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TDZ induction in somatic embryogenesis of natural tetraploid *Trifolium pratense* L.

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1 ABSTRACT

Plant regeneration of the natural tetraploid *T.pratense L.* cv. Elçi (red clover) could have been done only through the apical meristem calli. In order to proceed to the stage of production, other regeneration methods need to be experimented. One of these methods is production of somatic embryo from different explants. Hypocotyl, cotyledon, apical meristem, epicotyl and young primary leaves explants were used for the production of somatic embryo in Thidiazuron (TDZ) containing MS media. The best somatic embryo production was realized 95 % from apical meristem explants in 7.5 mg/L TDZ containing MS medium in 4-5 weeks. At the same time somatic embryos were produced from apical meristem, hypocotyl, epicotyl, cotyledon explants in same TDZ concentrations. The morphology of somatic embryos determined rarely abnormal structures including horn shaped, hyperhydration and vitrification embryoids.

2 INTRODUCTION

Legumes are one of the most important food crops due to their high nutrition value, medicinal properties (effects of phytoestrogen and cardiovascular) as well as their use in livestock feeding. *Trifolium pratense* L. (Red clover), one of the most studied diploid species, is widely grown worldwide. In addition to the classical methods, *in vitro* methods have a significant place in agricultural studies. For large-scale clonal propagation of elite cultivars, somatic embryogenesis provides an alternative approach to conventional micropropagation. It is a beneficial tool in plant biotechnology. Synthetic (artificial) seed can be improved from somatic embryos. Production of somatic embryos is comfortable method according to direct seeding of elite cultivars or providing a means of moving germplasm in a less fragile form than *in vitro* plantlets. Indirect embryogenesis via callus or secondary embryogenesis may support in the application of genetic improvements. Genetic selection can be made by production of somatic embryos. Somatic embryogenesis offers functional models for understanding molecular, regulatory and morphogenetic events during plant embryogenesis (Deo *et al.*, 2010). Natural tetraploid *T. pratense* L. is a special plant, which is grown in Turkey. The plant has high biomass efficiency and is an important medicinal plant (Elçi, 2005; Colgecen *et al.*, 2014). However, seed settings and hard seed problems hinder production of natural tetraploid *T. pratense*. In her Ph.D. thesis, Bakar (1995) assessed seed setting of natural tetraploid *T. pratense* by counting seeds in 100 capitatus each year throughout a period of 4 years and calculated seed setting ratio as 4.6%. Considering that each capitatus contains about 90-150 flowers, the calculated seed setting ratio is remarkably low (Bakar Büyükkartal, 2008). Previous plant regeneration of the natural tetraploid *T.pratense* could have been realized only through the apical meristem calli (Colgecen and Toker, 2008). In order to proceed to a lot of production, other regeneration methods need to be experimented. One of these methods for *in vitro* plant regeneration is somatic embryogenesis.

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Although, there are many researches on somatic embryogenesis in *Trifolium* species and Fabaceae species, a few studies has been completed for natural tetraploid *T. pratense*. The somatic embryos of natural tetraploid *T. pratense* were produced from hypocotyl (85%), cotyledon(75%), apical meristem (60%) explants in 0.3 mg/L 2,4-D and 2 mg/L Kinetin containing MS medium(Colgecen *et al*., 2016). Available many studies for somatic embryogenesis in literature are limited to diploid *Trifolium* varieties. Somatic embryo production from cultures of *T. pratense* and *T.repens* was reported with 2,4-D, adenine, BAP, kinetin (Phillps and Collins, 1980; Maheswaran and Williams, 1984, 1985, 1986; Radionenko *et. al*., 1994). In white clover (*Trifolium repens* L.), Cytokinin was reported to enhance embryogenic cell formation derived from immature zygotic embryos, hence, exogenous growth regulators were suggested to modify cell polarity by interfering with pH gradients or electro potential at a cellular level (Deo *et al*., 2010). Thidiazuron (TDZ; N-phenyl-N¢-[1,2,3 thidiazolyl]-urea), a substituted phenylurea, has been found to be more effective than all the adenine-type cytokinins in inducing *in vitro* organogenesis and somatic embryogenesis in some Fabaceae species (Lakshmanan and Taji, 2000; Iantcheva *et.al*., 2005; Chhabra *et al*., 2008; Saini and Chopra, 2012). The aims of this study were to produce somatic embryos faster and more efficient from aseptic seedlings by using different TDZ concentrations in natural tetraploid *T. pratense*.

3 MATERIALS AND METHODS

Trifolium pratense L. was collected from the Tortum vicinity of Erzurum, Turkey, by Elci (1982). This study examined natural tetraploid *T. pratense* L. E2 type (2n = 4x = 28 chromosomes). Natural tetraploid *T. pratense* L. was grown in the experimentation gardens of Bülent Ecevit University's Department of Biology in the Faculty of Arts and Sciences. Aseptic seedlings (15-day-old with unifoliate primary leaf) were used as the explant source. Seeds were first sterilized in 96% ethanol for one minute and then transferred to 10% sodium hypochlorite solution for 10 minutes (commercial sodium hypochlorite was used in the sterilization process). Then seeds were rinsed 3 times in autoclaved distilled water. After being scarified with autoclaved sandpaper, seeds were germinated on hormone-free MS medium (Murashige & Skoog, 1962). All the samples were incubated at 24±2°C with a 16/8-hour photoperiod (irradiance of 42-µmol m-2 s-1 provided by cool-white fluorescent tubes). Hypocotyl (0.5-1cm), cotyledon (whole and in two fragments), apical meristem (1mm), epicotyl (0.5-1cm) and young primary leaves (whole and divided into two fragments) of aseptically grown seedlings provided explant tissues. The explants were cultured in Petri dishes in the dark (100 mm x 15 mm) (Colgecen and Toker, 2008). Due to delayed response of embryogenic calli after the eighth week, the embryogenic calli were subcultured onto the media. Embryoid callus was produced in MS media supplemented with TDZ (2.5, 5, 7.5 mg/L) as plant growth regulators. Torpedo-stage somatic embryos were incubated at 24±2°C with a 16/8-hour photoperiod (irradiance of 42-µmol m-2 s-1 provided by cool-white fluorescent tubes). All media were adjusted to pH 5.8 before autoclaving (Sucrose 20 g, agar 7 g). Each treatment was replicated three times and arranged in a completely randomized design. The data given in percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967) before statistical analysis. The data were analyzed using one-way analysis of variance (ANOVA) and the differences among means were compared by Duncan's multiple-range test.

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3 RESULTS AND DISCUSSION

Callus formation started within 3-4 days in all explants of natural tetraploid *T. pratense*. The calli were yellow and formed clusters in later days of culture having nodular and compact appearance. Embryogenic calli were observed within 3-5 weeks. It is faster than 2, 4-D+Kinetin combination (4-6 weeks) (Colgecen *et al.*, 2016). First somatic embryo formation was observed in calli in all other trials after 4-5 weeks of incubation. Globular somatic embryos were helpful for observing the differences among the media. Globular somatic embryos were formed on calli in all trials. Somatic embryos proceeding through heart and torpedo stages were followed. A large number of somatic embryos in apical meristem explants were observed in the 7.5 mg/L TDZ containing MS medium (95%) (Table 1, Figure 1a). Actually in the same medium the apical meristems, hypocotyls and cotyledons were generated somatic embryos highly ratio. In 2.5 mg/L TDZ containing MS medium with the most successful somatic embryo production, average rate of hypocotyl explant-derived indirect somatic embryogenesis was calculated as 85% (Table 1, Figure 1b). Generally, hypocotyl explants were produced somatic embryos in all TDZ concentrations. Hypocotyl explants (aseptic seedlings were 15-day old) can be stimulated easily by low concentrations TDZ.

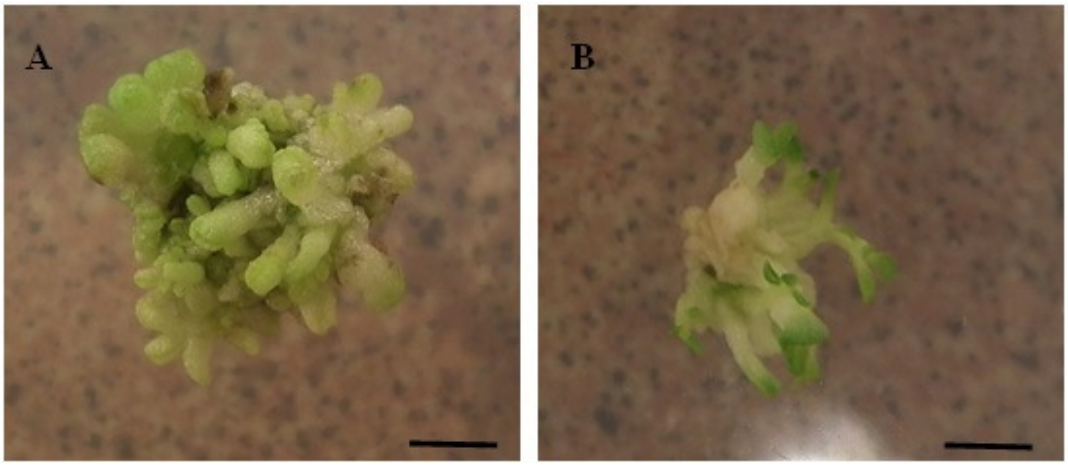

Figure 1: Different-stage somatic embryos of natural tetraploid *T. pratense* calli in 7.5(A- apical meristem) and 2.5 (B-hypocotyl) mg/L TDZ medium (Bars: 3mm)

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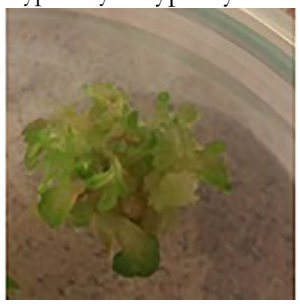
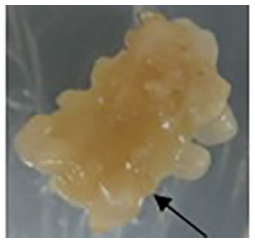
Table 1: Average somatic embryogenesis (SE) rates recorded in all explants of natural tetraploid *T. pratense* in different concentrations of TDZ.

TDZ (mg/L)	Embryoid/explant					Average indirect SE rates (%)					Abnormal observations	
	A	C	E	H	PL	A	C	E	H	PL		
2.5	1.8b	1.2b	1.6b	7.6a	1.1b	25b	18b	20b	**85a**	10b	Hypocotyl –hyperhydration 	Hypocotyl –Hormshape SE 
5	2.9b	2.1b	1.6c	6.3a	1.1d	30b	35b	22c	65a	15d	Hypocotyl –hyperhydration and vitrification 	
7.5	17.85a	7.2b	3c	6.8b	1.2d	**95a**	75b	28c	78b	11d		

A (apical meristem), C (Cotyledon), E (Epycotyl), H (Hypocotyl), PL (Primary leaf). Means were followed by the same letter are not significantly different in same column using Duncan's multiple range test at 0.01 level of significance.

Embryos were matured in 6-12 weeks. Somatic embryos left in the dark managed to proceed to torpedo stage but failed to proceed to mature embryo stage. When the indirect somatic embryos at torpedo stage were transferred under illumination, they were coloured and matured. Somatic embryos of natural tetraploid T. *pratense* (with TDZ) did not exhibit a monocotyledonary or multicotyledonary structure like the somatic embryos of *Glycine max* and natural tetraploid *T. pratense* (Fernando *et al*., 2002; Colgecen *et al*., 2016). Funnel-shaped somatic embryos of natural tetraploid T. *pratense* had such as displayed by the somatic embryos of *T. nigrescens* (Konieczny *et al*., 2012). Somatic embryos of natural tetraploid T. pratense (with TDZ) had such as displayed vitrification and hyperhydration but did not exhibit somatic embryo abortion (Colgecen *et al*., 2016). Very low rate of embryoid formation and somatic embryos could be obtained from apical meristem, young primary leaf, epycotyl, cotyledon explants derived calli in 2.5 and 5 mg/L TDZ containing MS media despite callus formation. Vitrification and hyperhydration were observed in early phase of embryogenesis in MS media in some explants (Table1). Plant regeneration was studied as an alternative to seed setting problem in natural tetraploid *T. pratense;* on the other hand somatic embryogenesis has not been explored (Bakar, 1995; Colgecen *et al*., 2008). About 40% direct somatic embryogenesis rate was achieved in the studies on diploid (2n=14) T. *pratense* varieties by using a variety of culture methods and nutrient media containing 2, 4-D, BAP, Kinetin, yeast or casein hydrolysate. Seedling pieces, zygotic embryos and mesophyll protoplasts were also used in other studies (Phillps and Collins, 1980; Maheswaran and Williams, 1984, 1985, 1986; Radionenko *et. al*., 1994). The somatic embryos of natural tetraploid *T. pratense* were produced from hypocotyl (85%), cotyledon(75%), apical meristem(60%) explants in 0.3 mg/L 2,4-D and 2 mg/L Kinetin containing MS medium(Colgecen *et al*., 2016). In the present study, 95% indirect somatic embryogenesis rate was achieved with TDZ. In this study, first time with TDZ production of somatic embryogenesis were recorded in apical meristem and hypocotyl, cotyledon of 15-day-old aseptic seedlings except young primary leaf, epycotyl of natural tetraploid *T. pratense* L. It was confirmed that young aseptic seedlings need to be used for somatic embryogenesis in natural tetraploid *T. pratense* as also reported by other studies. In the studies on diploid *T. pratense* and natural tetraploid *T. pratense*, the most successful results were obtained from SL medium supplemented with 0.01 mg/L 2,4-D+ 2 mg/L adenin, EC6 medium supplemented with 0.05 mg/L BAP, and KM8p medium supplemented with 0.5 mg/L 2,4-D+ 0.5 mg/L Kinetin, MS medium supplemented with 0.3 mg/L 2,4-D and 2 mg/L Kinetin (Phillps and Collins, 1980; Maheswaran and Williams, 1984, 1985, 1986; Radionenko *et. al*., 1994; Colgecen *et al*., 2016). In a study on *T. nigrescens*, 2, 4-D was more effective with inductive effect compared to NAA (Konieczny *et al*., 2009). Stimulation of somatic embryogenesis by TDZ has not been confirmed in *Trifolium* species. TDZ is an interesting plant growth regulator. It showed a significant regenerative ability in the entire legume. TDZ induced effectively direct somatic embryogenesis in peanut. TDZ elicits different morphogenic events in legumes. The mechanism is unexplained. It is suggested that TDZ promotes somatic embryogenesis by balancing endogenous levels of both auxins and cytokinins in peanut seedlings (Lakshmanan and Taji, 2000). TDZ is more effective than other cytokinins used for somatic embryogenesis. Compared to other cytokinins, TDZ, at lower concentrations, directly promotes growth due to its own biological activity or through inducing the synthesis and/or accumulation of endogenous cytokinins or auxins (Deo *et al*., 2010). In our study, hypocotyl explants can be more stimulated than apical meristem explants in all TDZ concentrations with endogenous hormones. TDZ can imitate the effects of both auxins and cytokinins (Hutchinson *et al*. 1996b). TDZ affects the metabolism of endogenous auxins causing a change in the tissue auxin: cytokinin ratio, which, eventually, stimulates somatic embryogenesis (Bespalhok and Hattori, 1998). In Fabaceae ***TDZ*** was found responsive for somatic embryogenesis

than BAP, 2, 4-D and Picloram in cotyledonary node explants of *Vigna umbellata*. The best result (18 somatic embryos per explant) was obtained with 12.5 µM TDZ in combination with 2.5 µM BAP **(**Saini and Chopra, 2012**)**. Direct somatic embryogenesis was promoted by TDZ in cotyledonary and petiole explants of *M. truncatula.* Transgenic plantlets were obtained for a short period of 60 days. MS medium for plant regeneration contained MS salts, Morel vit., 3% sucrose, 0.25% Phytogel and 0.5 mg/l TDZ (Iantcheva *et.al*., 2005). Direct shoot organogenesis and somatic embryogenesis procedure for inducing high frequency were realized efficiently in cotyledonary node explants of *Lens culinaris* (without both the cotyledons) with TDZ. TDZ at concentration lower than 2.0 µM induced shoot organogenesis whereas at higher concentration (2.5-15 µM-0.5-3.3 mg/L) it shifted the expression of regeneration from shoot organogenesis to somatic embryogenesis (Chhabra *et al*., 2008). In our study somatic embryogenesis of natural tetraploid *T., pratense* occurred in TDZ at higher concentrations than others. The different concentrations of TDZ were found to be favourable for somatic embryogenesis of natural tetraploid *T. pratense* from apical meristem and hypocotyl, cotyledon except young primary leaf, epycotyl. First with TDZ somatic embryos of natural tetraploid *T. pratense* appeared within 4-5 weeks on the calli obtained from apical meristem, hypocotyl, epicotyl, cotyledon explants. Globular, heart, torpedo and mature-stage somatic embryos were mostly produced in 7.5 mg/L TDZ. Hence, somatic embryogenesis can be considered as a new plant regeneration method as an alternative to seed setting problem in this valuable plant. The present study is expected to promote future studies on implementation of advanced biotechnological techniques and synthetic seed production.

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