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Ultrastructural observations in somatic embryogenesis of natural tetraploid *Trifolium pratense* L.

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Abstract: Previous reports of plant regeneration of natural tetraploid *T. pratense* L. 'Elçi' could be realized only through the apical meristem calli. In order to proceed to the production stage, other regeneration methods need to be tried. Aseptic seedlings were used for the production of somatic embryos through various 2,4-D and kinetin trials. Nonuniform external callus cells with translucent cytoplasm were observed in various developmental stages of somatic embryos. Beneath these cells, there were uniformly aligned, dark-stained embryo cells with dense cytoplasm. Despite the similar developmental stages and cell characteristics of zygotic and somatic embryos, the walls of somatic embryo cells revealed a highly wavy pattern. The nucleus generally contained only one nucleolus, which was spherical, dark stained, and electron-dense. Electron-dense droplets were seen in vacuoles. The cytoplasm consisted of starch-containing amyloplasts, mitochondria, plastids, ribosomes, endoplasmic reticulum, dictyosomes, lipid, and protein bodies. In some of the somatic embryos at the globular and heart stages, vacuole or electron-translucent zones were observed in the nucleolus. Additionally, a few embryo degenerations were recorded during developmental stages of the zygotic embryo. For the first time, the somatic embryos of natural tetraploid *T. pratense* were produced from hypocotyl (85%), cotyledon (75%), and apical meristem (60%) explants in 0.3 mg/L 2,4-D and 2 mg/L kinetin-containing MS medium. Our study developed an effective and efficient in vitro production method for using natural tetraploid *T. pratense* in biotechnological studies.

Key words: *Trifolium*, red clover, callus, somatic embryo, transmission electron microscope (TEM)

1. Introduction

Legumes are one of the most important food crops due to their high nutritional value and medicinal properties, 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. Genetic selection can be made by production of somatic embryos. Previously, a 40% somatic embryo production rate was achieved from cell suspension cultures of *T. pratense* Arlington using a basal medium supplemented with 0.01 mg/L 2,4-D and 2 mg/L adenine (Phillips and Collins, 1980). Direct somatic embryogenesis on immature zygotic embryos in vitro has been confirmed for *T. pratense* and *T. repens*. For both species, direct embryo cloning was achieved on an appropriate basal medium supplemented with 0.05 mg/L BAP and 1 g/L yeast (Maheswaran and Williams, 1984, 1985, 1986). Direct somatic embryogenesis was reported in mesophyll protoplast cultures of *T. pratense* using the nutrient medium of Kao and Michayluk supplemented with 0.5 mg/L 2,4-D, 0.5 mg/L kinetin, and 0.1% casein hydrolysate (Radionenko et al., 1994). Available studies in the literature are limited to diploid varieties.

Natural tetraploid *T. pratense* L. is an important plant that is grown in Turkey. The plant has high biomass efficiency; however, seed settings and hard seed problems hinder production of *T. pratense*. Bakar Büyükkartal (2008) observed that a mature embryo sac was formed in 18% of ovules examined, but the seed setting ratio was remarkably low (5.8%) in natural tetraploid *T. pratense*.

Previous plant regeneration of the natural tetraploid *T. pratense* could have been realized only through the apical meristem calli (84.56%) (Çölgeçen and Toker, 2008). In order to proceed to the stage of production, other regeneration methods need to be explored. An effective and efficient in vitro production method is needed for utilizing natural tetraploid *T. pratense* in different studies (for example, phytoestrogens production in bioreactor, genetic selection). One method for in vitro

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plant regeneration is somatic embryogenesis. Although, there is much research on somatic embryogenesis in *Trifolium* species and Fabaceae species like soy bean and alfalfa, no study has been completed for natural tetraploid *T. pratense*. For instance, somatic embryos were produced from leaf-derived protoplasts of *Medicago sativa* (Monteiro et al., 2003). A histological analysis was conducted on the somatic embryos obtained from cell-suspension cultures of *Vigna unguiculata* (Ramakrishnan et al., 2005). The embryological studies in natural tetraploid *T. pratense* examined the ultrastructure of the zygotic embryo and endosperm development, ultrastructure of the seed coat throughout developmental stages, apoximis, and factors causing poor seed setting (Algan and Bakar, 1996, 1997, 1999a, 1999b; Bakar and Algan, 1998).

The aim of this study was to develop an effective method for producing somatic embryos from aseptic seedlings by using different plant growth regulators and to examine the ultrastructural properties of somatic embryos of natural tetraploid *T. pratense.*

2. Materials and methods

2.1. Plant material and culture conditions

This study examined natural tetraploid E2 type (2n = 4x = 28 chromosomes) *Trifolium pratense* L. collected from Tortum in the vicinity of Erzurum, Turkey by Elçi (1982). The E2-type natural tetraploid *T. pratense* L. was grown in the experimentation gardens of Ankara University's Department of Biology in the Faculty of Science.

Fifteen-day-old aseptic seedlings with a unifoliate primary leaf were used as the explant source. Seeds were first sterilized in 96% ethanol for 1 min and then transferred to 10% sodium hypochlorite solution for 10 min (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 and Skoog, 1962). All the samples were incubated at 22–24 °C with a 16-/8-h photoperiod (irradiance of 42 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes). Then 25 explants were taken per hypocotyl (0.5–1 cm), cotyledon (whole and in two fragments), apical meristem (1 mm), epicotyl (0.5–1 cm), and young primary leaves (whole and divided into two fragments) of aseptically grown seedlings. The explants were cultured in petri dishes in the dark (100 mm × 15 mm) (Çölgeçen and Toker, 2008). The embryogenic calli were subcultured onto the same media after 8 weeks.

Embryoid callus was produced in MS-se media supplemented with combinations of 2,4-D and kinetin as plant growth regulators (Table 1). Samples of somatic embryos at different developmental stages were taken from the medium with the most successful somatic embryo production (MS-se3). Torpedo-stage somatic embryos were incubated at 22–24 °C with a 16-/8-h 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).

2.2. Ultrastructural analysis

Five somatic embryos of natural tetraploid *T. pratense* per development stage (globular, heart, torpedo, and mature) were taken from the medium and fixed with 3% glutaraldehyde (0.1 M Na-P buffer) and then 1% osmium tetroxide (0.1 M Na-P buffer) in the cold (+4 °C). The samples were dehydrated gradually by increasing concentrations of ethyl alcohol; then they were stained with uranyl acetate, saturated, and embedded into Epon 812 (Luft, 1961). For polymerization of the embedding medium, the blocks were kept in a drying oven at 30 °C for 1 night, at 45 °C for 1 day, and at 60 °C for 1 night. These blocks were then trimmed, and semithin sections were taken. Semithin sections, the first phase of TEM analyses, were stained with methylene blue and toluidine blue. The sections were examined and photographed with a DFC320 Leica camera. Ultrathin sections were stained with lead citrate (Stempak and Ward, 1964), examined with a JEOL CX 100 TEM, and micro-photographed.

2.3. Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA), and the differences among means were compared by Duncan's multiple-range test. 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.

Table 1. Experimental media used for somatic embryogenesis in natural tetraploid *T. pratense.*

Media	2,4-D (mg/L)	Kinetin (mg/L)
MS1	8	2
MS2	1	2
MS3	0.3	2
MS4	0.5	0.5

3. Results

3.1. Somatic embryogenesis

Callus formation started within 3 days in all explants of natural tetraploid *T. pratense*. The calli were yellow and formed clusters in the latter days of the culture that were nodular and compact in appearance. Embryogenic calli were observed within 4–6 weeks.

No somatic embryos could be obtained from young, primary leaf explant-derived calli. Vitrification and hyperhydratation were observed in the early phase of embryogenesis in MS1. A very low rate of embryoid formation was observed in MS2 despite callus formation. The first somatic embryo formation was observed in calli in all other trials after 5–6 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. A large number of globular somatic embryos were observed on the same callus in MS3 medium in all explants (Figure 1a). Somatic embryos proceeding through heart and torpedo stages were monitored. Only in MS3 medium were a large number of somatic embryos able to proceed to further stages. In MS3 medium had the most successful somatic embryo production, and the average rate of hypocotyl explant-derived indirect somatic embryogenesis was 85% (Table 2). Embryos matured in 6–12 weeks. Direct somatic embryogenesis was hardly observed (Figure 1b). 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 colored and matured. Samples of somatic embryos at different developmental stages were taken from MS3 medium, and ultrastructural analysis was performed (Figures 1a–1d).

3.2. Ultrastructural analysis of somatic embryos

Histological and cytological analysis of indirect somatic embryos produced in vitro were conducted by

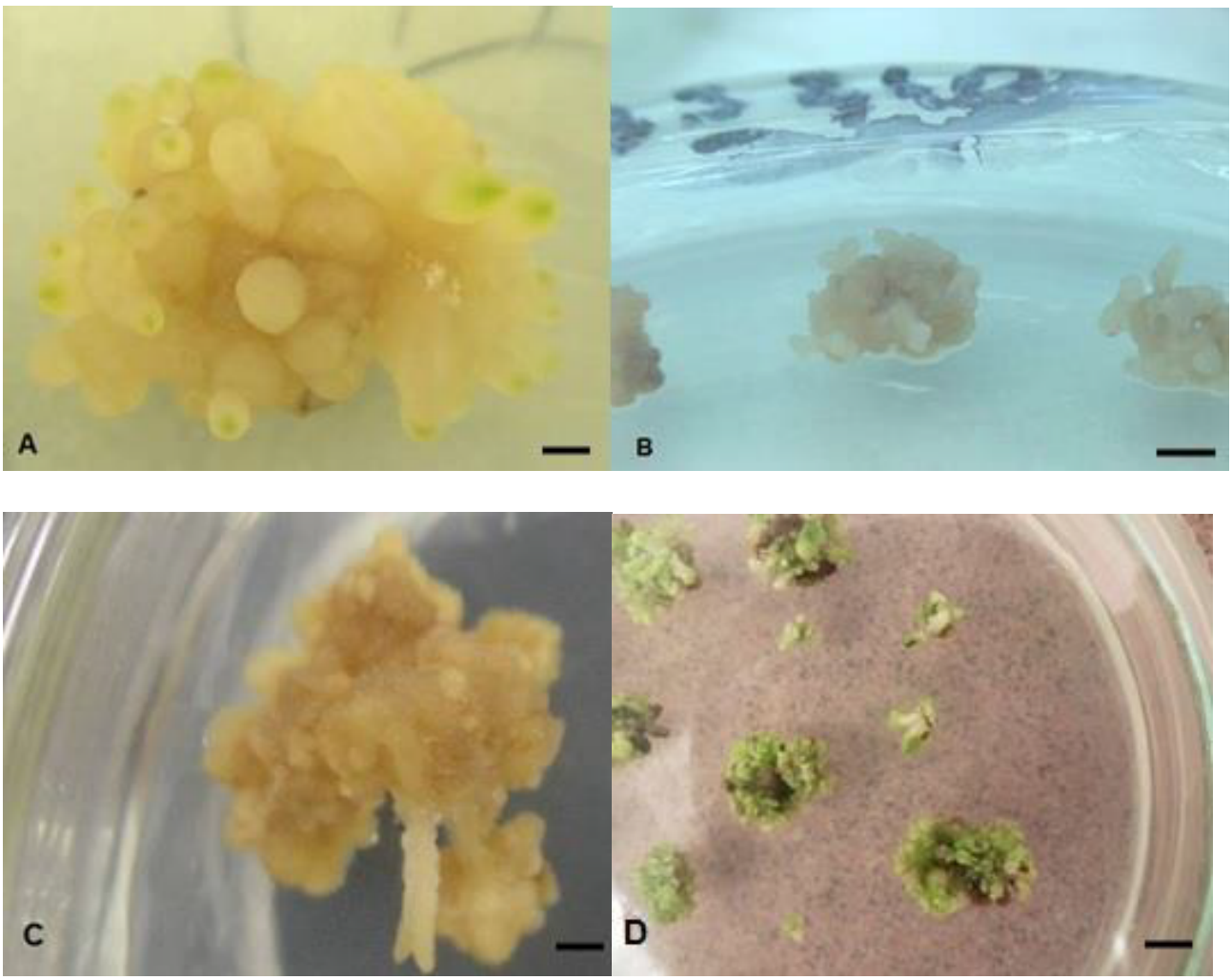
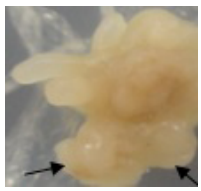
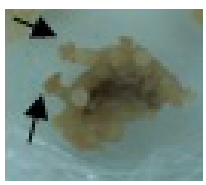


Figure 1. Globular stage (A), bar = 2 mm; heart stage (B), bar = 5 mm; torpedo stage (C), bar = 2 mm; mature stage (D), bar = 5 mm; somatic embryos of natural tetraploid *T. pratense* calli in MS-se3 medium.

Table 2. Average somatic embryogenesis (SE) rates recorded in all explants of natural tetraploid *T. pratense* in different media. A (apical meristem), C (cotyledon), E (epicotyl), H (hypocotyl), PL (primary leaf). Means followed by the same letter are not significantly different in same column using Duncan's multiple range test at a 0.01 level of significance.

Media	Embryoid/explant					Average indirect SE rates (%)					Direct SE number	Abnormal observations
	A	C	E	H	PL	A	C	E	H	PL		
MS1	1.8a	1.2a	1.6a	2.1a	1.1a	25a	18a	20a	33a	10b		
MS2	2.9b	2.1a	2.4b	3.7b	2b	30a	35b	22a	65c	15b		Cotyledon-hyperhydratation, primary leaf-vitrification 
MS3	4.85c	7.2b	3b	9.8c	1.2a	60b	75c	28a	85d	11b	Hypocotyl-1- 	Apical meristem- 1 multicotyledonary SE  Cotyledon- hyperhydratation 
MS4	1.9a	1.4a	1.5a	2.2a	0.9a	31a	21a	22a	45b	0.4a		Cotyledon-somatic embryo abortion 

transmission electron microscope (TEM). Like zygotic embryos, somatic embryos proceeded through spherical (globular), heart, torpedo, and mature stages. Two types of cells were observed during various developmental stages of embryo in the semithin sections: dispersed-shaped external callus cells with translucent cytoplasm and dark-stained, uniform cells with dense cytoplasm.

In all stages of embryo development, external callus cells were nonuniform, large, sometimes oval, and generally isodiametric-shaped. Cell walls were wavy or zig-zag shaped with translucent electrons (Figure 2a). Walls next to the intercellular spaces were thickened (Figure 2b). There was one nucleus in the center of the cell, which was small during early development and enlarged while advancing towards the mature stage. Cytoplasm formed a thin layer around the nucleus parallel to the cell walls. The nucleus was located near the center of the cell or just next to the cell wall. The nucleus had a very dark-stained and large nucleolus. The cells had large vacuoles. In some cells, vacuoles occupied almost the

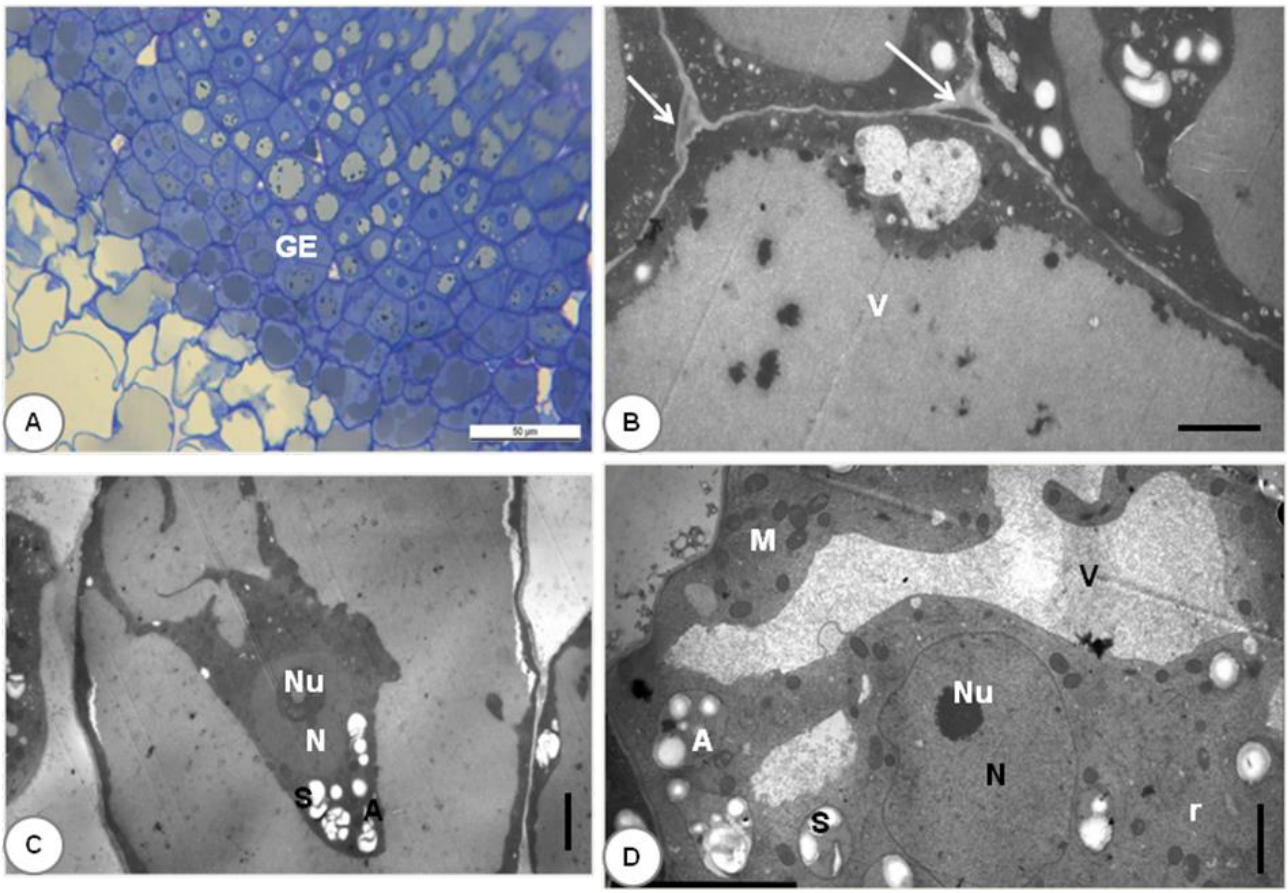


Figure 2. A. External callus cells during globular somatic embryo stage in semithin section, bar = 50 μm. B. Cell walls and intercellular spaces (indicated by arrow), bar = 3 μm. C. Electron micrograph of external callus cell, bar = 1 μm. D. Organelles in cytoplasm, bar = 5 μm (GE, globular embryo; N, nucleus; Nu, nucleolus; A, amyloplast; S, starch; r, ribosome; ER, endoplasmic reticulum; V, vacuole; M, mitochondria).

entire cell volume. Cytoplasm was pushed towards the cell wall. Large vacuoles located at the center of the cells contained tiny, granulated and electron-dense materials (Figure 2c). Cytoplasm pushed towards the cell wall was electron-dense in some of the cells. Cytoplasm consisted of various organelles such as starch-containing amyloplasts, mitochondria, plastids, ribosomes, endoplasmic reticulum, and dictyosomes. Amyloplasts contained a large number of electron-translucent starch grains. The amyloplasts in some of the cells contained large starch grains. During the torpedo and mature stages of the somatic embryo, the number of starch grains ranged between 1 and 6 and were large. Mitochondria were elongated, oval or spherical, and electron-dense. Mitochondria were concentrated around the nucleus. In some cells, mitochondria divided by splitting in half. There were plenty of ribosomes. Dictyosomes were located adjacent to cell walls and comprised 4–6 lamellae. Granular and agranular endoplasmic reticulum (ER) was dispersed throughout the whole cytoplasm. ER was located close to the nucleus and plasma membrane. It appeared as enlarged, short structures in some areas (Figure 2d).

Embryo cells in all somatic embryo stages were dark and stained intensively. Cells were uniformly aligned. In globular and heart-stage somatic embryos, embryo cells were small and isodiametric shaped and contained a large nucleus and dense cytoplasm (Figure 3a). In torpedo and mature-stage embryos, cells were rectangular, thin-walled, and contained a large nucleus and dense cytoplasm. The walls of the cells at this stage were slightly wavy or zig-zag shaped (Figure 3b). Walls next to the intercellular spaces were thickened. In spherical and heart-stage somatic embryos, highly thickened walls occurred next to the intercellular spaces. In all somatic embryo stages, cytoplasm was dispersed homogeneously throughout the cell.

In all somatic embryo stages, the nucleus was located at the center of the cell and consisted of a spherical and dark-stained nucleolus in some of the cells. In other cells, the nucleus was pushed towards the cell wall. The nucleus had a spherical, dark-stained, and electron-dense nucleolus. Nucleoplasma was homogeneous. In some of the cells at globular somatic embryo stage, there were one or more electron-transparent areas within the nucleolus. In some

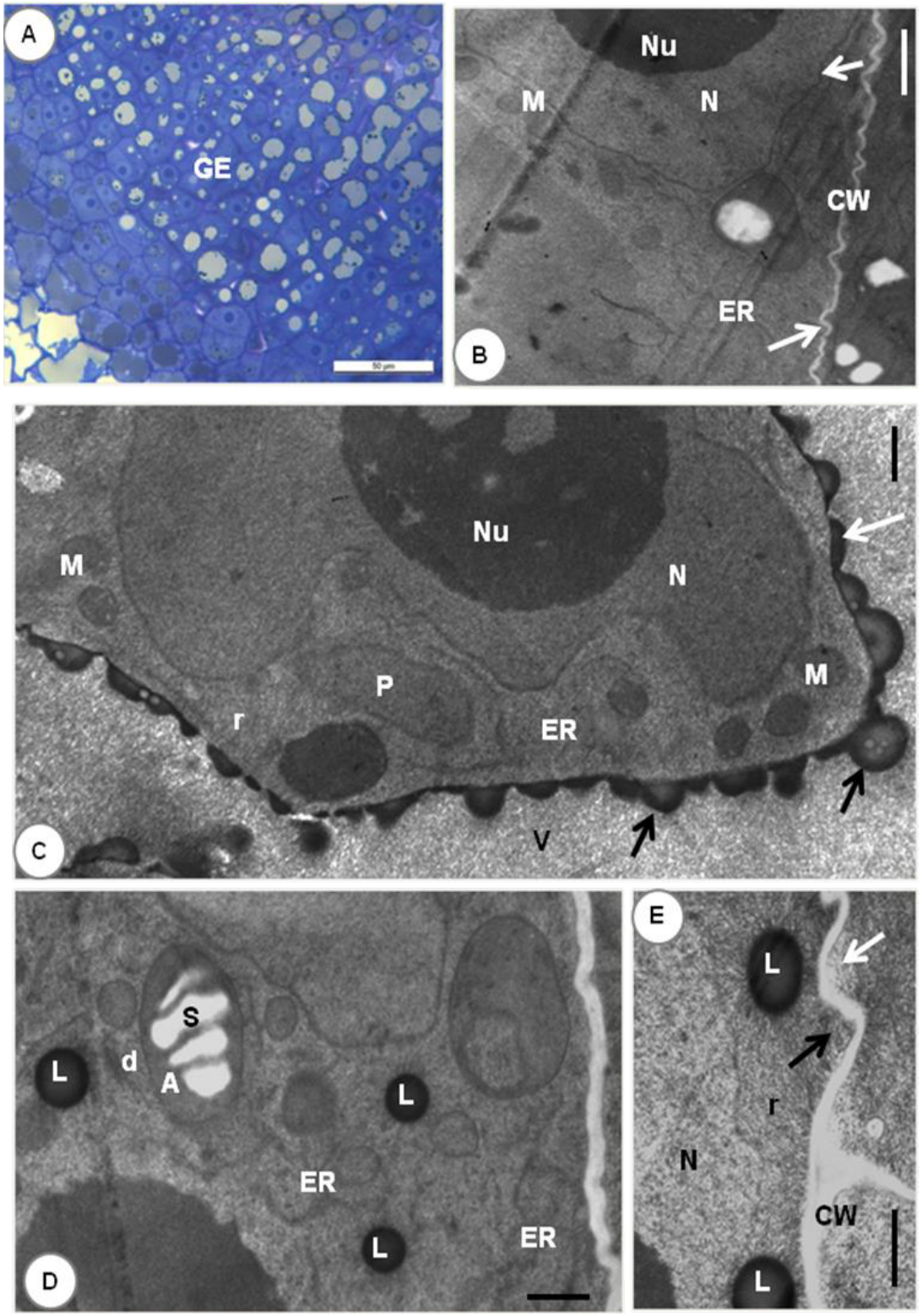


Figure 3. A. Semithin section of somatic embryo cells, bar = 50 µm. B. Organelles in cytoplasm of embryo cells and zig-zag–shaped cell wall (indicated by arrow), bar = 1 µm. C. Nucleus membrane was wavy, vacuole-containing nucleolus in heart-stage embryo, mitochondria also occurred around the nucleus; bar = 3 µm. D. Organelles in cytoplasm, bar = 2 µm. E. Lipid bodies and zig-zag–shaped cell wall (indicated by arrow), bar = 1 µm (GE, globular embryo; N, nucleus; Nu, nucleolus; A, amyloplast; S, starch; r, ribosome; Cw, cell wall; L, lipid bodies; M, mitochondria; ER, endoplasmic reticulum; d, dictyosome).

cells of the somatic embryos at heart-stage, vacuolization was observed at the center of the nucleolus (Figure 3c). Somatic embryos displaying such features may not be able to proceed to further stages. In heart, torpedo, and mature somatic embryo stages, the nucleus membrane was wavy or zig-zag shaped (Figure 3c). This can be seen in fast dividing cells.

During somatic embryo stages, most of the cytoplasm was located around the nucleus and was rich in organelles. There were one or more large vacuoles in the cells. Electron-dense bodies were observed in vacuoles. Large vacuoles were generally located at the center and filled with granulated material in spherical somatic embryo cells. It was remarkable that during heart, torpedo, and mature somatic embryo stages, there were storage materials in the form of dark structures along the whole tonoplast in parts of the vacuoles adjacent to the cell walls. These materials are thought to be tannin (Figure 3c).

In all somatic embryo stages, cytoplasm consisted of starch-containing amyloplasts, mitochondria, plastids, ribosomes, endoplasmic reticulum, and dictyosomes. The starch grains in amyloplasts were numerous, quite large, and electron-translucent. Mitochondria were oval or spherical and electron-translucent. Mitochondrial cristae were of short length. In spherical and heart-shaped somatic embryos, mitochondria were aligned parallel to the cell wall in some cells. During torpedo and mature stages, mitochondria also occurred around the nucleus (Figures 3c and 3d).

A small number of dictyosomes were situated near the cell wall during somatic embryo stages. Granular and agranular endoplasmic reticulum (ER) was located parallel to the cell wall in all somatic embryo stages. ER was located close to the nucleus and plasma membrane. It appeared as enlarged, short structures in some areas. ER surrounded some organelles. Numerous dispersed ribosomes were observed in both free and ER-bound cytoplasm. In spherical and heart-shaped somatic embryos, there were spherical, electron-dense, large lipid bodies situated close to nucleus and parallel to the cell wall. In torpedo and mature somatic embryo stages, small lipid and protein bodies were observed. Diminishing lipid size in further stages indicates they are being used and consumed (Figure 3e).

In general, in all somatic embryo stages, external callus cells and globular and heart-stage somatic embryo cells were isodiametric, and torpedo and mature-stage embryo cells were elongated and rectangular. While external callus cells were nonuniform, somatic embryo cells were uniform in all somatic embryo stages. External callus cells and somatic embryo cells had thin and zig-zag shaped walls in all somatic embryo stages. External callus cells had dense cytoplasm situated parallel to cell walls and around the nucleus. Somatic embryo cells had electron-dense, dark-stained cytoplasm dispersed homogeneously throughout the cell. In both types of cells, walls next to the intercellular spaces were thickened.

In most of the cells, the nucleus was located at the center of the cell and had a spherical and dark-stained nucleolus. In some of the cells, the nucleus was pushed to one side.

External callus cells had more numerous vacuoles compared to cells in all developmental stages of somatic embryos. Vacuoles contained granular, electron-dense materials. Storage materials appeared in the form of dark or gray structures along the whole tonoplast in parts of the vacuoles adjacent to the cell walls. These were probably multilamellar bodies or vesicles. One or more large vacuoles were present in somatic embryo cells. There were storage materials in the form of dark structures along the whole tonoplast in parts of the vacuoles adjacent to the cell walls. These materials are thought to be tannin. The cytoplasm contained starch-containing amyloplasts, mitochondria, plastids, ribosomes, endoplasmic reticulum, and dictyosomes.

4. Discussion

4.1. Somatic embryogenesis

MS3 medium enabled the most successful somatic embryo production and average rate of hypocotyl explant-derived indirect somatic embryogenesis. Some embryos were degenerated at torpedo stage. Such degeneration was also recorded in zygotic embryo stages in natural tetraploid *T. pratense* and reported to be one of the factors causing poor seed setting (Algan and Bakar, 1996). Somatic embryos of natural tetraploid *T. pratense* did not exhibit a monocotyledonary or multicotyledonary structure like the somatic embryos of *Glycine max* and did not have very different shapes, as displayed by the somatic embryos of *T. nigrescens* (Fernando et al., 2002; Konieczny et al., 2012).

Plant regeneration was studied as an alternative to the seed-setting problem in natural tetraploid *T. pratense*; however, somatic embryogenesis has not been explored (Bakar Büyükkartal, 2008; Çölgeçen et al., 2008). A direct somatic embryogenesis rate of approximately 40% was achieved in the studies in 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. In the present study, an 85% indirect somatic embryogenesis rate was achieved with 2,4-D + kinetin combinations. Seedling pieces, zygotic embryos, and mesophyll protoplasts were also used in other studies. In this study, for the first time high rates of somatic embryogenesis were recorded in all explants of 15-day-old aseptic seedlings, except in the young primary leaf of *T. pratense* L. It was confirmed that young aseptic seedlings need to be used for somatic embryogenesis in natural tetraploid *T. pratense*, as reported in other studies

(Phillips and Collins, 1980; Maheswaran and Williams, 1984, 1985, 1986; Radionenko et al., 1994). In the studies on diploid *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. In the present study, MS3 medium supplemented with 0.3 mg/L 2,4-D + 2 mg/L kinetin proved to be the most successful medium (Phillips and Collins, 1980; Maheswaran and Williams, 1984, 1985, 1986; Radionenko et al., 1994; Mclean and Nowak, 1998). Stimulation of somatic embryogenesis by high concentrations of 2,4-D has not been confirmed in every plant species (Babaoğlu et al., 2001). In a study on *T. nigrescens*, 2,4-D was more effective for an inductive effect than NAA. However, in this study, the 2,4-D + kinetin combination proved to be successful. Combined use of auxin and cytokinin supports normal somatic embryo development (Konieczny et al., 2010). In the present study, the combined low concentrations of 2,4-D with 2 mg/L kinetin stimulated somatic embryogenesis. Proportionally, kinetin was effective at higher concentrations compared to 2,4-D. An KM8p protoplast culture medium with a 0.5 mg/L 2,4-D + 0.5 mg/L kinetin (Radionenko et al., 1994) combination resulted in low somatic embryogenesis rates in this study. Yeast extract and casein hydrolysate were used in other research, but they were not used in the trials of our study. Culture medium was also used for subculture, and somatic embryos were matured. No embryonic structural abnormalities (multicotyledonary somatic embryos), as observed in *T. nigrenses*, were recorded; however, some of the embryos could not develop into mature somatic embryos (Phillips and Collins, 1980; Maheswaran and Williams, 1984, 1985, 1986; Radionenko et al., 1994; Konieczny et al., 2012). There are studies available in the literature on the ultrastructural analysis of somatic embryogenesis of *Trifolium* and other species. Proembryos or somatic embryos were formed in callus cultures from hypocotyl sections of *Trifolium incarnatum* L. and *T. ambiguum* Bieb. on Phillips and Collins L2 medium with 0.06 mg/L picloram and 0.1 mg/L 6-benzylaminopurine and *T. repens* L. and *T. vesiculosum* Savi. on Gamborg B5 medium with 1.25 mg/L 2,4-dichlorophenoxyacetic acid (2.4-D), 0.5 mg/L naphthaleneacetic acid (NAA), and 0.5 mg/L 6-furfurylaminopurine (kinetin) (Pederson, 1986). Media and plant growth regulators other than those used in our trials were used in these studies. In a departure from our study, immature zygotic embryos at torpedo and cotyledonary stage were cultured on MS media with 2,4-D in *T. nigrescens* (Viv.) for somatic embryogenesis (Konieczny et al., 2010). In addition, somatic embryogenesis was realized with immature zygotic embryos, leaf protoplasts of *Arabidopsis*, and transformed cotton by *Agrobacterium* (Luo et al., 1997; Shang et al., 2009).

4.2. Ultrastructural analysis of somatic embryos

Two types of cells were observed in somatic embryogenesis in the semithin sections: dispersed-shaped external callus cells and uniform cells. A single-row epidermal layer and 5–6 rows of cortical cells were observed during embryo development in direct somatic embryogenesis of *T. repens*. Cortical cells were smaller and meristematic. The cells had dense cytoplasm, tiny starch grains and vacuoles, and a visible nucleus and nucleolus. A single row of radially oriented epidermis cells with cortical cells beneath were observed in somatic embryos of *Quercus suber*, but they did not show any differences. The cells beneath external callus cells were similar in appearance in the present study. Cytoplasm consisted of various organelles such as starch-containing amyloplasts, mitochondria, plastids, ribosomes, endoplasmic reticulum, and dictyosomes. Subcellular structures did not exhibit any differences (Maheswaran and Willams, 1985; Puigderrajols et al., 2001).

Similar to the findings of this study, the presence of thin cell walls, a large nucleus, dense cytoplasm, large vacuoles, abundant starch, and other organelles was reported in former ultrastructural studies of other species in somatic embryos (Karlson and Vasil, 1986; Brisribe et al., 1993; Jasik et al., 1995; Puigderrajols et al., 2001). Vacuolization was also observed in the nucleolus of wheat somatic embryos (Wang et al., 1994). No lipids or proteins were reported in *Acrocomia aculeata* somatic embryos despite the presence of large vacuoles with electron-dense materials. This was considered an indicator of failure in embryo maturation by researchers (Moura et al., 2010). In the present study, despite their decreasing amounts in torpedo and mature stages, lipids and proteins were detectable in somatic embryos, as reported for *Q. suber*. Somatic embryos were matured. The decrease in storage materials in further developmental stages was attributed to increased mitotic activity and the differentiation and maturation process of the embryo (Puigderrajols et al., 2001). There are no studies on the ultrastructure of diploid or tetraploid *Trifolium pratense* somatic embryos.

First, somatic embryos of natural tetraploid *T. pratense* appeared within 5–6 weeks on the calli obtained from apical meristem, hypocotyl, epicotyl, and cotyledon explants planted on petri dishes. Explants produced embryo-generating calli. Globular, heart, torpedo, and mature-stage somatic embryos were mostly produced in MS3 medium, and their ultrastructure was examined. The combination of 0.3 mg/L 2,4-D and 2 mg/L kinetin was favorable for somatic embryogenesis of natural tetraploid *T. pratense* from all explants except young primary leaf. Hence, somatic embryogenesis can be considered as a new

plant regeneration method and an alternative to the seed-setting problem in this valuable plant. Our study developed an effective and efficient in vitro production method for using natural tetraploid *T. pratense* in biotechnological studies. The somatic embryos of natural tetraploid *T. pratense* were produced from hypocotyl (85%), cotyledon (75%), and apical meristem (60%) explants. Non-uniform external callus cells with translucent cytoplasm and uniformly ordered, dark-stained embryo cells with dense cytoplasm were observed in developmental stages of somatic embryos. The presence of vacuoles or transparent zones in the nucleoli during globular and heart stages indicates a failure of some somatic embryos to complete maturation. Despite similar developmental stages and cell characteristics of zygotic and somatic embryos, the walls of somatic embryo cells revealed a highly wavy pattern. The present study is expected to promote future studies on the implementation of advanced biotechnological techniques and synthetic seed production.

Acknowledgments

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