Response of five triticale genotypes to salt stress in in vitro culture

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Abstract: Salinity is one of the important abiotic stresses that restrict plant development. Triticale is a highly adaptable crop, known for its high-quality grain, yield potential, and resistance to disease. Responses of five cultivated genotypes (Ümran Hanım, Mikham 2002, Melez 2001, Tatlıcak, and Alper Bey) of triticale to salt stress were tested in callus and embryogenic callus formation in this study. Cotyledon explants were selected as explants for callus induction and embryogenic callus formation. The five tested triticale genotypes varied in their callus growth and embryogenic callus formation. Ümran Hanım, Tatlıcak, and Alper Bey were observed for better callus induction; similarly, the same genotypes responded with better embryogenic callus formation in the salt in vitro media. A significant decrease in embryogenic callus growth was observed under salt stress. Based on the responses to NaCl in terms of embryogenic callus, the five triticale genotypes were ranked in the order of Tatlıcak > Ümran Hanım > Alper Bey > Mikham 2002 > Melez 2001. More proline and sugar were accumulated in these five triticale genotypes than in control plants when all were subjected to salt stress. Proline level peaked at 200 mM while the lowest and highest content was obtained at 0–200 mM salt concentrations. The accumulation of soluble sugars was strongly linked to 0–200 mM salt concentrations. Antioxidant enzyme activities exhibited an increasing trend in response to the increasing concentration of NaCl.

Key words: Triticale, embryogenic callus, salt stress, proline, sugar

1. Introduction
Salinity is an abiotic stress that causes serious yield losses in various crops (Ashraf et al., 2004; Hussain et al., 2009; Semiz and Suarez, 2015; Nawaz et al., 2016). Subjection of the plants to salt stress can lead to serious injury to the plants (Zhao et al., 2009; Farooq et al., 2015; Nuriyeva et al., 2016). Different techniques have been suggested to protect plants from environmental stress. Therefore, new techniques should be developed to produce salt-resistant genotypes. Selection and improvement of desirable genotypes for this objective require suitable screening methods. Tissue culture can help in the efforts to produce new cultivars against environmental stress factors. In addition, in vitro culture studies permit relatively faster responses, shorter generation time, and regular environmental conditions as compared to classical breeding methods (Zhao et al., 2009; Elmaghribi et al., 2013). A high level of salt in soil or in tissue culture may lead to numerous genetic and biochemical changes, causing problems such as limitation in mineral nutrient uptake, nutritional imbalance, mineral deficiency, osmotic stress, ion toxicity, and oxidative stress (Rozema and Flower, 2008; Rahnama et al., 2010; James et al., 2011). It has been reported that oxidative and osmotic stresses affect the cellular membrane integrity, enzyme activity, DNA, and chlorophyll content (Lokhande et al., 2010), which inhibits the functioning of most plant species.

Proline is the most common metabolite that accumulates in response to salinity stress, shown to serve as a main osmotic regulator, and was reported in various plant species (Koca et al., 2007). The role of proline accumulation in plants’ osmotic regulation is still unclear (Koskeroglu and Tuna, 2010). Soluble sugar is known to commonly accumulate in higher plants against salinity stress, which could play a role in osmotic protection (Khedr, 2003). Salt stress usually inhibits the plant growth. When plants are exposed to different abiotic stresses, some reactive oxygen species (ROS) removing enzymes such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT) are produced (Li and Staden, 1998). The antioxidant enzyme activity is positively associated with salt tolerance in plants (Lokhande et al., 2011). Many researchers have indicated that the salt resistance mechanism is activated during the entire plant stage and this has been tested in both in vitro and ex vitro situations (Watanabe et al., 2000; Troncoso et al., 2002). A close relationship between physiological changes and callus cultures by salt stress has been previously reported in different genotypes (Piwowarczyk et al., 2016).
The effect of physiological changes to differentiate between salt-sensitive and salt-resistant cultivars or genotypes is routinely being tested (Djukic et al., 2013). A useful in vitro protocol for salt resistance using different concentrations of NaCl as selection tools has also been applied to many crops including sugarcane, potato, sweet potato, and beet (Prakash and Jack, 1993; Yang et al., 2005; Gandonou et al., 2006; He et al., 2009).

Triticale (× Triticosecale Wittmack) is a recognized artificial amphiploid cereal that has been remarkably developed by classical breeding and almost 3 × 10^6 ha of triticale is cultivated today in the world (Lelley and Gimbel, 1989; Machzynska et al., 2014; http://www.fao.org/faostat/en/). Intensive studies on triticale were started in the early 1950s (Kiss, 1966), followed by investigations on somatic tissue culture (Immonen, 1996), molecular genetics (Balatero et al., 1995), and transgenic studies (Zimny et al., 1995). Traditional breeding methods have been used extensively for improvement of cultivars resistant to stress conditions. However, these traditional breeding programs have some limitations, such as the lack of genetic sources (Rai et al., 2011). Utilization of in vitro methods for quantifying the salt stress tolerance of various crops has been increasing rapidly and these methods have obtained success in some plants such as tobacco (Nabors et al., 1980), rice (Lutts et al., 1999), and wheat (Barakat and Abdel-Latif, 1996). Intensification of breeding in triticale for resistance to salinity stress through a sustainable method would help produce stress-resistant cultivars. So far, triticale has not been applied in intense breeding methods or tissue culture techniques for salinity stress resistance. In vitro propagation strategies are the most effective ways for improving stress-resistant cultivars nowadays. This strategy can succeed under stress conditions with limited space and time (Elmagrahi et al., 2013) and it has the potential for improving stress-resistant cultivars using a low-cost laboratory setup. Despite triticale standing out from other cereals crops with its resistance to various abiotic stresses, the mechanisms of adaptation to these stresses at the physiological and biochemical levels are still not well known in this species. There is no available experiment on salt resistance in this crop under in vitro conditions. The aim of this study was to evaluate 5 triticale genotypes of landraces under different NaCl concentration conditions through in vitro callus cultures and also to determine salinity-induced changes in physiological processes that have been confirmed in other cereals.

2. Materials and methods
2.1. Plant material
Salt responses were tested in callus tissues and regenerated shoots of triticale. One triticale cultivar (Umran Hanım) was obtained from the East Anatolia Agricultural Research Institute (Erzurum, Turkey) and four triticale cultivars (Alper Bey, Meliz 2001, Mikhım 2002, and Tatlıcak) were kindly provided by the Bahri Dağdaş International Agricultural Research Institute (Konya, Turkey). In this study, mature seeds were surface-sterilized with 70% ethanol for 5 min, washed several times with sterile distilled water, treated with 33% commercial bleach for 20 min, and rinsed with several changes of sterile distilled water overnight at 4 °C. The mature embryos, aseptically excised from the imbibed seeds and placed scutellum up, were cultivated in petri dishes containing full-strength MS medium for 30 days at 26 ± 1 °C. The mature embryos were inoculated on MS medium supplemented with different concentrations of 2,4-D corresponding to a total of 4 treatments, i.e. 2 mg/L, 4 mg/L, 8 mg/L, and 12 mg/L, each replicated 4 times for callus induction. The lighting conditions were set as 16-h light/8-h dark photoperiod at 1500 lx illumination intensity.

2.2. Tissue culture media
The culture medium used in all stages of the experiment was MS medium with 2 mg L⁻¹ glycine, 100 mg L⁻¹ myo-inositol, 0.5 mg L⁻¹ nicotinic acid, 0.5 mg L⁻¹ pyridoxine HCl, 0.1 mg L⁻¹ of thiamine HCl vitamins, 1.95 g L⁻¹ of MES, 50 mg L⁻¹ of ascorbic acid, and 20 g L⁻¹ of sucrose, solidified with 7 g L⁻¹ of agar and the pH adjusted to 5.8 prior to autoclaving, according to Murashige and Skoog (1962). In order to sterilize the vitamins and hormones, 0.22-µm porous cellulose nitrate filters were used.

2.3. Salt stress treatment
The mature triticale embryos were cultured in MS medium containing 4 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) for 15 days in the dark. Later only embryogenic calli were transferred to MS medium containing NaCl in five different doses (0, 50, 100, 150, and 200 mM NaCl) for 60 days and subcultured for another 30 days. All calli were kept under fluorescent light at 62 µmol m⁻² s⁻¹ and a 16-h/8-h light/dark cycle at 26 ± 1 °C. The total culture duration was 75 days. Embryogenic calli were evaluated after 30 days of salt treatment for growth rate, accumulation of proline, total soluble sugars, and antioxidant enzyme activity.

2.4. Proline estimation
Proline content was measured with the method of Bates et al. (1973). Embryogenic calli material (100 mg) was homogenized in 5 mL of 3% aqueous sulfosalicylic acid and centrifuged at 4 °C for 15 min at 4800 × g. Extract (2 mL) was mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in test tubes. Samples were kept for 1 h at 100 °C. The reaction was terminated in an ice bath and 4 mL of toluene was used for the reaction of the mixture extraction. The absorbance of color reaction product was measured at 520 nm using toluene as a blank. The proline concentration was determined from a calibration curve.
2.5. Soluble sugar determination
For soluble sugar determination, 50 mg of tissue per embryogenic callus was ground in a mortar, homogenized in 1 mL of 80% ethanol, and centrifuged at 5000 × g at 4 °C for 10 min. Supernatants were transferred into other tubes and the pellets were homogenized again in 0.5 mL of 80% ethanol and centrifuged as above. The second supernatant was added to the first. Total soluble sugars were measured by a modified method of Watanabe et al. (2000). One milliliter of extract was reacted with 3 mL of freshly prepared anthrone reagent (50 mg anthrone + 50 mL of 95% H₂SO₄) at 100 °C for 10 min. After cooling on ice, the total sugar content was determined at 620 nm with a spectrophotometer using glucose as a standard.

2.6. Antioxidant enzyme activities
Leaf samples (0.5 g) were crushed with a mortar and homogenized in ice-cold 0.2 M phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA). The extract was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was utilized to measure the activity of CAT, APX, and SOD enzymes. CAT activity was measured by monitoring the decrease in absorbance at 240 nm in 50 mM phosphate buffer (pH 7.5) including 20 mM H₂O₂. One unit of CAT activity was defined as the amount of enzyme that used 1 µmol H₂O₂ per minute according to the method of Gong et al. (2001).

APX was analyzed by recording the decrease in absorbance at 290 nm in 3 mL of sample mixture including 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM sodium ascorbate, 0.1 mM EDTA, 0.2 mL of supernatant, and 6 mM H₂O₂ according to the method of Nakano and Asada, (1981). SOD activity was determined by monitoring the reduction in absorbance of nitro-blue tetrazolium (NBT) dye, according to method of Agarwall and Pandey (2004). The sample mixture included 13 mM methionine, 2 µM riboflavin, 75 µM NBT, 0.1 mM EDTA, 50 mM sodium carbonate, 50 mM phosphate buffer (pH 7.8), and 0.1 mL of the extract. Riboflavin was added at the end and the tubes were shaken and placed 30 cm below a light bank containing two fluorescent tubes. After 20 min, the reaction was finished by covering the tubes with a black cloth. The absorbance was recorded spectrophotometrically at 560 nm.

2.7. Statistical analysis
The experiment was conducted in a factorial design using a completely randomized design with 4 replications of 25 explants per petri dish. Each petri dish was considered as one experimental unit. Twenty-five explants were placed in each petri dish. Analysis of variance and the Waller–Duncan K-ratio t-test were used to determine significant differences. Statistical analysis was carried out using SPSS 20.0 (IBM Corp., Armonk, NY, USA).

3. Results
3.1. Genotypic capacity of callus induction
In the present study, different concentrations of 2,4-D (i.e. from 2 mg/L to 12 mg/L) were tested to find the best concentration for successful callus induction. It was found that a concentration of 4 mg/L 2,4-D was optimum for callus induction for all tested genotypes. The calli produced at 4 mg/L were compact, healthy, and fluffy (Figure 1). The callus induction ratio was calculated as follows: induced calli/number of total explants × 100. The results shown in Table 1 indicate that the triticale Ümran Hanım had the highest callus induction rate (99.07%) in terms of its callus formation when compared to the other genotypes. Among the cultivated triticale genotypes, Mikham 2002 (97.33%) and Tatlıcak (98.47%) had higher rates of callus formation as compared with Alper Bey (97.13%) and Melez 2001 (94.17%). Callus growth potential and embryogenic callus formation were greatly influenced by the genotype. Our results indicated that callus induction rates ranged from 94.17% to 99.07%, suggesting significant genotypic variations in the callus growth potential between the five genotypes (Table 1; Figure 1).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Callus induction (%)</th>
<th>Fresh callus weight (g)</th>
<th>Embryogenic callus weight (g)</th>
<th>Callus necrosis weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melez 2001</td>
<td>94.17 ± 3.69</td>
<td>0.19 ± 0.01</td>
<td>0.45 ± 0.04</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>Alper Bey</td>
<td>97.13 ± 11.58</td>
<td>0.17 ± 0.01</td>
<td>0.65 ± 0.06</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>Mikham 2002</td>
<td>97.33 ± 11.62</td>
<td>0.17 ± 0.01</td>
<td>0.48 ± 0.04</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>Tatlıcak</td>
<td>98.47 ± 11.45</td>
<td>0.23 ± 0.02</td>
<td>0.68 ± 0.06</td>
<td>0.52 ± 0.05</td>
</tr>
<tr>
<td>Ümran Hanım</td>
<td>99.07 ± 10.95</td>
<td>0.27 ± 0.02</td>
<td>0.64 ± 0.06</td>
<td>0.59 ± 0.05</td>
</tr>
</tbody>
</table>

Values with different superscripts in the same column are different at P < 0.05 based on the Duncan test.
3.2. In vitro embryogenic callus of triticale genotypes

A wide variation in the investigated parameters was determined among the genotypes tested in response to different concentrations of NaCl. Mean comparison of the traits measured for embryogenic callus formation under in vitro stress conditions (Table 2) displayed that significant effects of salt concentration, genotype, and the 5 different genotypes’ interactions were observed for all the studied parameters. The results indicated that embryogenic callus formation decreased significantly in high salinity stress. Embryogenic callus formation was reduced considerably in all the genotypes under 150 mM and 200 mM salt stress as compared with their control plants. In particular, the growth potential of the explants of the Melez 2001 and Mikham 2002 genotypes was reduced to a great degree under salt stress. Out of the 5 genotypes tested, Tatlıcak could resist by 85% at 200 mM NaCl. Ümran Hanım and Alper Bey could survive by 76% and 71%, respectively. The growth of Mikham 2002 and Melez 2001 was remarkably suppressed by 27% and 30% at 200 mM; moreover, these two genotypes necrotized and finally died in 2–3 weeks of treatment (Figure 1; Table 2).

3.3. Proline

The proline content of triticale embryogenic calli was shown to increase gradually in response to salt stress. All levels of salt were observed to cause slight increases in proline content and the highest proline level was seen in all genotypes exposed to 200 mM NaCl (Figure 2). It was observed that the level of proline in embryogenic calli was highly correlated with NaCl concentration under in vitro conditions and the lowest proline concentration was observed with 0 mM NaCl (Figure 2). Under the strongest salt stress conditions tested, proline contents reached about 3.87 µmol/g DW (200 mM NaCl), which represented an increase of 3.48-fold with respect to the nontreated controls.

Table 2. Effect of five triticale genotypes and NaCl doses on percentage of explants forming embryogenic calli in five triticale genotypes.

<table>
<thead>
<tr>
<th>Doses</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alper Bey</td>
</tr>
<tr>
<td>0 mM</td>
<td>95.00 ± 0.9aBC</td>
</tr>
<tr>
<td>50 mM</td>
<td>87.00 ± 0.8bB</td>
</tr>
<tr>
<td>100 mM</td>
<td>80.00 ± 0.8cC</td>
</tr>
<tr>
<td>150 mM</td>
<td>78.00 ± 0.7dC</td>
</tr>
<tr>
<td>200 mM</td>
<td>71.00 ± 0.7eC</td>
</tr>
</tbody>
</table>

The differences between the means shown with capital letters in the same column and the means shown with lowercase letters on the same line are significant (P < 0.05). Values are mean ± standard deviation.
3.4. Soluble sugar
Considerable variations were observed in the accumulation of sugar in the salt-treated and untreated calli. All the triticale cultivars displayed similar sugar accumulation in in vitro culture. The sugar level of the triticale cultivars in in vitro media compared to salt stress varied from 15.3 to 25.65 mg/g. When treated with salt stress, higher levels of sugar were accumulated in Tatlıcak compared to control plants, which increased nearly twofold, from 15.76 to 25.65 mg/g (Figure 3). The Tatlıcak cultivar showed the highest

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Figure 2. Changes of proline in five triticale genotypes treated with salt stress under in vitro conditions.

Figure 3. Changes of soluble sugar in five triticale genotypes treated with salt stress under in vitro conditions.
sugar accumulation in calli, followed by Ümran Hanım and then by Alper Bey. The sugar accumulation in salt-adapted calli increased significantly with the increase in the concentration of salt. The sugar content in Taticak was higher than that of the other genotypes under salt stress, which peaked at 200 mM salt stress. Compared with the control, the sugar content of all genotypes tested showed similar rises after salt stress.

3.5. Antioxidant enzyme activity
The levels of activities of antioxidant enzymes such as CAT, APX, and SOD were investigated in the 200 mM NaCl-stressed embryogenic calli in vitro culture at the end of 1 month. All tested enzyme activities were enhanced with the time of the in vitro culture. The activity of SOD was the highest in Taticak and had similar values in all the other genotypes (Figure 4). The APX activity in the salt medium was highest in Taticak and Alper Bey, and it was also relatively high in the Ümran Hanım genotype, whereas Melez 2001 and Mikham 2002 had low APX activity in the 200 mM NaCl-stressed embryogenic calli (Figure 5). CAT activity was substantially lower in Mikham 2002 than in the other genotypes and it was also relatively low in Melez 2001, while the activity of this enzyme was nearly the same in the other genotypes in the 200 mM NaCl-stressed embryogenic calli (Figure 6).

4. Discussion
Salinity is one of the main factors in reducing plant growth and productivity. Salinity resistance is a polygenic trait, difficult to obtain by using traditional breeding techniques under normal conditions (Richards, 1996). In vitro culture has been known to be a useful and rapid method to evaluate salt resistance and it provides a controlled and stable medium for studying physiological and biochemical pathways in plants, especially at the molecular level under different salt concentration levels (Lokhande et al., 2010). It has been suggested that in vitro culture is a stressful environment that could be responsible for the induction of salt stress during new cultivar improvement (Bang et al., 2015; Jo et al., 2016). Regeneration of plants in vitro culture conditions has been studied in several crops (Ghadakchiasl et al., 2016). Most of these studies were on salt stress and factors affecting callus formation and plant regeneration (Amini and Ehsanpour, 2006). In the present investigation, in vitro embryogenic callus formation responses of triticale under different salt concentrations were tested and the present study displayed considerable differences in their responses to the salt stress. The embryogenic callus induction for survival of the 5 triticale genotypes ranged between 27.0% and 95.0% (Table 2). Embryogenic callus formation and plant regeneration under salt stress conditions were shown to be strongly genotype-dependent. Formation of embryogenic calli was generally induced by auxins, especially 2,4-D (Lutts et al., 1999). The auxin 2,4-D alone was used for regeneration of embryogenic calli in many plants such as wheat, oat, and maize (He et al., 1986; Bregitzer et al., 1989; Naqvi et al., 2002). In the present study, a low concentration of 2,4-D was used in triticale for the induction of calli with embryogenic callus potential. Our results indicated that the percentage of embryogenic callus formation varied significantly depending on the NaCl level and genotype (Table 1), similar to those of earlier reports (Piwowarczyk et al., 2016). A similar response of callus induction and regeneration capacity was also reported in durum and wheat genotypes (Rashid et al., 2002). According to these authors, many factors such as medium composition, genotype, and source of explant affect at varying degrees the processes relating to the capacity of callus growth, embryonic differentiation, and plantlet regeneration. Significant genotypic differences in callus initiation response were observed among the wheat genotypes and
Callus induction capacity is related to salt medium (Özgen et al., 1996).

Embryogenic differentiation also demonstrated osmotic regulation against salt stress through the synthesis of compatible solutes like proline and soluble sugar contents (Patade et al., 2012). In this study, there was a strong relationship between NaCl and proline content in triticale, as reported by others in potato and quinoa (Ochatt et al., 1999; Hariadi et al., 2011). Proline content has been indicated to have protective effects against salinity stress (Ahmad et al., 2008). Our results also exhibited an increased accumulation of proline content in salt-resistant embryogenic differentiation as compared to control embryogenic differentiation of all tested genotypes. Embryogenic callus formation was very significantly negatively correlated with proline ($R = -0.484, P < 0.001$). There was a high positive correlation for embryogenic callus formation of calli ($R = 0.681, P < 0.001$). The level of proline accumulation continued to rise even when promotion of growth was diminished. Similarly, Zhao et al. (2009) found that different salt applications increased proline levels in mangrove Thellungiella halophila. Soluble sugar also imparts osmotic regulation of the cell under salt stress (Figure 3). The effects of salt stress on soluble sugar accumulation are evidently related to the salt resistance ability (Watanabe et al., 2000). Lokhande et al. (2011) also demonstrated that salt-stressed calli of Sesuvium portulaca retained more sugar than unstressed calli when both were treated with 200 mM NaCl. Moreover, the accumulation of soluble sugars in many crops has been well documented against abiotic stress. Our results indicated that the highest level of these sugars was found in the 200 mM NaCl-treated group, which could contribute to embryogenic callus formation. Thus, it seems that this trait is an effective marker of salt resistance in embryogenic calli derived from triticale genotypes.

Previous studies demonstrated that salt-tolerant species increased their antioxidant enzyme activities under stress conditions (Thounaojam et al., 2012; Shen et al., 2013; Long et al., 2014). In this study, salt stress increased SOD activity in embryogenic calli of all studied genotypes (Figure 4). A similar response to salt was found in wheat genotypes in terms of SOD activity (Sairam and Srivastava, 2002). These results suggest a significant positive relationship between salt stress and SOD under both salt treatment and nontreated conditions. Higher SOD activity in salt-resistant lines reflects better ROS scavenging capacity that helps in the detoxification of cells. APX activity is increased in both salt-tolerant and salt-sensitive cultivars under salt stress. A salt-tolerant cultivar of triticale has been shown to have higher APX activity than the salt-sensitive cultivar under salt stress conditions (Figure 5). This is in agreement with previous studies that showed that APX activity is directly related to salt stress (Santos et al., 2004; Sekmen et al., 2007). CAT activity was significantly increased in stressed embryogenic calli as compared to control calli (Figure 6), suggesting the enhancing capacity of calli tissue to scavenge $\text{H}_2\text{O}_2$ with increasing salt stress. The results are in disagreement with other reports that suggested the decrease in CAT activity under increasing salinity in callus cultures of Suaeda nudiflora (Cherian and Reddy, 2003) and Crithmum maritimum (Amor et al., 2005).

In conclusion, variations among triticale genotypes for embryogenic callus formation under in vitro conditions could be used as an alternative and rapid way to detect salt-resistant genotypes. The survival rate of embryogenic calli displayed the possibility of ranking the tested triticale genotypes as salt-resistant ones, including Tatlıcak and Ümran Hanım, and salt-sensitive ones, including Mikham 2002 and Melez 2001. Alper Bey seems to be intermediate in terms of sensitivity to salt stress. Therefore, induction of 200 mM salt stress may be considered as a stringent selection process in stress resistance studies in triticale. Proline and soluble sugars in stressed calli are related to high concentrations of NaCl. The responses of antioxidant enzymes to the salt conditions are different in the various cultivars of triticale. The results also showed that salt enhanced the activities of SOD, APX, and CAT enzymes. This in vitro selection method has great advantages for applications in selecting triticale embryogenic calli resistant to salt stress.

**Acknowledgment**

I would like to thank Prof Dr Tony J Fang, Department of Food Science and Biotechnology, National Chung Hsing University, Taiwan, for revising the English of the manuscript.
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