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SALT TOLERANCE OF TWO SUNFLOWER GENOTYPES: *HELIANTHUS ANNUUS* AND INTERSPECIFIC LINE *HELIANTHUS ANNUUS* × *HELIANTHUS MOLLIS*

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Summary: An experiment was carried out hydroponically in laboratory conditions for the identification of salt tolerant genotypes of sunflower (*Helianthus annuus* L.), as well as their characteristics. Two genotypes, cultivated cultivar 1114 and interspecific line *H. annuus* × *H. mollis* were studied at four different sodium chloride (NaCl) concentrations: 0 mM, 100 mM, 125 mM and 150 mM. Physiological and biochemical stress related parameters such as free proline content, hydrogen peroxide (H_2O_2) and malondialdechyde (MDA) were compared in seedlings of the two genotypes. The results indicated that both genotypes had similar responses at different salinity levels for the traits studied. All growth parameters such as germination percentage, root and shoot length, root and shoot fresh and dry weight decreased with increasing salinity level. With regard to the biochemical parameters evaluated, it was found that under different salinity treatments, the levels of free proline increased in comparison to control, while a smaller MDA increase was observed in both genotypes. H_2O_2 content increased significantly in *H. annuus* cv. 1114, while the interspecific line *H. annuus* × *H. mollis* accumulated a small amount of H_2O_2 under all tested stressful conditions. The data revealed that perennial wild *H. mollis* might be considered as an excellent candidate of salt tolerance genes.

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Key words: Biochemical markers; germination; interspecific crosses; salinity; sunflower; wide hybridization; wild sunflowers.

INTRODUCTION

Abiotic stresses, such as drought, salinity, low or high temperatures, flooding, metal toxicity, mineral deficiency, adverse pH, UV irradiation, ozone exposure or pollution decrease considerably growth of plants and yield of many crops all over the world (Ashraf and Wu, 1994; Tuteja et al., 2012). Therefore, increasing plant resistance to abiotic stresses is of great importance. Attempts to improve the salt

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tolerance through conventional breeding programmes have limited success due to the genetic and physiological complexity of this trait. Soil salinity is an important constraint to crop production world-wide (Munns and Tester, 2008). Salinity can disturb the physiological and biochemical functions of the plant cell, leading to cell death (Xiong and Zhu, 2002). In addition, salt stress also leads to oxidative stress through an increase in the production of reactive oxygen species.

Although some crops are moderately tolerant to saline conditions, many crops are adversely affected by even low levels of salt (Greenway and Munns, 1980). Salinity affects seed germination, vigor, reduces plant height, retards plant development, causes leaf damages and other physiological, biochemical and metabolic disorders. At the cellular and sub-cellular levels, stress-induced unique changes include increased unsaturation of the membrane lipids, increased levels of different osmolytes, general repression of protein biosynthesis and selective changes in K^+/Na^+ levels (Hare et al., 1998). During salt stress, plants adapt to oxidative stress by accumulating certain protective solutes like proline, glycine betaine, polyols, trehalose etc. (Sakamoto and Murata, 2002). Proline plays a predominant role in protecting plants from osmotic stress.

Various strategies have been adopted by plant scientists to overcome the harmful effect of salinity on plant development. Both genetic manipulation and conventional breeding will be required to develop salt-tolerant cultivars able to cope with the increasing soil salinity. In sunflower, wide hybridization (interspecific and intergeneric) is a useful technique for development of new genotypes with desirable agronomic traits (Jan and Fernadez-Martinez, 2002; Rönicke et al., 2004; Breton et al., 2012; Vassilevska-Ivanova et al., 2012; Kaya, 2014).

In this study, we expanded the information on the possible role of wild-type *H. mollis* to improve salinity tolerance of sunflower *via* hybridization by studying physiological and biochemical parameters under stress simulation by NaCl treatment.

MATERIALS AND METHODS

Plant material

Seeds of *H. annuus* L. cultivar 1114 and an advanced interspecific line *H. annuus* × *H. mollis* were used in this study.

Both genotypes were developed at the Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences.

Germination experiments

Twenty five seeds from each genotype in four replicates were surface sterilized with 5% sodium hypochlorite solution for 15 min and thoroughly washed three times with distilled water. The seeds were germinated in the dark at 25±1°C in rolled, moistened paper towels. Germination responses were visually scored as a percentage of germination (roots < 0.5 mm) at the following time intervals: 16, 20, 24, 32, 36, 40, 44, 48, 72, 96 h over a 4-day period and after final germination the germination percentage was estimated. In order to calculate the percentage of germination in the below-described stress conditions, the same experimental procedure was applied.

After germination, when cotyledons were fully emerged, healthy and uniform seedlings were selected and transferred

to 600 mL plastic bakers filled with half-strength Hoagland's solution to grow in a controlled environmental chamber "Forma Scientific" model 3744 at a day/night temperature of 25/18°C, relative humidity of 70%, 250 μmol m^{-2} s^{-1} irradiance and a 16/8 h photoperiod.

After 10 days of growth the length of roots and shoots was measured and seedlings were treated for 3 days with NaCl solution at a final concentration of 100 mM, 125 mM, 150 mM, or watered with half strength Hoagland's nutrient solution (control plants). NaCl was dissolved in half strength Hoagland's nutrient solution.

At the end of the experiment (14 days), the plants were harvested and their shoots and roots were separated. Their length, fresh weight (FW), dry weight (DW) and water content were measured. Water content was determined by changes in the weight. For DW determination samples were oven dried at 70°C for 72 h and then weighed. Each set of experiments was performed three times.

Proline content

Free proline content was extracted from 0.5 g of leaf and stems samples in 3% (w/v) aqueous sulphosalycylic acid and estimated by using ninhydrin reagent according to the method described by Bates et al. (1973). Proline concentration was determined using calibration curve and expressed as μmol proline g^{-1} FW.

Malonyldialdehyde (MDA) content

Lipid peroxidation (LP) was estimated based on thiobarbituric acid (TBA) reactivity. Samples were evaluated for MDA production using a spectrophotometric assay. The extinction coefficient of 155 mM^{-1} cm^{-1} at 532 nm for the chromophore was used to calculate the color intensity of MDA-TBA complex in the supernatant (Cakmak and Horst, 1991).

Determination of hydrogen peroxide (H_2O_2) content

The H_2O_2 level was colorimetrically measured as described by Alexieva at al. (2001). Leaf and stem tissues (about 500 mg) were homogenized in ice bath with 5 ml 0.1% (w/v) trichloracetic acid. The homogenate was centrifuged at 12 000×g for 15 min at 4°C. The enzymatic reaction started with 0.5 ml of supernatant and 0.5 ml of peroxidase reagent consisting of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1M KJ. The absorbancy of the supernatant was measured at 390 nm. The content of H_2O_2 was given on a standard curve.

Statistical analysis

Standard errors of means were calculated for all parameters studied. Data were analysed using ANOVA and Duncan's multiple range test at $P<0.05$ with the statistical package STATISTICA 7.0 (Stat-Soft, Inc., USA).

RESULTS

Background information on the germination behavior of the seed material used in the following experiments is given in Fig. 1. The seeds from the cultivated sunflower cv. 1114 germinated in darkness at 25°C by 98% within 28h after sowing. The seeds from the interspecific line *H. annuus* × *H. mollis* germinated slowly and higher germination occurred within 96h (nearly 84%), (Fig. 1A). NaCl treatment at all three concentrations had an inhibitory

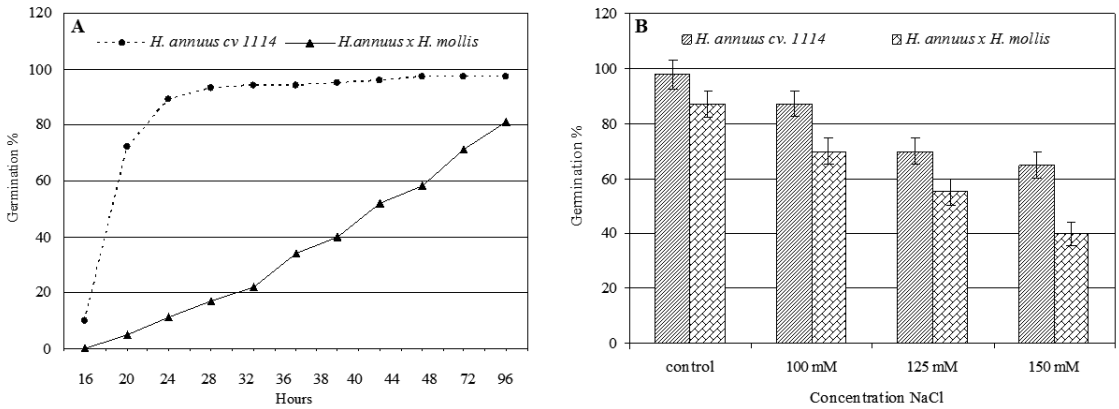

Figure 1. Germination responses of two sunflower genotypes under optimal (A) and high salinity conditions (B).

effect on sunflower seed germination as the degree of inhibition increased with the NaCl concentration (Fig. 1B). It can be noticed that the inhibitory effect of salinity on the germination was more obvious in the hybrid seeds *H. annuus* × *H. mollis* than in the *H. annuus* cultivar.

Root and shoot growth was followed by measuring length, FW and DW. Increasing concentrations of salinity from 100 mM to 150 mM considerably reduced root and shoot length in both sunflower genotypes and the lowest amount was recorded at 150 mM. The comparison of the effects of different salinity levels showed no significant difference in the mean length/seedling between *H. annuus* and the interspecific line *H. annuus* × *H. mollis* (Table 1).

There was a significant reduction in FW of shoots and roots of cultivated sunflower cv. 1114 and the interspecific line *H. annuus* × *H. mollis* and saline stress affected stronger the latter one (Table 2).

Table 1. Root and shoot growth response of two sunflower genotypes grown under optimal and high salinity conditions; (percentage control values are given in parenthesis).

Genotype	Treatment [NaCl]	Root length [cm]	Shoot length [cm]
H. annuus cv.1114	0	17.2±2.1 (100)^{a}	8.7±1.1 (100)^{a}
	100 mM	16.3±4.0 (94.8)^{a}	7.4±1.6 (85.0)^{ab}
	125 mM	15.3±2.0 (88.9)^{ab}	7.2±1.4 (82.8)^{ab}
	150 mM	13.8±2.5 (80.2)^{b}	6.9±1.7 (79.3)^{b}
	SED	1.2	0.8
H. annuus ×*H. mollis*	0	13.2±2.2 (100)^{a}	5.9±1.1 (100)^{a}
	100 mM	11.5±2.7 (87.1)^{a}	4.7±0.7 (79.7)^{b}
	125 mM	9.9±2.5 (75.0)^{b}	4.6±1.0 (78.0)^{b}
	150 mM	9.4±0.7 (71.2)^{b}	4.3±1.4 (72.9)^{b}
	SED	2.0	0.5

Table 2. Growth response of two sunflower genotypes grown under optimal and high salinity conditions.

Genotype	Treatment [NaCl]	Root fresh weight [g]	Shoot fresh weight [g]	Root dry weight [g]	Shoot dry weight [g]	Root water content [%]	Shoot water contetnt [%]
H. annuus cv 1114	0	1.5954±0.05^{a}	3.0729±0.07^{a}	0.1161±0.0108^{a}	0.3224±0.0536^{a}	93.38±0.03^{a}	93.33±0.03^{a}
	100 mM	1.5118±0.03^{b}	2.8839±0.05^{b}	0.1126±0.0168^{a}	0.2847±0.0492^{ab}	93.07± 0.01^{b}	90.19±0.03^{c}
	125 mM	1.5161±0.02^{b}	2.8295±0.02^{c}	0.1058±0.0143^{a}	0.2754±0.0266^{b}	92.58±0.02^{c}	90.28±0.02^{b}
	150 mM	1.4823±0.02^{c}	2.3245±0.03^{d}	0.1082±0.0236^{a}	0.2034±0.0255^{c}	92.17±0.01^{d}	86.12±0.04^{d}
	SED	0.0141	0.0197	0.0091	0.0218	0.01	0.01
H. annuus x *H. mollis*	0	0.8424±0.01^{a}	1.7022±0.01^{a}	0.0536±0.0064^{a}	0.1246±0.0184^{a}	93.80±0.03^{a}	93.10±0.04^{b}
	100 mM	0.7346±0.01^{b}	1.3386±0.01^{b}	0.0491±0.0089^{a}	0.1176±0.0136^{a}	93.03±0.03^{c}	91.40±0.02^{a}
	125 mM	0.6864±0.01^{c}	0.9433±0.01^{c}	0.0479±0.0057^{a}	0.0728±0.0065^{b}	93.22±0.02^{b}	90.96±0.01^{c}
	150 mM	0.4554±0.01^{d}	0.8180±0.01^{d}	0.0286±0.0078^{b}	0.0626±0.0073^{b}	91.12±0.02^{d}	84.80± 0.02^{d}
	SED	0.0051	0.0061	0.0039	0.0067	0.01	0.01

In the interspecific line, the maximum reduction (54.8%) of the root FW was observed at the high saline concentration (150 mM) compared to that of the controls; a reduction by about 25 and 52.4% compared to control was established for shoot FW. No differences were observed in the DW of the roots in cv. 1114 with increasing NaCl levels (Table 2). The root DW of the interspecific line *H. annuus* × *H. mollis* declined gradually with increasing salinity and reached 47.6% at a concentration of 150mM. Different levels of salinity revealed a weak inhibitory effect on the shoot DW. A significant reduction was established at a concentration of 150 mM NaCl. A similar trend of shoot DW reduction was observed also at lower salt concentrations and the interspecific line *H. annuus* × *H. mollis* was more strongly affected by salinity stress (Table 2).

There were no significant differences in the water content of the roots in the cultivated sunflower cv. 1114 and line *H. annuus* × *H. mollis* with increasing salt concentrations (Table 2). However, saline stress reduced the water content considerably in shoots of severely stressed seedlings. A maximum decrease was observed under high salinity (150mM NaCl).

The effect of salinity stress on free proline content in shoots depended on the concentrations of NaCl. It was found that the increasing level of salinity (from 100mM to 150 mM) increased proline content (Fig. 2). Compared to control, up to a 4- fold increase in proline content was registered in the cultivated sunflower and *H. annuus* × *H. mollis* shoots exposed to 150mM NaCl. However, at stress levels of 100 and 125 mM NaCl, accumulation of proline in the cultivated sunflower cv. 1114 was 2 times higher compared to control, while the amount of proline in the hybrid at 125 mM NaCl was equal to that found in the cultivated sunflower at 150 mM NaCl. The differential response of the two genotypes to saline stress was evident in severely stressed seedlings and the *H. annuus* × *H. mollis* hybrid line accumulated more proline than *H. annuus* (Fig 2).

In the present investigation, we found that increasing NaCl concentrations from 100 to 150 mM correlated with a strong

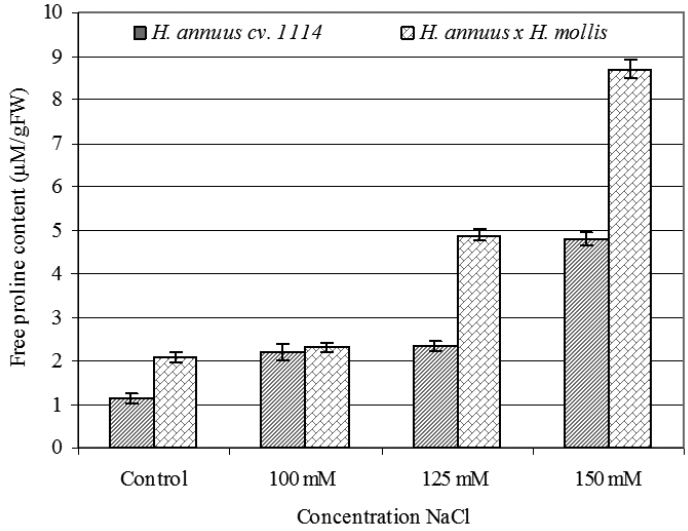

Figure 2. Effect of NaCl treatment on free proline content in seedlings of two sunflower genotypes.

increase in H_2O_2 content in the shoots of cv. *H. annuus* (Fig. 3). Greater accumulation of H_2O_2 due to NaCl treatment was observed even at 100mM (nearly 4 times compared to control) and it continued to increase, but much less. The interspecific line *H. annuus* × *H. mollis* accumulated a small amount of H_2O_2 under all tested NaCl concentrations and with the enhancement of the stress the concentration of H_2O_2 increased marginally (about 80%).

Membrane lipid peroxidation was estimated in shoots of the investigated sunflower genotypes after 3 days of NaCl treatment (Fig. 4) with MDA content being used as its index. In the cultivated sunflower, the amount of MDA slightly increased with enhancing stress severity, whereas the interspecific line showed a gradual increase of MDA content which was highest (nearly 1.3 times) at 150mM NaCl.

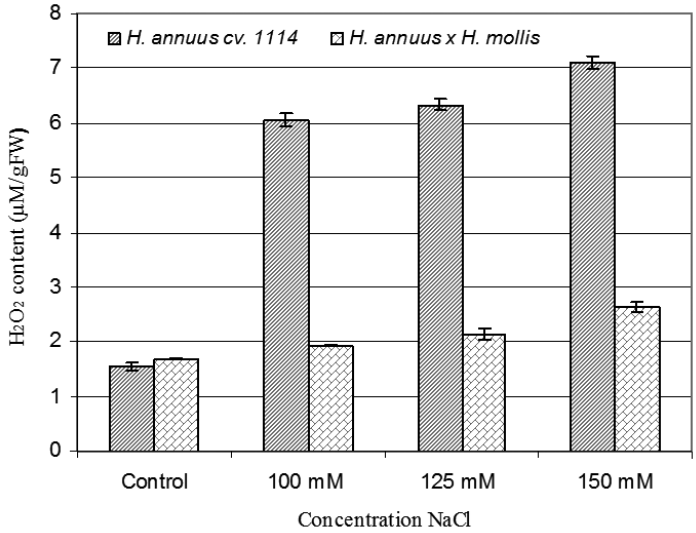

Figure 3. Effect of NaCl treatment on H_2O_2 content in seedlings of two sunflower genotypes.

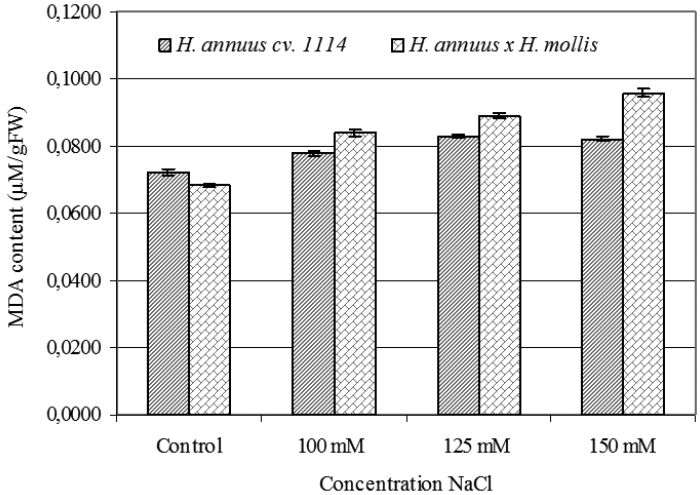

Figure 4. Effect of NaCl treatment on MDA content in seedlings of two sunflower genotypes.

DISCUSSION

The deleterious effects of salinity on plant growth are associated with (i) water stress (low osmotic potential of soil solution); (ii) nutrient ion imbalance; (iii) salt stress (specific ion effects); and (iv) a combination between these factors. All these factors cause adverse pleiotropic effects on plant growth and development at physiological, biochemical, molecular and whole plant levels (Rasool et al., 2013).

Generally, sunflower is classified as moderately salt tolerant (Steduto et al., 2000). Soil salinity up to EC 4.8 dS m-1(≈ 50 mM NaCl) didn't affect the sunflower seed productivity; further increasing of salt level, however, caused an yield reduction (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 (Akram and Ashraf, 2011; Shahbaz et al., 2011).

In the present study, the two sunflower genotypes tested – cultivated sunflower and line *H. annuus* × *H. mollis* revealed a wide range of responses to salinity stress that intensified with enhancing the severity of the stress. At a particular growth stage, sunflower plants grown in saline conditions exhibited water-deficient symptoms: reduced germination rate, root and shoot length, depressed shoot and root fresh and dry matter production, and water content (Tables 1-2). Seed germination in the sunflower genotypes was inhibited with increasing salt concentrations. At 150 mM NaCl, the percentage of seed germination decreased by 40 and 62%, respectively. The seeds of *H. annuus* showed higher tolerance to salinity stress, i.e. percentage of germination was higher at a more negative osmotic potential.

Albuquerque and Carvalho (2003) and Mwale at al. (2003) demonstrated that oxidative stress in sunflower caused irregular seed germination and poor and unsynchronized establishment of seedlings. Similar results were reported by Zhou and Xiao (2010). Salt and osmotic stresses are responsible for both inhibited or delayed seed germination and seedling establishment. Under these stresses there is a decrease in water uptake during imbibition and furthermore, salt stress may cause excessive uptake of ions (Murillo-Amador et al., 2002).

Shoot and root growth inhibition is a common response to salinity and one of the most important agricultural indices of salt stress tolerance (Munns, 2002). In order to define salt stress tolerance of both genotypes, growth parameters like length, fresh and dry weight of roots and shoots were measured under NaCl treatment. In the present study, the comparison of the effects of different salinity levels (100 and 125 mM NaCl) showed that salt stress inhibited shoot growth more strongly compared to root growth (Table 1). The magnitude of reduction was highly dependent upon both NaCl concentration and genotype. FW was significantly reduced in roots (about 50%) at salinity levels of 125-150 mM NaCl in the interspecific line compared to the cultivated sunflower cv. 1114. The reduction of DW in the interspecific line *H. annuus* × *H. mollis* was approximately 46% by increasing the concentration of salt, while in the cultivated sunflower cv. 1114 this value

was not affected with increasing salt concentration. Variation in growth might be associated with variations in other physiological attributes. The differential growth responses of the two sunflower genotypes might be due to their differential genetic behavior in absorption of water and changes in biochemical mechanisms under saline conditions.These results are consistent with those obtained for seed germination. Generally, the cultivated sunflower cv. 1114 seemed to be more tolerant to salinity stress than the interspecific line *H. annuus* × *H. mollis*.

The reduction in water uptake by germinating seeds in stress conditions resulted in decreases of seedling growth (Okçu et al., 2005). Our results are consistent with those already reported for different cultivars (Ashraf et al., 2012; Shahbaz and Zia, 2011).

Higher water retention was observed in roots than in leaves. Since roots are the first plant organ exposed to stress conditions, their response is associated with tolerance to abiotic stress. The water content in plant roots and leaves has been shown to be associated with stress tolerance in cultivated sunflower (Cellier et al., 1998).

In response to salinity and some other abiotic stresses, many plant species accumulate high levels of proline, which is thought to function in stress adaptation (Trovato et al., 2008). In the present work, the levels of proline increased in parallel with the severity of salinity stress in both sunflower genotypes. The effect of osmotic stress on proline content was shown to be more dramatic in the interspecific hybrid line *H. annuus* × *H. mollis* than in the cultivated sunflower genotype (Fig. 2). It could therefore be concluded that the hybrid line possessed a better potential to maintain osmotic balance and was more tolerant to salinity stress than the cultivated genotype.

The marked difference between sunflower genotypes in responding to oxidative stress is indicative for their key role for determining the plant's adaptation reaction to stress. This result is consistent with the observations that at different levels of salinity stress, each sunflower hybrid behaved differently according to its genetic makeup (Ŝcorić, 2009).

Despite the presence of a strong correlation between stress tolerance and accumulation of proline in higher plants, this relationship may not be universal. For example, in rice plants grown under salt stress, accumulation of proline in the leaf was deemed to be a symptom of salt injury rather than an indication of salt tolerance (Lutts et al., 1999). Further studies are needed to determine whether the relationship between stress tolerance and accumulation of proline is species-specific or it can be altered by experimental conditions. Currently, there is more evidence supporting the presence of a positive relationship.

H_2O_2 is a toxic ROS, which has deleterious effects in plant tissue (Salin, 1988). It is known that the accumulation of hydrogen peroxide is an early, common response to various stress factors. In the cultivated sunflower genotype, greater accumulation (nearly 4 times compared to control) of H_2O_2 was observed even at 100mM NaCl, and it continued to grow, but much less at concentrations of 125 mM and 150 mM. The interspecific line accumulated a small amount of H_2O_2 under all tested salt concentrations and

with the enhancement of the stress H_2O_2 content increased marginally. It may be therefore suggested that the increased level of H_2O_2 observed in the saline treated sunflower plants (Fig. 3) was due to oxidative damage, but eventually may also have a signal function.

One of the most representative markers for membrane destruction after free-radical chain reactions is the formation and accumulation of malondialdehyde (MDA), an endproduct of peroxidation of the unsaturated membrane fatty acids (Halliwell, 2006). It has been used as a promising criterion for plant sensitivity to saline stress (Ashraf et al., 2010). In the present study, the amount of MDA in the cultivated sunflower cv. 1114 slightly increased with enhancing stress severity, whereas the interspecific line *H. annuus* × *H. mollis* showed a gradual increase of MDA content, which was highest at 150mM NaCl. Therefore, our results showed that sunflower genotypes can tolerate salt stress (up to 150 mM NaCl). The lower level of MDA content in the sunflower genotypes tested indicated better protection against oxidative damage under saline stress. According to Labudda (2013) the high MDA accumulation has been correlated with water deficit stress sensitivity. Lower MDA content displays higher antioxidative ability, reflecting higher tolerance to stress.

In conclusion, our data strongly indicated that a number of physiological and biochemical features of the sunflower plants such as seed germination, shoot and root length, fresh and dry weight, water content and levels of proline, MDA and H_2O_2, were directly affected by the NaCl-mediated stress. Although the two genotypes showed similar responses to salinity, the cultivated sunflower cv. 1114 was less affected in respect to the growth parameters. With regard to parameters related to the antioxidant defense in stress conditions (accumulation of proline, H_2O_2 and MDA), the interspecific line *H. annuus* × *H. mollis* demonstrated better protection to salinity. These observations are in agreement with some reports demonstrating that the stress response of sunflower (*Helianthus* sp.) is strongly affected by genetical factors (Ŝcorić, 2009; Oraki et al., 2012). Genotypic differences in saline tolerance could be, at least in part, attributed to the ability of plants to acclimate and induce different defense mechanisms under severe salt stress in sunflower. The introgressed line *H. annuus* × *H. mollis* seemed to have greater potential to overcome the stress showing less membrane destruction and higher proline accumulation under stress conditions compared to the cultivated genotype cv.1114. This indicates that salinity-induced responses are dependent mostly on the genetic potential of the genotype. The current results suggest that the diploid perennial species *H. mollis* (2n=2x=34) might be considered to be an excellent candidate of salt tolerance genes.

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