Heat Stress Related Physiological and Metabolic Traits in Peanut Seedlings

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ABSTRACT

To maintain high yields under an increasingly hotter climate, high temperature resilient peanut cultivars would have to be developed. Therefore, the mechanisms of plant response to heat need to be understood. The objective of this study was to explore the physiological and metabolic mechanisms developed by virginia-type peanut at early growth stages in response to high temperature stress. Peanut seedlings were exposed to 40/35 C (heat) and 30/25 C (optimum temperature) in a growth chamber. Membrane injury (MI), the F_v/F_m ratio, and several metabolites were evaluated in eight genotypes at four time-points (day 1, 2, 4, and 7) after the heat stress treatment initiation. Even though we were able to highlight some metabolites, *e.g.*, hydroxyproline, galactinol, and unsaturated fatty acid, explaining specific differential physiological (MI) responses in peanut seedlings, overall our data suggested general stress responses rather than adaptive mechanisms to heat. Rather than individual metabolites, a combination of several metabolites better explained (41 to 61%) the MI variation in heat stressed peanut seedlings. The genotype SPT 06-07 exhibited lower MI, increased galactinol, reduced hydroxyproline, and higher saturated *vs*. unsaturated fatty acid ratio under heat stress compared to other genotypes. SPT 06-07 was also separated from the other genotypes during hierarchical clustering and, based on this and previous fieldwork, SPT 06-07 is proposed as a potential source for heat tolerance improvement of virginia-type peanut.

Key Words: Heat stress, peanut seedlings, membrane injury, chlorophyll fluorescence, metabolites.

The Virginia-Carolina (VC) region is the most important peanut production region for the large seeded, virginia-type peanut (*Arachis hypogaea* L.) in the United States. Although historically the region has had adequate climatic conditions for high peanut yields, recent occurrence of high temperatures has been frequently observed in particular during early growth stages of peanut. For example, frequent temperatures above 30 C have been reported during May and June in the VC region (NCDC, 2012) and predictions are for an additional 2.4 to 6.4 C increase by the end of 21st century (Meehl *et al.*, 2007). Optimum temperature for peanut growth is between 25 and 30 C but pod yields can be substantially reduced if temperatures exceed 33 C (Cox, 1979; Prasad *et al.*, 2003). During early growth stages, soil temperatures above 32 C reduced the early vegetative growth of peanut, with negative effects on pod yield and total plant biomass at maturity (Golombek and Johansen, 1997).

Under elevated abiotic stress, compatible solutes proline, glycine betaine, and mannitol and antioxidants ascorbate, and glutathione are produced to protect the cellular and thylakoid membranes (Alscher *et al.*, 2002; Apel and Hirt, 2004; Asada, 2006; Dat *et al.*, 2000; Foyer and Noctor, 2005; Kovtun *et al.*, 2000; McKersie *et al.*, 1990; Mittler *et al.*, 2004; Noctor and Foyer, 1998; Pei *et al.*, 2000; Xu *et al.*, 2006). Specifically, heat stress lead to increased pinitol levels in soybean (Guo and Oosterhuis, 1995), and galactinol and raffinose in *Arabidopsis* (Kaplan *et al.*, 2004). Heat tolerant cultivars of turfgrass accumulated higher levels of certain sugars, sugar alcohols, and organic acids in response to heat exposure and had reduced MI and higher F_v/F_m ratio (Du *et al.*, 2011).

To maintain high yields under an increasingly hotter climate, peanut cultivars adapted to high temperatures would have to be developed. Genetic variation for heat tolerance at post-flower stages has been reported worldwide (Awal *et al.*, 2003; Craufurd *et al.*, 1999; Prasad *et al.*, 2003; Vu, 2005), but little is known about pre-flower stage heat tolerance in peanut in general and in virginia-type peanut in particular (Selvaraj *et al.*, 2011). To our knowledge the data reported in this paper is the first attempt to understanding the physiological and metabolic mechanisms of the virginia-type peanut seedlings in response to high temperature stress. Our objectives were to identify the physiological and metabolic mechanisms developed by peanut at early growth stages in response to heat stress, evaluate the relationship between the physiological

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characteristics and metabolite levels, and assess the genetic variability for these mechanisms among eight virginia-type cultivars and breeding lines.

Materials and Methods

Plant Materials, Growth Conditions, and Temperature Treatments. Virginia-type peanut cultivars (CHAMPS, Bailey, and Phillips) and advanced breeding lines (N04074FCT, N05006, N05008, N05024J, and SPT 06-07) were selected for this experiment. They were selected based on their differential response to reduced soil moisture in the field, therefore potentially different response to heat (Singh *et al*., 2014). Seeds were surface sterilized with 70% ethanol for 1 min and rinsed twice with sterile water. Seeds were wrapped in moist germination paper and allowed to germinate in the dark for 6 d in a growth chamber at 30 C. The seedlings were then transferred to plastic boxes (29.2 cm × 18.7 cm × 15.2 cm) containing half strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Before imposing the heat treatment, seedlings were allowed to grow in a growth chamber for 7 d at 30/25 C (day/night) temperature and 16/8 hr (day/night) photoperiod, and light intensity of 300 to 400 μmol/m^2/s^1 at the plant level. Thirteen-day-old plants were then exposed to two temperature regimes 40/35 C (heat treatment) and 30/25 C (optimum temperature regime), sequentially using the same growth chamber. Plants were watered well to avoid drought stress during the heat treatment and the nutrient solution was replaced every 4 d to allow proper root aeration and to minimize microbial contamination. Each genotype within a temperature regime was replicated four times (single plant per replication) and two identical experiments were performed. Two leaflets from youngest, fully mature leaves per single plant replication were harvested at 1, 2, 4, and 7 d after beginning of the heat treatment for the physiological characteristics (membrane injury and the F_v/F_m ratio); three individual leaves from four single plants per each replication, genotype, and temperature regime were harvested at the same time-points for metabolite measurements.

Membrane Injury. We used a modified Membrane injury (MI) assay of Blum and Ebercon (1981) on two fully expanded youngest leaflets per replication. Leaf discs of 11.1 mm in diameter were cut from the two leaflets with a leaf disk sampler, rinsed twice with distilled water, and placed in 20 ml plastic vials containing 15 ml distilled water. After shaking the vials for 24 hr, initial conductivity (C_i) of the bathing solution was measured with a conductivity meter (Seven-multi conductivity module and InLab® 741 electrode, Mettler-Toledo Inc., Columbus, OH). Leaf discs were later autoclaved at 120 C for 45 min, and placed on a shaker for 24 hr before recording the final conductivity (C_f) of the bathing solution. The percentage MI was calculated using the formula:

$$MI = 100(C_i)/C_f \quad [1]$$

Chlorophyll Fluorescence. Two leaflets from the youngest fully expanded leaves were harvested for the chlorophyll fluorescence measurements with a modulated chlorophyll fluorometer (OS1p, OptiSciences Inc., NH, USA). Chlorophyll florescence was measured as the F_v/F_m ratio, the ratio of variable (F_v) to maximum (F_m) chlorophyll fluorescence as described (Burke, 2007) with incubation temperature modified from 39 to 45 C. Leaf discs were cut using an 11.1 mm disc sampler and subsequently arranged on a moist tissue paper in a Pyrex® glass dish, covered with GLAD® cling wrap transparent film (GLAD Products Company), and carefully pressed flat to remove air bubbles, and to ensure good contact between the leaf discs and film. Initial F_v/F_m values were recorded prior to incubation, in the dark at 45 C for 3 hr. The F_v/F_m ratios were recorded at hourly intervals. The percent change in F_v/F_m ratio was further calculated using the formula:

$$\text{Percent change in } F_v/F_m = [\{(F_v/F_m)_0 - (F_v/F_m)_3\}/(F_v/F_m)_0]100 \quad [2]$$

where $(F_v/F_m)_0$ and $(F_v/F_m)_3$ are the F_v/F_m readings after 0 and 3 hr of incubation at 45 C, respectively.

Metabolite Sampling, Extraction, and Analysis. Three individual leaves from four single plants per each replication, genotype, and temperature regime were harvested at four different time-points, snap-frozen in liquid nitrogen, and stored at −80 C until further GC-MS-FID based metabolite and lipid-derived fatty acid analyses (Duran *et al.*, 2003; Collakova *et al.*, 2008; 2013). Briefly, after lyophilizing, metabolites from 4.00 ± 0.05 mg of dry powderized tissue were extracted with equal volumes of chloroform and water (400 μl each) containing ribitol and heptadecanoic acid as internal standards. For untargeted polar metabolite profiling, 50 μl of the aqueous phase was dried under a stream of nitrogen gas and derivatized in two steps with methoxyamine.HCl and N-methyl-N-(trimetylsilyl) trifluoroacetamide containing 1% (*v*/*v*) trimethylchlorosilane (Thermo Fisher Scientific, Waltham, MA). Trimethylsilyl derivatives of metabolites were analyzed by GC-MS as described (Duran *et al.*, 2003; Collakova *et al.*, 2008; 2013). For lipid-derived fatty acid analysis, 200 μl of the organic phase was dried as for polar metabolites and

fatty acid methylesters were prepared and analyzed as described (Lu *et al.*, 2011; Collakova *et al.*, 2013).

The GC-MS-FID analyses were performed on an Agilent 7890A series GC-FID and 5975C series single quadrupole MS (Agilent Technologies, Santa Clara, CA) equipped with a DB-5MS-DG capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies) for untargeted polar metabolites and a 30-m DB-23 column (0.25 mm × 0.25 μm, Agilent Technologies) for fatty acid methylester analysis. Peak identification and data analysis were performed as described by Collakova *et al.* (2013) and by using the Agilent Enhanced Mass Selective Detector ChemStation software and three different libraries: FiehnLib spectral and retention time library [Kind *et al.*, 2009], our own custom-built spectral and retention time library, and the spectral NIST library (National Institute of Standards and Technology, Gaithersburg, MD). Final relative levels of polar metabolites and absolute levels of fatty acids were standardized for dry weight and internal standard recoveries.

Statistical Analysis. The two experiments were combined for statistical analysis because trends in seedling responses were similar. Data sets containing a total of eight replications per genotype and temperature regime were analyzed using JMP 9.0 software program (SAS Institute Inc., Version 9.0). Analysis of variance (ANOVA) for individual metabolites and physiological characteristics was carried out and the statistical model was:

$$y_{jkl} = \mu + g_j + t_k + d_l + (gt)_{jk} + (gd)_{jl} + (td)_{kl} + (gtd)_{jkl} + e_{jkl} \quad [3]$$

where μ is the overall mean effect, g_j the main effect of the j^{th} genotype ($j = 1$ to 8), t_k the main effect of the k^{th} temperature treatment ($k = 1, 2$), d_l the day of the l^{th} sampling effect ($l = 1$ to 4), $(gt)_{jk}$ the interaction of the j^{th} genotype and k^{th} treatment, $(gd)_{jl}$ the interaction of the j^{th} genotype and l^{th} day of sampling, $(td)_{kl}$ the interaction of the k^{th} treatment and l^{th} day of sampling, $(gtd)_{jkl}$ the interaction of the j^{th} genotype, k^{th} temperature treatment, and l^{th} day of sampling, and e_{jkl} the random error associated with the experimental unit.

Correlations between the physiological characteristics and changes in metabolite levels were calculated. Step-wise regression analysis was carried out to identify the most discriminating metabolites that explained the largest proportion of variation in the physiological data. These response variables were then used to construct a dendrogram of genotype clusters based on Ward's distance matrix.

Principal component analysis (PCA) was performed on the metabolite data. Loading and score plots were used to reveal the correlations and degree of variation present in the metabolite data. The first two principal components explained the highest percentage of variation between samples; hence further analyses were based on these two principal components.

Results

Membrane Injury. The ANOVA for MI showed temperature-by-genotype-by-day of measurement effect ($p < 0.05$) indicating that genotypes performed differently at each time-point and temperature treatment (Table 1). However, the three way interaction for MI could have been caused by a strong effect of line N05008 on Day 2 unusually showing twice more MI in control than in heat stressed leaves and twice more than for other genotypes; for the other genotypes on Day 2, MI was not significantly different between control *vs.* heat treated seedlings (Table 2), possibly due to initiation of heat acclimation processes (Alscher *et al.*, 2002; Apel and Hirt, 2004; Asada, 2006). Seedlings under heat stress had on average 14% more MI than control plants (Table 2). For the heat stressed plants, MI at Day 1 was 33%, at Day 4 30% and at Day 7 37% higher than for control. N05008 at Days 1 and 7, Phillips at Day 1, and N05024J at Day 7 showed higher MI in heat stressed than control plants. When averaged across the time-points, genotypes N05008 and Phillips showed the highest MI (20 and 18%), and Bailey and SPT 06-07 the lowest (14.5% each) MI under heat stress.

Chlorophyll Fluorescence. The F_v/F_m ratio decreased gradually over the 3 h of dark incubation period at 45 C for both control and heat stressed plants (Fig. 1). On Day 1, the F_v/F_m ratio decreased more for the control than for heat stressed plants ($p < 0.05$), but this trend was reversed on Day 2. After 4 d of differential temperature regime, the F_v/F_m ratio decreased similarly in control and heat stressed plants. Percent change in the F_v/F_m ratio from 0 to 3 hr of incubation 45 C was calculated for individual genotypes and temperature regimes. Because the temperature-by-genotype and temperature-by-genotype-by-day of measurement interactions were not statistically significant for the F_v/F_m (% change) (Table 1), the days of measurement or time-points were combined for each genotype. Overall, Bailey and Phillips exhibited the smallest (14 and 17%, respectively) and N05024J the largest (28%) decline in the F_v/F_m (% change) among the eight genotypes.

Table 1. F-ratios from the ANOVA for the physiological and metabolite characteristics of eight virginia-type peanut genotypes after 1, 2, 4, and 7 d of exposure to optimum (control) (30/25 C) and high temperature (40/35 C) in controlled conditions.

Characteristics	Source of variation						
	Temperature (T)	Genotype (G)	DAY[b] (D)	T × G	T × D	G × D	T × G × D
df[a]	1	7	3	7	3	21	21
F_v/F_m	0.00	2.94**	12.89**	0.45	12.98**	2.00**	1.50
Membrane injury	8.07**	3.47**	4.26**	0.48	9.17**	1.67*	1.69*
Organic acids	6.26*	1.58	0.07	2.15	2.33	0.85	1.21
Glycerate	0.00	0.94	3.42*	0.31	0.52	1.56	1.20
Fumarate	10.41**	0.78	2.41	0.38	0.93	0.95	0.60
Malate	0.10	1.83	8.32**	3.35**	1.43	0.70	1.11
α-ketoglutarate	2.18	0.63	19.60**	1.47	7.50**	0.66	0.64
Ascorbate	471.44**	1.24	36.57**	0.44	20.55**	0.82	0.54
Tartrate	14.64**	2.62*	25.11**	0.42	2.18	0.81	1.11
Shikimate	124.57**	3.21**	25.05**	3.78**	3.49*	0.89	1.15
Citrate	3.33	2.47*	11.72**	0.55	3.50*	1.72*	0.79
Quinate	63.17**	4.63**	26.96**	3.39**	6.57**	0.49	1.08
Sugar alcohols	143.40**	5.32**	20.95**	1.69	2.30	0.98	0.71
Threonate	1.57	1.14	18.57**	0.88	16.46**	0.48	0.55
Pinitol	46.09**	7.04**	21.54**	2.30*	5.97**	1.41	1.15
Inositol	20.89**	2.25*	2.29	0.99	9.35**	0.77	1.11
Galactinol	46.75**	0.82	1.22	0.80	1.19	0.40	0.40
Sugars	91.73**	7.11**	65.91**	2.94**	5.10**	2.09**	3.27**
Glucose	20.42**	4.36**	94.62**	1.36	8.86**	1.59*	2.72**
Fructose	50.41**	4.85**	91.19**	1.18	8.41**	1.74*	2.20*
Sucrose	98.28**	6.97**	1.70	4.55**	6.00**	1.54	2.04**
Ribose	58.36**	5.08**	3.62*	0.70	0.67	0.41	0.32
Maltose	22.08**	1.57	28.51**	1.17	2.20	2.49**	1.59*
Amino acids	3.54	7.64**	13.20**	1.49	0.53	1.41	0.58
Alanine	9.82**	2.21*	3.48*	0.34	0.58	0.75	0.95
Serine	3.43	0.70	3.26*	0.15	0.74	0.84	0.51
Threonine	7.88**	0.63	3.24*	0.13	0.83	0.73	0.48
Aspartate	19.96**	2.67*	10.67**	2.25*	0.18	1.11	0.68
Glutamine	17.23**	3.23**	8.88**	2.15*	0.19	1.05	0.95
Hydroxyproline	2.36	8.62**	22.83**	0.73	2.19	1.24	0.54
Glutamate	2.29	3.98**	5.34**	1.55	0.59	0.74	0.54
Asparagine	5.60*	8.94**	13.03**	1.24	1.80	1.40	0.75
Unsaturated Fatty acids	30.99**	0.53	1.58	0.76	4.83**	0.49	0.56
Saturated Fatty acids	7.96**	3.41**	14.04**	1.30	14.66**	1.63*	1.27

**,*Significant at the 0.01 and 0.05 probability levels, respectively
[a]Degrees of freedom
[b]Days after treatment initiation.

Metabolite Profiling. Out of the total peaks identified during the metabolite profiling analysis, the 26 most abundant metabolites were chosen for further analysis (Table 1). These metabolites belong to four major groups. There were eight amino acids, nine organic acids, five sugars, and four sugar alcohols and cyclic polyols. Significant within-treatment and between-time-point variations were observed with the first two principal components (PCs) explaining 37% of the total variance in the metabolite and fatty acid data (Fig. 2). The data cluster from Day 1 was separated from Day 7 by PC2, which was associated with tartrate, shikimate, citrate, quinate, threonate, myo-inositol, fructose, and glucose relative levels. The first two PCs along with their associated variables and component scores are presented in Table 3.

A total of nine organic acids were identified: glycerate, fumarate, citrate, malate, α-ketoglutarate, ascorbate, quinate, shikimate, threonate, and tartrate. For glycerate and fumarate, there were no or small changes in response to temperature regime or the number of days of heat exposure (Table 4). However, changes with the day of heat exposure were significant for the relative levels of malate, and citrate. Significant changes due to temperature

Table 2. Percentage membrane injury in leaves of eight virginia-type peanut cultivars and breeding lines after 1, 2, 4, and 7 d of exposure to optimum (control) (30/25 C) and high temperature (40/35 C) under controlled conditions.

	Day 1		Day 2		Day 4		Day 7	
	Control	Heat	Control	Heat	Control	Heat	Control	Heat
MI	%							
Bailey	15.95 abc[a]	17.89 abc	16.35 bcd	12.69 bcd	14.73 abc	14.31 bc	11.15 c	13.19 bc
CHAMPS	15.75 bc	16.21 c	14.33 bcd	13.58 bcd	15.20 abc	22.48 a	14.80 bc	14.33 bc
N04074FCT	13.35 c	18.59 abc	-	9.51 d	-	15.43 abc	10.60 c	-
N05006	16.50 abc	18.55 abc	13.50 bcd	13.86 bcd	12.10 bc	19.80 ab	10.88 c	13.68 bc
N05008	14.85 c	25.45 a	37.55 a	18.08 b	-	15.64 abc	10.93 c	19.96 a
N05024J	14.45 c	19.10 abc	17.13 bc	14.85 bcd	13.00 bc	11.96 c	11.98 bc	20.04 a
Phillips	14.75 c	24.74 ab	15.65 bcd	12.68 bcd	14.23 abc	20.99 ab	11.60 bc	15.74 b
SPT 06-07	11.73 c	16.94 bc	15.23 bcd	11.05 cd	10.33 c	17.10 abc	10.00 c	13.84 bc
Average	14.67 B[b]	19.68 A	18.53 A	13.29 B	13.26 B	17.21 A	11.49 B	15.75 A

[a]Means followed by different lower-case letters are significantly different for genotype × temperature regime interaction within each day of high temperature exposure ($P \leq 0.05$ Tukey-HSD).

[b]Means followed by different capital letters between temperature regimes and within a day are significantly different ($P \leq 0.05$ Student's t-test).

regime were recorded for α-ketoglutarate and ascorbate relative levels ($p < 0.05$). For shikimate, tartrate, quinate, and threonate, temperature regime and the number of days of heat significantly changed their relative levels in the peanut seedlings (Table 4). For example, significant decline with time under heat treatment were recorded for relative levels of shikimate (4.1 fold) and quinate (6.3 fold). Relative shikimate levels decreased in heat-stressed seedlings of all genotypes, except for N04074FCT (Table 5). Among genotypes, N05006 had the lowest and N05024J, Bailey, and N04074FCT the highest relative levels of α-ketoglutarate in heat-stressed seedlings. Relative quinate levels decreased ($p < 0.05$) in heat-stressed Bailey, N05006, N05008, and SPT06-07 and remained unchanged for all other genotypes.

Pinitol, inositol, and galactinol were the major cyclic polyols and sugar alcohols identified in peanut samples, and their relative levels increased in response to heat stress (Table 6). For example, galactinol was over 100 fold more in heat-stressed than in control plants at all time-points. No significant differences in relative galactinol levels were observed among genotypes in control plants. At high temperatures, Bailey, CHAMPS, N05024J, Phillips, and SPT 06-07 had approximately 66% more galactinol accumulation than N05006, N05008, and N04074FCT (data not shown). With the exception of N04074FCT and SPT 06-07, all other genotypes showed higher accumulation of

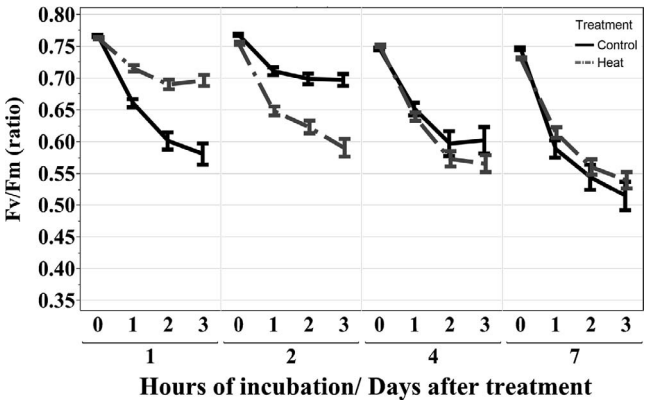

Fig. 1. The F_v/F_m chlorophyll fluorescence ratio of eight virginia-type peanut cultivars and breeding lines grown under two temperature treatments (heat, 40/35 C; control, 30/25 C), at four time-points after the temperature treatment initiation (day 1, 2, 4, and 7 combined), and after 3 hours of dark incubation at 45 C.

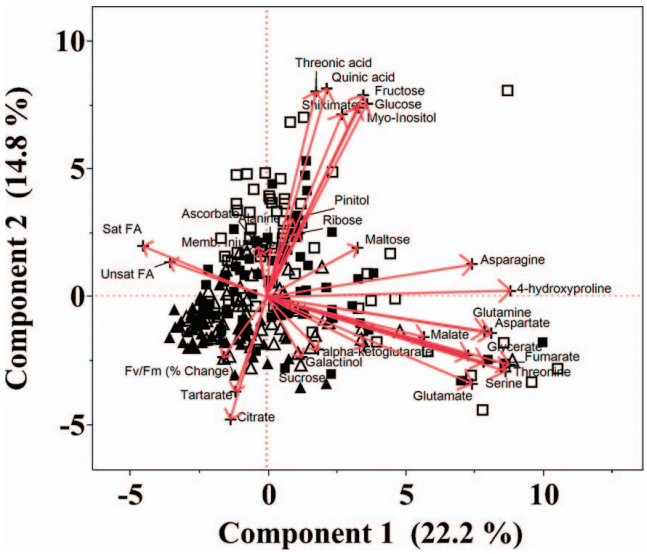

Fig. 2. Biplot from principal component analysis (PCA) of eight virginia-type peanut cultivars and breeding lines based on physiological characteristics and relative metabolite levels measured at day 1, 2, 4, and 7 of heat stress (40/35 C) under controlled conditions.

Table 3. Principal component scores of metabolites and fatty acids exhibiting variance in eight virginia-type peanut cultivars and breeding lines exposed to heat stress (40/35 C) under optimum (control) (30/25 C) conditions.

Metabolites (PC scores)	
PC1	PC2
Serine (0.86)	Tartrate (-0.39)
Threonine (0.86)	Shikimate (0.67)
Aspartate (0.79)	Citrate (-0.49)
Glutamine (0.76)	Quinate (0.76)
Hydroxyproline (0.79)	Threonate (0.78)
Glutamate (0.78)	Myo-Inositol (0.73)
Asparagine (0.61)	Fructose (0.81)
Glycerate (0.72)	Glucose (0.79)
Fumarate (0.78)	
Malate (0.60)	
α-ketoglutarate (0.43)	

pinitol in heat-stressed than in control plants. For example, N05008 and CHAMPS had almost 70% more pinitol compared to N04074FCT and N05006 under heat treatment (Table 5).

Glucose, fructose, and maltose also showed significant ($p < 0.05$) genotype-by-day of measurement and temperature-by-genotype-by-day interactions (Table 1). On average, barring ribose, relative levels of all other sugars decreased in response to heat stress (Table 6). Relative levels of sucrose and ribose changed only in response to temperature, whereas fructose, glucose and maltose levels changed in response to both temperature and number of days of exposure to high temperatures (Table 6). Relative fructose levels decreased at Days 1, 2, and 4 but no change was observed at Day 7 of the heat treatment. Relative fructose levels decreased for Bailey, CHAMPS, N05006, and SPT 06-07 in the heat-stressed compared to their respective controls (Table 5). N05006 and SPT 06-07 had the lowest and Phillips and N04074FCT the highest levels of fructose among the eight genotypes under heat stress.

The genotype-by-day of measurement and temperature-by-genotype-by-day of measurement interactions were absent for all amino acids (Table 1). In general, accumulation of amino acids increased in heat-stressed compared to the control plants (Table 7). For example, concentrations of alanine, serine, threonine, glutamine, hydroxyproline, and asparagine were higher in heat-stressed plants after one day of exposure to 40/35 C temperature regime. With the exception of alanine, aspartate, and glutamine, the other amino acids remained unchanged after Day 1 of the heat stress treatment initiation. Aspartate levels decreased gradually after 2, 4, and 7 d of heat stress whereas glutamate levels remained unchanged throughout the heat treatment time-course (Table 7). Total amino acid levels remained unchanged during Days 1, 2, and 4 and decreased significantly on Day 7 in both control and heat stressed seedlings. Among the genotypes, N04074FCT and Phillips had the highest and SPT 06-07 and N05008 the lowest levels of hydroxyproline in heat stressed plants (Table 5). Similarly, threonine levels were 65% higher for heat stressed seedlings of N04074FCT and Phillips than for SPT 06-07 (data not shown).

Fatty Acids. Saturated FA level was five times higher than unsaturated FA in control plants (1.42 vs. 0.27 μg mg^{-1}) and four times in heat stressed seedlings (1.29 vs. 0.34 μg mg^{-1}) (Table 7) so that the saturated/unsaturated FA ratio was on average 5.2 for control and 3.8 for heat-stressed plants, constantly throughout the temperature treatment duration. A smaller ratio for stressed than for control seedlings resulted from higher

Table 4. Mean relative organic acid levels (mg^{-1} of leaf dry weight) in the leaves of eight virginia-type peanut cultivars and breeding lines after 1, 2, 4, and 7 d of optimum (control) (30/25 C) and high temperature (40/35 C) under controlled conditions.

	Day 1		Day 2		Day 4		Day 7	
	Control	Heat	Control	Heat	Control	Heat	Control	Heat
Organic acid	mg^{-1} of leaf dry weight							
Glycerate	1.09 ab[a]	1.20 a	1.05 ab	0.85 abc	0.38 bc	0.71 abc	0.54 abc	0.28 c
Fumarate	0.86 ab	1.62 a	0.79 ab	1.43 a	0.41 b	1.63 b	0.39 b	0.57 b
Malate	162.60 ab	171.13 a	161.25 ab	180.28 a	178.12 a	177.71 a	144.14 bc	125.61 c
α-ketoglutarate	1.99 a	1.43 cd	1.92 ab	1.65 bc	1.53 cd	1.54 c	0.86 e	1.25 d
Ascorbate	8.30 a	0.87 e	8.96 a	2.94 c	4.56 b	1.72 d	4.84 b	1.08 de
Tartrate	34.75 e	36.61 e	41.60 de	45.47 cd	40.82 de	52.37 b	53.17 bc	67.68 a
Shikimate	17.93 ab	13.88 c	20.33 a	10.92 d	15.97 bc	7.02 e	13.24 cd	3.43 f
Citrate	215.90 b	237.50 b	223.23 b	248.89 b	214.39 b	343.24 a	385.40 a	346.59 a
Quinate	209.00 ab	200.47 b	244.89 a	142.35 c	215.25 ab	75.01 d	128.42 c	31.82 e

[a]Means followed by different letters within a row are significantly different within and between the days of treatment ($p < 0.05$, Student's t-test).

Table 5. Relative levels of shikimate, α-ketoglutarate, quinate, pinitol, fructose, and hidroxyproline (mg^{-1} of leaf dry weight) in the leaves of eight virginia-type peanut cultivars and breeding lines under optimum (control) (30/25 C) and high temperature (40/35 C) regimes. Data from days 1, 2, 4, and 7 of exposure to temperature regimes were combined.

	Shikimate		α-Ketoglutarate		Quinate		Pinitol		Fructose		Hydroxyproline	
Genotype	Control	Heat	Control	Heat	Control	Heat	Control	Heat	Control	Heat	Control	Heat
Bailey	20.55 ab[a]	10.16 de	1.53 ab	1.63 a	246.0 ab	135.0 ef	313.06 d-g	405.40 bc	152.77 ab	111.94 cd	3.54 b-f	4.59 abc
CHAMPS	15.23 c	8.94 e	1.62 ab	1.55 ab	208.4 bcd	147.1def	361.05 b-f	510.02 a	152.98 ab	111.97 cd	3.67 abc	4.75 abc
N04074FCT	14.23 cd	13.14 cd	1.64 a	1.60 a	161.4 c-f	124.0 efg	359.82 b-f	325.58 def	137.82 a-d	125.61 bcd	4.66 abc	5.33 a
N05006	22.57 a	7.37 e	1.71 a	1.19 b	294.7 a	102.0 ef	238.73 g	353.33 c-f	146.51 ab	105.35 de	3.13 c-f	2.90 def
N05008	16.79 bc	7.98 e	1.67 a	1.43 ab	239.9 abc	109.2 efg	404.73 bcd	513.58 a	135.76 a-d	112.48 cd	2.13 f	2.10 f
N05024J	14.07 cd	7.54 e	1.35 ab	1.64 a	140.2 d-g	105.0 efg	285.42 fg	417.60 bc	163.54 a	120.95 bcd	2.39 ef	4.06 a-e
Phillips	17.54 abc	8.72 e	1.74 a	1.38 ab	132.3 d-g	100.2 fg	307.98 efg	441.01 b	148.50 ab	135.20 abc	4.61 a-d	5.06 ab
SPT 06-07	13.97 cd	6.52 e	1.38 ab	1.34 ab	172.2 b-e	74.0 g	336.45 c-f	389.54 b-e	117.41 bcd	81.10 e	2.32 f	2.06 f
Average	**16.87 A**[b]	**8.80 B**	**1.58 A**	**1.47 A**	**199.4 A**	**111.9 B**	**325.91 B**	**419.51 A**	**144.41 A**	**113.08 B**	**3.43 A**	**3.87 A**

[a]Means followed by different lower-case letters are significantly different for genotype × temperature regime interaction ($P \leq 0.05$ Tukey-HSD).
[b]Means followed by different capital letters between temperature regimes are significantly different ($P \leq 0.05$ Student's t-test).

unsaturated and lower saturated FA content in stressed compared to control seedlings. SPT 06-07 had the highest saturated/unsaturated FA ratio in control (6.24) and heat-stressed treatments (4.11) among the genotypes. N04074FCT had the smallest saturated/unsaturated FA ratios, 4.31 in control and 3.48 in stressed plants (Table 8).

Correlations. The F_v/F_m decline was negatively correlated with the overall organic acids ($r = -0.24$, $p < 0.05$) and saturated fatty acid ($r = -0.35$, $p < 0.01$) levels at Day 1 of the heat treatment, but no other relationships were identified afterwards. Membrane injury was not correlated with any individual metabolite under heat stress.

A dendrogram was constructed based on the physiological characteristics and the most significant metabolites through step-wise variable selection (Fig. 3). Based on the metabolic and physiological similarities, the eight genotypes were classified into two distinct groups. Group I consisted of seven genotypes and was different from Group II with SPT 06-07 as the sole member. Within Group I, N05008 and Phillips were closely associated to each other, N05006 was similar phenotypically to N05024J, and Bailey was closely associated with N04074FCT.

Discussion

Membrane Injury. During abiotic stress, lipid peroxidation can cause severe membrane injury (MI) and as such, it can be measured to assess the degree of stress in crops including peanuts (Bajji *et al.*, 2002; Blum and Ebercon, 1981; Srinivasan *et al.*, 1996). MI and the F_v/F_m ratio have previously been used in screening peanut genotypes for salt, heat, and drought stress tolerance (Lauriano *et al.*, 2000; Qin *et al.*, 2011; Srinivasan *et al.*, 1996). Peanut seedlings exposed to a 40/35 C temperature treatment in this study showed on average 18% increased MI compared to control plants (Table 2). Genotypes Bailey, N04074FCT, and SPT 06-07 exhibited the least MI under heat stress in comparison with the other genotypes for which MI changes were inconsistent throughout the heat stress treatment. High levels of several amino acids, sugars, and sugar alcohols have been frequently associated with improved membrane stability during stress (Gounaris, 1984; Liu and Huang, 2000; Dhindsa, 1981). In this study we could not identify individual metabolites associated with reduced MI. However, when multiple regression analysis was used, the combination of fructose, glucose, maltose, inositol, pinitol and aspartate were positively associated with MI. In contrast, ribose, hydroxyproline and, surprisingly, saturated fatty acids were negatively correlated with MI. Combinations

Table 6. Mean relative sugar and sugar alcohol levels (mg^{-1} of leaf dry weight) in the leaves of eight virginia-type peanut cultivars and breeding lines after 1, 2, 4, and 7 days of control (30/25 C) and high temperature (40/35 C) under controlled conditions.

	Day 1		Day 2		Day 4		Day 7	
	Control	Heat	Control	Heat	Control	Heat	Control	Heat
Sugar alcohol	mg^{-1} of leaf dry weight							
Pinitol	378.9 ab[a]	788.1 a	395.8 ab	453.3 ab	345.5 ab	423.8 ab	183.5 b	376.4 ab
Inositol	25.5 a	23.7 a	29.4 a	27.3 a	15.6 a	32.9 a	11.6 a	32.4 a
Galactinol	0.2 d	31.2 c	0.2 d	66.3 a	0.2 d	41.3 bc	0.7 d	55.9 ab
Sugars								
Fructose	196.0 a[a]	170.1 b	168.7 b	119.5 c	135.3c	90.2 d	77.7 d	83.7 d
Glucose	404.9 a	346.8 b	349.1 b	274.4 c	271.5 c	212.9 d	165.9 e	202.7 d
Sucrose	124.8 a	9.6 d	89.9 ab	52.9 c	76.8 bc	26.3 d	121.7 a	24.8 d
Ribose	2.3 c	4.2 a	2.2 c	3.7 ab	2.0 cd	3.3 b	1.2 d	3.3 b
Maltose	4.5 a	2.7 b	4.4 a	2.5 b	2.0 bc	1.3 cd	1.0 cd	0.6 d

[a]Means followed by different letters within a row are significantly different within and between the days of treatment ($p < 0.05$, Student's t-test).

of these metabolites explained from 41 to 61% of the variation in the MI during the heat stress treatment.

Chlorophyll Fluorescence. Temperature effect was not significant for individual genotypes but overall Bailey and Phillips had the least (14 and 17%), and N05024J the greatest (28%) decline in the F_v/F_m % change among the eight genotypes after heat exposure (data not shown). Less F_v/F_m ratio decline under drought stress compared to well-watered plants has been reported in peanut (Burke, 2007; Kottapalli *et al.*, 2009). Burke (2007) explained this to be the result of stress-induced sucrose accumulation, which provided better respiratory metabolic substrate under high temperature stressed compared with non-stressed plants. In our experiment, a negative correlation between the F_v/F_m % change and total sugar alcohols was observed under heat stress, suggesting that for our genotypes accumulation of sugar alcohols was rather associated with decline in the F_v/F_m ratio in heat stressed peanut seedlings. Although seedlings exhibited symptoms of heat stress in terms of increased MI and change in the levels of the majority of metabolites, the F_v/F_m % change due to heat stress exposure was small for Bailey and absent for the other genotypes. The lack of genotypic differences may be due to the short duration (7 d) of the heat stress period which may have been insufficient to cause significant damage to the photosystem II complex, often reflected by

Table 7. Mean relative amino acid levels (mg^{-1} of leaf dry weight) and fatty acid content ($\mu g\ mg^{-1}$ leaf dry weight) in the leaves of eight virginia-type peanut cultivars and breeding lines after 1, 2, 4, and 7 days of optimum (control) (30/25 C) and high temperature (40/35 C) under controlled conditions.

	Day 1		Day 2		Day 4		Day 7	
	Control	Heat	Control	Heat	Control	Heat	Control	Heat
Amino acid	mg^{-1} of leaf dry weight							
Alanine	2.67 cd[a]	5.62 ab	3.88 bcd	4.59 bc	5.27 abc	7.32 a	1.81 d	4.72 bc
Serine	2.37 bc	4.88 a	1.96 bc	3.25 ab	1.21 bc	2.31 bc	1.03 bc	0.88 c
Threonine	0.69 bc	1.81 a	0.62 bc	1.31 ab	0.38 c	0.96 bc	0.26 c	0.38 c
Aspartate	34.63 ab	27.15 bc	37.20 a	26.27 bc	33.85 ab	21.60 c	20.75 c	9.89 d
Glutamine	59.05 cde	75.68 ab	67.80 bcd	87.14 a	59.43 cd	70.64 bc	41.75 e	58.04 d
Hydroxyproline	3.52 bc	4.96 a	4.57 ab	5.11 a	4.05 abc	3.47 c	1.57 d	1.93 d
Glutamate	73.31 ab	71.77 ab	68.28 bc	72.28 ab	72.53 ab	81.78 a	55.07 c	63.47 bc
Asparagine	63.21 bc	117.84 a	92.53 ab	115.32 a	88.20 ab	83.49 b	22.07 c	36.78 c
Fatty acid								
Saturated	1.41 b	1.27 b	1.03 c	1.29 b	1.35 b	1.31 b	1.90 a	1.29 b
Unsaturated	0.30 c	0.32 bc	0.23 d	0.35 ab	0.24 d	0.36 a	0.32 abc	0.34 abc

[a]Means followed by different letters within a row are significantly different within and between the days of treatment ($p < 0.05$, Student's t-test).

Table 8. Fatty acid content (μg mg^{-1} leaf dry weight) and the saturated *vs*. unsaturated fatty acid ratio in the leaves of eight virginia-type peanut cultivars and breeding lines after 1, 2, 4, and 7 days of optimum (control) (30/25 C) and high temperature (40/35 C) under controlled conditions.

	Saturated fatty acid		Unsaturated fatty acid		Saturated/unsaturated ratio	
	Control	Heat	Control	Heat	Control	Heat
Bailey	1.60 a[a]	1.25 cde	0.28 c-f	0.32 a-e	5.7	3.9
CHAMPS	1.40 a-e	1.39 a-d	0.26 def	0.36 ab	5.4	3.9
N04074FCT	1.12 de	1.15 e	0.26 ef	0.33 a-e	4.3	3.4
N05006	1.53 abc	1.31 b-e	0.30 b-f	0.35 abc	5.1	3.7
N05008	1.38 a-e	1.24 cde	0.29 c-f	0.35 abc	4.8	3.5
N05024J	1.24 b-e	1.25 cde	0.23 f	0.33 a-d	5.4	3.8
Phillips	1.54 ab	1.20 de	0.29 c-f	0.32 a-e	5.3	3.8
SPT 06-07	1.56 ab	1.52 ab	0.25 f	0.37 a	6.2	4.1
Average	1.42 A[b]	1.29 B	0.27 B	0.34 A	5.9	3.8

[a]Means followed by different lower-case letters are significantly different for genotype × temperature regime interaction ($P \leq 0.05$ Tukey-HSD).

[b]Means followed by different capital letters between temperature regimes are significantly different ($P \leq 0.05$ Student's t-test).

decreases in F_v/F_m ratio in stressed plants (Balota and Lichtenthaler, 1999; Lichtenthaler *et al.*, 1998; Maxwell, 2000).

Polar Metabolites. Principal component analysis showed low variance among time points, indicating a weak differentiation of time-points on the basis of physiological characteristics and metabolites. In general, organic acids decreased under heat stress (Table 4). For example, major organic acids including, ascorbate, shikimate, and quinate showed significant decreases in their relative steady-state levels in heat-stressed seedlings, especially during the last two time-points (Days 4 and 7). Similar results were reported in drought-stressed wheat (Bowne *et al.*, 2012) and field grown mature peanut plants (Singh, 2013). However, fumarate, malate, tartrate, citrate, and threonate increased either throughout entire heat stress exposure or in the first days of heat stress. Increased levels of organic acids were often positively related to cell growth and respiration (Glassop *et al.*, 2007; Vasquez-Robinet *et al.*, 2008). The relationship between organic acid levels and maintenance of high F_v/F_m ratio (low F_v/F_m % change) was positive ($p < 0.05$) at Day 1 during the heat treatment, suggesting that higher levels of organic acids during early heat stress could be an indicator of increased photochemical efficiency of peanut seedlings during stress as shown in other crops (Widodo *et al.*, 2009; Liu and Huang, 2000). Among the sugar alcohols identified during this study, galactinol levels increased over 100 fold as a result of heat stress (Table 6). The osmoprotective and anti-oxidation roles of galactinol during abiotic stresses have been documented before (Nishizawa *et al.*, 2008; Kaplan *et al.*, 2004; Peters *et al.*, 2007). In the present study, accumulation of galactinol in peanut plants is in agreement with this literature and highlights galactinol's role in heat stress tolerance of peanut seedlings (Kaplan *et al.*, 2004). Increased levels of pinitol are also known to confer tolerance to various abiotic stresses in other crop plants (Matos *et al.*, 2010; McManus *et al.*, 2000), but they were unchanged in our study. Indeed, in our research, a combination of several sugars, sugar alcohols, amino acids, and fatty acids better explained the variation in MI, 41 to 61% of the total.

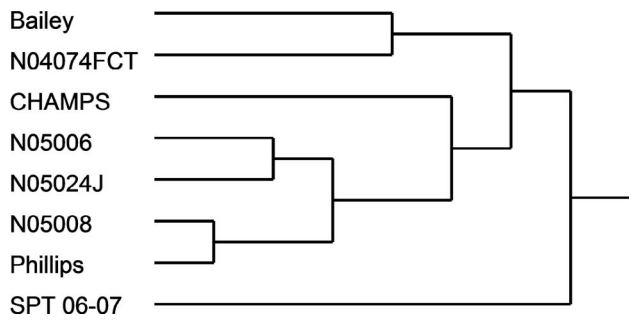

Fig. 3. Dendrogram of the relationship among eight virginia-type peanut cultivars and breeding lines based on the selected physiological and metabolic variables from stepwise variable analysis measured after 7 days of heat stress (40/35 C) under controlled conditions.

A steady decline in sugar content was observed following prolonged heat stress with the lowest levels at Day 7 of the heat treatment (Table 6). Sugars that contributed to this trend were glucose, fructose, and the disaccharide maltose. Abiotic stresses affect the process of photosynthesis through various means, *i.e.*, ROS-mediated membrane damage and reduced chlorophyll production (Chu *et al.*, 1974; Dhindsa *et al.*, 1981; Nishizawa *et al.*, 2008). Lower sugar levels can be a direct consequence of reduced photosynthesis (Shah and Paulsen, 2003) and altered carbon metabolism

under stress (Mangelsen *et al.*, 2011). Decline in sugar levels was seen in wheat seedlings exposed to drought (Bowne *et al.*, 2012). Heat-induced accumulation of soluble sugars such as glucose, fructose, and sucrose was also reported in *Arabidopsis* and other crops (Du *et al.*, 2011; Kaplan *et al.*, 2004; Rizhsky *et al.*, 2004). In the present study, a significant decrease in sugar levels during heat stress (7 d) was observed.

Several studies reported an increase in amino acid levels in response to various stresses in sensitive cultivars (Du *et al.*, 2011; Vasquez-Robinet *et al.*, 2008; Zuther *et al.*, 2007). The relative levels of hydroxyproline and asparagine were higher at Day 1, glutamate at Day 7, and glutamine throughout the time course in the heat-stressed than in control plants (Table 7). Their increase is sometimes attributed to stress-induced protein degradation due to tissue damage and senescence (Diaz *et al.*, 2005; Widodo *et al.*, 2009). For example, increased hydroxyproline and glutamine levels for N04074FCT could be indicative of sensitivity of this genotype to heat stress.

Fatty acids. As part of the cellular membrane complex, fatty acids play an important role in keeping the cellular membrane integrity during periods of environmental stress (McKersie *et al.*, 1990; Simon, 1974). In this study the unsaturated fatty acid content increased and saturated fatty acid content decreased during heat stress relative to the controls (Table 7). However, the ratio of saturated vs. unsaturated fatty acids was variable among genotypes. The highest saturated to unsaturated fatty acid ratio was observed in SPT 06-07; this possibly coincided with lower MI for this genotype compared to other genotypes.

Conclusions

This study investigated the effect of high temperature stress on seedlings of eight virginia-type peanut cultivars and breeding lines in a controlled environment. From the physiological perspective, MI increased and chlorophyll fluorescence (measured as the F_v/F_m ratio) decreased in response to high temperature. Comparative metabolomics revealed changes in the levels of specific metabolites in heat-treated relative to control peanut seedlings. Even though we were able to highlight some metabolites, *e.g.*, hydroxyproline, galactinol, and pinitol, that could explain specific differential physiological (F_v/F_m ratio and MI) responses in peanut plants, overall our data suggested general stress responses by seedlings rather than adaptive mechanisms under heat stress. Among the cultivars, Bailey seedlings seemed to have better tolerance to early season heat stress; MI was low (Table 2) and the F_v/F_m change due to the heat exposure was significantly less than for other genotypes (Singh, 2013). At least in part, this may explain the good crop stand and high yields observed in farmer fields for this cultivar, grown on over 85% peanut acreage in the VC region. Among the lines, SPT 06-07 exhibited lower membrane injury, increased galactinol, reduced hydroxyproline, and higher saturated *vs*. unsaturated fatty acids accumulation under heat stress compared to other genotypes. The uniqueness of SPT 06-07 in comparison with the other genotypes was also shown by the step-wise variable classification analysis, where SPT 06-07 was selected as the sole genotype of Group II, which was significantly different from Group I, that included the remaining genotypes. SPT 06-07 has exhibited improved drought tolerance of mature plants in field studies (Singh, 2013; Singh *et al*., 2014). Further, this shows that SPT 06-07 could be an important source for improvement of peanut tolerance to heat and drought stress at multiple growth stages.

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