	IBA (mg/l)	Frequency (%) of shoot regeneration	Number of shoots per explant	Shoot length (cm)
0.05	-	100.00^{a}	12.08^{c}	1.45^{cd}
0.10	-	100.00^{a}	13.08^{bc}	1.39^{cd}
0.20	-	100.00^{a}	13.58^{bc}	1.27^{cde}
0.40	-	100.00^{a}	22.21^{a}	1.10^{fg}
0.80	-	100.00^{a}	17.67^{b}	1.02^{g}
0.05	0.10	87.50^{b}	8.96^{d}	2.01^{a}
0.10	0.10	87.50^{b}	10.63^{cd}	1.79^{b}
0.20	0.10	79.17^{c}	11.67^{cd}	1.46^{c}
0.40	0.10	62.50^{d}	15.63^{bc}	1.28^{cde}
0.80	0.10	54.17^{e}	13.33^{bc}	1.19^{fg}

Values within a column followed by different letters are significantly different at the 0.05 level using Duncan test.

on MS medium containing 0.05 - 0.80 mg/l kinetin with maximum of 8.64 shoots which were obtained on MS medium containing 0.40 mg/l kinetin (Table 1). Shoots per explant on MS medium containing BA-NAA ranged 3.67 - 8.72 with maximum of 8.72 shoots on 1.0 mg/l BA with 0.20 mg/l NAA contained culture media (Table 2). TDZ with or without IBA produced more number of shoots when compared to kinetin and BA-NAA and ranged 8.96 - 22.21 (Table 3). Inclusion of IBA in the culture medium drastically decreased the number of shoots per explant. Though shoots produced on MS medium containing TDZ without IBA were hyperhydric (Figure 2a), normal shoots were obtained on MS medium containing kinetin (Figure 1b), BA-NAA (Figure 1c) and TDZ-IBA (Figure 2b).

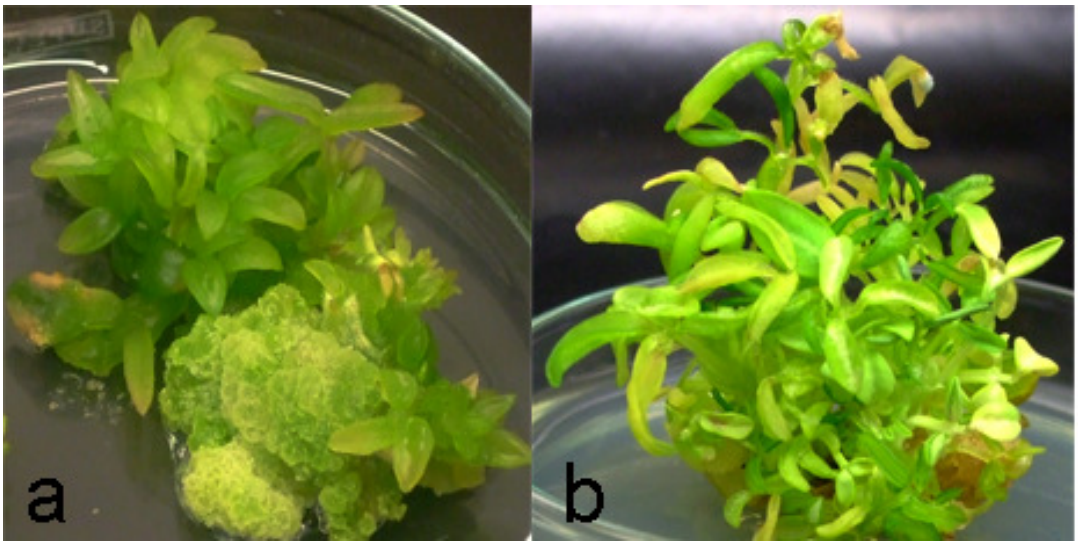

Figure 2. Shoot regeneration from cotyledon node explant. (a) Hyperhydric shoots on MS medium supplemented with TDZ and (b) normal shoots on MS medium supplemented with TDZ-IBA.

Shoot length on culture medium containing kinetin was statistically not significant and ranged 1.37 - 1.57 cm (Table 1). Shoot length increased with increase in BA concentration and decreased with increase in TDZ concentration irrespective of the presence of IBA in the culture media. However, addition of 0.1 mg/l IBA with TDZ showed promotory effect and resulted in longer shoots. Shoot length on MS medium containing BA-NAA and TDZ-IBA ranged 1.63 - 2.17 cm (Table 2) and 1.02 - 2.01 cm (Table 3), respectively.

Regenerated shoots from all cultures were isolated aseptically under *in vitro* conditions and rooted on MS medium containing 0.1 - 1.0 mg/l IBA or NAA. However, no shoot could be rooted and generated callus followed by secondary shoot regeneration from the base of shoot in the rooting medium containing IBA. More experiments are in progress to induce roots of this recalcitrant legume under *in vitro* conditions.

BA, TDZ and kinetin are generally used cytokinins for *in vitro* shoot regeneration, singly or in combination with auxins in legumes. Cotyledon node explant has been used for successful shoot regeneration of *Vigna radiata* (Gulati and Jaiwal, 1994), cowpea (Obembe et al., 2000; Van Le et al., 2002; Diallo et al., 2008), *Vicia narbonensis* (Kendir et al., 2008; Kendir et al., 2009), hungarian vetch (Sahin-Demirbag et al., 2008a), *Trifolium pannonicum* (Sahin-Demirbag et al. 2008b) and asiatic *Vigna* species (Avenido and Hattori, 2001) and in genetic transformation in lentil (Khawar et al. 2004, Dogan et al., 2005,), wild lentil (Sevimay et al. 2005), cowpea (Popelka et al., 2006., Chaudhary et al., 2007), chickpea (Sanyal et al., 2003) and *Pisum sativum* (Svabova et al., 2005).

The results of this study showed that cotyledonary node explant was more responsive when compared to hypocotyl explant. Hypocotyl explant induced callus only on MS medium containing BA-NAA concentrations with no shoot regeneration on all culture media. Cotyledonary node explant responded well but variably to cytokinin type concentration and presence or absence of auxins in the culture medium. MS medium supplemented with BA devoid of NAA resulted in total failure of cotyledon node explant to induce shoots. However, addition of NAA exploited the explant and 50.0 - 66.67% shoot regeneration frequency was achieved. However, other culture media contained kinetin and TDZ-IBA. TDZ singly induced 100% shoot regeneration which decreased significantly with the presence of IBA. Malik and Saxena (1992), Khawar and Özcan (2002), Khawar et al. (2004,) and Sevimay et al. (2005) achieved the highest shoot regeneration in lentil on MS medium containing TDZ. Addition of IBA with TDZ resulted in decreased shoot regeneration frequency. Aasim et al. (2009a) also reported inhibitory effect of IBA with TDZ on shoot regeneration from apical meristem of fenugreek.

Likewise, shoot regeneration frequency and number of shoots per explant were more on MS medium supplemented with TDZ with or without IBA when compared with kinetin and BA-NAA. However, TDZ concentration promoted the hyperhydric or vitrified shoots which are in line with Aasim et al. (2009a) who also reported vitrification due to TDZ in fenugreek. However, inclusion of IBA along with TDZ positively overcame the vitrification problem and no vitrified shoots were recorded on MS medium containing TDZ-IBA. Aasim et al. (2009a) also reported positive effect of IBA on overcoming the problem of vitrification in fenugreek. There was no sign of vitrification on shoots regenerated on either kinetin or BA-NAA. Ziv (1991) reported cytokinin concentration in the

culture media as one of the factors for vitrification.

Results further showed that shoot length was dependant on cytokinin type and concentration and presence or absence of auxin in the culture medium. Shoot length on MS culture medium containing BA-NAA gradually increased with increase in BA concentration. These results arecontrary to the findings of Aasim et al. (2009b) who reported adverse effects of increased BA concentration on shoot length. Each increase of TDZ concentration decreased the shoot length (Aasim et al., 2009a) and positively increased with the addition of IBA in the culture medium. Brar et al. (1997) and Aasim et al. (2009a,b) also reported the promotry effect of auxins in the culture media on shoot length in cowpea.

In vitro rooting studies focuses on application of phyto-hormones, especially auxins, to induce a rooting response. The recalcitrant nature of legumes towards rooting has slowed down the application of biotechnological tools in legume crops (Fratini and Ruiz, 2003). Regenerated shoots cultured on MS medium containing 0.1 - 1.0 mg/l IBA or NAA failed to induce rooting in fenugreek. No rooting in fenugreek is due to carry over effects of cytokinins and are in line with the findings of Polanco and Ruiz (1997). Similarly, Fratini and Ruiz (2002) reported negative effect of TDZ and BA during the shoot induction phase on induction of roots in lentil. Huetteman et al., (1993) and Aasim et al. (2009a) also reported negative effects of TDZ on rooting. However, Aasim et al.(2008b) in red squill and Aasim et al. (2009c) in cowpea did not find carry over effect of TDZ on rooting.

On the basis of these results, it is concluded that TDZ alone or in combination with IBA is more potent for *in vitro* shoot regeneration and produced more number of shoots when compared to kinetin and BA-NAA. However, TDZ alone can cause vitrification and presence of IBA helps in solving vitrification in fenugreek. Regenerated shoots failed to root and more studies are needed to overcome this problem in fenugreek.

REFERENCES

Aasim M, Khawar KM, Özcan, S (2008a). *In vitro* Micro Propagation From Shoot Meristems of Turkish Cowpea (*Vigna unguiculata* L.) Cultivar Akkız. Bangladesh J. Bot. 37: 149-154.

Aasim M, Khawar KM, Özcan S (2008b). *In vitro* Regeneration of Red Squill *Urginea maritima* (L.) Baker. Using Thidiazuron. Biotech. Biotech. Eq. 22(4): 925-928.

Aasim M, Khawar KM, Sancak C, Özcan S (2009a). *In vitro* shoot regeneration of Fenugreek (*Trigonella foenumgraceum* L.). Am-Eu J. Sustain. Agric. 3: 135-138.

Aasim M, Khawar KM, Ozcan S (2009b). *In vitro* micropropagation from plumular apices of Turkish cowpea (*Vigna unguiculata* L.) cultivar Akkiz. Sci. Hortic. 122: 468-471.

Aasim M, Khawar KM, Ozcan S (2009c). Comparison of shoot regeneration on different concentrations of TDZ from shoot tip explant of cowpea on gelrite and agar containing medium. Not. Bot. Hort. Agrobot. Cluj, 37(1): 89-93

Avenido RA, Hattori K (2001). Benzylaminopurine preconditioning in germinating mungbean seedlings stimulated axillary buds in cotyledonary nodes resulting in multiple shoot regeneration. Breed. Sci. 51: 137-142.

Billaud C, Adrian J (2001). Fenugreek composition, nutritional value and Physiological properties. Sci. Aliments, 21: 3-26.

Brar MS, Al-Khayri JM, Shamblin CE, McNew RW, Morelock TE, Anderson EJ (1997). *In Vitro* shoot tip multiplication of cowpea *Vigna unguiculata* (L.) Walp. *In Vitro* Cell. Dev. Biol. 33: 111-118.

Chantry CJ, Howard CR, Montgomery A, Wight N (2004). Use of glactogogues in initiating or augmenting maternal milk supply. The academy of breastfeeding medicine.

Chaudhary D, Madanpotra S, Jaiwal R, Saini A, Kumar P, Pawan JK (2007). *Agro bacterium tumefaciens*-mediated high frequency genetic transformation of an Indian Cowpea (*Vigna unguiculata* L.Walp) cultivar and transmission of transgene into progeny. Plant Sci. 172: 692-700.

Chhibba IM, Nayyar VK, Kanwar JS (2007). Influence of Mode and Source of Applied Iron on Fenugreek (*Trigonella corniculata* L.) in a Typic Ustochrept in Punjab, India. Int. J. Agric. Biol. 9: 254-256.

Diallo M S, Ndiaye A, Sagna M, Gassama-Dia YK (2008). Plants regeneration from African cowpea variety (*Vigna unguiculata* L. Walp.). Afr. J. Biotechnol. 7: 2828-2833.

Dogan D, Khawar KM, Özcan S (2005). *Agrobacterium* Mediated tumor and hairy root formation from different explants of lentils derived from young seedlings. Int. J. Agric. Bio. 7:1019-1025.

Fratini R, Ruiz ML (2002). Comparative study of different cytokinins in the induction of morphogenesis in lentil (*Lens culinaris* Medik.). *In Vitro* Cell. Dev. Biol. Plant. 38: 46-51.

Fratini R, Ruiz ML (2003). A rooting procedure for lentil (*Lens culinaris* Medik.) and other hypogeous legumes (pea, chickpea and Lathyrus) based on explant polarity. Plant Cell Rep. 21: 726-732

Gulati A, Jaiwal PK (1994). Plant regeneration from cotyledonary node explants of mungbean (*Vigna radiata* L. Wilczek). Plant Cell Rep. 13: 523-527.

Huetteman SE, Schotz AH, Wardley-Richardson T, Spencer D, Higgins TJV (1993). Transformation and regeneration of two cultivars of pea *Pisum sativum* L. Plant Physiol. 101: 751-577.

Kendir H, Sahin-Demirbag N, Khawar KM, Aasim M (2008). *In vitro* plant regeneration from Narbon Vetch (*Vicia narbonensis* L.) using cotyledonary node explants. Afr. J. Biotechnol. 7: 2491-2494.

Kendir H, Sahin-Demirbag N, Aasim M, Khawar KM (2009). *In vitro* plant regeneration from Turkish Narbon Bean (*Vicia narbonensis* L.). Afr. J. Biotechnol. 8: 614-618.

Khan MB, Khan MA, Sheikh M (2005). Effect of phosphorus levels on growth and yield of fenugreek. Int. J. Agric. Biol. 7: 504-507.

Khawar KM, Özcan S (2002). High frequency shoot regeneration from cotyledonary node explants of different lentil (*Lens culinaris* Medik) genotypes and *in vitro* micrografting. Biotech. Biotech. Eq. 16: 12-17.

Khawar KM, Sancak C, Uranbey S, Özcan S. (2004). Effect of Thidiazuron on shoot regeneration from different *explants of le*ntil (*Lens culinaris* M*edik) vi*a organogenesis. Turk. J. Bot. 28: 421-426.

Malik KA, Saxena PK (1992). Thidiazuron induces high frequency shoot regeneration in intact seedlings of pea (*Pisum sativum*), chickpea (*Cicier arietinum*) and lentil (*Lens culinaris*). Aust. J. Plant Physiol. 19: 731-740.

Murashige T, Skoog F (1962). A revised medium for ra p id g rowt h a nd bioas s ays with tobacco tissue cultures. Physiol. Plant, 15: 473-497.

Obembe OO, Kadiri M, Machuka J (2000). Induction of multiple shoots and regeneration from cotyledonary nodes and epicotyls. (Abstracts) Afr. J. Plant Sci. 108: p. 20. (World cowpea Research Conference III, 4-7 September 2000, Ibadan, Nigeria. p. 32).

Polanco MC, Ruiz ML (1997). Effect of benzylaminopurine on *in Vitro* and *in vivo* root development in lentil (*Lens culinaris* Medik.). Plant Cell Rep. 17: 22-26

Popelka JC, Gollasch S, Moore A, Molvig L, Huggins TJV (2006). Genetic transformation of cowpea and stable transmission of the transgenes to progeny. Plant Cell Rep. 25: 304-312.

Pribacl C, Ardelean A (2008). *In Vitro* culture of *Trigonella foenum-graecum* plantules and their anatomic characterization. EMC 2008 14th European Microscopy Congress 1-5 September 2008, Springer Berlin Heidelberg, Aachen, Germany, 3: 181-182.

Sahin-Demirbag N, Kendir H, Khawar KM, Aasim M (2008a). *In vitro*

plant regeneration from Hungarian vetch (*Vicia pannonica* Crantz) using cotyledonary node explants. Biotech. Biotech. Eq. 22: 929-932.
Sahin-Demirbag N, Kendir H, Khawar KM, Aasim M, (2008b). *In vitro* regeneration of Turkish endemic *Trifolium pannonicum* JACQ. Subsp. elongatum (WILLD). Biotech. Biotech. Eq. 22 (4): 921-924.
Sanyal I, Singh AK, Amla DV (2003). *Agrobacterium tumefaciens* mediated transformation of chickpea (*Cicer arietinum* L.) using mature embryogenic axis and cotyledonary nodes. Indian J. Biotechnol. 2: 524-532.
Sevimay CS, Khawar KM, Yuzbasioglu E (2005). Adventitious shoot regeneration from different explants of wild lentil (*Lens culinaris* subsp. *orientalis*) Biotech. Biotech. Eq. 19: 46-49
Svabova L, Smykal P, Griga M, Ondrej V (2005). *Agrobacterium*-mediated transformation of *Pisum sativum in vitro* and *in vivo*. Biologia Plantarum, 49: 361-370.
Van Lee BUI, De Carvalho MHC, Zuily-Fodil Y, Thi ATP, Van KTT (2002). Direct whole plant regeneration of cowpea (*Vigna unguiculata* (L.) Walp) from cotyledonary node thin cell layer explants. J. Plant Physiol. 159: 1255-1258.
Ziv M (1991). Quality of micropropagated plants-vitrification. *In Vitro* Cell. Dev. Biol. 27: 64-69.