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Salt Stress Response of Sunflower Breeding Lines Developed After Wide Hybridization

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Abstract

Response of sunflower germplasms viz. cultivated sunflower *H. annuus* and two breeding lines *H. annuus* × *T. rotundifolia* and *H. annuus* × *V. encelioides* developed after wide hybridization were used for identification of salt tolerant sunflower genotypes at the seedling growth stage. Two levels of salinity stress (100 and 200 mM NaCl) were created and performances were monitored against a control. Physiological and biochemical stress, determining parameters such as root and shoot lengths, fresh weight, and antioxidant enzyme activities superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPO), and ascorbate peroxidase (APX) were compared between seedlings of all three genotypes. The results indicated that all three genotypes had similar responses at different NaCl concentrations for seedling growth parameters studied which decreased with increasing salinity. The data obtained on antioxidant enzymes such as CAT, SOD, GPO, and APX have shown significant differences among examined genotypes. Such differential responses of the enzymatic antioxidant systems of the tested sunflower genotypes with different origin put in evidence that the contrast between these genotypes in terms of salt tolerance exists. Of the three genotypes examined, hybrid line *H. annuus* × *V. encelioides* followed by line *H. annuus* × *T. rotundifolia* were more salt-tolerant as compared to the cultivated cultivar *H. annuus* cv 1114.

Key words: Antioxidant enzymes, *Helianthus annuus* L., intergeneric hybridization, interspecific hybridization, *Tithonia rotundifolia*, *Verbesina encelioides*

Abbreviations: SOD (Superoxide dismutase); CAT (Catalase); GPO (Guaiacol peroxidase); APX (Ascorbate peroxidase); DPPH (2, 2-diphenyl-1-picryl-hydrasyl); PEG (Polyethylene glycol-6000); F_1 (First generation hybrid); BC (Backcross generation); cms (Cytoplasmic male sterility).

Introduction

Salinization of soils is one of the main abiotic stresses in nature that limits plant productivity involving an inhibition of growth and development, reduction in photosynthesis, respiration and protein synthesis in sensitive species (Meloni et al., 2003; Chinnusamy et al., 2005; Ashraf et al., 2008; Ahmad et al., 2008; Ahmad and Prasad, 2012; Tuteja et al., 2012). Like other abiotic stresses, salt stress also leads to oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide ($O_{2\bullet}^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH_\bullet$) (Alscher et al., 1997; Mittler, 2002). ROS are regarded as the main source of damage of cells under biotic and abiotic stress causing lipid peroxidation, protein denaturating and DNA disorders (Mittler, 2002). During salt stress, plants adapt to oxidative stress eliminating or reducing ROS by accumulation certain protective solutes like proline, glycine, betaine, polyols, trehalose etc. (Sakamoto and Murata, 2002; Beak and Skinner, 2003; Ahmad et al., 2010; Gill and Tuteja, 2010). Also, an antioxidant protective system that includes both nonenzymatic metabolites (ascorbate, glutathione, α-tocopherol, carotenoids and phenolic) and antioxidant enzymes (superoxide dismutase, different specific

peroxidases, catalase and enzymes of ascorbate-glutathione cycle) has been reported to exist (Sharma et al., 2012).

Helianthus annuus is classified as moderately tolerant to salinity (Steduto et al., 2000); seed productivity is unaffected by salinity up to EC 4.8 dS m^{-1} (≈ 50 mM NaCl) in the saturation soil extract and declines by approximately 5% per unit increase in salinity thereafter (Flagella et al., 2004). It has been found that salinity induces a number of adverse effects on growth, yield and some physiological and biochemical processes taking place within the sunflower plant tissues (Ashraf and Tufail, 1995, Muralidharudu et al., 1999, Akram and Ashraf, 2011; Shahbaz et al., 2011, Gaballah et al., 2012). In Bulgaria, sunflower is widely grown and ranks first in terms of importance among oil crops. There has been considerable interest in the developing plant varieties with improved salt tolerance and agronomic inputs. Besides conventional breeding techniques, wide (interspecific and intergeneric) hybridization is also used as an alternative for improvement of desired characters in agricultural crops. Breeding progress resulting from wide hybridization in sunflower is well documented (Breton et al., 2010; Breton et al., 2012; Vassilevska-Ivanova et al., 2013; Kaya, 2014; Kantar et al., 2014).

Although previous researches discussed wide hybridization in sunflower as one of the most important strategies having the potential to combine useful traits of both parents that could not be achieved by crossing within a single species (Faure et al. 2002; Rönicke et al. 2004; Breton et al. 2010; Breton et al. 2012; Vassilevska-Ivanova et al. 2013; Vassilevska-Ivanova et al. 2014), so far there has been no side-by-side comparison of hybrid plants with regard to their origin on salt stress. Therefore, we have analyzed the behavior of three different sunflower genotypes–cultivated *H. annuus* and two advanced lines developed after intergeneric hybridization with species of related genera *Verbesina* and *Tithonia* in the early developmental stages under experimental salt stress conditions. The response of sunflower plants was characterized with reference to salt stress on growth parameters, antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPO), and ascorbate peroxidase (APX).

Materials and Methods

Reagents: All chemicals used were of analytical grade. All of them were obtained from Sigma Chemical Co. (St Louis, MO, USA) and Merck (Darmstadt, Germany).

Plant materials, growth conditions and stress treatments

Three sunflower (*Helianthus annuus* L.) genotypes with diversified genetic background were chosen on the basis of speculation of the potential of wild *Helianthus* species to impact the adaptation of the introgressed hybrids under salinity conditions. Seeds of *H. annuus* L. cultivar 1114 and two advanced intergeneric lines *H. annuus* × *Tithonia rotungifolia* and *H. annuus* × *Verbesina encelioides* were used in the study. These genotypes were developed at the Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria following a research program using the potential of wide hybridization for producing and evaluating new sunflower germplasms. The intergeneric line *Helianthus* × *Verbesina* originated from pollination of cytoplasmic male sterile (cms) line HA89 (female), a public oilseed sunflower inbred maintainer line released by US Department of Agriculture–Agricultural Research Service, with bulked pollen from wild annual *Verbesina encelioides* (male). The cms line L 1234 of *H. annuus*, released by Dobrudza Institute of Wheat and Sunflower, Bulgaria was pollinated with bulked pollen from *T. rotundifolia*. The populations of both *V. encelioides and T. rotundifolia* species were grown from seeds originally obtained from the Botanical Garden of Lisbon, Portugal. The crosses were made by conventional hybridization method. Each experiment was carried out on flower heads which had been protected from foreign pollen by bagging. First generation hybrid plants were verified using morphological and cytological methods, and F_1 hybrids were back-crossed to the common sunflower to obtain BC_1 and BC_2. The main agronomic and morphological characteristics of these plants as well as their inheritance were described in our previous works (Vassilevska-Ivanova et al. 1999; Vassilevska-Ivanova and Tcekova, 2002). Seeds from advanced plant generations were produced after self-pollination under a bag. They were stored dry in paper bags at 5 °C for at least 9 months before the experiments and were non-dormant.

Phenotypic study of salinity tolerance at seedling stage

The genotypes were screened for salt tolerance at seedling stage in hydroponic system using the standard protocol as follow: twenty-five seeds of each genotype were pretreated with 5% sodium hypochlorite for 15 min and then germinated in rolled moistened paper towels in darkness (25 ± 1°C) as previously described

(Vassilevska–Ivanova et al., 2000). After germination, when cotyledons fully emerged, the healthy and uniform seedlings were transferred to 600 mL plastic beakers filled with half-strength Hoagland's solution (Hoagland and Arnon, 1950) and grown in a controlled growth chamber "Forma Scientific" model 3744 at 25 ± 2 °C with a 16-h light (250 µmol m^{-2} s^{-1}) and 8-h dark photocycle. The nutrient solution was salinized by adding crude salt to half-strength Hoagland's solution to obtain desired experimental concentration (100 and 200 mM). The salinity stress period created by NaCl was 3 days. Each set of experiments was performed three times.

At the end of the experiment (14 days), the plants were harvested. For phenotypic observation like shoot and root length as well as their fresh weight were recorded in both normal (control) and salinized conditions.

Estimation of enzyme activity

Enzyme extracts were prepared by homogenizing plants tissue in a pre-chilled mortar in 20 ml chilled extraction buffer (pH 7.8) contained 100 mM K_2HPO_4, 5 mM EDTA, 2% Polyclar AT, 1mM PMSF (phenyl methyl sulfonyl fluoride). Extracts were then centrifuged at 12 000 g for 30 min at 4°C. Enzyme assays were conducted immediately following extraction.

Superoxide dismutase (SOD) (EC 1.15.1.1) was measured by photochemical method described by Giannopolitis and Ries (1977). Assays were carried out under illumination. Reaction mixture (total volume of 3.0 ml) contained the following parameters: 2.35 ml 50 mM Na_2HPO_4; 0.10 ml 33 µM p-nitro blue tetrazolium chloride, 0.30 ml 10 mM L-methionine, 0.20 ml 0.66mM Na_2EDTA and 0.05 ml 3.3 µM riboflavin. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of p-nitro blue tetrazolium chloride reduction at 560 nm.

Catalase (CAT) (EC 1.11.1.6) activity was assayed in a method following Beers and Sizer (1952) with minor modifications. The following reaction mixture (total volume of 3.0 ml) was used: 2.77 ml 0.05 M Na_2HPO_4 (pH 7.0) containing 0.1 mM EDTA; 0.20 ml enzyme extract; 0.03 ml 3% H_2O_2. In the blank sample 0.03 ml d.H_2O instead of H_2O_2 were added. Activity was determined by following decomposition of H_2O_2 (extinction coefficient, 39.4 mM^{-1} cm^{-1}) at 240 nm. The enzyme activity was expressed in catalytic units-mmol H_2O_2 mL^{-1} min^{-1} per mg protein.

Guaiacol peroxidase (GPO) (EC 1.11.1.7) activity was determined according to Urbanek et al. (1991). Reaction mixture (total reaction volume of 3 ml) contained 2.8 ml quaiacol, 0.1 ml enzyme extract and 0.1 ml 2% H_2O_2. Blank sample contained 0.1 ml extraction buffer instead of H_2O_2. The oxidation of guaiacol in the presence of H_2O_2 was measured as the increase in absorbance recorded at 470 nm. The enzyme activity was expressed as nmol H_2O_2 mg $protein^{-1}$ min^{-1} (Plewa et al. 1991).

Ascorbate peroxidase (APX) (EC 1.11.1.1) activity was determined measured according to the method of Nakano and Asada (1981). Total volume of 3 ml reaction mixture was used. One ml of reaction mixture contained a 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 200 µM of enzyme extract. The following ratio between the components was complied: 2.8 ml buffer: 0.05 ml ascorbic acid: 0.1 ml enzyme extract: 0.05 ml H_2O_2. The concentration of oxidized ascorbate was calculated by the decrease in absorbance at 290 nm. Enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 mM^{-1} cm^{-1}), and was expressed as moles of ascorbate oxidized per milligram of protein per minute (McKersie and Leshem 1994).

Soluble protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

Twenty plants were raised for each treatment and all the experiments were repeated twice. Data were subjected to one-way ANOVA analysis of variance for comparison of means, and significant differences were calculated according to Fisher LSD test at the 5% level using a statistical software package (Statigraphics Plus, version 5.1 for Windows). Data were reported as means ± standard error. Standard errors were represented as vertical bars, which were the mean of three values (n=3), and similar letters in figures represent non-significant differences among the treatments within each genotype. The figures were performed with the OriginPro 8 SRO, Company OriginLab Corporation program package.

Results and Discussion

Effect of salt stress on seedling growth in the three sunflower genotypes

The results revealed that the root and shoot length as well as the fresh weight were strongly affected by salt treatments. Increased NaCl concentration caused a decrease in these characters as the response to salinity varied among genotypes. The data presented in Table 1 indicate clearly that average length of shoots and roots as well as their fresh weight significantly decrease

with increasing sodium chloride concentrations. Decrease in fresh weight of root was more pronounced as compared to shoot in all NaCl treatment in all three sunflower genotypes. However, this decrease was more prominent in *H. annuus* cv 1114 and *H. annuus* × *T. rotundifolia* genotypes.

Table 1. Effects of salt stress on root and shoot growth responses (mean length per seedling in cm) and fresh weight (g per plant) of cultivated sunflower *H. annuus* cv. 1114 and two intergeneric *lines H. annuus × Tithonia rotundifolia* and *H. annuus × Verbesina encelioides*; percentage control values are given in parenthesis.

	Treatment (NaCl)	Genotypes		
		H. annuus cv 1114	*H. annuus* × *T. rotundifolia*	*H. annuus* × *V. encelioide*
		Length, cm		
Root	0	20.9 (100) ± 1.85^{a}	18.2 (100) ± 2.06^{a}	21.2 (100) ± 2.12^{a}
	100 mM	16.9 (80.8) ± 1.71^{b}	15.5 (85.2) ± 1.50^{ab}	19.6 (92.4) ± 1.82^{ab}
	200 mM	11.9 (56.9) ± 1.27^{c}	12.4 (68.1) ± 1.45^{c}	17.0 (80.2) ± 1.44^{bc}
Shoot	0	15.8 (100) ± 2.08^{a}	11.2 (100) ± 1.15^{a}	9.8 (100) ± 1.42^{a}
	100 mM	12.8 (81.0) ± 1.58^{ab}	7.4 (66.1) ± 0.95^{b}	8.4 (85.7) ± 1.13^{ab}
	200 mM	10.6 (67.1) ± 1.24^{bc}	5.9 (52.7) ± 0.72^{c}	7.3 (74.5) ± 0.83^{bc}
		Fresh weight (g per plant)		
Root	0	0.9813 ± 0.03^{a}	0.8010 ± 0.04^{a}	0.4796 ± 0.01^{a}
	100 mM	0.8143 ± 0.04^{b}	0.6539 ± 0.02^{b}	0.3969 ± 0.01^{b}
	200 mM	0.3480 ± 0.04^{c}	0.2916 ± 0.03^{c}	0.2164 ± 0.01^{c}
Shoot	0	0.8597 ± 0.08^{a}	0.6992 ± 0.04^{a}	0.3629 ± 0.03^{a}
	100 mM	0.7163 ± 0.05^{b}	0.3987 ±0.02^{b}	0.3102 ± 0.01^{b}
	200 mM	0.4964 ± 0.04^{c}	0.3015 ±0.01^{c}	0.1969 ± 0.01^{c}

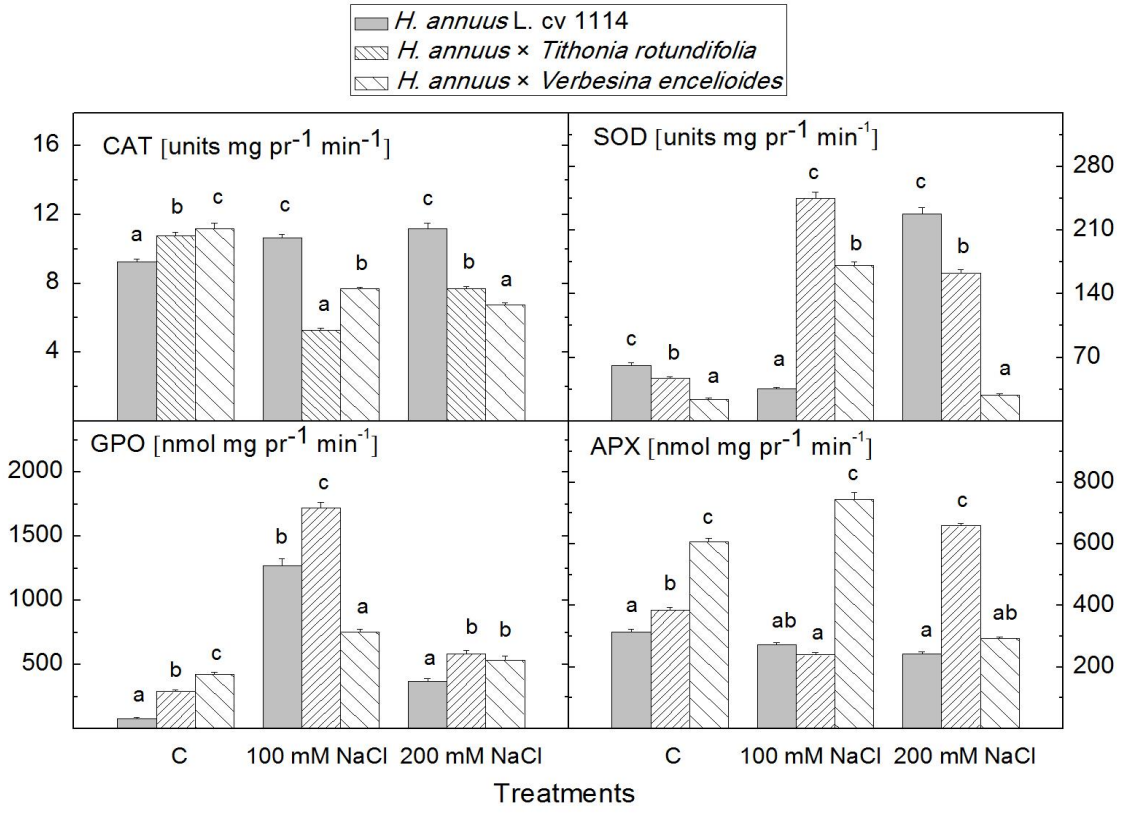

Figure 1. Activity of antioxidant enzymes in the leaves of cultivated sunflower *H. annuus* cv. 1114 and two intergeneric lines *H. annuus* × *Tithonia rotundifolia* and *H. annuus* × *Verbesina encelioides* at different salt concentrations.

Determination of the level of CAT, SOD, GPO, and APX in the three sunflower genotypes after NaCl treatment

Figure 1 shows the effect of salinity stress on the activity of antioxidant enzymes participating in the scavenging of ROS in examined sunflower genotypes. In both intergeneric lines *H. annuus* × *V. encelioides* and *H. annuus* × *T. rotundifolia*, catalase (CAT) activity in the leaves of unstressed plants was significantly higher than in *H. annuus* cv 1114 (Fig. 1). Under salinity conditions CAT activity in *H. annuus* significantly increased. CAT activity, on the other hand, decreased in both intergeneric lines in response to salt stress. Leaf-CAT activity from stressed plants of both lines *H. annuus* × *T. rotundifolia* and *H. annuus* × *V. encelioides* lines was lower than in the control (Figure 1).

SOD that catalyzes the conversion of the superoxide anion to H_2O_2, performs the first step in the removal of ROS (Rout and Show, 2001). In the present study, we observed a differential response in SOD activity in the all three sunflower genotypes (Fig. 1). Total SOD activity mostly increased in salt stressed plants; but it was higher in the control treatment in cultivated sunflower than in the intergeneric lines. *H. annuus* cv 1114 exhibited an 4-fold increase in SOD activity at 200 mM NaCl and about 6 and 3.5 increase, respectively, at 100 and 200 mM NaCl in *H. annuus* × *T. rotundifolia.* In *H. annuus* × *V. encelioides,* maximal increase of SOD activity as a result of salt treatment was measured at 100 mM NaCl.

Guaiacol peroxidase (GPO) activity increased under salt stress in all the genotypes (Fig. 1). The greatest increase was established in hybrid line *H. annuus* × *T. rotundifolia* after treatment with 100 mM NaCl (Fig. 1). Ascorbate peroxidase (APX) is another important enzyme that is effective in ascorbate-glutathion cycle. APX activities showed a decrease in cultivated genotype cv 1114 under salt stress (Fig. 1). There was an increase in APX activity in *H. annuus* × *V. encelioides* line at 100 mM NaCl compared with its control level (Figure 1).

Salinity (NaCl) adversely affected the seedling growth parameters of sunflower seedlings. In the present study, the root and shoot length as well as fresh weight were decreased in all three sunflower genotypes subjected to 100 and 200 mM NaCl compared with measurements from control plants. At particular growth stage, sunflower plants grown in saline conditions exhibited water-deficient symptoms: depressed root and shoot length, and root fresh production (Table 1). The deleterious effects of NaCl on these growth characters were caused by osmotic reduction of water absorption (Maas et al., 1977) and by specific toxic effects of sodium and chloride ions. The results of the current investigation are consistent with the generally accepted idea that osmotic stress reduces growth of plant tissue (Rauf *et al*. 2012). Similar results were reported in soybean (Hosseini et al., 2002), in wheat (Almansouri et al., 2001, Rauf et al., 2010), in race (Jamil et al., 2012), in hyacinth (Koksal et al, 2014) and many other species. Growth decreases was attributed to reductions in the shoots and roots length and their fresh weight as significant genetic differences were found among three genotypes. Hybrid line *H. annuus* × *V. encelioides* was found to be the most tolerant to salinity. This finding might suggest the presence of salt tolerance qualities in the wild relative of sunflower, *V. encelioides*, that could lead to an increase in salt tolerance in progeny derived from intergeneric crosses. However, additional data would be required to support this interpretation.

The results obtained on antioxidant enzymes such as CAT, SOD, GPO and APX have shown significant differences among examined genotypes treated with different NaCl concentrations. Increased SOD and GPO were observed, which showed that these were major enzymes in scavenging cellular H_2O_2 (Vranova et al., 2002). Stimulating effect of NaCl on SOD activity has been reported for many other plant species, particularly the salt-tolerant ones (Dionisio-Sese and Tobita, 1998, Rout and Shaw, 2001, Jebara et al., 2005; Kim et al., 2005; Hadiye et al., 2007; Fedina et al., 2009; Zagorchev et al. 2014). The highest GPO activity was recorded in the leaves of *H. annuus* × *T. rotundifolia* (Fig. 1c) where the activity of CAT was low. It might be suggested that in this genotype with increased GPO activity peroxyl radicals were accumulated. The CAT activity after treatment with NaCl in different sunflower genotypes decreased as compared to the control treatment. The only exception was genotype *H. annuus* cv 1114 in which the CAT activity slightly increased with the increased NaCl concentration (Fig. 1a). Our results are in agreement with the findings of Lee et al. (2001), who reported that in the rice the activity of CAT generally decreases in response of NaCl treatment. The current data showed that the activity of APX was reduced under salt stress (100 mM) in both genotypes *H. annuus* × *T. rotundifolia* and *H. annuus* cv 1114 but significantly increased in *H. annuus* × *V. encelioides* line (Fig. 1d). However, similarly increased APX level was found in *H. annuus* × *T. rotundifolia* line subjected to 200 mM salt stress. These results could be due to the differences of sunflower genotypes included in the study, as supported by Malenčić et al. (2004), who

reported that sunflower genotypes expressed different antioxidant systems in response of stress treatments.

In conclusion, the data here strongly suggest that a number of physiological and biochemical features of the sunflower plants, such as shoot and root length, fresh weight and the enzymatic antioxidant (CAT, SOD, GPO, and APX) systems are directly affected by the NaCl - mediated stress. Although three genotypes show similar reactions to salinity, the intergeneric hybrid line *H. annuus* × *V. encelioides* was less affected in respect of the growth parameters. With regard to parameters, concerning the antioxidant defense of the stress (SOD, GPO, and APX), intergeneric lines *H. annuus* × *V. encelioides* and *H. annuus* × *T. rotundifolia* indicated better protection to salinity. Current results revealed that salinity induced responses mostly depended on the genetic potential of the plants and therefore, wide hybridization (interspecific and intergeneric) could be consider as an useful tool for creation new sunflower germplasms using as a source of favorable traits the wild *Helianthus* species and species from related generation.

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