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CULTIVATED *Helianthus annuus* DIFFERS FROM TWO WILD RELATIVES IN GERMINATION RESPONSE TO SIMULATED DROUGHT STRESS

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SUMMARY

Wild sunflowers are a source of desirable traits for improving cultivated sunflower *Helianthus annuus* (ANN). Two wild species of sunflower, *H. argophyllus* (ARG) native to coastal sand dunes and *H. niveus* ssp. *tephrodes* (TEPH) native to the desert sand dunes, have been hypothesized to be drought resistant. We tested the expectation that these wild species would have higher percent germination and more uniform germination under simulated drought stress conditions compared to cultivated ANN. In a growth chamber study, we assessed germination of ANN, ARG and TEPH (three representative accessions for each species) under four osmotic stress treatments imposed using polyethylene glycol to simulate varying levels of drought stress: 0, -0.4, -0.8 and -1.2 MPa. As expected, all three species responded to increasing simulated drought stress with decreased percent germination assessed after seven days and delayed germination timing and uniformity assessed with logistic regressions. ARG responded marginally better than ANN with a greater percent germination at -1.2 MPa and greater uniformity at 0 and -0.4 MPa. Although the ARG advantage was small, this suggests that ARG × ANN recombinant hybrids may deserve further investigation to see if desirable alleles can be identified for improving cultivated ANN germination and establishment under mild drought conditions. In contrast, TEPH responded with substantially lower percent germination and less uniformity than ANN, suggesting that it is unlikely that TEPH will be useful as a donor of desirable drought resistance alleles.

Key words: Algodone dunes sunflower, osmotic, PEG, polyethylene glycol, silverleaf sunflower

INTRODUCTION

Global climate change projections include significant changes in precipitation patterns throughout the world, which may lead to an increase in the frequency and

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severity of drought in many areas (IPCC, 2007). These changes are expected to exacerbate the complex relationship between crop productivity and abiotic stressors such as drought (Boyer, 1982; Blum, 1996; Passioura, 1996; Verslues *et al.*, 2006). The development of cultivars with improved drought resistance may help maintain crop yield under marginal agricultural conditions. Although many cultivated plants have lost stress resistance during domestication, wild congeners may serve as a source of genetic variation for breeding for stress resistance (Seiler, 1992; Tanksley and McCouch, 1997; Koziol *et al.*, 2012; Khoury *et al.*, 2013).

The relationship between crop phenology and water availability is an important consideration in determining drought resistance (Passioura, 1996). In many crop species the two most sensitive times for susceptibility to drought are the germination-to-seedling stage and the flowering stage. Drought can impact germination by reducing the total extent of germination and altering the timing of germination, resulting in loss of robust and uniform stand establishment and ultimately yield (Harris *et al.*, 1999; Iqbal and Ashraf, 2006; Ahmad *et al.*, 2009). One method of enhancing germination under water stress imposed by both drought and soil salinity is to apply pre-sowing treatments such as soaking (priming) with water, inorganic salt solution, or organic osmotic solutions (Hegarty, 1978; Kaya *et al.*, 2006). However, presowing treatments can be both economically costly and time-consuming and have varying success depending on crop and soil moisture at sowing (Taylor and Harman, 1990). The incorporation of alleles for drought resistance from wild congeners provides an additional avenue for improving crop germination characteristics under drought stress.

Helianthus annuus (ANN) is a globally valuable oilseed crop and an important source of confectionery seeds. There is a growing need to breed cultivars with greater drought resistance (Lambrides *et al.*, 2001; Rauf, 2008; Škorić, 2009; Anonymous, 2013). The *Helianthus* genus includes 51 wild species which contain useful allelic variation for agronomically important traits such as yield and abiotic and biotic stress resistance (Seiler, 1992; Jackson and Koch, 1997; Seiler and Rieseberg, 1997; Seiler *et al.*, 2006a; Rauf, 2008; Škorić, 2009; Vear, 2011; Koziol *et al.*, 2012). Two wild species, the silverleaf sunflower *H. argophyllus* (ARG) and the Algondone dunes sunflower *H. niveus* ssp. *tephrodes* (TEPH), have been hypothesized to be drought resistant based on characteristics of their native habitats and their dense, white, and pubescent leaves, a trait often associated adaptation to water-limited environments (Gausman *et al.*, 1977; Seiler, 1992; Sandquist and Ehleringer, 1998; Seiler *et al.*, 2006a; Seiler *et al.*, 2006b; Rauf, 2008; Škorić, 2009). ARG, an annual sunflower native to the Texas coast on the Gulf of Mexico in North America, grows in saline dune habitats suggesting the potential for salt and drought resistance (Seiler and Rieseberg, 1997; Munns, 2002). ARG and some ARG × ANN hybrids have demonstrated physiological and growth characteristics that may be promising for increasing drought resistance of ANN, although the postive resutls are not consistent and require further investigation (Morizet *et al.*, 1984;

Martin *et al.*, 1992; Baldini and Vannozzi, 1998; Baldini, 1999; Škorić, 2009). TEPH, a facultative perennial, is endemic to the Algodones dunes region of the Sonoran Desert in Western North America (Bowers, 1996; Seiler and Rieseberg, 1997). Adaptation to such a hot dry climate suggests that it may be drought resistant, but this has not been experimentally tested. Water is not necessarily the primary factor limiting sunflower establishment and growth in desert dunes that can store precipitation (Rosenthal *et al.*, 2005; Ludwig *et al.*, 2006). We are not aware of any studies that have experimentally assessed ARG or TEPH germination in response to drought.

PEG (polyethylene glycol) can be used to experimentally create osmotic stress treatments that simulate drought stress for germination and short-term growth studies, although its use in longer-term growths studies can be controversial (Lawlor, 1970; Michel and Kaufmann, 1973; El Midaoui *et al.*, 2003; Munns *et al.*, 2010). Studies of cultivated sunflower germination in response to simulated drought stress with PEG have generally found that germination declines at osmotic potentials in the range of -0.6 to -0.8 MPa, although the thresholds for the initial decline and complete inhibition of germination varied among cultivars (Somers *et al.*, 1983; El Midaoui *et al.*, 2001; Iqbal and Ashraf, 2006; Kaya *et al.*, 2006; Ahmad *et al.*, 2009). For cultivated sunflowers, germination percentages in PEG solutions correlate with seedling emergence in drying soils with different matric potentials, supporting the use of PEG for screening for drought resistance at early stages of crop development (Somers *et al.*, 1983).

The objective of this study is to compare wild ARG and TEPH to cultivated ANN to determine if the wild species show promise as a germplasm resource for improving cultivated ANN germination in response to drought. We quantify the extent of germination as percent germination after 7 days, and assess the timing and uniformity of germination with logistic regression analyses for all three species under control conditions (0 MPa) and mild, medium and severe simulated drought stress treatments (-0.4, -0.8, and -1.2 MPa, respectively). We hypothesize that increasing simulated drought stress will reduce percent germination and decrease the rate and uniformity germination in all three species, but that wild species will have a greater percent germination and greater uniformity compared to ANN under increasing stress.

MATERIALS AND METHODS

Germination response to simulated drought stress was assessed in a growth chamber study at the University of Georgia greenhouse facility, Athens, GA, in January of 2012, using seeds germinated in Petri dishes. The growth chamber conditions were dark and 25°C. The experimental design was a randomized complete block design with three species, three accessions per species, four simulated drought stress treatments, three spatial blocks within the growth chamber, and two

Petri dishes per species/line/block (Petri dishes within block were averaged to a single data point) for a total of n=108 experimental units for statistical analysis.

The three study species were ARG, TEPH, and cultivated ANN. All achenes (hereafter referred to as seeds) were obtained from the USDA National Plant Germplasm System, National Genetic Resources Program (GRIN, http://www.ars-grin.gov/). The ANN accessions are PI-642777 (HA-412-HO), PI-560141 (RHA-373) and PI-578872 (HA-383). The ARG accessions are PI-468651 (ARG-1575), PI-435623 (ARG-400) and PI-649862 (No. 81). The TEPH accessions are PI-664653 (AMES-27850 and NIV-2442), PI-613758 (NIV-1243), and PI-650018 (AMES-27422). Seeds were scarified by excising the blunt end with a razor blade prior to treatments and then dehulled to enhance imbibition and eliminate any dormancy affects associated with the seed coat (Chandler and Jan, 1986). Ten seeds per accession per species were placed in a 100×15 mm Petri dishes containing two sheets of Whatman #1 filter paper and 6 ml of treatment solution. Seeds were transferred to new Petri dishes with filter paper and solution every other day to minimize fungal growth.

The simulated drought stress treatments were created with polyethylene glycol, molecular weight 6000 (PEG-6000), at four osmotic stress levels: 0 (control), -0.4, -0.8, and -1.2 MPa (Michel and Kaufmann, 1973). For osmotic treatments, PEG-6000 was dissolved into deionized water solutions totaling 1 l, utilizing 165.72 g (-0.4 MPa), 222.84 g (-0.8 MPa), and 265.56 (-1.2 MPa). PEG-6000 treatment solutions were assessed for osmotic potential by measuring the osmolality in a Wescor Vapro 5520 vapor pressure osmometer (Westcor Inc. Logan, Utah) and then converting osmolality to solute potential ($\Psi\pi$) using the van't Hoff relationship at 25°C (Nobel, 1999).

Seeds were scored for germination on daily basis for a total of seven days, based upon radical length. To account for species differences in seed weight and size, successful germination was as defined as reaching a radicle length of 2, 1.5, and 1.5 mm for ANN, ARG, and TEPH, respectively. For each Petri dish, the percent germination for the entire 7 day interval was calculated as the total number of seeds germinated per plate divided by the total number of viable seed per plate, excluding a few seeds lost to mold before germination.

Statistical analyses of percent germination was carried out using a general linear model (PROC GLM) in the statistical software package SAS (PROC GLM; SAS Institute, Cary, NC, USA). Main effects comparisons for among-species means were analyzed with analysis of variance (ANOVA) using an α of 0.05. Additionally, differences among accessions were assessed with ANOVA using the SLICE command to partition osmotic stress treatment comparisons to within species. Significant differences among accessions within species for a given treatment were assessed by comparing least squared means.

To further investigate the relationship between germination and drought stress, germination times for each species and treatment combination were modeled with logistic regressions (SAS; PROC GLM; SAS Institute, Cary, NC, USA) according to the following formula:

$$\mathrm{Logit}(y) = mx + b \quad (1)$$

Logit(y) is the log of the probability of germination; m and b are the regression slope and intercept, respectively; and x represents day. The cumulative probability of germination is given by the formula:

$$y = \frac{e^{mx+b}}{(1+e^{mx+b})} \quad (2)$$

To compare species-specific responses to drought treatments, logistic regressions were statistically compared using likelihood ratio tests. A significant result indicates a difference in the germination time regressions that can be assessed examining the shape of the response curve.

RESULTS AND DISCUSSION

We expected cultivated ANN and the two wild *Helianthus* species to have decreased percent germination and a slower rate of germination that would contribute to less uniformity in response to increasing simulated drought stress, but that the effects would be more pronounced for the cultivated species. As expected, all three species demonstrated decreased percent germination with increasing simulated drought stress (Figure 1, Table 1).

Table 1: Percent germination (mean ± SE) for *Helianthus annuus* (ANN), *H. argophyllus* (ARG) and *H. niveus* ssp. *tephrodes* (TEPH) after seven days of polyethylene glycol – 6000 osmotic stress treatments simulating drought stress: 0, -0.4, -0.8, and -1.2 MPa. Significant *P*-values are indicated in bold.

Treatment	ANN	ARG	TEPH	F	df	P
0 MPa	99.44 ± 0.56	99.44 ± 0.56	99.38 ± 0.62	0.00	2, 3	0.9998
-0.4 MPa	98.27 ± 1.20	100.00 ± 0.00	97.16 ± 1.23	0.25	2, 3	0.7770
-0.8 MPa	97.42 ± 1.39	98.88 ± 0.73	75.34 ± 4.85	21.51	2, 3	**<0.0001**
-1.2 MPa	75.99 ± 4.81	84.44 ± 3.95	41.23 ± 5.18	64.80	2, 3	**<0.0001**

Compared to the 0 MPa control, increasing intensity of simulated drought resulted in a decline in percent germination and a decrease in rate of germination (Figure 2). There were species differences, but the expectation that both wild species would be substantially more resistant to simulated drought stress than ANN was not well-supported. Compared to ANN, germination response to increasing stress was only marginally better for ARG and substantially poorer for TEPH.

For cultivated ANN, our germination responses are generally consistent with reports from other experiments that used PEG to simulate drought stress (Somers *et al.*, 1983; El Midaoui *et al.*, 2001; Iqbal and Ashraf, 2006; Kaya *et al.*, 2006; Ahmad *et al.*, 2009). In our study, there was no decline in percent germination in response to simulated drought stress in the -0.4 and -0.8 MPa treatments, indicting a threshold for response below -0.8 MPa (Figure 1). This is comparable to several other studies which found a threshold for cultivated ANN germination response in a similar range (Somers *et al.*, 1983; El Midaoui *et al.*, 2001; Kaya *et al.*, 2006), although two studies found a more sensitive threshold for response (Iqbal and Ashraf, 2006; Ahmad *et al.*, 2009). In our study, ANN germination in the most stressful treatment (-1.2 MPa) was relatively high at 76%. This is comparable to levels found in a some studies (Somers *et al.*, 1983; Ahmad *et al.*, 2009), but much higher germination was found in other studies (0-40%) for comparable treatments (El Midaoui *et al.*, 2001; Iqbal and Ashraf, 2006; Kaya *et al.*, 2006). Our study also found differences among ANN accessions for percent germination in the -1.2 MPa treatment ($F(2, 2)=19.84$, $P<0.001$) consistent with cultivar differences found in previous studies (Somers *et al.*, 1983; El Midaoui *et al.*, 2001; Ahmad *et al.*, 2009). Differences among studies are likely due to a combination of cultivar effects (i.e. genetic) and differences in seed age and storage conditions (Seiler, 1998; Iqbal and Ashraf, 2006).

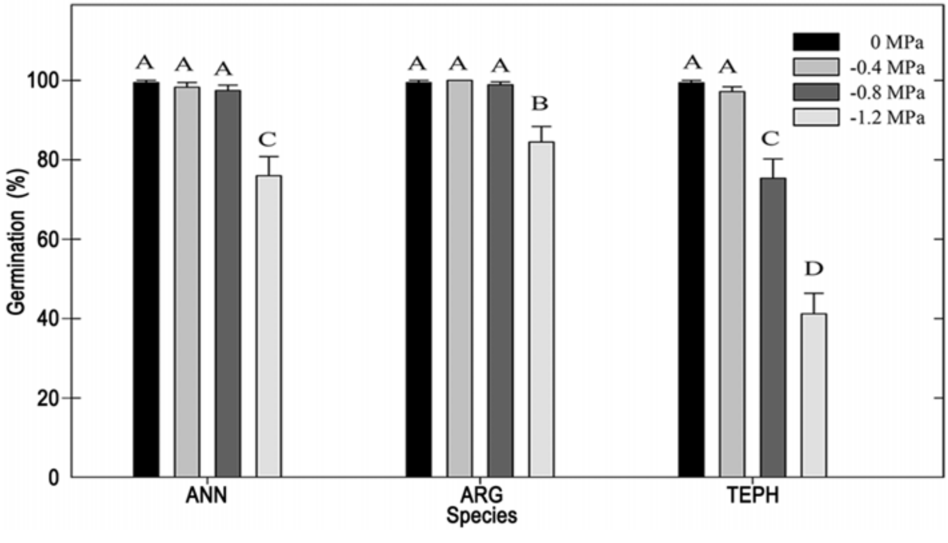

Figure 1: Percent germination of Helianthus annuus (ANN), H. argophyllus (ARG) and H. niveus ssp. tephrodes (TEPH) after seven days of polyethylene glycol – 6000 osmotic stress treatments simulating drought stress: 0, -0.4, -0.8, and -1.2 MPa. Different letters represent significant differences based on least square means at P<0.05 level, error bars represent SE. See Table 1 for statistical details.

ARG had a higher percent germination than ANN under the severe simulated drought treatment (-1.2 MPa), but the enhanced drought resistance was of small magnitude and only evident at a stress level that may not be agriculturally relevant (Figure 1, Table 1). Additionally, contrasts of germination time regressions detected

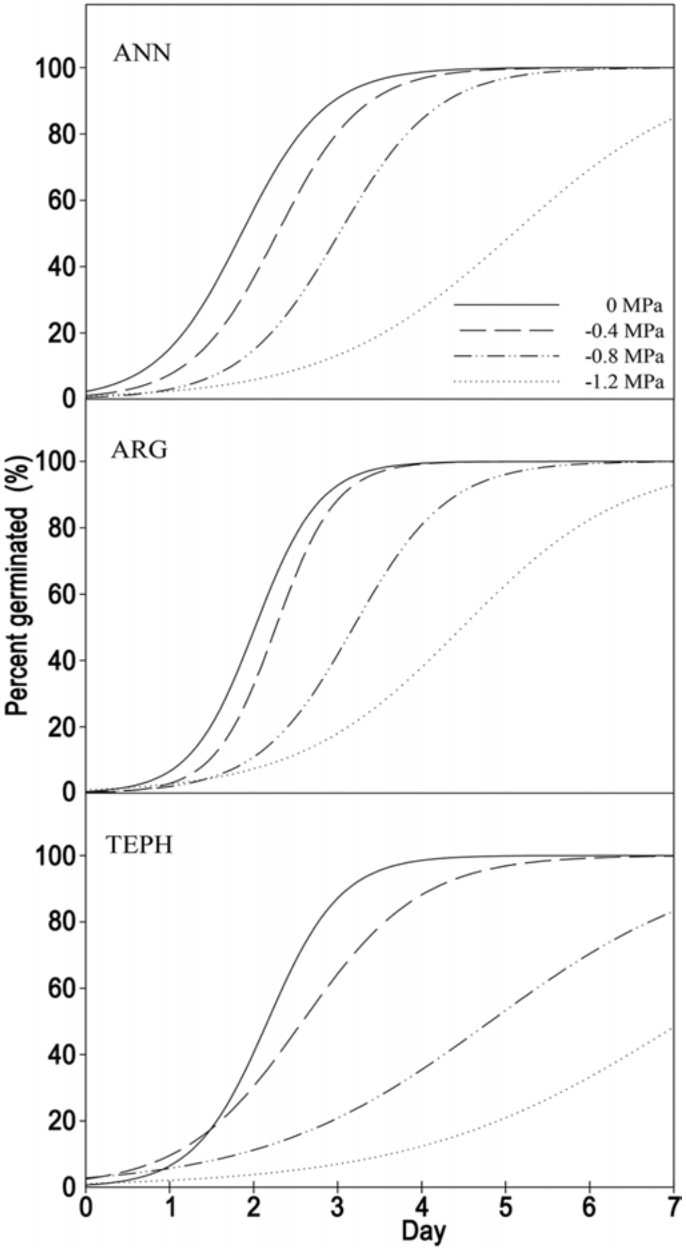

Figure 2: Cumulative probability density functions for observed germination frequency by day for cultivated Helianthus annuus (ANN), H. argophyllus (ARG) and H. niveus ssp. tephrodes (TEPH) for seven days of polyethylene glycol – 6000 stress treatments simulating drought stress: 0, -0.4, -0.8, and -1.2 MPa. See Table 2 for likelihood ratio contrasts of species in each treatment.

species differences for germination percentage on a given day in the 0 and -0.4 MPa treatments (Table 2). Examination of the germination time plots (Figure 2) indicates that ANN had a slightly faster rate of germination than ARG on days one and two, but that ARG compensates with higher germination on day three, with the end result showing no difference for extent of germination (Figure 2, Table 2).

Table 2: Likelihood ratio contrasts of germination time regressions for *Helianthus annuus* (ANN), *H. argophyllus* (ARG) and *H. niveus* ssp. *tephrodes* (TEPH) after seven days of polyethylene glycol – 6000 osmotic stress treatments simulating drought stress: 0, -0.4, -0.8, and -1.2 MPa. Significant *P*-values are indicated in bold, *df*=1

Treatment	ANN vs. ARG		ARG vs. TEPH		ANN vs. TEPH	
	$\chi 2$	P	$\chi 2$	P	$\chi 2$	P
0 MPa	6.8300	**0.0090**	1.6600	0.1974	1.8800	0.1703
-0.4 MPa	12.5600	**0.0004**	52.1800	**<0.0001**	13.7600	**0.0002**
-0.8 MPa	0.1400	0.7047	120.8000	**<0.0001**	105.7000	**<0.0001**
-1.2 MPa	2.7100	0.1000	27.5100	**<0.0001**	13.6000	**0.0002**

The steeper slope for ARG on day three can be interpreted as indicating greater uniformity or synchronization of germination, although again the differences between ANN and ARG for the germination timing were of relatively small magnitude. We also found a trend for differences among ARG accessions for percent germination in the -1.2 MPa treatment ($F(2, 2)=3.26$, $P=0.058$. Although the differences between ARG and ANN were relatively small, they suggest that it may be worth evaluating ARG × ANN recombinant hybrids to see if any desirable alleles are revealed for germination characteristics. This approach has been used to investigate physiological and growth characteristics beyond the germination stage for ARG and ARG × ANN hybrids (Morizet *et al.*, 1984; Martin *et al.*, 1992; Baldini and Vannozzi, 1998; Baldini, 1999; Škorić, 2009). For example, one study demonstrated that an inbred line derived from ARG × ANN had a higher water use efficiency and harvest index under drought compared to cultivated ANN, although it performed more poorly under well-watered conditions (Baldini and Vannozzi, 1998; Baldini, 1999). Another comparison of ARG × ANN hybrid seedlings for short term root and shoot growth under osmotic stress (PEG) found that ARG derived lines performed similarly to cultivated ANN (El Midaoui *et al.*, 2003). Thus, the evidence to date is mixed for ARG as a useful source of drought resistance alleles.

Although TEPH is adapted to the desert dune habitats, we found it less resistant to simulated drought than ANN and ARG. TEPH had a more sensitive threshold for response (less negative than -0.8 MPa) and substantially lower germination than ANN and ARG in -1.2 MPa (Figure 1, Table 1).

Additionally, the rate of TEPH germination was more sensitive to simulated drought than either ANN or ARG, resulting in more delayed and less uniform or synchronous germination (Figure 2, Table 2). We found no differences among TEPH accessions in each treatment. These results suggest that it is relatively unlikely that

TEPH will be a desirable source for drought resistant alleles useful in cultivated sunflower, although this should be definitively assessed using hybridization and recombination to reveal the effects of individual alleles. The germination patterns do suggest, however, that TEPH germination may respond to environmental cues in a manner that provides an adaptive advantage in its native desert dune habitat.

For example, a study of xerophytic species from arid and semi-arid regions in China found that species from most arid regions experienced the greatest decline in percent germination in response to increasing simulated drought stress with PEG (Zeng et al. 2010). Semi-arid species were capable of germination at osmotic stress of -1.5 to -2.4, while species from more arid environments were only capable of germination at less stressful levels of -1.2 to -1.8MPa. For TEPH, germination in the natural environment has been observed following relatively large precipitation events for this area in the coolest part of the year (Bowers, 1996; Seiler et al., 2006a; Seiler et al., 2006b). TEPH germination appears to be triggered by conditions of higher soil water availability that would be more conductive to seedling survival in this desert dune habitat.

Overall, the aim of this study was to evaluate drought resistance at the germination stage for two wild species of sunflower hypothesized to be drought resistant. The results provide some evidence that ARG may deserve further investigation as potential donor for desirable alleles for improved drought resistance in cultivated ANN. In contrast, there was no support for TEPH as a potential donor for desirable genetic variation for drought resistance. Further studies of hybrids between the wild species and ANN are required to uncover the genetic architecture of germination responses and the potential usefulness of any favorable alleles for molecular breeding.

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