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BRIEF COMMUNICATION

Adventitious shoot regeneration and micropropagation in *Calendula officinalis* L.

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Abstract

Hypocotyl, cotyledon and cotyledonary node explants of *Calendula officinalis* L were cultured on Murashige and Skoog (MS) media supplemented with various concentrations of thidiazuron (TDZ), kinetin (KIN), α-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) to induce adventitious shoot regeneration and micropropagation. The highest frequency of adventitious shoot regeneration was achieved from hypocotyl and cotyledon explants on MS media supplemented with 0.75 mg dm^{-3} TDZ and either 0.25 or 0.50 mg dm^{-3} IBA. Efficient *in vitro* clonal propagation was also induced from cotyledonary nodes on a range of media supplemented with 0.75 mg dm^{-3} TDZ and 0.05 mg dm^{-3} NAA or 2 mg dm^{-3} KIN and 1 mg dm^{-3} NAA. Regenerated shoots were excised and rooted in MS medium supplemented with 1 mg dm^{-3} NAA. The rooted plantlets were finally transferred to pots.

Additional key words: clonal propagation, cotyledon, cotyledonary node, hypocotyl, indole-3-butyric acid, kinetin, α-naphthalene-acetic acid, organogenesis, thidiazuron.

Calendula (*Calendula officinalis* L.) is a native plant of the Mediterranean region (Earle *et al*. 1964) and is grown widely across Europe and North America as an ornamental and medicinal plant. This species is known to have antiseptic and anti-inflammatory activities (Boucard-Maitre *et al*. 1988). *In vitro* micropropagation allows the production of pathogen-free uniform plants and conservation of germplasm. Large amount of secondary metabolites can also be produced from *in vitro* cultures. *In vitro* shoot regeneration and micro-propagation from *Calendula* has not been reported previously. Here, we describe, for the first time, a rapid and prolific adventitious shoot regeneration and *in vitro* micropropagation system from various explants of *Calendula*.

Seeds of *Calendula officinalis* L were obtained from the Department of Field Crops, Faculty of Agriculture, University of Ankara, Turkey. Seeds were surface-sterilized in 50 % commercial bleach (*Axion*) for 15 min and washed 3 times in sterile distilled water. Surface sterilized seeds were germinated in Petri dishes containing Murashige and Skoog (1962; MS) medium supplemented with 3 % sucrose and 0.7 % agar. For adventitious shoot regeneration studies, hypocotyl (0.5 cm) and half cotyledon explants were excised from *in vitro* grown seedlings after 1 week of culture. Cotyledon explants were cut across by discarding the petiole and the lower 1 - 2 mm of cotyledon base and divided into two equal parts. Edges of cotyledons were also trimmed off. Cotyledonary nodes used in micropropagation studies were excised from *in vitro* developing seedlings after 10 d of culture. All explants

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Abbreviations: IBA - indole-3- butyric acid; KIN - kinetin; MS - Murashige and Skoog medium; NAA - α-naphthalene-acetic acid; TDZ - thidiazuron.
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were cultured in glass Petri dishes containing 35 cm^3 of MS regeneration medium containing 3 % sucrose, 0.8 % agar and various concentrations of thidiazuron (TDZ), kinetin (KIN), α-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA). The pH of medium was adjusted to 5.7 with 1 M NaOH or 1 M HCl before autoclaving at 121 ºC, 1.4 kg cm^{-2} for 20 min. All cultures were kept at 24 ± 2 ºC under cool white fluorescent light (irradiance of 35 µmol m^{-2} s^{-1}) with 16-h photoperiod. Well developed shoots were transferred to MS medium supplemented with 1 mg dm^{-3} NAA in *Magenta* vessels for rooting. The rooted shoots were then transferred to compost in pots. Each treatment had 3 replicates containing 10 explants for adventitious shoot regeneration and 5 explants for micropropagation experiments. Significance was determined by analysis of variance (*ANOVA*) 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 arcsine (√X) transformation (Snedecor and Cochran 1967) before statistical analysis.

Table 1. Adventitious shoot regeneration from hypocotyl and cotyledon explants of *Calendula officinalis* on MS media containing various concentrations of TDZ, KIN, NAA and IBA [mg dm^{-3}]. Values within a column followed by different letters are significantly different at the 0.05 level.

TDZ	KIN	NAA	IBA	Explants producing shoots [%]		Number of shoots [$explant^{-1}$]	
				hypocotyl	cotyledon	hypocotyl	cotyledon
0.50				16.7 c	50.0 cd	3.34 bc	4.34 cde
0.50		0.05		66.7 b	76.7 bc	4.00 bc	6.67 bc
0.50		0.10		70.0 b	80.0 bc	6.34 b	3.34 de
0.75				13.3 c	43.3 cd	1.34 cd	2.67 e
0.75		0.05		0.0 d	10.0 e	0.00 d	6.00 bcd
0.75		0.10		80.0 b	56.7 cd	3.34 bc	3.34 de
0.75			0.25	100.0 a	100.0 a	6.34 b	8.01 ab
0.75			0.50	80.0 b	93.3 ab	9.34 a	10.34 a
	1.0	0.50		73.3 b	53.3 cd	3.34 bc	2.34 e
	1.0	1.00		53.3 b	33.3 de	4.67 b	4.67 cde
	2.0	0.50		66.7 b	53.3 cd	5.67 b	3.01 de
	2.0	1.00		66.7 b	86.7 ab	5.01 b	6.67 bc

Table 2. *In vitro* clonal propagation of calendula from cotyledonary nodes on MS media containing various concentrations of TDZ, KIN, NAA and IBA [mg dm^{-3}]. Values within a column followed by different letters are significantly different at the 0.05 level.

TDZ	KIN	NAA	IBA	Number of shoots [$explant^{-1}$]	Shoot length [cm]
0.50				4.00 def	1.83 g
0.50		0.05		5.67 cde	2.17 fg
0.50		0.10		5.33 de	2.00 fg
0.75				10.00 a	2.00 fg
0.75		0.05		8.67 ab	4.00 cd
0.75		0.10		6.33 bcd	1.90 g
0.75			0.25	3.33 ef	2.50 fg
0.75			0.50	4.33 def	2.63 efg
	1.0	0.25		3.33 ef	5.33 ab
	1.0	0.50		5.00 de	3.33 def
	1.0	1.00		5.00 de	5.28 abc
	1.0		0.25	3.67 ef	5.50 a
	1.0		0.50	2.33 f	3.87 de
	2.0	0.50		4.33 def	4.50 abcd
	2.0	1.00		8.00 abc	4.17 bcd

After 3 weeks of culture, hypocotyl and cotyledon explants produced morphogenic callus at wound sites. Green shoot initials formed on this callus and subsequently developed into normal shoots after 6 - 7 weeks of culture initiation. Table 1 summarizes the shoot regeneration from hypocotyl and cotyledon explants on MS medium containing various concentrations of TDZ, KIN, NAA and IBA. Considering both percentage of explants producing shoots and the mean number of shoots per explant, the highest shoot regeneration capacity was achieved from both explants on MS media supplemented with 0.75 mg dm^{-3} TDZ and either 0.25 or 0.50 mg dm^{-3} IBA. In general, addition of KIN and NAA to growth media reduced the frequency of shoot organogenesis. Results clearly showed that TDZ induced high frequency of shoot regeneration underlining the importance of TDZ in efficient morphogenesis from cultured explants. Wang *et al*. (1991) found changes in key enzymes and other regulatory elements in response to TDZ. Wang and Faust (1988) observed that TDZ induces accumulation of unsaturated polar membrane fatty acids, which strongly propose the active involvement of TDZ in inducing metabolic changes and suggested that it is not a

simple substitute of purine based cytokinins. Vinocur *et al*. (2000) while investigating various levels of benzyl-aminopurine (BAP) and thidiazuron on bud regeneration in root explants found that TDZ had a more marked effect on bud development as compared with BAP, inducing a tenfold increase in the number of buds regenerated from various root explants in *Populus tremula*.

Prolific shoot meristems were observed within 3 weeks on cotyledonary nodes which were later developed into normal shoots after 4 - 5 weeks of culture initiation. All cotyledonary nodes produced shoots on all media tested. The mean number of shoots per explant varied significantly with the varying concentrations of TDZ, KIN, NAA and IBA in the basal medium (Table 2; $P < 0.05$). The highest number of shoots per explant was achieved on media supplemented with 0.75 mg dm^{-3} TDZ alone or 0.75 mg dm^{-3} TDZ and 0.05 mg dm^{-3} NAA or 2 mg dm^{-3} KIN and 1 mg dm^{-3} NAA. The longest shoots were produced on a MS medium containing 1 mg dm^{-3} KIN and 0.25 mg dm^{-3} IBA (Table 2). Addition of TDZ to the media reduced the shoot length significantly ($P < 0.05$). Although TDZ induces shoot proliferation (Vinocur *et al*. 2000, Khawar and Özcan 2002), it has inhibited shoot elongation in several woody plants (Huetteman and Preece 1993, Lu 1993) as found in the present study. Multiple shoot formation has also been reported from cotyledonary nodes of lentil (Polanco 2001, Khawar and Özcan 2002). The results obtained in previous studies and in the current work confirm the regeneration potential of cotyledonary nodes.

Regenerated shoots (10 - 20 mm in length) were excised and rooted readily within 3 weeks in half-strength MS medium containing 1 mg dm^{-3} NAA. Approximately 80 % of regenerated shoots rooted on this medium within four weeks. The rooted shoots were transferred to pots and acclimatized to ambient conditions in growth cabinet and later established under greenhouse conditions.

In conclusion, to our knowledge the present study is the first report for *in vitro* shoot regeneration in *Calendula*. The procedure presented here provides a rapid and prolific shoot regeneration system that may be used for rapid propagation and transformation of the plant.

References

Boucaurd-Maitre, Y., Algernon, O., Raynaurd, J.: Cytotoxic and antitumoral activity of *Calendula officinalis* extracts. - Pharmazie **43**: 220-221, 1988.

Earle, F.R., Miklojajczak, K.L.,Wolf, I.A.: Search for new industrial oils: Seed-oils of the *Calendula*. - J. amer. Oil chem. Soc. **41**: 345-347, 1964.

Huetteman, C.A., Preece, J.E.: Thiadiazuron: a potent cytokinin for woody plant tissue culture. - Plant Cell Tissue Organ Cult. **33**: 105-119, 1993.

Khawar, K.M., Özcan, S.: High frequency shoot induction from cotyledonary node explants of different lentil (*Lens culinaris* Medik) genotypes and *in vitro* micrografting. - Biotechnol. Biotechnol. Equipment **16**: 12-17, 2002.

Lu, C.Y.: The use of thidazuron in tissue culture. - *In vitro* cell. dev. Biol. Plant **29**: 92-96, 1993.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Polanco, M.C.: Factors that affect plant regeneration from *in vitro* culture of immature seeds in four lentil genotypes. Plant Cell Tissue Organ Cult. **66**: 133-139, 2001.

Snedecor, G.W., Cochran, W.G.: Statistical Methods. - The Iowa State University Press, Iowa 1967.

Vinocur, B., Carmi, T, Altman, A., Ziv, M.: Enhanced bud regeneration in aspen (*Populus tremula* L) roots cultured in liquid media. - Plant Cell Rep. **12**: 1146-1154, 2000.

Wang, S.Y., Faust, M.: Changes of fatty acids and sterols in apple buds during the break induced by a plant bioregulator, thidiazuron. - Physiol. Plant. **82**: 231-236, 1988.

Wang, S.Y., Jiao, H.J., Faust, M.: Changes in activities of catalase, peroxidase and polyphenyl oxidase in apple bud break induced by thidiazuron. - J. Plant Growth Reg. **10**: 33-39, 1991.