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Cent. Eur. J. Biol. • 9(12) • 2014 • 1206-1214
DOI: 10.2478/s11535-014-0355-5

Central European Journal of **Biology**

Response of sunflower (*Helianthus annuus* L) genotypes to PEG-mediated water stress

Research Article

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Received 21 October 2013; Accepted 13 December 2013

Abstract: Drought tolerance of two sunflower (*Helianthus annuus* L.) genotypes, cultivated cultivar 1114 and interspecific line *H. annuus* × *H. mollis,* was studied under laboratory conditions using PEG-6000. Four levels of osmotic stress (-0.4, -0.6, -0.8 and -1.0 MPa) were created and performances were monitored against a control. Physiological and biochemical stress determining parameters such as malondialdechyde (MDA), proline content, and hydrogen peroxide (H_2O_2) were compared between seedlings of both genotypes. The results indicated that both genotypes have similar responses at four osmotic potentials for all traits studied. All seedling growth parameters such as germination percentage, root length, shoot length, root and shoot dry weight decreased with increasing osmotic stress. MDA, proline, and H_2O_2 were found to be increased at different osmotic gradients in comparison to control. Cultivar 1114 was less affected than the interspecific line under these stress conditions. The data observed in the experiments revealed that perennial wild *H. mollis* can hardly be considered to be an excellent candidate of drought tolerance genes.

Keywords: *Drought • Germination • Helianthus mollis • Hydrogen peroxide • Malondialdehyde • Proline • Wide hybridization*

1. Introduction

Sunflower (*Helianthus annuus* L., *Asteraceae*) is one of the most cultivated oil crops in the world after soybean, rapeseed, and peanut [1]. In Bulgaria, sunflower is a crop of economic significance as a source of edible vegetable oil that is more healthful than other types of oil [2]. Although sunflower is categorized as a low to medium drought sensitive crop [3] its production suffers substantially from the effect of water stress. In this sense, after an analysis of water availability over a period of 39 years, Bosnjak [4] has reported that drought causes up to 50% yield losses in sunflower and predicted that severe droughts may be expected in the future with global climatic changes. The degree of yield reduction resulting from water stress depends on the growth stage of the crop (germination, seedling, and flowering), the severity of the drought, and drought tolerance of the plant genotype [5,6]. Therefore, it is essential to develop and identify drought-tolerant germplasm that will allow an expansion of the cultivated area.

In sunflower, wide hybridization (interspecific and intergeneric) is a useful technique for development of new genotypes with desirable agronomic traits [7-12]. For drought tolerance breeding, wild annual species *H. argophyllus* has been reported to be a potential source for genes for drought resistance and is therefore extensively used by sunflower breeders [13,14]. Wide crosses between cultivated sunflower *H. annuus* and diploid perennials such as *H. mollis* (2n=2x=34) are difficult to carry out, and successful hybridization is usually obtained through the use of *in vitro* embryo

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rescue methods or appropriate cultivated genotype as mother plant [7,12,15]. It has been established that *H. mollis* is a potential source of a number of useful traits such as resistance against *Sclerotinia sclerotiorum* (Lib.) de Bary stem and rot infections [9], *Orobanche cumana* Wallr. [16]. However, to the best of our knowledge, data indicating drought tolerance genes in *H. mollis* have not been presented. Here, we seek to expand information on the possible capability of *H. mollis* to improve drought tolerance of sunflower *via* hybridization, by studying physiological and biochemical parameters under stress simulated by polyethylene glycol.

In the present study, we have analyzed the behavior of two *H. annuus* genotypes in the early developmental stages under experimental drought conditions. The response of sunflower plants was characterized with reference to water deficit on seed germination, growth parameters, relative water content, lipid peroxidation, proline content, and H_2O_2 level during stress.

2. Experimental Procedures

2.1 Plant material

Two sunflower (*Helianthus annuus* L.) genotypes were chosen on the basis of speculation of the potential of wild *Helianthus* species to impact the adaptation of the introgressed hybrids under drought conditions. 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, Sofia, Bulgaria following a research program using the potential of wide hybridization for producing and evaluating new sunflower germplasms. The origin of the interspecific line and its characteristics is as in our previous works [12,15]. The seeds were stored dry in paper bags at 5°C for at least 9 months before the experiments and were non-dormant.

2.2 Germination experiments

Two sunflower genotypes were evaluated against drought stress at germination and seedling growth stages for 14 days under laboratory conditions. Twenty-five seeds of both genotypes 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 [17]. Number of seeds germinated was counted gradually from 16 to 96 hours (4 hrs interval). Germination was considered to have occurred when the seed had developed a radicle at least 5 mm long. The experiment was laid out with four replicates for each experimental unit. Total germination was expressed as percent of that in the control treatment for each genotype and then data underwent statistical analysis.

One-week-old seedlings were transferred to 600 mL plastic beakers filled with half-strength Hoagland's solution [18] 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. Polyethylene glycol-6000 (PEG-6000) was used as a drought stimulator. Four water stress levels with different osmotic potentials of -0.4 (10% w/v), -0.6 (15% w/v), -0.8 (20% w/v), and -1.0 (25% w/v) MPa were generated by dissolving PEG to half-strength Hoagland's solution [19]. The drought stress period created by PEG-6000 was 3 days. The osmotic potential of control solution was 0 MPa. Each set of experiments was performed three times.

At the end of the experiment (14 days), the plants were harvested and their shoots and roots were separated. Lengths were measured and fresh weight (FW), dry weight (DW) and water content were recorded. Water content of the roots and shoots was determined by weight change. For dry weight determination samples were oven dried at 70°C for 72 h and then weighed.

2.3 Proline content

Free proline content was extracted from 0.5 g of leaf and stem samples in 3% (w/v) aqueous sulphosalycylic acid and estimated by using ninhydrin reagent according to the method described by Bates *et al*. [20]. The absorbance of the fraction with toluene aspired from liquid phase was read at 520 nm. Proline concentration was determined using calibration curve and expressed as µmol proline g^{-1} FW.

2.4 Lipid peroxidation

The level of lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 500 mg in leaf and stem fresh weight according to Cacmak and Horst [21]. MDA is a product of lipid peroxidation by thiobarbituric acid reaction. The concentration of MDA was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) by using extinction coefficient of 155 mM^{-1} cm^{-1}.

2.5 H_2O_2 content

The H_2O_2 level was colorimetrically measured as described by Sergiev at al. [22]. About 500 mg of leaf and stem tissues 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 4ºC. Enzymatic reaction was 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 supernatant was measured at 390 nm. The content of H_2O_2 was given on a standard curve.

The data collected were analyzed by analysis of variance technique. Duncan's New Multiple Range test at 5% level of probability was used to test the significance of means [23].

3. Results

Background information on the germination behavior of the seed material used in the following experiments is given in Figure 1. The seed germination from cultivated sunflower cv 1114 was 98% at 28 h after sowing. The seeds from interspecific line *H. annuus* × *H. mollis* germinated slowly and higher germination occurred within 72 h.

The PEG solution of all four concentrations had an inhibitory effect on sunflower seed germination, and the degree of inhibition increased with the PEG concentration (Table 1). The inhibitory effect of PEG on the seed germination was more obvious on the hybrid seeds *H. annuus* × *H. mollis* than the cultivar 1114 (*H. annuus*).

Root and shoot growth was followed by measuring length, fresh weight (FW) and dry weight under PEG treatment. Increasing concentrations of PEG from -0.4 to -1.0 MPa progressively reduced root and shoot length

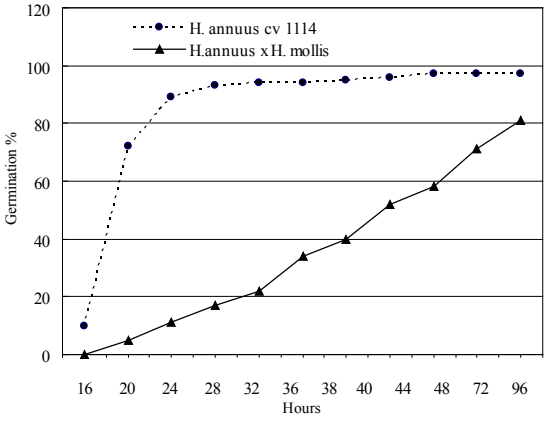

Figure 1. Germination responses of two sunflower genotypes tested at optimal conditions.

in both sunflower genotypes as the lowest values were recorded at -1.0 MPa (Table 2). The comparison between the effects of PEG at iso-osmotic concentrations showed that *H. annuus* × *H. mollis* hybrid seeds were more inhibited than cultivated sunflower. The results of the present investigation are consistent with the generally accepted idea that osmotic stress reduces growth of plant tissue.

There was significant reduction in the fresh weight of shoots and roots of the cultivar 1114 and hybrid, and the PEG-mediated osmotic stress had a stronger effect on the latter one (Table 3). Maximum reduction (49-36%) of the fresh weight of roots was observed at high osmotic potential (-1.0MPa) compared to that of the controls. Similar trend of reduction to about 39 and 25% down from the control was established for shoot fresh weight.

A marked adverse effect of PEG-6000 treatments on shoot and roots dry weight was observed in both sunflower genotypes (Table 3). The roots dry weight of sunflower cultivar 1114 declined gradually with increasing level of PEG. Twenty-seven and eighty percent reduction in root dry weight was observed

Table 1. Effect of osmotic stress induced by PEG-6000 on the germination of two sunflower genotypes; percentage control values are given in parenthesis.

Osmotic potential (MPa)	Germination (%)	
	*H. annuus cv. 1114**	*H. annuus x H. mollis*
0	98.0 (100) ± 3.0	87.0 (100) ± 1.7
-0.4	95.0 (97) ± 2.6	75.0 (86) ± 2.6
-0.6	86.0 (88) ± 2.5	70.0 (80) ± 2.9
-0.8	83.0 (85) ± 2.7	36.0 (41) ± 3.2
-1.0	57.0 (58) ± 2.2	21.0 (24) ± 3.6

**Each value is expressed as mean ± SD (n=3)*

Table 2. Root and shoot growth responses (mean length per seedling in cm) of two sunflower genotypes grown under optimal and stress conditions; percentage control values are given in parenthesis.

	Osmotic potential (MPa)	Length (cm)	
		H. annuus cv. 1114*	*H. annuus* x *H. mollis*
Root	0	17.2 (100) ± 2.1 a	13.2 (100) ± 2.2 a
	-0.4	16.2 (94) ± 1.2 ab	11.4 (86) ± 1.8 ab
	-0.6	15.4 (89) ± 2.0 bc	10.7 (81) ± 0.9 b
	-0.8	14.4 (84) ± 1.9 c	9.5 (72) ± 2.1 bc
	-1.0	12.1 (70) ± 2.1 d	8.7 (66) ± 1.3 c
	SED	0.85	0.95
Shoot	0	8.7 (100) ± 1.1 a	5.9 (100) ± 1.1 a
	-0.4	7.7 (88) ± 0.9 ab	5.0 (85) ± 0.9 ab
	-0.6	7.0 (80) ± 1.7 bc	4.6 (78) ± 0.3 bc
	-0.8	6.7 (77) ± 1.0 bc	4.4 (74) ± 1.0 bc
	-1.0	6.3 (72) ± 1.4 c	4.0 (68) ± 0.8 c
	SED	0.56	0.49

**Each value is expressed as mean ± SD (n=3)*

at high PEG level (-1.0 MPa). All the levels of PEG treatments had inhibitory effect on the shoots dry weight but they were less affected by PEG-treatment than the roots (Table 3).

The water content increased gradually in the roots of cultivated sunflower cv 1114 and in hybrid *H. annuus* × *H. mollis* with increasing osmotic potential (Figure 2a). However, the osmotic stress reduced the water content considerably in shoots of severely stressed seedlings (Figure 2b). A maximum decrease was observed under high osmotic potential (-1.0 MPa).

The effect of osmotic stress on free proline content of shoots depended on the concentrations of PEG as increasing levels of PEG (-0.4 to -1.0 MPa) increased the level of proline over control (Figure 3). Up to a 13- fold increase in proline content was registered in cultivated sunflower shoots exposed to -1.0 MPa PEG. In hybrid seedlings, free proline content also increased significantly in response to osmotic stress, but its value was up to 4-fold of the control. However, at -0.4 MPa and -0.6 MPa osmotic potential, a comparable amount of proline accumulation in shoots of *H. annuus* and

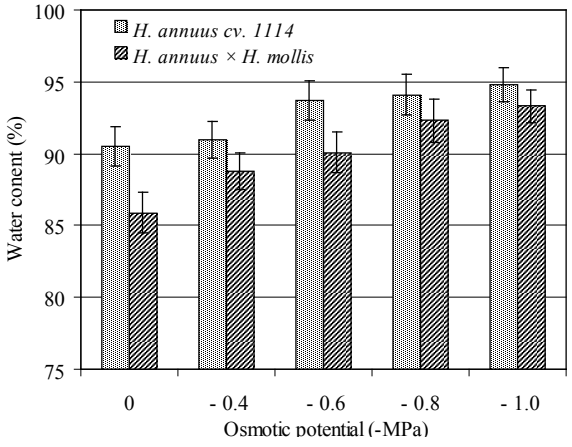

Figure 2a. Effect of osmotic stress on root water content in two sunflower genotypes (*H. annuus* cv. 1114 and *H. annuus* × *H. mollis*). Data represent the average of two experiments with three replicates. Vertical bars indicate ± SD.

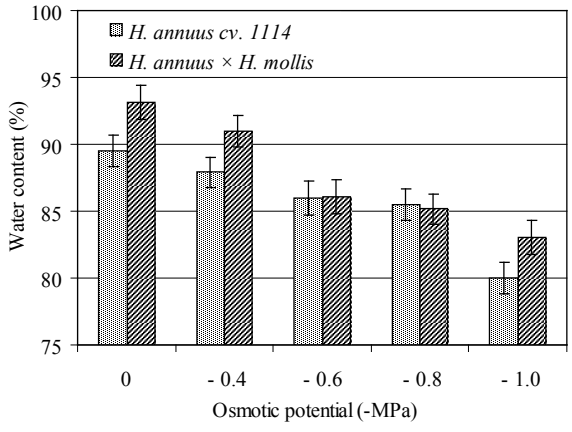

Figure 2b. Effect of osmotic stress on seedling water content in two sunflower genotypes (*H. annuus* cv. 1114 and *H. annuus* × *H. mollis*). Data represent the average of two experiments with three replicates. Vertical bars indicate ± SD.

Table 3. Effects of osmotic stress induced by PEG-6000 on fresh and dry weight (g/5 seedlings) of two sunflower genotypes; percentage control values are given in parenthesis.

	Osmotic potential (MPa)	Fresh weight (g)		Dry weight (g)	
		H. annuus cv. 1114*	*H. annuus* x H. mollis	*H. annuus* cv. 1114	*H. annuus* x H. mollis
Root	0	1.595 (100) ± 0.05 a	0.842 (100) ± 0.01 a	0.152 (100) ± 0.005a	0.118 (100) ± 0.009a
	-0.4	1.506 (94) ± 0.01 b	0.725 (86) ± 0.01 b	0.119 (78) ± 0.024b	0.082 (69) ± 0.006b
	-0.6	1.486 (93) ± 0.01 b	0.612 (73) ± 0.03 c	0.094 (62) ± 0.008c	0.061 (52) ± 0.005c
	-0.8	0.977 (61) ± 0.05 c	0.314 (37) ± 0.02 d	0.057 (37) ± 0.006d	0.022 (19) ± 0.005d
	-1.0	0.787 (49) ± 0.01 d	0.300 (36) ± 0.01 d	0.04 1 (27) ± 0.005e	0.021 (18) ± 0.005d
	SED	0.0152	0.0076	0.006	0.0034
Shoot	0	3.073 (100) ± 0.07 a	1.702 (100) ± 0.01 a	0.323 (100) ± 0.009a	0.123 (100) ± 0.014a
	-0.4	2.308 (75) ± 0.03 b	1.139 (67) ± 0.01 b	0.280 (87) ± 0.035b	0.108 (88) ± 0.020b
	-0.6	2.208 (72) ± 0.01 c	0.826 (48) ± 0.01 c	0.266 (82) ± 0.022b	0.084 (68) ± 0.003c
	-0.8	1.734 (56) ± 0.02 d	0.495 (29) ± 0.02 d	0.251 (78) ± 0.031bc	0.074 (60) ± 0.005cd
	-1.0	1.190 (39) ± 0.03 e	0.435 (25) ± 0.01 e	0.232 (72) ± 0.033c	0.064 (52) ± 0.005d
	SED	0.0158	0.0073	0.0147	0.0062

**Each value is expressed as mean ± SD (n=3)*

in hybrid was observed. The difference in response to water stress of both genotypes was evident in severely stressed seedling, where *H. annuus* cv 1114 accumulated more proline than *H. annuus* × *H. mollis* hybrid (Figure 3).

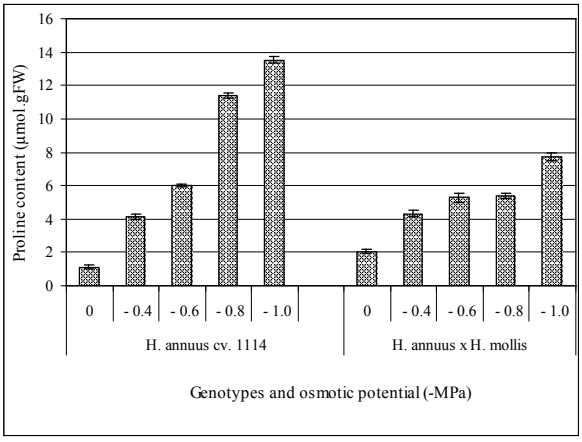

Figure 3. Effect of osmotic stress on free proline content in seedlings of two sunflower genotypes (*H. annuus* cv. 1114 and *H. annuus* × *H. mollis*). Data represent the average of two experiments with three replicates. Vertical bars indicate ± SD.

Lipid peroxidation level in the shoots of the two sunflower genotypes, measured as the content of MDA, is given in Figure 4. In each genotype, the level of MDA was not significantly affected by drought treatments upon exposure to -0.4 and -0.6 MPa osmotic potential. A gradual increase in lipid peroxidation levels both in *H. annuus* cultivar and hybrid *H. annuus* × *H. mollis* was found at -0.8, while a statistically significant stress-dependent increase in lipid peroxidation level became apparent at-1.0 MPa.

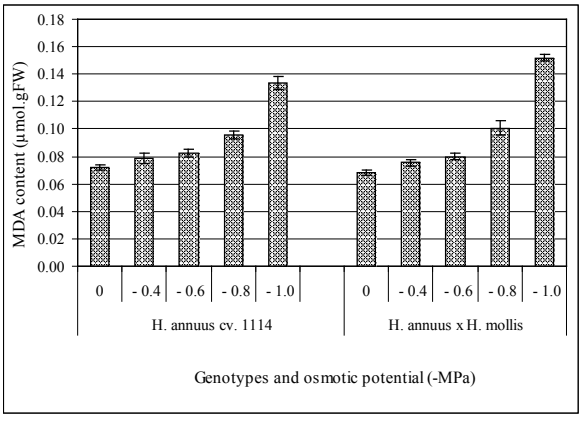

Figure 4. Effect of osmotic stress on MDA content in seedlings of two sunflower genotypes (*H. annuus* cv. 1114 and *H. annuus* × *H. mollis*). Data represent the average of two experiments with three replicates. Vertical bars indicate ± SD.

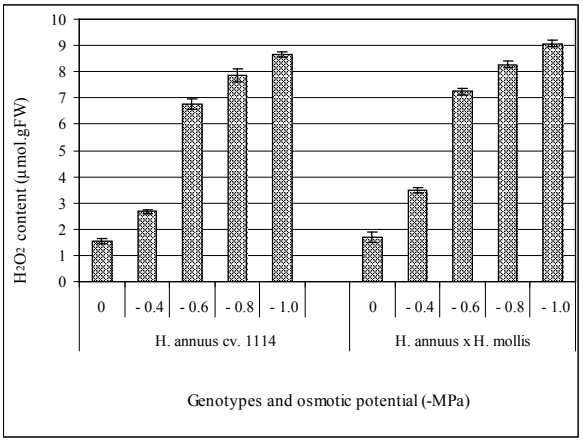

Figure 5. Effect of osmotic stress on H_2O_2 content in seedlings of two sunflower genotypes (*H. annuus* cv. 1114 and *H. annuus* × *H. mollis*). Data represent the average of two experiments with three replicates. Vertical bars indicate ± SD.

In the present investigation, we found that increasing concentrations of PEG from -0.4 to -1.0 MPa correlated with a strong increase in H_2O_2 level in shoots of both sunflower genotypes (Figure 5). Greater accumulation of H_2O_2 due to PEG was very evident at higher osmotic potential-from -0.6 to -1.0 MPa.

4. Discussion

In the present study, both sunflower cultivar 1114 and a hybrid *H. annuus* × *H. mollis* revealed a wide range of responses to PEG-induced osmotic stress that intensify with the severity of the treatments. At particular growth stage, sunflower plants exhibited water-deficient symptoms: depressed germination rate, root and shoot length, depressed shoot and root dry and fresh matter production, and water content (Table 1-3). Albuquerque and Carvalho [24] and Mwale *at al*. [25] have demonstrated that water stress in sunflower caused irregular seed germination and poor and unsynchronized establishment of seedlings. In the current study, the germination was inhibited in the presence of PEG-6000. The seeds of sunflower cultivar 1114 showed higher tolerance to osmotic stress, *i.e*. percent of germination was higher at more negative osmotic potential. Higher water retention was observed in roots than in leaves. Since roots are the first part of the plant exposed to stress conditions, their reaction is associated with tolerance to abiotic stress. This result is in accordance with data reported by Kaya *et al*. [26]. Maintenance of favorable plant water relations is vital for the development of drought resistance in crop plants

[27-29]. The water content in plant roots and leaves has been shown to be associated with drought tolerance in cultivated sunflower [30]. The results suggested that sunflower is sensitive to water deficiency during early vegetative stage, which could lead to severe loss in agricultural production of sunflower grown in drought conditions. As the whole, under PEG treatment, cultivated sunflower cultivar 1114 was more tolerant to water deficit stress than the interspecific line *H. annuus* x *H. mollis* on all the studied traits.

It is already known that proline content in plant tissues is both a reflection and measure of stress-induced damage at the cellular level [31,32]. Accumulation of proline under stress protects the cell by balancing the osmotic strength of cytosol with that of the vacuole and external environment [33]. Also, it may interact with cellular macromolecules such as enzymes and stabilize the structure and function of such macromolecules [34]. In the present work, the levels of proline increased in parallel with the severity of water stress in both sunflower genotypes. The effect of osmotic stress on proline content was shown to be more dramatic in cultivated sunflower genotype cv 1114 than in interspecific hybrid *H. annuus* × *H. mollis* (Figure 3). It could therefore be concluded that the cultivated genotype is more tolerant to drought stress than the hybrid line. The marked difference between sunflower genotypes in responding to water 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 level of water stress, each sunflower hybrid behaved differently according to their genetic makeup [6,35,36].

Results in the present study also revealed that the exposure of water deficit of severe nature led to differential H_2O_2 accumulation (Figure 5) of both the sunflower genotypes. In contrast, during PEG-treatment, no significant difference in the malondialdehyde level was observed at lower osmotic potential (-0.4 and -0.6 MPa). The increases of MDA content was evident under severe water stress (-1.0 MPa) (Figure 4), thus showing that the water deficit is associated with lipid peroxidation mechanisms.

In conclusion, the data here strongly suggest that a number of physiological and biochemical features of the sunflower plants, such as seed germination, shoot and root length, FW and DW, water content and level of proline, MDA and H_2O_2, are directly affected by PEG-mediated water stress. Although both genotypes had similar responses to the PEG-exposure, the cultivated cultivar was less affected by various parameters. Results from this study indicate that *H. annuus* cv 1114 is more tolerant to osmotic stress than interspecific hybrid line *H. annuus* × *H. mollis.* These observations are in agreement with some reports demonstrating that drought response of sunflower (*Helianthus* sp.) is strongly affected by genetic factors [6,35–37]. Genotypic differences in drought tolerance could be, at least in part, attributed to the ability of plants to acclimate and induce different defense mechanisms under severe water stress in sunflower. The cultivated genotype exhibited higher drought tolerance than introgressed line *H. annuus* × *H. mollis,* indicating that drought induced responses are mostly dependent on the genetic potential of the genotype. These observations suggest to us that the diploid perennial species *H. mollis* (2n=2x=34) can hardly be considered an excellent candidate of drought tolerance genes. Further experiments are needed to elucidate if, and how, the investigated sunflower genotypes are associated with the oil yield and fatty acid composition.

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