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Wild and Cultivated Sunflower (*Helianthus annuus* L.) Do Not Differ in Salinity Tolerance When Taking Vigor into Account

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Abstract: Cultivated crops are expected to be less stress tolerant than their wild relatives, leading to efforts to mine wild relatives for traits to increase crop tolerance. However, empirical tests of this expectation often confound tolerance with plant vigor. We assessed whether wild and cultivated *Helianthus annuus* L. differed for salinity tolerance with 0 and 150 mM NaCl treatments. Salinity tolerance was assessed as the proportional reduction in biomass and as the deviation from expected performance based on vigor. Cultivated accessions had a greater proportional decline in biomass than wild accessions, but proportional decline was positively associated with vigor in both. Thus, wild and cultivated *H. annuus* did not differ for tolerance when variation in vigor was corrected for statistically. For traits potentially related to tolerance mechanisms, wild and cultivated accessions differed for elemental content and allocation of N, P, K, Mg, Ca, S, Na, Fe, Mn, B, Cu, and Zn for some tissues, biomass allocation, specific leaf area, and leaf succulence. However, these traits were generally unrelated to tolerance corrected for vigor. Osmotic adjustment was associated with tolerance corrected for vigor only in wild accessions where more osmotic adjustment was associated with greater tolerance. Our results for *H. annuus* suggest that efforts to use wild relatives to enhance crop abiotic stress tolerance will benefit from greater knowledge of traits related to plant growth responses decoupled from vigor, in order to get beyond potential growth-tolerance trade-offs.

Keywords: salinity stress; tolerance; osmotic adjustment; sunflower

1. Introduction

During plant domestication, humans selectively bred for higher productivity and ease of harvest [1]. This has resulted in a host of morphological and physiological changes in crops compared to their wild progenitors, defined by a suite of desirable traits shared by many crops, referred to as the “Domestication Syndrome”, which often includes loss of seed dispersal and dormancy, reduced defensive compounds, and an increase in plant size [2–5]. It is generally thought that along with these changes, crops developed reduced abiotic stress tolerance [6–9]. Thus, wild germplasm could serve as a resource for increasing crop stress tolerance [10–14]. However, our ability to do this is limited by our knowledge of the traits underlying tolerance.

Stress tolerance has often been assessed as relative performance under stressed and non-stressed conditions, where a lower reduction in absolute, percentage or proportional performance indicates higher tolerance [7,8,15,16]. However, this alone neglects differences in plant vigor (defined here as performance under non-stressed conditions), which is generally greater for crop cultivars than their wild progenitors and may influence how plants respond to stress [2,4,17]. Several stress tolerance indices exist that weight the stress-induced reduction in growth by vigor in some manner, in order

to identify lines that do relatively well in both environments [18–20]. However, these indices do not explicitly assess plant vigor as a trait that could influence tolerance. To do so, we developed a tolerance metric that accounts for vigor. Accessions are scored on their deviation from the expected response to stress, or expectation-deviation tolerance, so that traits associated with vigor may be separated from traits associated with tolerance [21]. When comparing stress tolerance of crops and wild relatives, it is useful to take into account any trade-off between vigor and stress tolerance, so that the genetic basis of traits responses independent of those associated with vigor can be identified and incorporated into breeding programs.

The ability to tolerate stress is proposed to be antagonistic to the potential for growth under optimal conditions [22]. Thus, traits contributing to vigor, such as high relative growth rate, have been implicated in trade-offs with abiotic stress tolerance [23]. Cultivated sunflower shows this trade-off between plant growth or size and drought and salinity stress tolerance [7,8,24,25]. In rice, more vigorous varieties have a lower mortality rate than less vigorous varieties while experiencing the same proportional reduction in growth due to salinity stress, potentially because increased vigor dilutes accumulated salt [26]. For domesticated plants, the increase in whole plant size is accompanied by changes in the size of individual organs [4,5,17]. Cultivated plants generally have a higher allocation to above ground tissue (especially leaf size), which serves to increase overall plant size through increased photosynthesis [5,17]. Since plant size and mass allocation influence plant performance under stress, they are worth considering in determining stress tolerance and associated traits.

Crops face many abiotic stresses, including salinity, drought, and low nutrients. As twenty percent of the world's irrigated agricultural land is currently affected by salt, a focus on salinity stress is important [16]. Increasing productivity on salinized land will require more salinity tolerant crops, [16]. A better understanding of the physiological responses to salinity stress can aid in achieving greater salinity tolerance in crops. Salinity stress initially occurs as a physiological drought [16,27,28]. In the following phase, disruptions in ion homeostasis lead to ion toxicity, where salt ions accumulate such that older leaf senescence outpaces the rate of newly produced leaves [16]. Survival is highly dependent on the ability to balance water uptake ion accumulation [16,29]. As such, mechanisms that confer resistance to reduced water availability and salt accumulation are important for long term growth [16,30].

Studies of salinity effects on plant growth have often focused on leaf Na content due to its impact on photosynthetic tissue [16]. Generally, these involve analyzing a single leaf or bulked leaf tissue. Lower leaf Na has been associated with greater tolerance in some species, such as cultivated sunflower, potentially reflecting a greater ability to exclude Na from the shoot [16,24,28]. However, it can be useful to distinguish between younger leaves that are still growing and older leaves that are more likely to serve as a site of salt ion accumulation, providing information about tissue tolerance [16]. Additionally, salinity tolerance may be dependent not only on leaf Na content but also on the Na content in other tissues, the content of other elements, and the relative distribution of elements across tissues [15,16,26,30–32]. Thus, analyzing a broader array of elements across all plant tissues can provide insights into mechanisms underlying different responses to salinity.

A mechanism that can prevent intracellular water loss during salinity stress and be related to Na uptake is osmotic adjustment [16,28,29]. For osmotic adjustment, plants accumulate osmotic compounds (also known as compatible solutes) that do not interfere with normal metabolic functions in order to lower cell water potential [16,27,33]. With a high enough concentration of these compatible solutes, plants can continue to absorb soil water from a saline environment [27]. Osmotic adjustment can help maintain metabolic activity growth under water deficits and enable regrowth under higher water availability [33]. Some species have the capacity to accumulate Na and Cl in the vacuole as an energetically favorable way to balance the water potential effects of compatible solutes in the cytoplasm [29]. Thus, it is interesting to assess osmotic adjustment as a possible mechanism underlying different responses to salinity.

In this study, we investigated salinity tolerance in sunflower (*Helianthus annuus* L.), a moderately salt tolerant crop with known genotypic differences in response to salinity [15,25,34–36]. We compared wild and cultivated *H. annuus* for salinity tolerance with two different metrics, (1) as proportional reduction in biomass in response to salinity (proportional-reduction tolerance), and (2) as the deviation from expected performance based on vigor (expectation-deviation tolerance). We also examined traits potentially associated with salt tolerance, including elemental content by tissue type (young leaves, old leaves, stem, and root), elemental allocation to each tissue type, size-independent morphological traits, and osmotic potential. Specifically, we asked the following questions. (1) Do wild and cultivated *H. annuus* differ in salinity tolerance assessed both as proportional-reduction and as expectation-deviation tolerance (i.e., when vigor is taken into account)? (2) Do wild and cultivated *H. annuus* differ in traits potentially related to salinity tolerance? (3) Do wild and cultivated *H. annuus* differ for associations between traits and salinity tolerance?

2. Materials and Methods

2.1. Experimental Design

We grew ten cultivated and ten wild accessions of *H. annuus* L. in the University of Georgia Botany Greenhouses from June to July of 2018 (Table S1). The cultivated accessions were inbred genotypes of sunflowers that were chosen from a sunflower association mapping population to encompass a range of performance under abiotic stress based on previous phenotypic screens [25,37]. These inbred lines are parental lines used to generate commercial lines, but are not hybrids themselves [37]. The wild accessions were natural populations chosen from a geographically diverse spread across the range within the United States.

The cultivated and wild accessions were arranged within a split-plot experimental design where each of four plots contained two subplots that either received a 0 mM or 150 mM sodium chloride (NaCl) solution, for the control and stress salinity treatments, respectively. There were two replicates of each accession within one treatment subplot, for a total of 320 (20 accessions $\times$ 2 replicates/accession/subplots 2 subplots/plot $\times$ 4 plots) plants. Subplots consisted of a 1.5 m $\times$ 1.5 m wooden frame covered in black plastic lining to keep the bottom ten centimeters of the pots submerged in the treatment solution. Each subplot held 40 pots in the treatment solution that was replaced every other day for the duration of the experiment.

Different germination procedures were used for achenes (hereafter “seeds”) of wild and cultivated *H. annuus* in order to generate sufficient numbers of seedlings at the same developmental stage for study initiation. Germination of wild *H. annuus* seeds was initiated with scarification on 10 June 2018. The blunt end of the seedcoat was removed, and seeds were sown on wet filter paper treated with a 0.45 g/L solution of Banrot fungicide (Everris NA112 Inc., Dublin, OH, USA). Germination of cultivated seeds was initiated five days later by sowing intact seeds in seed trays with a soil substrate of a 3:1 ratio sand to calcinated clay (Turface Athletics, PROFILE Products, LLC, Buffalo Grove, IL, USA). Seedlings with fully expanded cotyledons were transplanted into individual 5-L pots, with cultivated seedlings staggered behind wild seedlings by five days, to account for the difference in germination time. Pots were filled the same sand and Turface substrate, each supplemented with 40 g of 15-9-12 (N-P-K) Osmocote Plus with micronutrients and calcium in the form of 2.5 mL of gypsum and 2.5 mL of garden lime mixed into the sand and Turface prior to transplanting.

Both wild and cultivated seedlings were allowed to acclimate in their pots in fresh water until every individual reached the first pair of true leaf stage (non-cotyledon leaf stage), at which point, the fresh water was exchanged for a salinity treatment of 0 mM or 150 mM NaCl solution. In addition to bottom watering, each individual was top watered by the 0 mM or 150 mM NaCl solution to avoid buildup of salt due to evaporation from the soil. Plants were top watered daily for the first seven days of treatment, and then every other day for the rest of the experiment. Salinity concentrations of each treatment were monitored daily with an electric conductivity (EC) probe (HI 8733, Hanna Instruments Inc.,

Woonsocket, RI, USA). Plants were harvested for both above and belowground tissue after 21 days of treatment.

2.2. Measurements

Each harvested plant had their biomass separated into the most recently fully expanded leaf (MRFEL), later categorized as young leaf tissue, leaves at the level of or above the MRFEL including any reproductive tissue (young leaves), leaves below the MRFEL (old leaves), stem, and roots. Roots were washed on a metal screen to remove the soil substrate. All tissue types were oven-dried at 60 °C for 72 h, then weighed. Total biomass was calculated as the sum of all tissues per individual. Young leaf mass fraction (YLMF), old leaf mass fraction (OLMF), stem mass fraction (SMF), and root mass fraction (RMF) were calculated by dividing the young leaf, old leaf, stem, and root biomass values by the total biomass values for each plant sample, respectively.

At harvest, the MRFEL was weighed for fresh weight then scanned as a digital image with 300 dpi resolution for area. To calculate osmotic adjustment, we measured the osmotic potential on 120 MRFELs from a randomly chosen subset from each accession and treatment combination. Immediately after scanning, the selected MRFELs were rehydrated by submerging the petiole in deionized water in the dark for 24 h to achieve 100% relative water content (RWC) while the rest of the MRFELs were dried separately. Their dry weights were added to the young leaf tissue category. After rehydration, a disc 6 mm in diameter was punched one inch from the base of each leaf blade on the right-hand side and immediately stored in a −80 degree °C freezer. The remainder of the leaf was dried and weighed for MRFEL dry weight (with a negligible reduction in mass from the removal of a leaf disc). Leaf discs were frozen for 48 h before measurement to ensure leaf cell walls had ruptured. The osmotic potential (MPa) of the cell sap of each leaf disc was measured with a vapor pressure osmometer (Wescor Vapro 5520, Wescor Inc., Logan, UT, USA). Osmotic adjustment was calculated as the difference between the mean stressed and control osmotic potential values for each accession. Leaf area for the MRFEL was determined with ImageJ (NIH, Bethesda, DE, USA, <http://rsb.info.nih.gov/ij/>) by converting the scanned images through a pixel to cm scale. From that, specific leaf area (SLA, mm²/g) was calculated by dividing the leaf area by the leaf dry weight. Leaf succulence was calculated as a MRFEL's dry weight from its fresh weight then divided by its area.

We designated a group of non-elemental traits as size-independent traits, including each of the four tissue type mass fractions (YLMF, OLMF, SMF, RMF), leaf SLA and succulence. Osmotic potential and osmotic adjustment were analyzed separately from the size-independent trait group due to insufficient data (fewer than three replicates) to calculate an estimated marginal mean for each accession in each treatment, as required to calculate osmotic adjustment (difference between the two treatment values).

2.3. Ion Analysis

The dried mass of each of the four tissue types (young leaves, old leaves, stem, and roots) was bulked for every accession and treatment combination, then ground into a powder with a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) and then a Qiagen tissue lyser (Qiagen, Venlo, The Netherlands) with a steel bead. Each sample of powder was placed in a 2 mL Eppendorf tube and sent to Midwest Laboratories (Omaha, NE, USA) for an analysis of nitrogen (via Dumas method) and elements (Inductively Coupled Argon Plasma Optical Emission, ICP). The analysis yielded element concentrations per gram of dried matter in four tissue types for: nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sulfur (S), sodium (Na), iron (Fe), manganese (Mn), boron (B), copper (Cu), and zinc (Zn).

We also calculated mass relative allocated amount (MRAA), a measure for quantifying preferential elemental allocation, as the difference between the ion fraction (fraction of elemental content in the tissue relative to the total amount of element in the whole plant) and the mass fraction (fraction of a tissue biomass relative to whole plant biomass) [24,38]. Positive values indicate preferential allocation

of an element into that tissue (more than expected by neutral distribution) while negative values indicate preferential exclusion from that tissue (less than expected by neutral distribution) [24].

2.4. Statistical Analysis

A mean trait value was generated as an estimated marginal mean for each accession using a nested mixed model to account for the split plot design [39,40]. All total biomass means were natural log (ln) transformed prior to generating a genotypic mean to reduce the effect of the larger individuals skewing a genotypic mean under control conditions. The estimated effect of domestication status, salinity treatment, and their interaction were generated using Wald's Chi-square test on the mixed model [41]. A principal component analysis (PCA) of the elemental concentrations per tissue type was used to assess the variation between wild and cultivated groups within each treatment. Differences between domestication status and tissue types were tested using Hotelling's-t test on the first two principal components. All statistics were carried out using R (version 3.4.3) in Rstudio (version 1.1.383), all figures were made using ggplot2 [42].

Salinity tolerance was defined in two ways: by the proportional reduction in total biomass, and by its expectation-deviation tolerance, which is the performance of an accession compared to its expected performance based on vigor which we defined as biomass under control conditions. When using the proportional reduction in biomass as tolerance, a lower relative decrease under stress was associated with greater tolerance. The expectation-deviation tolerance was calculated by taking the residuals of each accession from the linear regression of proportional reduction in biomass vs. the natural log of plant vigor. Greater salinity tolerance corresponded to accessions with a more positive residual, performing better than expected for their size, and lower salinity tolerance corresponded to accessions with a more negative residual, performing worse than expected. To investigate trait associations with variation in tolerance, we conducted linear regressions between both metrics of tolerance and the first two principal components of our stress and difference between treatment (Delta) based PCAs. The traits examined were elemental content by tissue type, elemental allocation to each tissue type, size-independent morphological traits, and osmotic potential. We also regressed both metrics of tolerance and osmotic potential under stress and osmotic adjustment separately due to the exclusion of these traits from the non-elemental size independent traits. These traits were examined for the plants in the salinity treatment, and as the response to the salinity treatment (control minus treatment).

3. Results

3.1. Differences in Tolerance Between Wild and Cultivated

Salinity stress imposed in this study decreased total biomass in both wild and cultivated *H. annuus*, but cultivated accessions had a larger proportional decrease (difference after natural log transformation) than wild accessions (Figure 1A, Table 1, $p = 0.001$ for interaction between domestication status and salinity treatment). Using the proportional reduction in biomass as the metric for salinity tolerance, cultivated accessions were less tolerant than wild accessions. However, increased vigor was associated with a proportionately larger decrease in biomass under salinity stress for all accessions, but did not differ for by domestication status (Figure 1B, $p = 0.002$ for vigor, $p = 0.935$ for domestication status, $p = 0.960$ for interaction between vigor and domestication status). Thus, the difference in proportional tolerance to salt between domestication status evident in Figure 1A can be attributed to the lower overall vigor of the wild accessions. The expectation-deviation tolerance of each accession, which statistically corrects for vigor, was then calculated by comparing the performance deviation from expectation based on its vigor (biomass under control conditions), i.e., as the residuals in Figure 1B.

Figure 1. The effect of salinity stress on biomass in wild and cultivated *H. annuus*. Points indicate accession means. (A) Accession means of natural log (ln) transformed total plant biomass by domestication status and salinity treatments. (B) The relationship of vigor (biomass under control conditions) and the proportional decrease in biomass. The residuals (gray lines) of each accession reflect expectation-deviation tolerance. (C) Accession means of proportional reduction in total biomass by domestication status. (D) Accession means of expectation-deviation tolerance (residuals from 1B) by domestication status. Purple circles indicate cultivated accessions and orange triangles indicate wild accessions. Different lower-case letters denote significant differences ($p < 0.05$) based on post-hoc contrasts from Tukey-HSD tests (Table 1).

Table 1. Mean values of non-elemental plant traits and *p*-value significance of Wald's Chi-square test on treatment, domestication status, and their interaction (treatment × domestication status).

Trait	Cultivated Control Mean	Wild Control Mean	Cultivated Stress Mean	Wild Stress Mean	Treatment Effect	Domestication Effect	Interaction
In Total Biomass (g)	2.011	1.235	0.51	0.187	<0.001	<0.001	0.001
Young Leaf mass (g)	0.956	0.568	0.39	0.343	<0.001	<0.001	<0.001
Old Leaf mass (g)	3.483	1.639	0.594	0.552	<0.001	<0.001	<0.001
Stem mass (g)	2.872	1.405	0.451	0.3	<0.001	<0.001	<0.001
Root mass (g)	0.997	0.438	0.348	0.238	<0.001	<0.001	<0.001
Young Leaf Mass Fraction (YLMF)	0.131	0.147	0.222	0.242	<0.001	0.172	0.834
Old Leaf Mass Fraction (OLMF)	0.412	0.391	0.34	0.386	0.003	0.49	0.009
Stem Mass Fraction (SMF)	0.335	0.35	0.246	0.209	<0.001	0.544	<0.001
Root Mass Fraction (RMF)	0.122	0.113	0.192	0.163	<0.001	0.01	0.042
Specific Leaf Area (SLA) (mm ² /g)	323.988	311.44	247.851	206.81	<0.001	0.006	0.027
Leaf Succulence (g/mm ²)	0.025	0.028	0.026	0.031	0.061	<0.001	0.112
Osmotic Potential (MPa)	−0.847	−0.819	−1.143	−1.008	<0.001	0.034	0.067

3.2. Differences in Traits Between Wild and Cultivated

In order to compare how salinity affects levels of essential elements in structural and photosynthetic tissue between wild and cultivated accessions, we examined variation in elemental content across the four tissue types (young leaves, old leaves, stem, and root) and both domestication statuses using principal component analysis (PCA). In the control treatment, 86% of the variation in tissue elemental content was captured by the first two PCA axes (Figure 2A). Young and old leaves differed from roots primarily along PC1, which was dominated by variation in N, Ca, and Na (Figure 2A, Table S2). Both young and old leaves had considerably higher N and Ca and less Na content than the roots. Stems were differentiated from other tissues primarily along PC2, dominated by K and S. Stem tissue had the highest K and lowest S content. However, there was no differentiation between wild and cultivated accessions in each tissue type in the control treatment (Figure 2A, $p = 0.576$ for young leaves, $p = 0.565$ for old leaves, $p = 0.067$ for stem, $p = 0.063$ for roots).

To assess the effect of the salinity treatment, we looked at both the elemental content of plant tissues in the salinity treatment and the response of the elemental content to the treatment (Delta, calculated as control minus salinity treatment). In the salinity stress treatment, a major portion of the variation in tissue elemental content was captured by the first two PCA axes (79%, Figure 2B). Tissue types still differed from each other, but there was additional differentiation between wild and cultivated accessions in some tissues. Young and old leaves differentiated from roots primarily along PC1, with leaves having more Ca, Mg, and B, and roots having more Z (Figure 2B and Figure S4, Tables S2 and S4). Stems were differentiated from other tissues primarily along PC2, with stems having the highest Na content of all the tissue types. Elemental content was different by domestication status in young leaf, old leaf, and root tissue, but not stem tissue ($p = 0.011$, 0.043 , 0.037 , and 0.320 , respectively). When examining the difference in elemental content between the treatments (Delta), the tissue types were less differentiated from each other (Figure 2C). Domestication status significantly affected Delta for young leaf and stem tissues ($p = 0.036$ and $p = 0.003$, respectively), but not old leaf and root tissues ($p = 0.188$ and 0.068 , respectively).

We also assessed MRAA (mass relative allocated amount) to determine whether relative allocation of elements differed across tissue types and by domestication status (Figure S5 and Table S4). Under control conditions, MRAA results were similar to those of elemental content. Tissue types differed from each other, but wild and cultivated accessions did not differ for any tissue type (Figure 2D, $p = 0.475$ for young leaves, $p = 0.104$ for old leaves, $p = 0.735$ for stem, $p = 0.189$ for roots). Under salinity stress, wild and cultivated accessions differed in elemental allocation only in young leaf tissue (Figure 2E,

$p = 0.034$). For Delta, wild and cultivated differed for MRAA for each tissue type (Figure 2F, $p < 0.001$ for young leaves, $p = 0.018$ for old leaves, $p = 0.015$ for stem, $p = 0.040$ for roots).

Figure 2. Principal component analyses (PCA) of elemental concentration (A–C) and the mass relative allocated amount (MRAA) of elements (D–F) in the four tissue types (young leaf, old leaf, stem, and root) for wild and cultivated *H. annuus*. Panels represent control treatment (A,D), stress treatment (B,E), and Delta, the difference between treatments (C,F). Different lower-case letters denote significant Hotelling's-T2 test for differences ($p < 0.05$) among the eight tissue type and domestication status groups.

To set the stage for determining whether the relationships of traits and tolerance differed by domestication status, PCA analyses of elemental content and MRAA were additionally analyzed by tissue type (Figures S1 and S2). The PC1 and PC2 values for each accession and tissue type were then used in tests of trait associations with tolerance (both proportional-reduction tolerance and expectation-deviation tolerance) and domestication status.

We also assessed variation of non-elemental traits that we designated as size-independent morphological traits. This trait group included mass fractions of the four tissue types (YLMF, OLMF, SMF, RMF), specific leaf area (SLA), and leaf succulence. A PCA of the size independent morphological traits showed that in each treatment, over 60% of the variation was captured in the first two principal components (Figure 3A,B). In the control treatment, the wild and cultivated accessions did not differ for the first two PC axes (Figure 3A, $p = 0.062$). In the salinity treatment, however, the domestication status differed primarily by PC1, dominated by succulence, RMF, and SLA (Figure 3B, $p < 0.001$). For the shift in response to the stress treatment (Delta), wild and cultivated accessions were also different, but now largely by RMF and OLMF (Figure 3C, $p < 0.001$). Looking at some of the dominant traits individually, SLA decreased more in wild accessions than in cultivated accessions, succulence was higher in wild than cultivated accessions but this did not differ by treatment, and RMF increased less in wild than cultivated accessions (Figure 4A–C, Table 1).

Figure 3. Principal component analyses (PCA) of the six size-independent morphological traits, which include specific leaf area (SLA) and leaf succulence per area and the four tissue type mass fractions (Young Leaf Mass Fraction (YLMF), Old Leaf Mass Fraction (OLMF), Stem Mass Fraction (SMF), and Root Mass Fraction (RMF)) under (A) Control conditions, (B) Stress conditions, and (C) Delta (the change between treatments). Purple circles indicate cultivated accessions and orange triangles indicate wild accessions. Different lower-case letters denote significant Hotelling's-T2 test for differences ($p < 0.05$) between the two domestication status groups.

Figure 4. Estimated marginal means of wild and cultivated accessions under the control and stress treatments (grouped by domestication status) for (A) Specific leaf area (SLA), (B) Leaf succulence on a per area basis of a most recently fully expanded leaf (MRFEL)), (C) Root mass fraction (RMF) (calculated by root mass/total biomass) and (D) Osmotic potential taken from a rehydrated MRFEL. Different lower-case letters denote significant differences ($p < 0.05$) based on post-hoc contrasts from Tukey-HSD tests.

We also assessed variation in the osmotic potential of the MRFEL. We detected the osmotic adjustment is response to salinity stress, as indicated by the decreased osmotic potential (more negative) of the MRFELs for both wild and cultivated accessions in response to the salinity treatment (Figure 4D,

Table 1). However, wild and cultivated did not differ for the amount of osmotic adjustment, as indicated by no significant interaction of treatment and domestication status for osmotic potential.

3.3. Differences in Associations Between Traits and Tolerance

To further explore potential mechanisms of salinity tolerance, we investigated which traits were associated with salinity tolerance, and whether this differed by domestication status. For elemental content, proportional-reduction tolerance was associated with the treatment response for PC1 of young leaf, stem, and root elemental content, as well as salinity treatment PC2 of root tissue (Table S3). Proportional-reduction tolerance was also associated with domestication status in all four tissue types (Table S3). In contrast, expectation-deviation tolerance, or salinity tolerance corrected for vigor, was associated with only one elemental content trait, which was the treatment response for PC1 of old leaf tissue ($p = 0.015$, Table S3).

Accessions with a higher tolerance when vigor was corrected for statistically had increased Ca and Mg. However, these trait-tolerance relationships did not differ by domestication status (Table S3). For MRAA, proportional-reduction tolerance was associated with values in the salinity treatment for stem PC2 ($p = 0.027$, Table S3) and for the change in treatment response of young leaf PC2 ($p < 0.001$, Table S3), but wild and cultivated accession did not differ for these relationships ($p = 0.111$ and $p = 0.114$, respectively, Table S3). Expectation-deviation tolerance was associated with the salinity treatment values of young leaf PC2 ($p = 0.022$, Table S3) and the change in treatment response of young leaf PC2 ($p = 0.014$, Table S3) and stem PC2 ($p = 0.013$, Table S3).

For the size-independent morphological traits, such as the tissue mass fractions, leaf SLA and succulence, proportional-reduction tolerance was associated with PC1 under stress conditions and with the difference between treatments, but this did not differ by domestication status ($p = 0.026$ and $p = 0.039$, respectively, Table 2). Proportional-reduction tolerance was also associated with PC2 in the difference between treatments and differed by domestication status as well ($p = 0.011$ and $p = 0.015$, respectively, Table 2), but not by the interaction ($p = 0.907$, Table 2). In contrast, expectation-deviation tolerance was associated with PC1 under stress and this association did differ by domestication status ($p = 0.001$, Table 2). For PC1 under stress, accessions with a higher expectation-deviation tolerance had lower SLA and higher succulence (Figure 5A and Figure S3, Table S3). However, this strong, negative relationship was highlighted in the wild accessions ($R^2 = 0.58$, Figure 5B) and not in cultivated accessions ($R^2 = 0.14$, Figure 5A).

Table 2. Associations of tolerance (proportional reduction tolerance and expectation-deviation-tolerance) with traits (PC1 and PC2 scores from PCAs of size-independent morphological traits (Morph PCA), and osmotic potential) and domestication status, for traits reported as measured in the salinity stress treatment (stress) and as the difference between the control and salinity treatment (Delta).

Trait Type	Response to	Trait	Proportional-Reduction Tolerance			Expectation-Deviation Tolerance		
			Trait Effect	Domestication Effect	Interaction	Trait Effect	Domestication Effect	Interaction
Morph PCA	Stress	PC1 Axis	0.026	0.660	0.154	0.091	0.140	0.001
Morph PCA	Stress	PC2 Axis	0.390	0.029	0.865	0.799	0.968	0.850
Morph PCA	Delta	PC1 Axis	0.039	0.405	0.145	0.681	0.756	0.451
Morph PCA	Delta	PC2 Axis	0.011	0.015	0.907	0.145	0.913	0.091
Osmotic Potential	Stress	Stress Value	0.429	0.313	0.538	0.288	0.844	0.059
Osmotic Potential	Delta	Delta Value	0.283	0.328	0.142	0.061	0.851	0.003

Figure 5. The relationships between expectation-deviation tolerance metric and (A) size-independent morphological traits PC1 under stress, or (B) osmotic adjustment (OA). Purple circles indicate cultivated accessions and orange triangles indicate wild accessions.

Leaf osmotic potential in the stress treatment was not associated with either metric of tolerance (Table 2). However, the response of osmotic potential to the treatment, which is osmotic adjustment, did show a significant interaction of domestication status with expectation-deviation tolerance (Figure 5B, Table S3). Results showed a strong negative relationship between osmotic adjustment and expectation-deviation tolerance for wild accessions ($R^2 = 0.7$, Figure 5B), but not for cultivated accessions ($R^2 = -0.06$, Figure 5B). Wild accessions with greater osmotic adjustment had higher salinity tolerance when vigor was corrected for statistically.

4. Discussion

Crops differ from their wild progenitors in many traits, with a general expectation that crops are less tolerant to abiotic stress than their wild relatives. However, few empirical studies test this expectation, despite proposals to use wild germplasm to increase stress tolerance in crops. Furthermore, wild and cultivated groups have differences in vigor. This inherent difference is rarely considered in tolerance studies. Though it is unknown if conclusions found in the literature would change if plant vigor was taken into account, it has been shown in cultivated sunflower that vigor correlates with the effect of salinity stress [24,25]. We compared wild and cultivated *H. annuus* for salinity tolerance and found for proportional-reduction tolerance, crops were less salt tolerant than wild accessions. However, when tolerance was assessed as expectation-deviation tolerance, which accounts for the trade-off between vigor and proportional decline in biomass, wild and cultivated accessions did not differ in tolerance. Our results suggest that domestication status and vigor should be considered separately when predicting salinity tolerance in sunflowers. This also suggests that responses to salinity are complex, and if a trade-off between vigor and proportional decline in biomass exists, then efforts to mine wild relatives for traits to improve crop tolerance should take this into consideration.

For sunflower, leaf Na concentration under salinity stress has been found to be higher in a wild accession than in a cultivated accession [43]. Prior work has shown strong differentiation in elemental content among tissue types in cultivated *H. annuus* under salinity stress [24]. We build on this finding by showing that wild and cultivated accessions differ for a whole suite of elements in young and

old leaves, although elemental content differed more by tissue type rather than domestication status. This reinforces that it is important to consider more elements and tissues when comparing wild and cultivated accessions for relationships of traits to tolerance. It may also be of interest to further examine differential sodium ion sequestration within cells, e.g., compartmentalization of Na in the vacuole, which increases the cytosolic K:Na ratio [16,44]. Nonetheless, it is useful to additionally assess the relative distribution of elements across tissues.

To consider the effect of differential allocation of elemental content, we calculated the relative contribution of elements with respect to the given size of each tissue type (MRAA). The tissue types once again separated into distinct groups but did not differentiate by domestication status in most tissue types, except in young leaves under stress. Wild and cultivated accessions differed in allometry, especially under stress, which could affect the allocation of elemental content within tissues via dilution [17]. However, wild and cultivated accessions were largely similar in preferential element allocation in both treatments, even when not correcting for multiple comparisons. For both wild and cultivated *H. annuus* under salinity stress, Na accumulates in the stem, suggesting they share the ability to accumulate some Na in the stem as a first line of defense to keep Na away from photosynthetic tissue. As the storage tissue meets its limit, transporters at the upper root and lower leaf level of the xylem may be additionally required to intercept the flood of Na ions from reaching younger leaf tissue [16,29].

We found an association of expectation-deviation tolerance with the interaction between the size-independent morphological traits loading on PC1 under stress and domestication status. The dominant traits on this axis were two leaf traits known to be affected by salinity stress: specific leaf area (SLA) and succulence [45–47]. Under stress, SLA decreased in all accessions, but more so in wild populations. This larger decrease in SLA within wild sunflowers is similar to that found in other crop-wild comparisons under water deficits [48]. A decrease in SLA is expected to curb water loss, as thicker leaves are associated with decreased transpiration [49]. Our results showed wild *H. annuus* had greater leaf succulence per unit area than cultivated accessions, with no significant treatment effect. Succulence has previously been linked to salinity tolerance in both wild and cultivated tomatoes [50]. It is worthwhile to further investigate succulence as part of a suite of leaf traits beneficial to salinity tolerance. For example, in *Populus* species, a decrease in SLA is associated with an accumulation of osmolytes, suggesting these are coordinated responses to stress [51]. Since multiple traits differ by domestication status, these could be considered in their relationship to stress tolerance.

Both wild and cultivated accessions exhibited osmotic adjustment within the range reported in previous sunflower studies [35,46]. Although the magnitude of osmotic adjustment did not differ between wild and cultivated accessions, the association of osmotic adjustment and expectation-deviation tolerance differed by domestication status. Higher osmotic adjustment was associated with higher expectation-deviation tolerance in wild *H. annuus*, but not in cultivated *H. annuus*. This supports that osmotic adjustment occurs in response to salinity stress, but surprisingly, that osmotic adjustment is associated with tolerance only in wild accessions. Studies of crops often hypothesize that osmotic adjustment is an important mechanism of salinity tolerance, but results showing an association between the two are not consistently observed [52–55]. Our results suggest it would be interesting to characterize the biochemical basis of osmotic adjustment in wild and cultivated *H. annuus* to determine if there is anything unique about the wild accessions with a higher expectation-deviation tolerance that could be incorporated into vigorous cultivated lines.

Additionally, it is of great agricultural interest to further investigate differences between stress tolerance of wild and cultivated plants across the life cycle. It has been observed in multiple other crops that susceptibility to salinity stress is dependent on the stage at which plants experience stress, notably that susceptibility to salinity decreases with increasing plant maturity [56,57].

Overall, our results for *H. annuus* suggest that when variation in vigor is corrected for statistically, crops are not necessarily less salt tolerant than their wild relatives. However, the complex and intertwined nature of how traits relate to stress responses suggests that the relationship we found

between vigor and tolerance could differ when other species, stresses, or the timing of stresses are considered. Thus, it is worth comparing wild and cultivated accessions of additional life stages or species and other abiotic stresses, such as drought and low nutrients, to further assess whether any generalities can be drawn for the association of vigor with stress tolerance. And, moving forward, efforts to mine wild relatives to improve cultivated salinity tolerance would benefit from knowledge of traits that are decoupled from vigor to avoid potential growth-tolerance trade-offs.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/7/1013/s1>, Figure S1: Elemental Content within Tissue Type, Figure S2: Element MRAA within Tissue Type, Figure S3: Top Three Size-Independent Traits from PC1 Axis under Stress Treatment against Expectation-deviation Tolerance, Figure S4: Elemental Content by Tissue Type of Twelve Elements, Figure S5: Elemental Allocation by Tissue Type of Twelve Elements, Table S1: Accession List, Table S2: Elemental PC Loading Contributions, Table S3: Associations of Both Metrics of Tolerance with Elemental Content and Allocation for Twelve Elements, Table S4: Mean Elemental Content and Allocation of Twelve Elements and *p*-value Significance of Wald's Chi-square Test on Treatment, Domestication Status, and their Interaction.

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