Australian Journal of Crop Science AJCS
AJCS 5(6):668-673 (2011) ISSN:1835-2707

Direct shoot regeneration from stem nodal explants of two wild *Medicago* species- *Medicago scutellata* and *Medicago rigidula*

Somaieh Maleki Band, Mortaza Ghadimzadeh, Morad Jafari* and Iraj Bernousi

Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Urmia, Urmia, Iran

***Corresponding author: m.jafari@urmia.ac.ir**

Abstract

Adventitious shoot-bud regeneration from nodal explants of two diploid *Medicago* species was established. Nodal explants isolated from *in vitro* germinated seedlings (30-day-old) were cultured on Murashige and Skoog (MS) medium with Gamborg's (B5) vitamins supplemented with different concentrations of 6-Benzylaminopurine (BAP)/α-Naphthaleneacetic acid (NAA) or thidiazuron (TDZ)/Silver nitrate ($AgNO_3$). The potential of shoot-bud induction and shoot regeneration per nodal explants significantly ($P < 0.05$) intensified with decreasing concentration of TDZ (from 0.5 to 0.1 mg l^{-1}) or with increasing level of BAP (from 0.5 to 1 mg l^{-1}). However, we found no significant difference between the two species in view of shoot development and regeneration capacity. TDZ at 0.1 mg l^{-1} in combination with 3 mg l^{-1} $AgNO_3$ gave the maximum shoot-buds induction (average of 5.57 shoot buds per explant) and shoot regeneration response (average of 5.34 shoots per explant) in both species. Our results showed that the incorporation of $AgNO_3$ into the medium promoted both the parameters recorded. Whereas, Augmenting NAA level at the same levels of BAP did not show significantly ($P < 0.05$) improvement of shoot-bud induction. Regenerated shoots were elongated properly on MS medium fortified with 0.05 mg l^{-1} TDZ in a short time (10-14 days). Profuse rooting was achieved in 67-89% of plantlets on ½ strength MS medium containing 1.5 mg l^{-1} indole-3-butyric acid (IBA) and 1.5 mg l^{-1} NAA. The rooted plantlets were acclimatized successfully in the greenhouse with a survival rate of 95% and no visible morphological alteration was observed among these plants.

Keywords: cytokinins; diploid *Medicago* species; medium; shoot-bud induction; regeneration.
Abbreviations: $AgNO_3$-Silver nitrate; BAP-6-Benzylaminopurine; IBA- indole-3-butyric acid; NAA- α-naphthaleneacetic acid; PGRs- plant growth regulators; SEM- shoot elongation medium; SIM-shoot induction medium; TDZ- thidiazuron.

Introduction

One-third of the genus *Medicago* are comprised of annual medic species, majority of which are diploid, self-pollinated and are superior to perennial alfalfa species from the viewpoints of faster growth, feed quality, and crude protein value (Zhu et al., 1996). More than two hundred fifty phyto-geographical regions of Iran have been delineated for the potential distribution of these alfalfa species (Heidari-Sharifabad and Torknejad, 1990). Annual alfalfa medics would be a valuable source of the genes for resistance or tolerance to biotic and abiotic stresses (Iantcheva et al., 1999). Therefore, they are more valid for molecular and genetic studies in comparison to the perennial alfalfa species (possess very high level of heterozygosity and allogamous nature). Development of a proper and efficient method for fast and massive *in vitro* regeneration of plant is one of the most important aims of tissue culture systems and is a prerequisite for successful applications of genetic transformation technology (Zare et al., 2009). The regeneration of annual medics is more difficult than those perennials. For a limited number of these species, most of the protocols have been reported indirect regeneration method via callusogenesis and somatic embryogenesis. Nolan et al. (1989) reported the first protocol of M. truncatula regeneration through indirect somatic embryogenesis and similar protocols have also been used for regeneration of other annual alfalfa species such as *M. polymorph, M. littoralis*, *M. suffruticosa, and M. lupulina* (Iantcheva et al., 2005). Compared with the direct regeneration protocols, indirect regeneration is so time-consuming and laborious process that can exhibit somaclonal variation and abnormal morphological characteristics. The direct regeneration has been reported in only a limited numbers of annual medics such as *M. truncatula, M. littoralis, M. murex, M. polymorpha* (Iantcheva et al., 1999), *M. arabica, M. orbicularis* (Iantcheva et al., 2005). There is no information about the regeneration capability of annual medics, especially in the species growing in Iran hemisphere. Therefore, this study describes a rapid and efficient regeneration protocol for two wild *Medicago* species, *M. rigidula* and *M. scutellata* by induction of multiple shoots from stem node explants on media containing different concentrations of BAP and TDZ that is applicable for a wide range of annual medics.

Materials and methods

Seed germination and explant preparation

Seeds of *M. rigidula* and *M. scutellata* were provided from Agriculture and Natural Resources Research Centre of West Azerbaijan, Iran. The seeds were surface sterilized by immersion in 70% (v/v) ethanol for 1 min and 5% (v/v) sodium hypochlorite for 10 min with gentle shaking followed by 3 times rinses in sterilized water for 5 min. Sterilized seeds were germinated on 0.8% water-agar solidified in jars

Table 1. Effects of different concentrations of BAP and NAA on induction of shoot-bud and regeneration of shoots derived from nodal stem of two annual medics, *M. rigidula* and *M. scutellata* after 2 weeks of culture.

Medium	Concentrations (mg l^{-1})		Mean no. of induced shoot-buds per explants		Mean no. of regenerated shoots per explants	
	BAP	NAA	*M. rigidula*	*M. scutellata*	*M. rigidula*	*M. scutellata*
HF	0	0	1.00 c	1.00 c	0.38 d	0.37 d
SIM I-1	0.5	0.02	3.29 b	2.46 b	3.13 c	2.50 c
SIM I-2	0.5	0.2	3.79 b	2.77 b	3.21 bc	2.66 c
SIM I-3	1	0.02	4.47 a	4.16 a	4.13 a	3.99 b
SIM I-4	1	0.2	4.92 a	4.66 a	4.55 a	4.33 a

Values followed by different lower case letters within a column indicate significant differences at $P < 0.05$ according to DMRT.

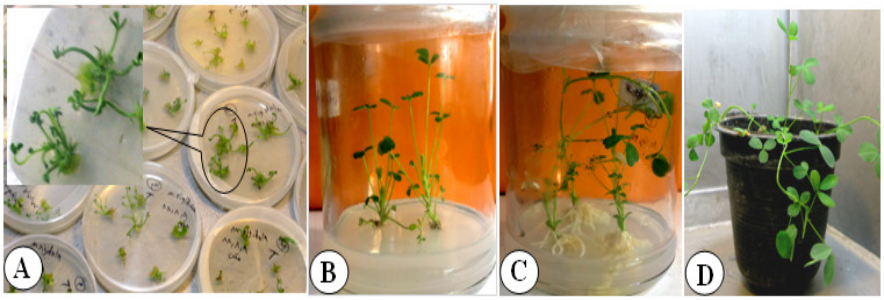

Fig 1. Different stages of shoot-bud induction, shooting, and rooting in two annual medic species, *M. rigidula* and *M. scutellata*. A) Multiple shoot-bud induction on stem node explants and shoot regeneration, B) shoot growth in growing media, C) shoot rooting in root induction media, D) An in vitro derived acclimatized plants after 4-week of the transfer to plastic pot containing 2:1:1 mixture of soil, peat and perlite.

at 25 ± 2°C in the darkness. Nodal stem segments (1 cm size) with small adjoining parts of petiole base were prepared from 30-day-old *in vitro*-growing plants by discarding internodes as well as trifoliates and were then used as the experimental materials.

Basal medium and Culture conditions

In all of the experiments, MSB medium comprised of MS (Murashige and Skoog, 1962) salts and B5 (Gamborg et al., 1968) vitamins containing 3% sucrose and 0.8% plant agar was used as basal medium. The pH of the media was adjusted to 5.8 prior to the inclusion of agar and autoclaved for 20 min at 121°C. TDZ and $AgNO_3$ were added to the medium after autoclaving by filter sterilization (0.22 µm Millipore). All the cultures were kept in growth chambers at 20 ± 2°C under a 16/8 h (light/dark) photoperiod at a photon flux rate of 60 µmol m^{-2} s^{-1} provided by cool daylight fluorescent lamps.

Shoot-bud induction and regeneration

To investigate the effects of plant growth regulators (PGRs) in shoot-bud induction, two independent experiments were carried out. In the first experiment, the explants were cultured in Petri dishes (100 × 10 mm) containing shoot induction medium I (SIM I) supplemented with different concentrations and combinations of BAP (0.0, 0.5 and 1 mg l^{-1}) and NAA (0.0, 0.02 and 2 mg l^{-1}). In the second experiment, shoot induction medium II (SIM II) containing different concentrations of TDZ (0.0, 0.1 and 0.25 mg l^{-1}) with or without 3 mg l^{-1} $AgNO_3$ was used for culture of the explants. In both experiments, explants were subcultured on the same shoot induction media at 2 weeks interval. After four weeks, differentiated micro-shoots (1.5–2 cm) were excised and individually transferred on shoot elongation medium (SEM) supplemented with 0.05 mg l^{-1} TDZ till the shoots attain a suitable height. The number of shoot-bud per explant and the number of regenerated shoot per explant were calculated for each treatment.

Rooting and acclimatization

For *in vitro* rooting studies in both the species, elongated shoots (3-4 cm long) were transferred to half-strength MSB basal medium supplemented with factorial combinations of TDZ and NAA at two different concentrations (1 and 1.5 mg l^{-1}). Some shoots were maintained on auxin free ½ MSB medium as control. The percentage of rooted shoots and length of roots were recorded for each treatment. For *ex vitro* acclimatization, well rooted plants were gently rinsed with tap water to remove the remnants of agar and transferred to plastic filled with a mixture of autoclaved peat, perlite and soil (1:1:2) moistened with MSB basal liquid medium and kept in a mist chamber with 80–90% relative humidity at a day/night temperature regime of 20 ± 2°C under a 16 h photoperiod for four weeks. The regenerates were finally transferred into greenhouse conditions.

Statistical Analysis

Each treatment had 4 replicates consisting of Petri dishes containing 10 explants for shoot-bud induction and regeneration studies. For rooting experiment, each treatment had 4 replicates consisting of jars containing 3 shoots each. The experiments were repeated two times and the results were pooled. In all experiments, plant species and phytohormonal treatment as the two factors studied were considered. Factorial analysis of variance (ANOVA) based on completely randomized design (CRD) was performed on the data with the General Linear Model procedure using SAS 9.1 software and the means were compared using Duncan's multiple range test (DMRT) at the 5% probability level.

Table 2. Effects of different concentrations of TDZ and $AgNO_3$ on induction of shoot-bud and regeneration of shoots derived from nodal stem of two annual medics, *M. rigidula* and *M. scutellata* after 2 weeks of culture.

Medium	TDZ (mg l^{-1})	$AgNO_3$ (mg l^{-1})	Mean no. of induced shoot-buds per explants		Mean no. of regenerated shoots per explants	
			M. rigidula	*M. scutellata*	*M. rigidula*	*M. scutellata*
HF	0	0	1.00 f	1.00 e	0.38 f	0.37 e
SIM II-1	0.1	0	4.46 b	4.66 b	4.33 b	4.00 b
SIM II-2	0.1	3	5.64 a	5.50 a	5.28 a	5.39 a
SIM II-3	0.25	0	3.19 d	3.29 c	2.92 d	3.12 c
SIM II-4	0.25	3	3.86 c	3.67 c	3.66 c	3.33 c
SIM II-5	0.5	0	2.33 e	2.28 d	2.09 e	1.88 d
SIM II-6	0.5	3	2.66 e	2.49 d	2.33 e	2.28 d

Values followed by different lower case letters within a column indicate significant differences at $P < 0.05$ according to DMRT.

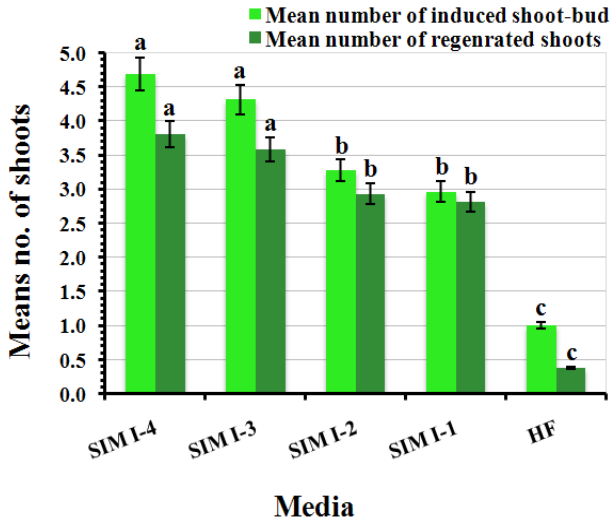

Fig 2. Effect of SIM I containing different concentrations of BAP combined with NAA on induction of shoot-bud and shoot regeneration of two annual medics, *M. rigidula* and *M. scutellata*. The bars represent means ± SE. Bars (separately for each species) followed by the same letter are not significantly different ($P < 0.05$) according to DMRT.

Results

Effect of growth regulator on shoot-bud Induction and Regeneration

Nodal segments cultured on MSB medium without any PGRs (HF medium) were differentiated into single shoot primordia per an explant within 1-2 weeks. Multiple shoot buds were induced at the periphery of the petiole base on nodal segments cultured on both SIM media supplemented with various PGRs and shoots with developing trifoliate were visible by 2 weeks of culture (Fig. 1A). Based on the results of the first experiment, shoot-bud development and shoot regeneration were significantly affected by SIM I media containing various levels of BAP in combination with NAA and analysis of variation revealed significant ($P < 0.05$) differences between the media in both medics (Table 1). Nonetheless, increasing concentration of NAA from 0.02 to 0.2 mg l^{-1} at the same level of BAP did not significantly intensify shoot-bud induction. The maximum number of induced shoot-bud and shoot regeneration was achieved on SIM I-3 and SIM I-4 media containing 1 mg l^{-1} of BAP irrespective of NAA (Fig. 2). The mean number of induced shoot-buds per explant on SIM I ranged from 2.64 to 4.66 in *M. scutellata* and 3.29 to 4.92 in *M. rigidula*. In a second set of experiments, the effects of SIM II media containing different concentrations of TDZ with or without 3 mg l^{-1} of $AgNO_3$ were examined for adventitious shoot regeneration from nodal stem segments. Analysis of data indicated that number of shoot-buds and regenerated shoots significantly affected by different levels of TDZ (Table 2). The highest number of shoot-bud (total mean number, 5.55 shoot-buds per explant) was induced on SIM II-2 containing 0.1 mg l^{-1} of TDZ and 3 mg l^{-1} $AgNO_3$ within 7-10 days and in this combination, shoot regeneration was as high as (total mean number, 5.36 shoot per explant) (Fig. 3). We found out that appending $AgNO_3$ (3 mg l^{-1}) into SIM II media at the same TDZ level had a significant effect on both shoot-bud development and shoot regeneration, especially in *M. rigidula*. However, Increasing concentration of TDZ significantly resulted ($P < 0.05$) in steady decreases for both the parameters recorded regardless of $AgNO_3$. The number of induced shoot-buds per explant varied from 2.33 to 5.64 in *M. rigidula* and 2.28 to 5.50 in *M. scutellata* and no significant difference was found in their capacity of shoot development. Any vitreous shoots appeared during regeneration and the regenerated shoots were elongated properly (4-8 cm length) in media containing 0.05 mg l^{-1} of TDZ within 10-14 days (Fig. 1B).

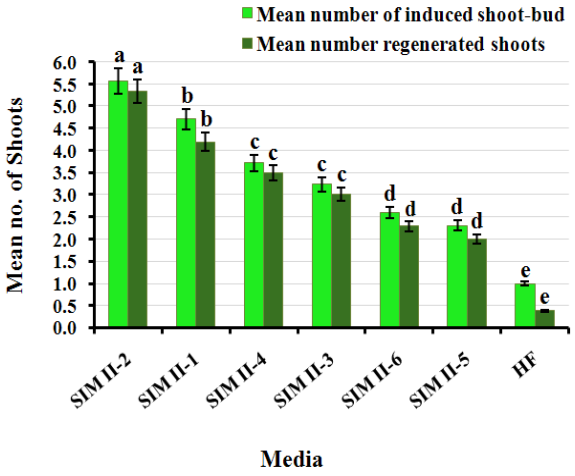

Fig 3. Effect of SIM II media supplemented with different concentrations of TDZ with or without $AgNO_3$ on shoot-bud induction and shoots regeneration of two annual medics, *M. rigidula* and *M. scutellata*. The bars represent means ± SE. Bars (separately for each species) followed by the same letter are not significantly different (P < 0.05) according to DMRT.

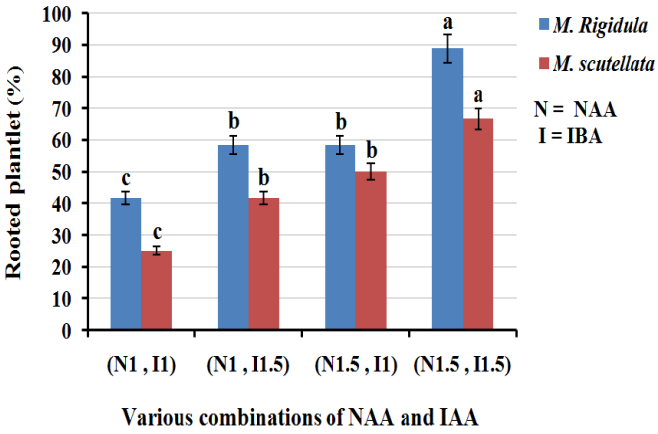

Fig 4. Rooting response of the regenerated shoots of two annual medics, *M. rigidula* and *M. scutellata* cultured on ½ MSB medium supplemented with combinations of NAA and IAA at two various levels. The results are expressed as percentage rooting. The bars represent means ± SE. Bars (separately for each species) followed by the same letter are not significantly different ($P < 0.05$) according to DMRT.

Root induction and recovery of complete plants

Roots emerged from the basal end of the shoots of both medics in all the cultures on ½ MS medium supplemented with various combinations of NAA and IBA within 3-4 weeks (Fig. 1C). The rooting treatments showed significantly ($P < 0.05$) difference in terms of percentage rooting (Fig. 4) and root forming capacity was also found to be statistically significant ($P < 0.05$) between the medics (data not shown). The highest frequency of root induction (67% and 89% for *M. rigidula* and *M. scutellata*, respectively) was achieved from medium supplemented with 1.5 mg l^{-1} NAA and 1.5 mg l^{-1} IAA. All the regenerants acclimatized well in the greenhouse and then under outdoor conditions (Fig. 1D). A survival of 95% was achieved when the rooted shoots were transferred to pots containing soil, peat and perlite in the proportion of 2:1:1 supplemented with ¼ MS salts solution and under regular irrigation with tap water. The acclimatized plants did not show any visible variations from the mother plants.

Discussion

Shoot-bud induction using combination of BAP and NAA

In the natural status, the axillary buds of the higher plants are dormant due to apical dominance and the mechanism of apical dominance has been demonstrated to be under the control of various growth regulators specially auxin (Cline, 1996). Cutting the stem into segments and culturing them on medium supplemented with suitable PGRs can break the dormancy of the bud (Dai et al., 2006; Punyarani and Sharma, 2010). In general, Bud induction and development of multiple shoots from stem node explants is a function of cytokinin activity (Sahoo and Chand, 1998). The role of BAP in bud breaking has already been reported in many plants (Sivanesan and Jeong, 2007; Safdari and Kazemitabar, 2010; Alam et al., 2010). It has been reported that a combination of cytokinin and auxin is well suitable for the shoot regen - eration and morphogenesis of the annual medic species (Smolenskaya and Ibragimova, 2002). Combinations of cytokinin such as BAP and Kin with low level of auxin (e.g. IAA or NAA) have also been used to induce shoot formation in numerous other plants (Patnaik and Debata, 1996; Chen, 2001; Sivanesan and Jeong, 2007; Sunil, 2009). The present study also revealed that shoot-bud induction and shoot regeneration from nodal segments in both medic species improve with increasing level of BAP, Whereas these parameters no significantly affected by increasing NAA level (Table 1). In consistent with our results, Safdari and Kazemitabar (2010) reported the unsuitability of BAP at low concentrations for shoot regeneration from nodal segments of *Portulaca grandiflora.*

Shoot-bud induction using TDZ in combination with $AgNO_3$

TDZ is among the most active cytokinin–like substances and it induces greater *in vitro* shoot induction and proliferation than many other cytokinins in many plant species (Khawar, 2004). Adventitious bud regeneration under TDZ affect might depend on the plant's internal growth regulators and it balances the internal oxine level of the plant (Hutchinson and Saxena, 1996). Recent researches have been reported high adventitious bud and regeneration ratio under TDZ affect in several legume plants (Luo, 1993; Kim et al., 1997; Hosseini-Nasr and Rashid, 2003; Uranbey, 2005; Sajid and Aftab, 2009). Our results indicated promotion of multiple shoot formation by both SIM media augmented with TDZ or BAP. But reducing the concentration of TDZ in the media significantly increased (more than 50%) the number of shoot induction and the number of shoot regeneration per nodal explant and vice versa. This was in contrast to the results of BAP treatment. TDZ at lower concentration (0.1 mg l^{-1}) proved to be the best treatment for bud induction and regeneration from nodal explants of both medics. Recent studies on the shoot induction and regeneration of *Medicago sativa* (Li et al., 2009) and *Holarrhena antidysenterica* (Mallikarjuna and Rajendrudu, 2007) also reported the

effectiveness of TDZ at lower concentrations. The established procedure for regeneration promoted by TDZ, efficient for two others annual *Medicago species* (Iantcheva et al., 1999, 2005). Ethylene is considered to suppress shoot organogenesis and callus embryogenesis *in vitro* (Beyer, 1976). $AgNO_3$ is a potent inhibitor of ethylene action and has been shown to increase shoot proliferation when used with cytokinins such as TDZ (Chi, 1990; Cogbill et al., 2010). The beneficial effects of $AgNO_3$ on shoot organogenesis have been reported in many plant species (Mohiuddin et al., 1997; Zhang et al., 2001; Akasaka-Kennedy, 2005; Anantasaran and Kanchanapoom, 2008; Cogbill et al., 2010; Thiruvengadam et al., 2010). Li et al. (2009) also reported the positive effect of $AgNO_3$ on adventitious shoot formation from cotyledonary nods of perennial alfalfa (*Medicago sativa*). In consistent with these reports, our results also indicated that the inclusion of $AgNO_3$ in the medium improved the potential of shoot formation and regeneration in both medic species to some extent and this effect was significantly appeared for *M. rigidula* (Table 2). This genotype-dependent effect of $AgNO_3$ has also been reported in *Phoenix dactylifera* (Al-Khayri and Al-Bahrany, 2004), *B. napus* (Uliaie et al., 2008) *and Zinnia* cultivars (Anantasaran and Kanchanapoom, 2008). The regenerated shoots well elongated at lower level of TDZ (0.05 mg l^{-1}) since a high concentration of cytokinins produce inhibitory effect on shoot elongation and it can also lead to shoot vitrification. Shoot vitrification did not appear in this study. Root formation was observed on all ½ MSB media containing different concentrations and combinations of NAA and IBA. But most plants rooted significantly on medium supplemented with high concentration of NAA and IBA combination (each 1.5 mg l^{-1}) in both medics (Fig. 4). Poor root initiation was observed form the shoots regenerated on higher concentration of TDZ and BAP. This is in agreement with findings by Naik et al. (2000) and Mohamed et al. (2006), who have shown that long exposure of the shoots to high concentration of either TDZ or BAP, suppressed the capacity of shoots to form adventitious roots. The good response of small genome sized annual medics to *in vitro* culture media was reported for callus induction (Iantcheva et al., 2003) and direct regeneration (Iantcheva et al., 2005). In this study, the *M. rigidula* species with small genome size (2n= 14) showed better response in culture media compared to *M. scutellata* with bigger (two times) genome size (2n= 30).

Conclusion

The simple and reliable regeneration protocol presented in this study was successfully applied for other annual and perennial *Medicago* species (Data not shown). It can be used for very advantageous for a variety of purposes, including mass multiplication of annual *Medicago* species close to extinguish, plant breeding studies and transgenic plant production. The desired transgenic plants can be generated from annual medics with using mentioned method within a period of 45 days, since the regeneration of large number of transformants in short time interval is a major pre-requisite for genetic transformation of plants.

Acknowledgements

We are thankful to Mr. Odisho at the Agriculture and Natural Resources Research Centre, Jahad-e-Keshavarzi Organization, West Azarbaijan, Iran for providing the seeds.

References

Akasaka-Kennedy Y, Yoshida H, Takahata Y (2005) Efficient plant regeneration from leaves of rapeseed (*Brassica napus* L.): the influence of $AgNO_3$ and genotype. Plant Cell Rep 24, 649–654

Alam MJ, Alam I, Sharmin SA, Rahman MM, Anisuzzaman M, Alam MF (2010) Micropropagation and antimicrobial activity of *Operculina turpethum* (syn. *Ipomoea turpethum*), an endangered medicinal plant. POJ 3(2):40-46

Al-Khayri JM, Al-Bahrany AM (2004) Genotypedependent in vitro response of date palm (*Phoenix dactylifera* L.) cultivars to silver nitrate. Sci Hortic-Amsterdam 99 (2): 153–162

Anantasaran J, Kanchanapoom K (2008) Influence of medium formula and silver nitrate on *in vitro* plant regeneration of zinnia cultivars. Songklanakarin J Sci Technol 30 (1): 1–6

Beyer EM (1976) A potent inhibitor of ethylene action in plants. Plant Physiol 58 (3): 268–271

Chen CC, Chen SJ, Sagare AP, Tsay HS (2001) Adventitious shoot regeneration from stem internode explants of *Adenophora triphylla* (Thunb.) A. DC. (Campanulaceae) -An important medicinal herb. Bot Bull Acad Sin 42: 1–7

Chi GL, Barfield DG, Sim GE, Pua EC (1990) Effect of silver nitrate and aminoethoxyvinylglycine on in vitro shoot and root organogenesis from seedling explants of recalcitrant *Brassica* genotypes. Plant Cell Rep 9: 195–198

Cline MG (1996) Exogenous auxin effects on lateral bud outgrowth in decapitated shoots. Ann Bot 78:255–266

Cogbill S, Faulcon T, Jones G, McDaniel M, Harmon G, Blackmon R, Young M (2010) Adventitious shoot regeneration from cotyledonary explants of rapid-cycling fast plants of *Brassica rapa* L. Plant Cell Tiss Organ Cult 101:127–133

Dai Y, Wang H, Li B, Huang J, Liu X, Zhou Y, Mou Z, Li J (2006) Increased expression of mapkinase kinase causes deficiency in polar auxin transport and leads to plant architectural abnormality in Arabidopsis. Plant Cell 18:308–320

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Heidari-Sharifabad H, Torknejad A (1990) Annual *Medicago* species. 1st edn. Research Institute of Forest and Rangelands, Tehran, IRI

Hosseini-Nasr M, Rashid A (2003) Thiadiazuron-induced high frequency shoot regeneration from root region of *Robinia pseudoacacia* L. seedlings. Biol PLANTARUM 47: 591–596

Hutchinson MJ, Saxena PK (1996) Acetylsalicylic acid enhances and synchronizes thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium* × *hortorum* Bailey) tissue culture. Plant Cell Rep 15**:** 512–515

Iantcheva A, Brown S, Atanassov A (2003) Flow cytometric analysis in diploid *medicago* species from algeria: Relationship between genome size and competence for direct somatic embryo formation. Biotechnol. & Biotechnol., 17(2): 44-49

Iantcheva A, Vlahova M, Atanassov A (2005) Investigation of the potential of two wild *medicago* species–*medicago orbicularis* and *medicago arabica* for *in vitro* callusogenesis and direct organogenesis. Biotechnol. & Biotechnol. Eq 19(3): 27–31

Iantcheva A, Vlahova M, Bakalova E, Kondorosi E, Elliott MC, Atanassov A (1999) Regeneration of diploid annual medics via direct somatic emberyogenesis promoted by thidiazuron and benzylaminopurine. Plant Cell Rep 18:904–910

Khawar KM, Sancak C, Uranbey S, Zca S (2004) Effect of thidiazuron on shoot regeneration from different explants of lentil (*lens culinaris* medik.) via organogenesis. Turk J Bot 28: 421–426

Kim MK, Sommer HE, Bongarten BC, Merkle SA (1997) High frequency induction of adventitious shoots from hypocotyl segments of *Liquidambar styraciflua* L. by thidiazuron. Plant Cell Rep 16: 536–540

Li JJ, Wu YM, Wang T, Liu JX (2009) *In vitro* direct organogenesis and regeneration of *Medicago sativa.* Biol PLANTARUM 53 (2): 325–328

Luo CY (1993) The use of thidiazuron in tissue culture. In Vitro Cell Dev Biol-Pl 29: 92–96

Mallikarjuna K, Rajendrudu, G (2007) High frequency *in vitro* propagation of *Holarrhena antidysenterica* from nodal buds of mature tree. Biol Plant 51: 525–529

Mohamed SV, Sung JM, Jeng TL, Wang CS (2006) Organogenesis of *Phaseolus angularis* L.: high efficiency of adventitious shoot regeneration from etiolated seedlings in the presence of N6-benzylaminopurine and thidiazuron. Plant Cell Tiss Org 86 (2): 187–199

Mohiuddin AKM, Chowdhury MKU, Zaliha CA, Suhaimi N (1997) Influence of silver nitrate (ethylene inhibitor) on cucumber *in vitro* shoot regeneration. Plant Cell Tiss Org 51: 75–78

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–476

Naik SK, Pattnaik S, Chand PK (2000). *In vitro* propagation of pomegranate (*Punica granatum* L. cv. Ganesh) through axillary shoots proliferation from nodal segments of mature tree. Scientia Hort 79: 175–183

Nolan KE, Rose RJ, Gorst JR (1989) Regeneration of *Medicago truncatula* from tissue culture increased somatic embryogenesis using explants from regenerated plants. Plant Cell Rep 8:278–281

Patnaik J, Debata BK (1996) Micropropagation of Hemidesmus indicus (L.) R. Br. through axillary bud culture. Plant Cell Rep 15: 427–430

Punyarani K, Sharma GJ (2010) Micropropagation of *Costus speciosus* (Koen.) using nodal segment culture. Not Sci Biol 2 (1): 58–62

Safdari Y, Kazemitabar SK (2010) Direct shoot regeneration, callus induction and plant regeneration from callus tissue in Mose Rose (*Portulaca grandiflora* L.). POJ 3(2):47-51

Sahoo Y, Chand PK (1998) *In vitro* multiplication of medical herb *Tridax procumbens* L. (Mexican daisy, coat button) influence of explanting season, growth regulator, synergy, culture passage and passing substrate. Phytomorphology 48: 195–205

Sajid ZA, Aftab F (2009) Effect of thidiazuron (TDZ) on *in vitro* micropropagation of *solanum tuberosum* l. cvs. desiree and cardinal. Pak J Bot 41(4): 1811–1815

Sivanesan I, Jeong BR (2007) Direct shoot regeneration from nodal explants of Sida cordifolia Linn. In Vitro Cell Dev- Pl 43:436–441

Smolenskaya, SA, Ibragimova, SS (2002) Restorative Morphogenesis in vitro in the Annual Medics in the Presence of Benzylaminopurine, Russian Journal of Developmental Biology 33 (6): 349–354

Sunil B, Abdullah JO, Sreeramanan S, Karuthan C (2009) Shoots Induction from *Hibiscus Rosa-sinensis* Nodal Explant Using *N*6-benzylaminopurine (BAP). Res J Agr Biol Sci 5(4): 403–410

Thiruvengadam M, Rekha KT, Yang CH, Jayabalan N, Chung IM (2010) High-frequency shoot regeneration from leaf explants through organogenesis in bitter melon (*Momordica charantia* L.). Plant Biotechnol Rep 4: 321–328

Uliaie ED, Farsi M, Ghreyazie B, Imani J (2008) Effects of genotype and $AgNO_3$ on shoot regeneration in winter cultivars of rapeseed (*Brassica napus*). Pak J Biol Sci 11(16): 2040–2043

Uranbey S (2005) Thidiazuron induced adventitious shoot regeneration in henbane (*Hyoscyamus niger* L.). Biol Plant 49 (3): 427–430

Zare N, Valizadeh M, Tohidfar M, Mohammadi SA, Malboobi MA, Habashi AA (2009) Selection of regenerative genotypes from Iranian alfalfa cultivars. J Food Agric Environ 7 (3&4): 567–572

Zhang P, Phansiri S, Puonti-Kaerlas J (2001) Improvement of cassava shoots organogenesis by the use of silver nitrate *in vitro*. Plant Cell Tiss Org 67: 45–54

Zhu, YP, Sheaffer CC, Barnes, DK, Zhu YP (1996) Forage yield and quality of six annual *Medigaco* species in the north central USA. Agron J 88: 955–960