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MICROSCOPIC AND PHYSIOLOGICAL ANALYSIS OF SOMATIC EMBRYOS UNDER IN VITRO CULTURE IN *TRITICALE*

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ABSTRACT

Somatic embryogenesis is a developmental process during which plant somatic cells, under in vitro conditions, produce embryogenic cells that develop into somatic embryos. Somatic embryogenesis is an efficient method clonal propagation in vitro of plants. Mature embryos were used as explant source for embryogenic callus formation and the callus produced compact, healthy and most mature embryos were induced embryogenic callus. Somatic embryos showed a similar morphologically to their non-somatic counterparts in their development. In a comparative study, total protein, DNSA and activities of some antioxidant enzymes including H_2O_2, MDA of somatic embryos of three *triticale* cultivar '*Tatlıcak, Alper Bey* and *Mikham*' at embryogenic callus stages were analyzed. However; somatic embryos displayed the highest level of DNSA, MDA, H_2O_2 and total protein in all tested cultivars. Our results showed that the *triticale* somatic embryo maturation process was complete within two years in vitro conditions.

Key words: Somatic embryos, triticale, tissue culture, enzyme activities, *in vitro* assay

1. INTRODUCTION

Plant tissue culture is an efficient method for the in vitro regeneration of plants. Its developmental stages different in vivo development, which is a ideal material for analyzing molecular biology and physiological in callus tissues and embryogenesis (Morel et al., 2014). Somatic embryogenesis has powerful biotechnological tools which allows study of morphology, physiology, and molecular mechanisms of embryo development. Also, somatic embryos studies can provide insight into cell differentiation, totipotency, and plant regeneration (Zimmerman, 1993; Egertsdotter, 2019). Somatic embryogenesis can likely be induced for all plant species provided that the suitable initial cell type, culture conditions and environmental factors are employed. Several reports have displayed the process of uembryogenesis in triticale strongly influenced by factors including explant type, medium properties and genotypes. Bezirganoglu (2017) found that triticale callus cells could be regenerated through somatic embryogenesis in plant tissue culture conditions. However, pyhsiological mechanism of somatic embryogenesis process is unknown yet, but it is clear that several genes are specially activated or repressed during somatic embryogenesis. Sugars are very important energy resources and essential for embryonic development (Bartos et al., 2018), and it can involving proliferation of leaves. Sugars can adjust osmotic pressure and protect cell water loss (Iraqi and Tremblay, 2001). Many studies have indicated that the alters in sugars contents could be used as a indicator of various somatic embryogenesis phase (Cangahuala-Inocent et al., 2014). Plants have an efficient enzymatic and non-enzymatic antioxidant mechanisms as a source of exogenous (Kasote et al., 2015). Antioxidants can be synthesized peroxidase, catalase, ascorbate peroxidase and superoxide dismutase. Energy organelles are the two essential central station and sites of reactive oxygen species production within plant cells. These organelles are also participated in maintenance of a fine balance between energy linked functions and control of ROS production. In recent years, alters in the activities of POX, APX, SOD and CAT had been indicated to be greatly linked with ROS elimination at various somatic embryos phase (Cui et al., 1999). A close relationship between callus cultures and biochemical by exogenous hormones has been previously reported in different plant species. Exogenous hormones applications modify the callus formation and structures by inducing alters in their endogenous hormones levels (Guo et al., 2017). Auxin is a group of plant hormones, which contributes to the regulation of stimulating cell elongation in stems. Moreover, auxins also acts a role in maintaining apical dominance. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a synthetic phytohormones that plants cannot degrade *in vivo* (Wright et al., 2010). It is absorbed through the leaves and is translocated to the meristems of the plant. Endogenous plant growth regulator are strictly controlled through different pathways, such as promote cell elongation, division, degradation, transportation, and biosynthesis (Korasick et al., 2013). However, it is not clear whether the effects of exogenous hormones on plant growth are direct or whether they are connected with their effects on endogenous hormones (Szalai et al., 2011). Investigation of the physiological and microscopic changes during somatic embryogenesis could provide new knowledge about embryogenic events in *Triticale*. In this plant, a few physiological observations were reported for *triticale* callus culture (Yazıcılar et al., 2021; Bezirganoglu, 2017). Physiological characterics in the formation of somatic embryo in *triticale* were determined to gain a better understanding of the role of inductive

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produce embryogenic cells, enzymatic activity process and the energy substance, and they have functional significance in the determination of various phase of somatic embryo development. However, very little is known about the specific physiological parameters that might affect somatic embryogenesis, such as ROS and the antioxidant system. The objective of the current study was to measure the levels of sugar and the antioxidant system in *triticale* callus. Moreover; an overview of morphology (using SEM analysis) of embryogenic cultures was also provided.

2. MATERİAL AND METHODS

2.1. Somatic embryo development medium

In our study, three *Triticale* cultivars (*Mikham*, *Alper Bey*, and *Tatlıcak)* were used as the material for the response to somatic embryo. The callus were cultured in MS medium containing 4 mg L 2,4-dichlorophenoxyacetic acid (2,4- D) for 30 days in the growth chambers. Later only embryogenic calli were transferred to MS medium containing 2,4-D in four different doses (0, 4, 8, 12: 2,4-dichlorophenoxyacetic acid) for 60 days and subcultured for another 30 days. All calli were kept under fluorescent light at 62 µmol m 16-h/8-h light/dark cycle at 26 ± 1 °C. The total culture duration was 2 years. Somatic embryos were obtained from embryogenic callus maintaince medium. Somatic embryos were evaluated after 2 years of 2,4-D treatment for somatic embryo development rate, accumulation of protein, total soluble sugars, and antioxidant enzyme activity.

2.2. Soluble Sugar Determination

100 mg callus was homogenized with 5mL 2.5N HCl cold. It was centrifuged at 9000 rpm for 10 minutes. The pellet part was discarded and 2 mL of supernatant was taken and transferred to the glass tube and 2 mL of DNSA (3,5- dinitrosalicylic acid) was added. It was incubated in a 90 $^{\circ}$C water bath for 20 minutes. It was kept in the ice bath until it cools. For each sample, 100 µL per well was added in triplicate to 96 well plate. As a blank, 2 mL of DNSA (3,5- dinitrosalicylic acid) and 2 mL of 2.5N HCl were made in triplicate. Measurements were made at 550 nm at the NanoDrop.

2.3. MDA (Malondialdehyde)

Malondialdehyde was measured using the method of (Heath and Packer, 1968) using liquid nitrogen. 0.4 grams of ground callus material was dispersed in 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) tricholoroacetic acid. The sample was boiled at 98°C for 30 min and then quickly taken into an ice bath. The sample content was centrifuged at 3000 ×*g* for 10 min and the value of the supernatant was monitored at 532 and 600 nm (Heath and Packer 1968; Jaleel et al., 2007; Erdal, 2012).

2.4. H_2O_2 (Hydrogen peroxide)

H_2O_2 (Hydrogen peroxide) content was measured using the method of Sergiev et al. (1997). 0.4 g of callus material was homogenized in 4 ml of trichloroacetic acid and centrifuged at 4°C for 15 min at 13000 rpm. 2 ml of extract was mixed with 0.8 ml of KH_2PO_4 and 1.6 ml of KI in test tubes. The absorbance of the callus sample product was measured at 390 nm using a standard curve with H_2O_2 solutions (Velikova et al., 2000).

2.5. Scanning Electron Microscopy

Somatic embryos were prefixed in 5% buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for 2 h at room temperature. After dehydration through a graded ethanol series, samples were dried with a CPD (CO_2 critical-point drying) system, sputter-coated with gold (Jeol JFC-1100 E ion-sputtering system) and observed with a scanning electron microscope (HITACHI S-4700).

2.6. Statistical Analysis

Each experiment was repeated three times. Analysis of variance was conducted using a one-way ANOVA test using SPSS 13.0 and means were compared by Duncan test at the 0.05 confidence level.

3. RESULTS

3.1. DNSA

Among all tested cultivars, a remarkably higher DNSA activity was determined in *Tatlıcak* somatic

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embryos (1.650 nmol g-^{1} FW), whereas *Alper Bey* somatic embryos (1.366 nmol g-^{1} FW) and *Mikham* somatic embryos (1.566 nmol g-^{1} FW) exhibited a lower trend in long-term 2,4-D treatments in vitro conditions. There was a detectable difference of DNSA between control, somatic embyos and non somatic embryos in *Tatlıcak* and *Mikham* cultivars. Whereas there was no a detectable difference of DNSA between control, somatic embyos and non somatic embryos in *Alper Bey* cultivar (Figure 1).

3.2. MDA

The levels of activities of MDA were investigated in the 2,4-D - long terms in in vitro culture at the end of 2 years. The MDA content of triticale somatic embryo was shown to change gradually in terms of somatic embryos. The activity of MDA was the highest in *Mikham* somatic embryos and had similar values in all the other genotypes (Figure 2).

3.3. H_2O_2

The H_2O_2 content in three of the cultivars of *Triticale* (*Mikham*, *Alper Bey*, and *Tatlıcak*) were investigated under 2,4-D treatments in vitro conditions. H_2O_2 values showed a large range of variation among somatic embryos, non somatic embryos and control treatments for long terms cultivation, ranging from 0.0220 to 1.404 nmol g-^{1} FW. The cultivar *Alper Bey* non somatic embryos (0,146 g-^{1} FW) exhibited the highest value followed by *Tatlıcak* somatic embryos (0,131 g-^{1} FW) and *Mikham* somatic embryos (0,119 g-^{1} FW) (Figure 3).

3.4. Protein

Among all tested cultivars, a remarkably lower protein activity was determined in *Tatlıcak* somatic embryos (0.582 nmol g-^{1} FW), whereas *Alper Bey* non-somatic embryos (1.174 nmol g-^{1} FW) and *Mikham* non-somatic embryos (1.274 nmol g-^{1} FW) exhibited a higher trend in long-term 2,4-D treatments in vitro conditions. There was a detectable difference of protein between control, somatic embyos and non somatic embryos in *Tatlıcak* and *Mikham* cultivars. Whereas there was no a detectable difference of protein between control, somatic embyos and non somatic embryos in *Alper Bey* cultivar (Figure 4).

3.5. SEM

SEM analysis was conducted to verify the structures of somatic embryos, non somatic embryos and control callus, and the results are shown in Figure 5**.** Three *Triticale* cultivars, *Mikham*, *Alper Bey*, and *Tatlıcak* were evaluated for somatic embryos development stages in the presence of 2,4-D in various concentrations using scanning electron microscope. The analysis indicated that the somatic embryos development is easily detectable in *triticale* callus. Globular of callus are visible in non somatic callus. Membranous and fibril of callus are visible in somatic callus.

4. DISCUSSION

Somatic embryogenesis is a complex process, which is the outcome of a series of biochemical, physiological and molecular alters taking place in callus tissues. Somatic embryogenesis necessaries embryogenic capacities via differentiation, dedifferentiation, events of gene expression and chromatin remodeling (Krishnan and Siril, 2017). In general the somatic embryo formation contains a multiple of bilateral patways that participates changes in the degrees of endogenous hormones and stress factors (Mozgová et al., 2017). Various reports contribute the hypothesis that the earlier stages of somatic embryogenesis are demonstrated by the formation of many genes related to different plant growth regulation and abiotic stress (Nic-Can et al., 2016; Nowak and Gaj, 2016). Recent proved in wheat (Adero et al., 2019), in potato (Kaur et al., 2018), *barley* (Orlowska, 2021), has indicated that the presence of various types of hormones plays an active role in the formation of somatic embryos. The main factor for callus tissues during the formation of somatic embryos is the presence of high auxin concentration in the culture medium. Other factors used for the formation of somatic embryos are higher pH, heat-shock exposure or treatment with different chemical substances. Under experimental analysis in our study, formation of somatic embryogensis in the presence of 2,4-D was based on the determination of MDA (malondialdehyde), H_2O_2 (hydrogen peroxide), DNSA and protein as well as laser scanning electron analysis. Embryogenic callus formation was the essential of somatic embryogenesis. The degrees of DNSA, MDA and H_2O_2 were higher in embryogenic callus than non embryogenic callus, similarly to finding reported in *Ormosia Henry Prain* (Wu et al., 2021) *alfalfa* (Martin et al., 2000), since it was'nt easy for non embryogenic callus to improve into the somatic embryo. These findings indicated that the flowing of energy events was the material basis for embryogenic callus transformation into the somatic embryo. However, significant genotypic differences in DNSA, MDA and H_2O_2 response were observed among the *triticale* genotypes and somatic embryo induction capacity is related to genotype. Our results exhibited an decreased accumulation of DNSA, MDA in H_2O_2 embryogenic differentiation as compared to non embryogenic differentiation of *Alper Bey* genotypes (Figure 1, 2,3). This confirms us that the embryogenic callus induction of long-term callus maybe gradually developmental

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stages of genotype. In *Alper bey* callus, DNSA, MDA in H_2O_2 embryogenic differentiation was greatly decreased under 2,4-D treatments and the the effects of plant growth regulations on DNSA, MDA and H_2O_2 accumulation are well linked to the genotype. The H_2O_2 and MDA could trigger the stress response, play a role as a secondary messenger participated in cell signaling and improve somatic embryo formation. Compared with embryogenic callus, the amounts of protein were lower in somatic embryos of all tested genotypes except protein amounts of somatic embryos of *Tatlıcak* cultivar, protein amount between the three different *Triticale* cultivars, protein different significantly in somatic in *Tatlıcak and Mikham* somatic embryos, but a similar protein amounts was observed in somatic, non somatic embryos and control callus of *Alper Bey*, which may explain the embryogenic callus formation stages. The results indicate that the somatic embryos in the *Alper Bey* was mainly turned necrosis and extensively brown color during the culture medium. Somatic embryos could not further improve into unhealhty callus, which confirmed that the accumulation of energy substances affected the cell activity and morphogenesis. Proteins influence embryo formation, embryo differentiation, embryo maturation and cell signal transduction (Cangahuala-Inocente et al., 2014). Some studies have reported that the alters in protein amounts could be used as a marker of different somatic embryos stages (Jiménez, 2001;Cangahuala-Inocente et al., 2014). In our cases, somatic embryo development was at various levels in the three *triticale* cultivars under the scanning electron microscopy following treatments with long-terms, including 2,4-D. The result may have verified by the microscopy studies with a SEM in which display a reduction in the formation of continous surface in presence of somatic embryo. The first globular structures resembling embryos at the somatic embryo stage of *Tatlıcak* callus development (Figure 5e). In fact, the presence of somatic embryos in the three *triticale* cultivars, the formation of globular structures as well as some membranous and amorphous compounds were observed (Figure 5 a,c,e). SEM observations demonstrated that each genotype callus type had different callus structures in the non somatic embryos. The wrinkled and mucilage character of the callus in non-somatic embryos *Tatlıcak* and *Mikham* convert to granular-soft and compact resembles under SEM detections (Figure 5 b,d,f). Conversely, there was a continuous amorphous sphere, termed extracellular matrix, on the callus surface both somatic and non somatic embryos of *Alper Bey*. It was also observed that cultivars belonging to the same cultivars share mainly similar cell structure and shapes (Figure 5a,b). This confirms that *Tatlıcak* and *Mikham* cultivars has an effect on converting somatic embryos after long-terms and thus promoting antioxidant enzyme, DNSA and protein activities. It seems that this results of SEM analysis is an effective marker of somatic embryos in callus structures derived from *triticale* genotypes. Similar results have been reported in *Triticale* (Yazıcılar et al., 2021) and in *Picea sitchensis* SE (Liu, 2009). In conclusion, the embryogenic callus formation and frequency of embryogenic callus transformation into a somatic embryo in *triticale* cultivars could be regulated and developed by adjusting the external conditions, such as the genotypes and culture durations.

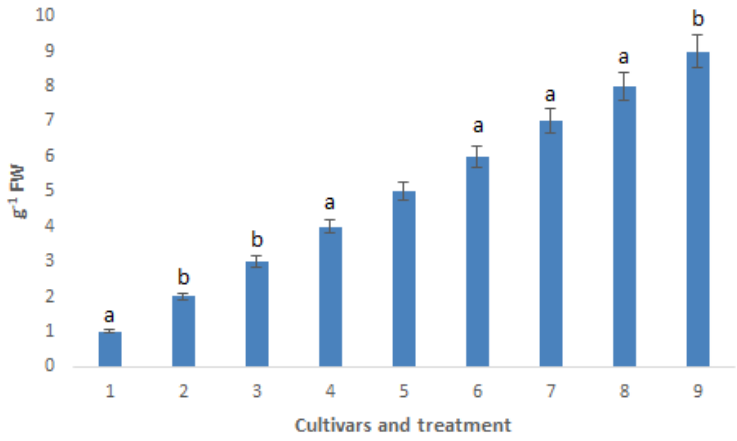

Figure1: DNSA analysis results(*Tatlıcak ,Alper bey, Mikham* 1,4,7:control,2,5,8:Somatic embryo 3,6,9:Non-somatic embryo)

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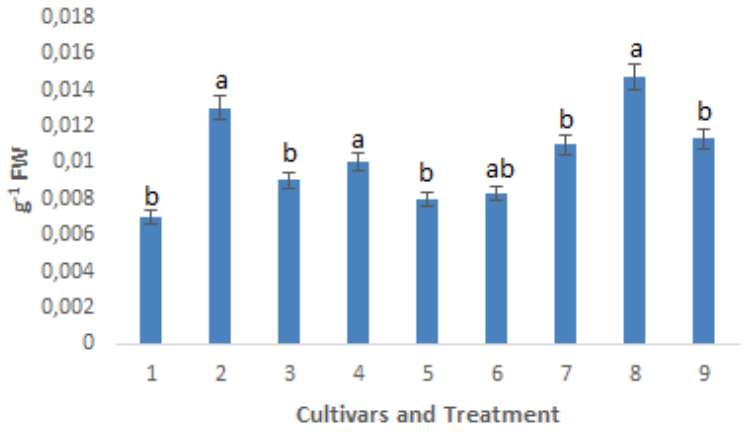

Figure 2:MDA analysis results(*Tatlıcak ,Alper bey, Mikham* 1,4,7:control,2,5,8:Somatic embryo 3,6,9:Non-somatic embryo)

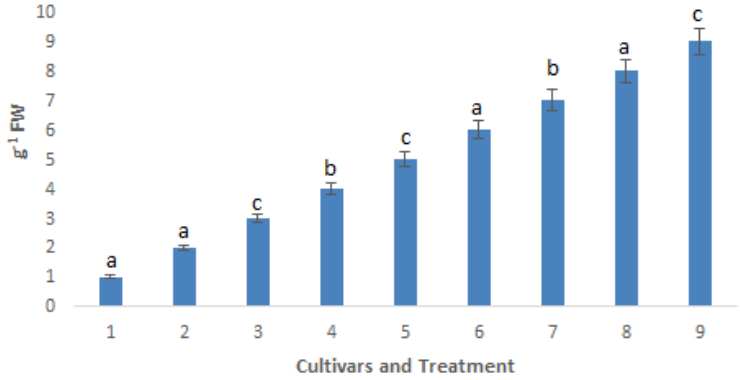

Figure 3: H_2O_2 analysis results(*Tatlıcak ,Alper bey, Mikham* 1,4,7:control,2,5,8:Somatic embryo 3,6,9:Non-somatic embryo)

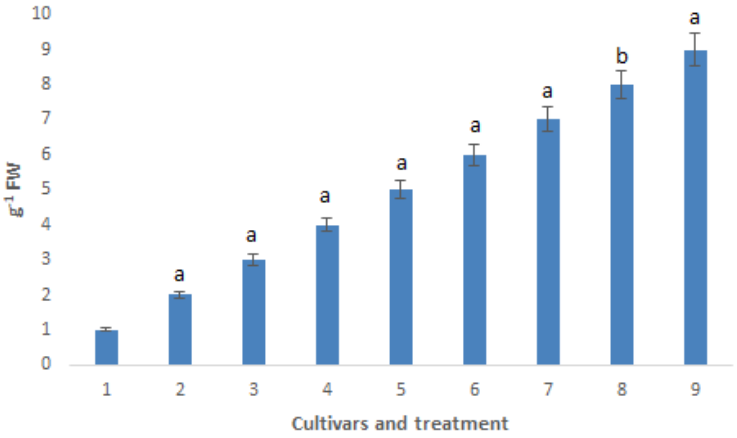

Figure 4: Protein analysis results(*Tatlıcak ,Alper bey, Mikham* 1,4,7:control,2,5,8:Somatic embryo 3,6,9:Non-somatic embryo)

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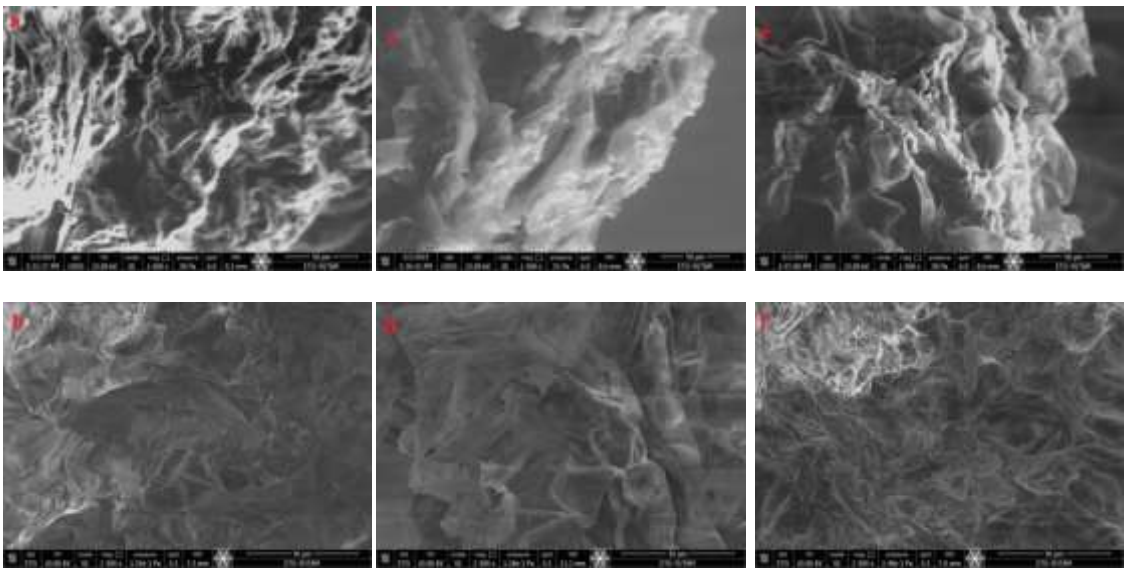

Figure 5: *Alper Bey*, *Mikham*, *Tatlıcak* somatic and non-somatic embryo SEM analysis results

a:*Alper bey* somatic embryo, **b:** *Alper bey* non-somatic embryo, **c:***Mikham* somatic embryo **d:***Mikham* non-somatic embryo, **e:***Tatlıcak* somatic embryo **f:***Tatlıcak* non-somatic embryo

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