HortScience 55(9):1446–1452. 2020. https://doi.org/10.21273/HORTSCI15058-20

Cell Membrane Stability and Relative Cell Injury in Response to Heat Stress during Early and Late Seedling Stages of Diverse Carrot (*Daucus carota* L.) Germplasm

Aneela Nijabat
Department of Botany, University of Sargodha, Sargodha-40100, Pakistan

Adam Bolton
Department of Horticulture, University of Wisconsin—Madison, 1575 Linden Drive, Madison, WI 53706

Muhammad Mahmood-ur-Rehman
Institute of Horticultural Sciences, University of Agriculture Faisalabad-38000, Pakistan

Adeel Ijaz Shah, Rameez Hussain, Naima Huma Naveed, and Aamir Ali
Department of Botany, University of Sargodha, Sargodha-40100, Pakistan

Philipp Simon
Department of Horticulture, University of Wisconsin—Madison, 1575 Linden Drive, Madison, WI 53706; and Institute of Horticultural Sciences, University of Agriculture Faisalabad-38000, Pakistan

Additional index words. abiotic stress, carrot breeding, developmental variation, genetic diversity, heat tolerance, high temperature

***Abstract*. Heat waves occur with more regularity and they adversely affect the yield of cool season crops including carrot (*Daucus carota* L.). Heat stress influences various biochemical and physiological processes including cell membrane permeability. Ion leakage and increase in cell permeability are indicators of cell membrane stability and have been used to evaluate the stress tolerance response in numerous crops and inform plant breeders for improving heat tolerance. No study has been published about the effects of heat stress on cell membrane stability and relative cell injury of carrot. Therefore, the present study was designed to estimate these stress indicators in response to heat stress at the early and late seedling developmental stages of 215 diverse accessions of wild and cultivated carrot germplasm. The article identifies the relationship between early and late stages of seedling tolerance across carrot genotypes and identifies heat-tolerant genotypes for further genetic analysis. Significant genetic variation among these stress indicators was identified with cell membrane stability and relative cell injury ranging from 6.3% to 97.3% and 2.8% to 76.6% at the early seedling stage, respectively; whereas cell membrane stability and relative cell injury ranged from 2.0% to 94.0% and 2.5% to 78.5%, respectively, at the late seedling stage under heat stress. Broad-sense heritability ranged from 0.64 to 0.91 for traits of interest under study, which indicates a relatively strong contribution of genetic factors in phenotypic variation among accessions. Heat tolerance varied widely among both wild and cultivated accessions, but the incidence of tolerance was higher in cultivated carrots than in wild carrots. The cultivated carrot accessions PI 326009 (Uzbekistan), PI 451754 (Netherlands), L2450 (USA), and PI 502654 (Pakistan) were identified as the most heat-tolerant accessions with highest cell membrane stability. This is the first evaluation of cell membrane stability and relative cell injury in response to heat stress during carrot development.**

Carrot (*Daucus carota* L.) is a cross-pollinating, diploid ($2n = 18$), biennial root vegetable belonging to the Apiaceae. It ranks among the top-ten vegetable crops globally and is an important source of prebiotics, minerals, fiber, and especially vitamin A. It is an important cool-season crop that demonstrates optimum growth and sustainable production under low-temperature regimes i.e., 18 to 22 °C (Rubatzky et al., 1999). Heat stress is the most adverse abiotic constraint that significantly affects plant growth, physiology, yield, and productivity for most crops (Bilal et al., 2015; Lobell et al., 2015) including carrot; because carrot is a cool-season crop, sustained production under heat stress may be anticipated to be particularly challenging.

Heat stress response is a complex phenomenon (Chinnusamy et al., 2004) that involves various physiological changes, resulting in damage to the cell membrane. Cell membrane damage due to exposure to heat results from membrane protein denaturation, enzyme inactivation, and, in turn, resulting changes in membrane permeability and integrity causing reduced ion flux, leakage of electrolytes, changes in relative water content, production of toxic compounds, and a general disruption of homeostasis that reduces cell viability. This reduced viability inhibits plant growth and induces leaf wilting, leaf area reduction, and leaf abscission, (Bartels and Sunkar, 2005; Mafakheri et al., 2010). Given the critical role that the cell membrane plays in cellular integrity, measures of membrane stability before and after exposure to heat stress serve to provide quantitative data that have been found to be well-correlated with heat tolerance (Wahid et al., 2007). The occurrence of wide variation in the physiological responses to abiotic stress among and within plant species suggests that the genetic improvement of the stress tolerance response may be effective in breeding programs, and membrane stability response to heat stress has been used to identify genetic sources of thermotolerance for wheat improvement (Almeselmani and Deshmukh, 2012; Ayalew et al., 2015; Lobell et al., 2015). Relative water content, ''stay green'' character, photosynthate reserve mobilization, and total chlorophyll content are all influenced by cell membrane stability and relative cell injury, and these are important physiological characters that have been demonstrated to respond to effective selection and breeding for abiotic stress tolerance response of several crops (Blum et al., 2001; Cossani and Reynolds, 2012; Farooq et al., 2009).

Among various targeted physiological traits that respond to heat stress, membrane stability and relative cell injury are observed to be affected at various growth stages (Seidler-Lozykowska et al., 2010). Cell membrane stability varies with the age of plant tissue, growth stage, growing season, plant species, and intensity of heat stress. Heat stress disturbs the native conformation of membrane proteins that may affect the integrity and function of biological membrane system. Dysfunction of cell membranes in response to heat stress is best estimated by measure of electrolyte leakage from cell membrane in aqueous medium (Ibrahim and Quick, 2001).

Increase in solute leakage is an indication of disruption of cell membrane stability and is an indirect measure of heat-tolerance response in diverse plant species (Blum et al., 2001; Khajuria et al., 2016; Wahid et al., 2007). Plants with high cell membrane stability and relative water content under heat stress at several growth stages ranging from seedling to harvest tend to sustain higher yield (Khakwani et al., 2012), and genetic variation in cell membrane stability in response to abiotic stress has been reported in several cereal crops including wheat, barley, rice, and maize (Khajuria et al., 2016; Kumari et al., 2009; Swapna and Shylaraj, 2017). However, while wide variation for

heat tolerance during seed germination of diverse collections of carrot has been observed (Bolton et al., 2019; Nascimento et al., 2008), no such evaluation for cell membrane stability at any growth stage has been reported for carrot. Therefore, the present study was undertaken 1) to estimate cell membrane stability and relative injury of cells in response to heat stress (35 °C) at early and late seedling developmental stages of diverse carrot germplasm, 2) to evaluate the relationship between early and late stages of seedling tolerance across carrot genotypes, and 3) to identify heat-tolerant genotypes for further genetic and physiological analysis.

Materials and Methods

Germplasm. Diverse carrot germplasm comprised of 166 wild and 49 cultivated accessions provided by the U.S. Department of Agriculture's National Plant Germplasm System collection of Plant Introductions (PIs) was evaluated in the present study. Storage root color varied among cultivated carrots, while root color of all wild carrots is white. Accessions were selected by previous evaluation of heat tolerance during seed germination (Bolton et al., 2019) and geographic diversity. The 215 accessions selected originated from 34 countries in 12 geographic regions (northern Africa, North America, South America, Central Asia, eastern Asia, southern Asia, western Asia, eastern Europe, northern Europe, southern Europe, western Europe, and Oceania).

Controlled exposure to heat stress. Germplasm was evaluated for two consecutive cropping seasons (2016 and 2017) using a randomized complete block design (RCBD) with three replicates for each accession in each of three heat treatments. Plants grown for 30 d were referred to as being in the early seedling stage, or early seedlings (ES); while plants grown for 60 d were referred to as being in the late seedling stage, or late seedlings (LS). Carrot seeds were primed by soaking in water at 4 °C for 24 h in complete darkness before sowing for more uniform and rapid germination. Thirty seeds of each accession were sown in 13-L pots filled with a mixture (80%:20%) of soil and cow manure and watered, as necessary, to avoid wilting. Potted plants were grown outdoors at the Agriculture Research Station in Sargodha, Pakistan, during December to February, where the average daily temperature over 60 d was maintained at 24 °C ± 3 °C both years. Plant populations were thinned to 10 plants per pot at 15 d after planting (DAP) and then to five plants per pot at 30 DAP. To raise and maintain the average temperature of 35 °C ± 3 °C during the stress periods, which was the temperature used to evaluate heat stress during seed germination (Bolton et al., 2019), frames were placed over plants and covered with polyethylene sheeting to create tunnels as described for controlled temperature production of vegetables (Sethi et al., 2009) and selection of heat-tolerant rice (Poli et al., 2013) The targeted temperature was maintained by raising or lowering side panels.

All plants were grown at a mean temperature of 24° ± 3 °C for an initial 15 DAP followed by three heat-stress treatments (Table 1). Control plants were kept at 24 °C ± 3 °C, while the air temperature for ES plants was raised to 35 °C ± 3 °C for up to 30 DAP when leaves were sampled for both treatments. Remaining control plants and LS plants were grown at 24 °C ± 3 °C for up to 30 DAP, at which time control plants were kept at 24 °C ± 3 °C, while the air temperature for LS plants was raised to 35 °C ± 3 °C up to 60 DAP when leaves were sampled for both treatments.

Leaf samples were collected for cell membrane stability estimation twice from the control plants, after 30 and 52 d of planting at both ES and LS developmental stages, respectively. In addition, leaves were collected from heat-stress experiments after 30 d of planting (ES) and 52 d after planting (LS).

Estimation of cell membrane stability. Young leaves were used for estimation of cell membrane stability because they are more sensitive to heat stress than other plant parts (Rehman et al., 2016). Cell membrane stability of heat-treated and nontreated plants of each accession was estimated during ES and LS stages by measuring electrolytic leakage from cellular membranes of leaves following the method of relative conductivity (Ibrahim and Quick, 2001) with some modifications. Briefly, five leaves of ≈3.5 cm length were collected from each of five heat-treated and five nontreated plants of each accession-replicate. Leaf samples were washed with tap water, rinsed with double distilled water, and placed in vials containing 10 ml of deionized water for 18 h at 10 °C. Then the control accession vials were moved to 25 °C; whereas heat-treated accession vials were heated in a water bath at 52 °C for 1 h. All leaf samples of control and heat-stressed plants were again incubated at 10 °C for 24 h for diffusion of electrolytes from leaf tissue to aqueous media, brought to room temperature, shaken by hand, and initial conductance (E_1) was measured. Samples were then autoclaved at 0.10 MPa and 121 °C for 15 min to kill plant tissue and release electrolytes. Samples were cooled to 25 °C, contents shaken, and final conductance (E_2) was measured.

Cell membrane stability and relative cell injury were calculated with the formula reported by Ibrahim and Quick (2001):

$$\text{Cell membrane stability}\,(\%) = 1 - (E_1 / E_2) \times 100$$

$$\text{Relative cell injury}\,(\%) = [1 - (CMS_2) / 1 - (CMS_1) \times 100]$$

While CMS_1 is cell membrane stability for control samples, CMS_2 is for heat-treated samples.

Statistical data analysis. The mixed linear model was used for analysis of variance (ANOVA) for traits of interest on the basis of carrot accessions, geographic origin, and heat treatments

$$Y_{ij} = \mu + R_i + A_j + \varepsilon_{ij}$$

where Y_{ij} = value of the measurements for the j^{th} carrot genotype in the i^{th} replicate; where $i = 1, \ldots, 6$; and $j = 1, \ldots, 215$; μ = total mean (constant); R_i = effect of the i^{th} replicate (random effect) on the response measurement; A_j = effect of the j^{th} accession (fixed effect) on the response measurement, and ε_{ij} = effect of the experimental error associated with the ij^{th} observation. All analyses were performed in R studio version 3.4.4 (R Core Team, 2018) using the "lmer function" in the "lme4 package" (Bates et al., 2018). The mean separation analysis based on geographic origin, domestication status, and root color for traits under study was performed using the "LSD test function" in the "agricolae package" with $\alpha = 0.05$ (De Mendiburu, 2014).

The broad-sense heritability (H^2) was calculated by using the following equation derived from Falconer and Mackay (1996),

$$H^2 = (\sigma^2_G / \sigma^2_P) = \left[(\sigma^2_G / (\sigma^2_G + (\sigma^2_E / r) + (\sigma^2_R / r)) \right]$$

where σ^2_G = genotypic (accessions) variance, σ^2_P = phenotypic variance, σ^2_E = variance due to experimental error, σ^2_R = variance due to replication, and r = the number of replications for each treatment. Variance components were derived using these three formulas: σ^2_G = (MSA – MSE) / r; σ^2_E = MSE; σ^2_R = (MSR – MSE) / n (with MSA = mean square accession, MSE = mean square error, MSR = mean square replication, r = number of replications, and n = number of accessions).

Results and Discussion

Evaluation of the effects of heat treatments on cell membrane stability. Two-way ANOVA revealed highly significant treatment effects ($F = 4481$, $P < 0.0001$) and accession effects ($F = 18.43$, $P < 0.0001$) on cell membrane stability at ES. Similarly, a highly significant effect of heat treatments ($F = 36.32$, $P < 0.0001$) and accessions ($F = 1086$, $P < 0.0001$) on cell membrane stability was noted at LS. Binary interaction of accessions and heat treatments was also significant for cell membrane stability during carrot ES and LS (Table 2). These

Received for publication 8 Apr. 2020. Accepted for publication 26 June 2020.
Published online 6 August 2020.
We thank the Global Crop Diversity Trust Project GS14014 for providing financial support.
We also thank Kathleen Reitsma and the U.S. Department of Agriculture's National Germplasm System for their capable assistance in providing the Plant Introduction carrot collection accessions.
P.S. is the corresponding author. E-mail: psimon@wisc.edu.

results indicate that cell membrane stability is sensitive to heat stress at ES and LS, as was reported during vegetative growth in rice (Kumar et al., 2016).

Cell membrane stability at the ES stage. Average cell membrane stability ranged from 26.4% to 94.5%, with a mean of 67.3% and a standard deviation of 13.1 for 215 carrot accessions at ES under the nonstress condition. When these plants were subjected to heat stress for 15 d, cell membrane stability was reduced to 41.2%, ranging from 8.4% to 91.8%, with a standard deviation of 12.8 for all accessions under study. Relative cell injury in response to heat stress of carrots ranged from 2.8% to 76.6%, with a mean of 39.1% and a standard deviation of 11.4%. These evaluations indicated that cell membrane stability was significantly reduced by high temperature in the majority of accessions, and that result ultimately led to cell injury (Table 3); but heat tolerance was observed in several accessions. Significant variation for cell membrane stability (F = 11.55, P < 0.0001) was observed among carrot accessions under nonstress conditions at ES (Table 4). PI 326009 (Uzbekistan_C, 94.5%), PI 652235 (Bulgaria_W, 92.8%), and PI 306810 (New Zealand_C, 92.4%) had the highest cell membrane stability percentages; whereas PI 652120 (USA_W, 26.4%), PI 652306 (Greece_W, 33.2%), and Ames 30259 (Tunisia_W, 35.4%) exhibited the lowest cell membrane stability percent values at ES under nonstress conditions (where _C and _W indicate cultivated and wild carrots, respectively) (Supplemental Table 1). Cell membrane stability also significantly varied (F = 9.90, P < 0.0001) among carrot accessions during the ES stage under heat-stress conditions (Table 3). PI 326009 (Uzbekistan_C, 91.8%), PI 451754 (Netherlands_C, 86.7%), PI 390887 (Israel_W, 80.8%), L2450 (USA_C, 79.7%), and PI 502654 (Pakistan_C, 74.7%) had high values of cell membrane stability, indicating a high level of heat-tolerance response during ES. On the other hand, PI 652120 (USA_W, 8.4%), PI 652306 (Greece_W, 11.4%), and PI 478873_W (Italy, 15.0%) were found to be highly heat sensitive during ES, with the lowest cell membrane stability percentages among all accessions (Supplemental Table 1). Relative cell injury in response to heat stress during ES significantly varied (F = 3.43, P < 0.0001) among carrot accessions (Table 4). PI 326009 (Uzbekistan_C, 2.8%), PI 451754 (Netherlands_C, 5.5%), L2450 (USA_C, 6.0%), and PI 502654 (Pakistan_C, 9.67) had the lowest values for relative cell injury in response to heat stress; while PI 451754 (Netherlands_C, 76.6%), PI 652120 (USA_W, 68.3%), and PI 652306 (Greece_W, 66.0%) had the highest relative cell injury percentages at ES (Supplemental Table 1). Previous studies reported that germplasm accessions with higher cell membrane stability were heat tolerant in chrysanthemum (Yeh and Lin, 2003), tomato (Din et al., 2015), and wheat (ElBasyoni et al., 2017); and stability of cell membranes under heat stress was linked to proline content (Din et al., 2015), heat-induced chaperon production, and ratio of saturated to unsaturated phospholipids (Torok et al., 2001). In response to heat stress, the level of membrane stabilizers, i.e., osmolytes such as proline, heat-shock proteins, and saturated lipids, increased in heat-tolerant accessions to maintain the membrane permeability and integrity (Niu and Xiang, 2018). It would be interesting to determine the relationship between cell membrane stability and membrane stabilizing factors such as osmolyte concentration (proline) and saturated structural lipids (phospholipids and glycolipids) to better understand the heat-tolerance mechanism in carrot germplasm.

In this study, the most heat-tolerant accessions were cultivated PIs from diverse geographic origin (PI 326009; Uzbekistan_C, PI 451754; Netherlands_C, L2450; USA_C and PI 502654; Pakistan_C); whereas heat-sensitive accessions were mainly wild PIs from diverse origins (PI 652120; USA_W, PI 652306; Greece_W, PI 478873_W; Italy and Ames 29087; Tunisia_W), although exceptions were not uncommon. The identification of heat-tolerant germplasm accessions and breeding lines based on membrane stability and relative cell injury in this study provides valuable direction to inform carrot breeders improving carrot thermotolerance. Interestingly, similar trends of abiotic stress tolerance were reported in carrot during seed germination under salinity (Bolton and Simon, 2019) and heat stress (Bolton et al., 2019). Previous studies reported that wild relatives were the main source of abiotic stress tolerance in several crops including

Table 1. Temperatures (±3 °C) and time periods for treatments used to evaluate heat stress in carrots.

	Time period		
Treatment	Days 1–15	Days 16–30	Days 31–60
No stress	24 °C	24 °C	24 °C
Early seedling stress	24 °C	35 °C	24 °C
Late seedling stress	24 °C	24 °C	35 °C

Table 2. Two factorial ANOVA (accessions and treatments) for cell membrane stability during ES (ES_CMS) and cell membrane stability during LS (LS_CMS) for 215 carrot accessions.

Measurements	Source	df	Sum of square	Mean square	F ratio	Prob. > F
Cell membrane stability during ES (ES_CMS)	Accessions	214	387,868	1,812	18.43	<2.2E-16***
	Treatment	1	440,543	440,543	4,481	<2.2E-16***
	Accessions × Treatment	214	43,068	201	2.04	<2.5E-15***
	Error	2,150	211,349	98		
Cell membrane stability during LS (LS_CMS)	Accessions	214	315,269	1,473	10.86	<2.2E-16***
	Treatment	1	492,641	492,641	3,632	<2.2E-16***
	Accessions × Treatment	214	77,195	361	2.65	<2.2E-16***
	Error	2,150	291,619	136		

df = degree of freedom; ES = early seedling; LS = late seedling.
***Significant at P < 0.001.

Table 3. Descriptive statistics for cell membrane stability without heat stress (ES_CMS, NS), cell membrane stability with heat stress (ES_CMS, HS), relative cell injury (ES_RCI), cell membrane stability without heat stress (LS_CMS, NS), cell membrane stability with heat stress (LS_CMS, HS), and relative cell injury (LS_RCI) at early and late seedling stages of diverse carrot germplasm.

	Avg			Standard deviation			Range		
Measurements	Overall	Cultivated	Wild	Overall	Cultivated	Wild	Overall	Cultivated	Wild
Cell membrane stability without stress at ES (ES_CMS, NS)	67.3	75.10	65.1	13.1	7.60	13.5	26.3–94.5	60.4–94.5	26.3–92.9
Cell membrane stability with heat stress at ES (ES_CMS, HS)	41.2	46.90	39.5	12.8	14.50	11.8	8.3–91.8	16.4–91.9	8.3–80.8
Relative cell injury at ES (ES_RCI)	39.1	36.90	39.7	11.4	14.80	10.2	2.8–76.6	2.8–76.6	10.1–68.3
Cell membrane stability without stress at LS (LS_CMS, NS)	67.2	69.00	66.7	12.3	13.30	12.1	15.4–94.5	15.5–92.2	23.8–94.5
Cell membrane stability with heat stress at LS (LS_CMS, HS)	39.6	47.20	37.4	12.2	14.20	10.5	8.3–70.1	8.3–70.0	9.1–68.7
Relative cell injury at LS (LS_RCI)	40.5	31.20	43.3	13.2	15.90	11.0	2.5–78.6	2.5–65.7	7.13–78.6

Table 4. ANOVA among 215 carrot accessions for cell membrane stability without heat stress (ES_CMS, NS), cell membrane stability with heat stress (ES_CMS, HS), relative cell injury (ES_RCI), cell membrane stability without heat stress (LS_CMS, NS), cell membrane stability with heat stress (LS_CMS, HS), and relative cell injury (LS_RCI) at early and late seedling stages of diverse carrot germplasm accessions.

Measurements	Source	df	Sum of square	Mean square	*F* ratio	Prob. > *F*
Cell membrane stability without stress at ES (ES_CMS, NS)	Accessions	214	221,986	1,037.32	11.55	<2.2E-16***
	Rep	5	653	130.61	1.45	0.2018
	Error	1,070	96,021	89.74		
Cell membrane stability with heat stress at ES (ES_CMS, HS)	Accessions	214	208,967	976.48	9.90	<2.2E-16***
	Rep	5	9,207	1,841.45	18.6	<2.2E-16***
	Error	1,070	105,468	98.75		
Relative cell injury during ES (ES_RCI)	Accessions	214	158,236	739.4	3.43	<2.2E-16***
	Rep	5	19,226	3,845.2	17.6	<2.2E-16***
	Error	1,070	230,345	215.3		
Cell membrane stability without stress at LS (LS_CMS, NS)	Accessions	214	203,394	950.44	7.60	<2.2E-16***
	Rep	5	2,574	514.78	4.12	0.001**
	Error	1,070	133,664	124.92		
Cell membrane stability with heat stress at LS (LS_CMS, HS)	Accessions	214	189,070	883.5	7.60	<2.2E-16***
	Rep	5	31,095	6,219.0	53.5	<2.2E-16***
	Error	1,070	124,268	116.2		
Relative cell injury during LS (LS_RCI)	Accessions	214	226,182	1,056.9	4.02	<2.2E-16***
	Rep	5	62,137	12,727.4	47.3	0.134
	Error	1,070	281,067	262.7		

df = degree of freedom; ES = early seedling; LS = late seedling.
**, ***Significant at $P < 0.01$ or 0.001.

tomato, rice, and barley (Hajjar and Hodgkins, 2007). In contrast, this study along with results reported by Bolton et al., (2019) demonstrated that a wider range of variation was observed in cultivated carrot germplasm than had been noted in cultivated germplasm of most other crops.

Cell membrane stability at the LS stage. Average cell membrane stability ranged from 15.4% to 94.5% with a mean of 67.2% and a standard deviation of 12.3 for 215 carrot accessions during LS under the nonstress condition. When plants were exposed to heat stress for 30 d, cell membrane stability was reduced to 39.6%, ranging from 15.7% to 70.1% with standard deviation of 12.2 for all accessions under study. Relative cell injury in response to heat stress in carrot accessions ranged from 2.5% to 78.6%, with a mean of 40.5% and a standard deviation of 13.2 (Table 3). This evaluation indicated that cell membrane stability was more highly influenced by high temperature in the majority of accessions at LS than at ES.

Significant variation for cell membrane stability ($F = 7.60$, $P < 0.0001$) was recorded among carrot accessions under nonstress conditions during LS (Table 4). PI 478875 (Italy_W, 94.5%) PI 652242 (India_C, 92.2%), and PI 478369 (China_W, 90.9%) had the highest cell membrane stability; whereas AR0377 (USA_C, 15.4%), Ames 17826 (Estonia_W, 23.7%), and PI 652398 (Turkey_W, 25.4%) exhibited the lowest values for cell membrane stability during LS under nonstress conditions (Supplemental Table 1). Cell membrane stability also varied significantly ($F = 7.60$, $P < 0.0001$) among carrot accessions during LS under heat-stress conditions (Table 3). PI 326009 (Uzbekistan_C, 70.1%), PI 451754 (Netherlands_C, 69.6%), and PI 652242 (India_C, 69.5%) had high values of cell membrane stability, indicating a high level of heat tolerance during LS. On the other hand, PI 652220 (Poland_W, 15.7%), PI 652290 (Poland_W, 19.7%), and Ames 25771 (Syria_W, 25.9%) were found to be highly heat sensitive during LS, with the lowest membrane stability values among all accessions (Supplemental Table 1). These results demonstrate that variation exists in carrot accessions during multiple stages of growth under both control conditions and heat stress where germplasm accessions representing the highest and lowest levels of heat tolerance overlapped to some extent, but they differed between ES and LS stages. Furthermore, the germplasm accessions representing extremes in heat tolerance at germination (Bolton et al., 2019) differ at the early (ES) and late (LS) stages of growth. Consequently, this indicates that evaluation of carrot germplasm for membrane thermo-stability at a single stage of growth is not adequate to broadly predict heat tolerance throughout the life cycle of carrot. Relative cell injury in response to heat stress is a more effective criterion to evaluate the heat-tolerance level of a carrot accession than cell membrane stability, because it characterizes the percentage decrease in performance under abiotic stress relative to that under nonstress conditions. Significant variation for relative cell injury ($F = 4.02$, $P < 0.0001$) was recorded among carrot accessions during LS (Table 4). PI 326009 (Uzbekistan_C, 2.5%), PI 451754 (Netherlands_C, 3.1%), L2450 (USA_C, 6.0%), and PI 502654 (Pakistan_C, 7.1%) had lowest values for relative cell injury in response to heat stress; while PI 652220 (Poland_W, 78.6%), PI 652290 (Poland_W, 70.9%), and Ames 25771 (Syria_W, 69.1%) had the highest relative cell injury at ES (Supplemental Table 1). Like carrot seed germination under heat stress (Bolton et al., 2109), this evaluation also indicated that heat-tolerant accessions were more often observed to be cultivated PIs, whereas heat-sensitive accessions were more often observed in wild PIs during both ES and LS. Interestingly, cultivated accessions PI 326009 (Uzbekistan_C), PI 451754 (Netherlands_C), L2450 (USA_C), and PI 502654 (Pakistan_C) were found to be among the most heat-tolerant accessions during both ES and LS. Their heat tolerance at germination stage was 0.12, 0.71, and 0.34, respectively.

Relative cell injury in response to heat stress at the ES and LS stages according to geographic origin. Significant differences were observed for cell membrane stability and relative cell injury under control and heat-stress conditions during both ES and LS among 12 different geographic origins of carrot accessions ($P < 0.0001$) (Table 5). When comparing accessions from diverse origins, accessions from New Zealand and North Africa were heat sensitive at ES with a relative cell injury of 57.6% and 45.1%, respectively. Beyond these two geographic regions, the level of heat tolerance was statistically similar in accessions from northern Europe, Central Asia, North America, eastern Europe, western Europe, south Asia, and western Asia at the ES stage (Table 6). Significant variation among accessions from diverse origins was observed when compared for relative cell injury in response to heat-stress exposure during LS. Accessions from eastern Asian and northern European origin were heat tolerant at LS; whereas accessions from North America, South America, south Europe, eastern Europe, western Europe, and south Asia were moderately heat tolerant (Table 5). Significant variation in heat tolerance during seed germination of accessions from diverse origin was also reported (Bolton et al., 2019); and like this study, accessions from central and eastern Asia were generally heat tolerant, while accessions from northern Africa and southern Europe were heat sensitive, perhaps reflecting differences in founding carrots between these two geographic regions.

Cell membrane stability and relative cell injury in response to heat stress at the ES and LS stages as a function of domestication status and storage root color. Significant differences were observed for mean cell membrane stability under nonstress conditions

Table 5. ANOVA based on geographic origin among 215 carrot accessions for cell membrane stability without heat stress (ES_CMS, NS), cell membrane stability with heat stress (ES_CMS, HS), relative cell injury (ES_RCI), cell membrane stability without heat stress (LS_CMS, NS), cell membrane stability with heat stress (LS_CMS, HS), and relative cell injury (LS_RCI) at early and late seedling stages of diverse carrot germplasm.

Measurements	Source	df	Sum of square	Mean square	*F* ratio	Prob. > *F*
Cell membrane stability without	Region	11	19,888	1,807.98	7.72	<4.8E-13***
stress at ES (ES_CMS, NS)	Rep	5	653	130.61	0.55	0.7325
	Error	1,273	298,119	234.19		
Cell membrane stability with heat stress at ES (ES_CMS, HS)	Region	11	14,457	1,314.26	5.57	<8.7E-09***
	Rep	5	9,207	1,841.45	7.81	<2.9E-07***
	Error	1,273	299,979	235.65		
Relative cell injury during ES (ES_RCI)	Region	11	10,153	923.0	3.10	<0.0003***
	Rep	5	19,226	3,845.2	12.9	<2.6E-09***
	Error	1,273	378,427	297.3		
Cell membrane stability without	Region	11	8,266	751.47	2.90	<0.008***
stress at LS (LS_CMS, NS)	Rep	5	2,574	514.78	1.99	0.07
	Error	1,273	328,791	258.28		
Cell membrane stability with	Region	11	9,721	883.7	3.70	<3.23E-05***
heat stress at LS (LS_CMS, HS)	Rep	5	31,095	6,219.0	26.0	<2.2E-16***
	Error	1,273	303,635	238.5		
Relative cell injury during LS	Region	11	12,795	1,163.2	2.99	<0.0006***
(LS_RCI)	Rep	5	62,137	12,427.4	31.9	<2.2E-16***
	Error	1,273	494,454	388.4		

df = degree of freedom; ES = early seedling; LS = late seedling.
***Significant at $P < 0.001$.

Table 6. Mean separation for relative cell injury during early and late seedling stages of 215 accessions from 12 geographic origin.

Region of origin	No. of genotypes	Relative cell injury during ES	Relative cell injury during LS
Northern Europe	7	35.99 C[z]	33.87 B
Central Asia	6	33.59 C	35.69 AB
Oceania	1	57.63 A	22.08 B
North America	23	36.43 C	37.14 AB
Eastern Asia	2	42.23 ABC	25.24 B
South America	1	40.71 BC	42.54 AB
Eastern Europe	30	37.27 C	40.34 AB
South Asia	16	38.04 C	37.93 AB
Western Europe	39	38.62 C	42.28 AB
Southern Europe	52	42.08 BC	41.97 AB
Western Asia	29	39.32 C	43.08 A
North Africa	9	45.14 AB	43.55 A

[z]Means with the same letter are not significantly different using Fisher's least significant difference test at $\alpha = 0.05$.

across cultivated PIs (75.06%) and wild PIs (65.09%) at ES. Under heat stress, cultivated accessions demonstrated a relatively higher heat tolerance than the wild accessions. At ES, the mean cell membrane stability and mean relative cell injury for cultivated accessions were 46.9% and 39.5%, respectively, in response to heat stress (Table 7). It is important to note that a similar trend of heat tolerance was observed at LS in cultivated and wild PIs. Cultivated accessions demonstrated higher cell membrane stability under nonstress (69.0%) and heat-stress (47.2%) conditions, with lower relative cell injury (31.2%) than wild accessions (Table 6). Similarly, open-pollinated cultivated accessions usually demonstrated more heat tolerance during seed germination stage than the inbred lines and wild accessions (Bolton et al., 2019). There were, however, some exceptions to the general trends based on domestication status. For example, the cultivated accessions PI 451753 (Netherlands_C), PI 261650 (Netherlands_C), and PI 256066 (Afghanistan_C) had more than 60% relative cell injury, ranging from 61.8% to 76.6%; while wild accessions PI 297762 (Denmark_W), PI 390887 (Israel_W), PI 478866 (Germany_W), and PI 478867 (Germany_W) had less than 15% relative cell injury, ranging from 10.1% to 13.5% at ES. There were also two cultivated PIs with high levels of relative cell injury in response to heat stress at LS, namely Ncw4459 (USA_C) and PI 271384 (India_C), which had more than 65% relative cell injury. In contrast, two wild accessions, PI 390887 (Israel_W) and PI 652193 (Poland_W), both had less than 15.0% cell injury under heat-stress conditions.

The relative cell injury did not vary significantly among cultivated accessions, based on their root color, with average values ranging from 35% to 42% at ES. On the other hand, relative cell injury did vary significantly among accessions based on root color, with values ranging from 26% to 55% at the LS stage of development. Yellow-rooted carrots were significantly more heat tolerant than the other colors, with relative cell injury of 26.0% (Table 7). In seed germination evaluation under heat stress, yellow-rooted carrots were also more heat tolerant than other colors (Bolton et al., 2019). It is important to note that the number of samples for each color category were not equal and were not equally distributed across geographic origins; thus the trends reported here should be confirmed with larger sample sizes.

Broad-sense heritability. Broad-sense heritability (H^2) ranged from 0.84 to 0.91 for cell membrane stability at ES and LS under nonstress and heat-stress conditions. These values suggest a significant genetic basis for these traits in carrot. Heritability values for relative cell injury at ES (H^2 = 0.69) were like LS (H^2 = 0.71). Similar values of heritability, ranging from 0.64 to 0.88, were reported for seed germination percent and other measurements in heat tolerance evaluation during seed germination (Bolton et al., 2019). Low heritability values for relative cell injury at both ES and LS, compared with cell membrane stability under nonstress and heat-stress conditions, suggests that genetic factors have less effect on these traits of interest than do environmental factors. As there is no previous information available for abiotic stress tolerance in carrot except seed germination under salt (Bolton and Simon, 2019) and heat stress (Bolton et al., 2019; Nascimento et al., 2008), there is a need to investigate the genetic basis of stress tolerance to identify significant genomic regions and candidate genes responsible for these traits of increasing interest.

Correlation among cell membrane stability and relative cell injury in response to heat stress at the ES and LS development stages. Pearson rank correlation coefficients calculated for each trait of interest under study revealed a very strong positive correlation between cell membrane stability under nonstress and heat stress conditions at ES (r = 0.80), but correlations for the same traits were lower at LS (r = 0.61). Relative cell injury had a negative high correlation with cell membrane stability under nonstress at ES (r = –0.38) and at LS (r = –0.16). On the other hand, a negative, but strong, correlation was observed between relative cell injury and cell

Table 7. Mean separation based on domestication status and root color for cell membrane stability (CMS) and relative cell injury (RCI) at ES and LS.

Factor	Category	No. of genotypes	CMS-ES (nonstress)	CMS-ES (heat stress)	RI ES	CMS-LS (nonstress)	CMS-LS (heat stress)	RI LS
Domestication status	Cultivated	49	75.06 A[z]	46.93 A	37.33 B	69.00 A	47.15 A	31.23 B
	Wild	166	65.09 B	35.54 B	39.84 A	66.71 B	37.37 B	43.25 A
Primary root color	Orange	30	75.66 A	47.91 A	36.46 AB	65.05 C	44.59 B	31.35 C
	Purple	9	74.04 A	47.51 A	35.67 AB	75.08 AB	53.18 A	28.00 C
	Red	3	78.10 A	44.76 AB	42.42 A	66.87 BC	28.18 C	55.56 A
	Yellow	7	73.89 A	43.69 AB	41.04 A	77.33 A	56.32 A	26.04 C
	White	166	65.11 B	39.54 B	39.85 A	66.78 C	37.40 B	43.26 B

[z]Means with the same letter are not significantly different using Fisher's least significant difference test at $\alpha = 0.05$.

Table 8. Correlations among cell membrane stability (CMS) and relative cell injury (RCI) in response to heat stress at ES and LS; and between seed germination (SG) with (HS) and without (NS) heat stress, and heat tolerance index (Bolton et al., 2019).

Trait	ES_CMS (NS)	ES_CMS (HS)	LS_CMS (NS)	LS_CMS (HS)	ES_RCI	LS_RCI	SG (NS)	SG (HS)
Cell membrane stability without stress during ES [ES_CMS (NS)]	1							
Cell membrane stability with heat stress during ES [ES_CMS (HS)]	–0.80**	1						
Cell membrane stability without stress during LS [LS_CMS (NS)]	0.02	0.08	1					
Cell membrane stability with heat stress during LS [LS_CMS (HS)]	0.20**	0.30**	0.61**	1				
Relative cell injury during ES (ES_RCI)	–0.38**	–0.84**	–0.16**	–0.24**	1			
Relative cell injury during LS (LS_RCI)	–0.29**	–0.32**	–0.04	–0.80**	0.18**	1		
Seed germination % without stress [SG (NS)]	–0.02	0.12	–0.01	0.02	–0.19	0.01	1	
Seed germination % with heat stress [SG (HS)]	0.22	0.23	0.22	0.01	–0.17	–0.04	0.44	1
Heat tolerance index for seed germination	0.20	0.23	0.21	0.03	–0.18	–0.07	0.61	0.97

**Significant at $P < 0.01$.

membrane stability under heat-stress condition ($r = –0.80$) (Table 8). In heat-tolerance evaluation of carrot germplasm during seed germination, traits of interest were not strongly correlated to each other under nonstress; whereas correlation was significant between traits under heat stress, especially with heat-tolerance index (Bolton et al., 2019). Pearson correlation was determined between seed germination percentage and heat tolerant index with cell membrane stability and relative cell injury in response to heat stress during ES and LS of 56 PIs evaluated in recent study by Bolton et al. (2019) and the present study. This analysis indicated a very weak correlation between all traits, ranging from –0.01 to 0.22, suggesting that accessions that were heat tolerant during seed germination may not be tolerant to heat stress during subsequent developmental stages of seedling. Therefore, it is important to evaluate the heat-tolerance response of accessions from seed germination to maturity.

Conclusions

This study identified a wide range of variation in the heat-tolerance response of diverse wild and cultivated carrot germplasm in terms of cell membrane stability and relative cell injury at early and late seedling stages. Heat-tolerance levels observed at ES were not consistent with levels at LS, indicating independent genetic control for carrot heat tolerance at different stages of plant development, as has been observed for abiotic stress tolerance in other crops (e.g., Ashraf et al., 1994; Khajuria et al., 2016; Kumar et al., 2016; Shivhare and Lata, 2019; Ulloa et al., 2010; Wahid et al., 2007). The cultivated accession PI 326009 (Uzbekistan_C), PI 451754 (Netherlands_C), L2450 (USA_C), and PI 502654 (Pakistan_C) were found to be among the most heat-tolerant accessions, while the wild accessions were mainly heat sensitive during both ES and LS. Relative cell injury in response to heat stress was confirmed to be an important measure of heat tolerance to be used to identify, select, and screen for genetic variation in heat tolerance among and within carrot germplasm accessions and breeding stocks. This in turn suggests that, as in other crops, strategies of carrot genetic improvement for warmer growing conditions will need to evaluate and select breeding stocks that are most thermotolerant at germination, if carrots are planted in the hottest season of the year; most tolerant at ES, if carrots are at early stages of development in the hottest season of the year; and most tolerant at LS, if carrots are at late stages of development in the hottest season of the year.

The observations of this study also suggest that carrot growers in regions experiencing seasonal heat stress should evaluate and characterize seasonal variation in thermotolerance among their locally used cultivars to schedule their production cycle, to the extent possible, to match cultivar-specific variation in developmental tolerance with seasonal variation in growing temperature, to avoid exposure of the cultivars they grow to the period of seasonal heat stress. Our observations of greater negative impact of high temperatures on membrane stability on more germplasm accessions late in the growing season than early in the season point to a need to place a greater focus on identifying cultivars and breeding stocks with late season heat tolerance.

This study also provides basic information about heat tolerance in carrot crop at early and late seedling growth stages. It will be interesting to determine the relationship between variation in membrane stability and relative cell injury observed in this study, and membrane stabilizing factors such as concentrations of proline and phospholipids, carrot yield, and quality in future studies.

Literature Cited

Almeselmani, M. and P.S. Deshmukh. 2012. Effect of high temperature stress on physiological and yield parameters of some wheat genotypes recommended for irrigated and rain fed condition. Jordan J. Agr. Sci. 8(1): 66–78.

Ashraf, M., M.M. Saeed, and M.J. Qureshi. 1994. Tolerance to high temperature in cotton (*Gossypium hirsutum* L.) at initial growth stages. Environ. Exp. Bot. 34(3):275–283.

Ayalew, H., X. Ma, and G. Yan. 2015. Screening wheat (*Triticum* spp.) genotypes for root length under contrasting water regimes: Potential sources of variability for drought resistance breeding. J. Agron. Crop Sci. 201:189–194.

Bartels, D. and R. Sunkar. 2005. Drought and salt tolerance in plants. CRC Crit. Rev. Plant Sci. 24(1):23–58.

Bates, D., M. Maechler, B. Bolker, S. Walker, R.H.B. Christensen, H. Singmann, B. Dai, F. Scheipl, G. Grothendieck, and P. Green. 2018. Linear mixed-effects models using 'Eigen' and S4. R. package, version 17, p. 42–44. <https://arxiv.org/abs/1406.5823>.

Bilal, M., R.M. Rashid, S.U. Rehman, F. Iqbal, J. Ahmed, M.A. Abid, Z. Ahmed, and A. Hayat. 2015. Evaluation of wheat genotypes for drought tolerance. J. Green Physiol. Genet. Genom. 1:11–21.

Blum, A., N. Klueva, and H.T. Nguyen. 2001. Wheat cellular thermotolerance is related to yield under heat stress. Euphytica 117:117–123.

Bolton, A.L. and P.W. Simon. 2019. Variation for salinity tolerance during seed germination in diverse carrot [*Daucus carota* (L.)] germplasm. HortScience 54:38–44.

Bolton, A.L., A. Nijabat, M.M. Rehman, N.H. Naveed, A.T.M.M. Mannan, A. Ali, M.A. Rahim, and P.W. Simon. 2019. Variation for heat tolerance during seed germination in diverse carrot [*Daucus carota* (L.)] germplasm. HortScience 54:1470–1476.

Chinnusamy, V., K. Schumaker, and J. Zhu. 2004. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J. Expt. Bot. 55:225–236.

Cossani, C.M. and M.P. Reynolds. 2012. Physiological traits for improving heat tolerance in wheat. Plant Physiol. 160:1710–1718.

De Mendiburu, F. 2014. Agricolae: Statistical procedures for agricultural research. R package version 1, p. 1–6.

Din, J.U., S.U. Khan, A. Khan, A. Qayyum, K.S. Abbasi, and M.A. Jenks. 2015. Evaluation of potential morpho-physiological and biochemical indicators in selecting heat-tolerant tomato (*Solanum lycopersicum* Mill.) genotypes. Hort. Environ. Biote. 56(6):769–776.

ElBasyoni, I., M. Saadalla, S. Baenziger, H. Bockelman, and S. Morsy. 2017. Cell membrane stability and association mapping for drought and heat tolerance in a worldwide wheat collection. Sustainability 9(9):1606–1621.

Falconer, D.S. and T.F.C. Mackay. 1996. Introduction to quantitative genetics. 4th ed. Pearson, Essex, England.

Farooq, M., A. Wahid, O. Ito, D.J. Lee, and K.H.M. Siddique. 2009. Advances in drought resistance of rice. CRC Crit. Rev. Plant Sci. 28:199–217.

Hajjar, R. and T. Hodgkins. 2007. The use of wild relatives in crop improvement: A survey of developments over the last 20 years. Euphytica 156:1–13.

Ibrahim, A.M. and J.S. Quick. 2001. Heritability of heat tolerance in winter and spring wheat. Crop Sci. 41(5):1401–1405.

Khajuria, P., A.K. Singh, and R. Singh. 2016. Identification of heat stress tolerant genotypes in bread wheat. Elec. J. Plant Breeding 7(1):124–131.

Khakwani, A.A., M.D. Dennett, M. Munir, and M.S. Baloch. 2012. Wheat yield response to physiological limitations under water stress condition. J. Anim. Plant Sci. 22:773–780.

Kumar, N., S.C. Shankhdhar, and D. Shankhdhar. 2016. Impact of elevated temperature on antioxidant activity and membrane stability in different genotypes of rice (*Oryza sativa* L.). Indian J. Plant. Physiol. 21(1):37–43.

Kumari, S., V.P. Sabharwal, H.R. Kushwaha, S.K. Sopory, S.L. Singla-Pareek, and A. Pareek. 2009. Transcriptome map for seedling stage specific salinity stress response indicates a specific set of genes as candidate for saline tolerance in *Oryza sativa* L. Funct. Integr. Genomics 9:109–123.

Lobell, D.B., G.L. Hammer, K. Chenu, B. Zheng, G. McLean, and S.C. Chapman. 2015. The shifting influence of drought and heat stress for crops in northeast Australia. Glob. Change Biol. 21:4115–4127.

Mafakheri, A., A. Siosemardeh, B. Bahramnejad, P.C. Struik, and Y. Sohrabi. 2010. Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. Aust. J. Crop Sci. 4:580–585.

Nascimento, W.M., J.V. Vieira, G.O. Silva, K.R. Reitsma, and D.J. Cantliffe. 2008. Carrot seed germination at high temperature: Effect of genotype and association with ethylene production. HortScience 43:1538–1543.

Niu, Y. and Y. Xiang. 2018. An overview of biomembrane functions in plant responses to high-temperature stress. Front. Plant Sci. 9:915–932.

Poli, Y., R.K. Basava, M. Panigrahy, V.P. Vinukonda, N.R. Dokula, S.R. Voleti, S. Desiraju, and S. Neelamraju. 2013. Characterization of a Nagina22 rice mutant for heat tolerance and mapping of yield traits. Rice 6(1):36–45.

R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rehman, S.U., M. Bilal, R.M. Rana, M.N. Tahir, M.K.N. Shah, H. Ayalew, and G. Yan. 2016. Cell membrane stability and chlorophyll content variation in wheat (*Triticum aestivum*) genotypes under conditions of heat and drought. Crop Pasture Sci. 67:712–718.

Rubatzky, V.E., C.F. Quiros, and P.W. Simon. 1999. Carrots and related vegetable Umbelliferae. CABI Publishing, New York.

Seidler-Lozykowska, K., H. Bandurska, and J. Bocianowski. 2010. Evaluation of cell membrane injury in caraway (*Carum carvi* L.) genotypes in water deficit conditions. Acta Soc. Bot. Pol. 79:95–99.

Sethi, V.P., R.K. Dubey, and A.S. Dhath. 2009. Design and evaluation of modified screen net house for off-season vegetable raising in composite climate. Energy Convers. Manage. 50(12): 3112–3128.

Shivhare, R. and C. Lata. 2019. Assessment of pearl millet genotypes for drought stress tolerance at early and late seedling stages. Acta Physiologiae Plantarum 41:1.

Swapna, S. and K.S. Shylaraj. 2017. Screening for osmotic stress responses in rice varieties under drought condition. Rice Sci. 24(5): 253–263.

Torok, Z., P. Goloubinoff, I. Horvath, N.M. Tsvetkova, A. Glatz, G. Balogh, V. Varvasovszki, D.A. Los, E. Vierling, J.H. Crowe, and L. Vígh. 2001. Synechocystis HSP17 is an amphitropic protein that stabilizes heat-stressed membranes and binds denatured proteins for subsequent chaperone-mediated refolding. Proc. Natl. Acad. Sci. 98(6):3098–3103.

Ulloa, S.M., A. Datta, and S.Z. Knezevic. 2010. Tolerance of selected weed species to broadcast flaming at different growth stages. Crop Prot. 29(12):1381–1388.

Wahid, A., S. Gelani, M. Asharf, and M.R. Foolad. 2007. Heat tolerance in plants: An overview. Environ. Exp. Bot. 61:199–223.

Yeh, D.M. and H.F. Lin. 2003. Thermostability of cell membranes as a measure of heat tolerance and relationship to flowering delay in chrysanthemum. J. Amer. Soc. Hort. Sci. 128(5):656–660.