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Physiological responses of c_4 grasses to prolonged heat stress

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Abstract: C_4 grasses are best adapted to the transition, warm-arid, and warm-humid climatic zones and have the ability to acquire thermotolerance by exposure to acute heat stress. Exposure to sub-lethal temperatures results in changes in physiological, biochemical, metabolic, and molecular processes. The response of two warm-season grasses to prolonged heat stress was investigated. Plants of hybrid bermudagrass (*Cynodon dactylon* × *C. transvaalensis* 'Tifway') and Japanese lawn grass (*Zoysia japonica* Steud. 'Meyer') were exposed for 168 h to supraoptimal temperature conditions (47°C) in controlled-environment chamber. Compared with zoysiagrass, bermudagrass showed greater damage. Metabolite profiles were affected by prolonged heat exposure, with significant differences between these species. Consistent differences were found in total soluble sugars accumulation over the study period and severity of plant organ senescence. Bermudagrass roots were more affected, as compared to leaves. Leaf proteins expression determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis showed an early degradation in zoysiagrass, as thermal exposure proceeded. A significant net decline in protein content was observed after 48 h of exposure, while in bermudagrass an analogous decline was not detected until 96 h of treatment. Although heat stress is not considered a detrimental factor to C_4 grass species, the two species showed significant differences in their physiological response to continuous high temperatures.

1. Introduction

Higher plants exhibit a photosynthetic limitation as a function of temperature. Warm-season grasses, characterized by the Hatch-Slack pathway (C_4), evolved a RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) type that more efficiently utilizes high CO_2 at high temperature, and have an optimum growth temperature range of 27 to 35°C, which is approximately 10°C higher than in C_3 plants (Leegood, 1993). C_4 grasses are best adapted to the transition, warm-arid, and warm-humid climatic zones and usually have the ability to acquire thermotolerance by exposure to acute heat stresses (DiPaola and Beard, 1992).

Plants compensate for their sessile nature by developing specific responses to abiotic conditions. Exposure to sub-lethal temperatures results in changes in physiological, biochemical, metabolic, and molecular processes, developing an active regulatory mechanism for maintaining cellular homeostasis, as well as enhanced tolerance (Kislyuk *et al.*, 2004, 2007; Guy *et al.*, 2008; Kumar *et al.*, 2013). The severity of damage to cellular and subcellular structures mainly depends on the intensity, duration, and rate of temperature increase. Changes in protein metabolism have been correlated with the thermotolerance mechanism. In particular, transcription and translation of specific heat shock proteins (HSPs) are induced or enhanced when plants are exposed to supraoptimal temperature, playing a crucial role in adaptive mechanism. Most of the HSPs are molecular chaperones, functioning by binding and stabilizing proteins at intermediate stages of folding, assembly, degradation, and translocation across membranes (Xu *et al.*, 2011).

Furthermore, carbohydrate metabolism was found to be affected by heat shock on *Arabidopsis*, with an accumulation of maltose, sucrose, galactinol, myo-inositol, raffinose and monosaccharide cell-wall precursors (Rizhsky *et al.*, 2004). Induction of the triose phosphate and starch hydrolytic pathways of carbohydrate metabolism provides precursors, leading to raffinose biosynthesis and accumulation of galactinol and raffinose. Soluble sugars are known osmolytes that are beneficial during heat stress conditions, providing protection of cell membranes during stress exposure (Diamant *et al.*, 2001).

Bermudagrass (*Cynodon* spp. Rich.) and zoysiagrass (*Zoysia* spp. Willd.) provide excellent surfaces for functional, recreational, and ornamental areas, including golf course fairways, ornamental lawns and parks. The ability of these perennial, warm-season grasses to tolerate heat

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stress is reported to be excellent compared to cool-season grasses (Beard, 1973). In an investigation, conducted to determine the relative effects of drought and heat on cool-season tall fescue (*Festuca arundinacea* Schreb.) and Manilagrass [*Zoysia matrella* (L.) Merr.], the superior heat and drought tolerance recorded for zoysiagrass has been associated with its ability to maintain photochemical activity and cellular membrane stability (Du *et al.*, 2008). This characteristic was also associated with maintenance of more active antioxidant enzymes and lower membrane lipid peroxidation (Du *et al.*, 2009). Additionally, comparing the ability of three warm-season turfgrass species to mitigate heat accumulation during a prolonged 60-day summer drought, zoysiagrass increased rates of leaf damage and maintained significantly higher canopy temperatures during drought conditions, as compared to bermudagrass and St. Augustinegrass (*Stenotophrum secundatum* (Walt.) Kuntze), suggesting these latter species have an enhanced heat dissipation through greater evapotranspiration (Steinke *et al.*, 2009). Amongst the species, few significant differences were observed during cultivar comparisons, although Manilagrass tended to accumulate and retain heat more quickly than cultivars of Japanese lawn grass (*Zoysia japonica* Steud.). The differential heat tolerance after prolonged stress, exhibited by bermudagrass as compared to *Poa pratensis* (L.), was attributed to a higher accumulation of organic acids, amino acids, soluble sugars, and inositol (Du *et al.*, 2011).

Differences in photosynthetic response in C_4 plants do exist. RuBisCO in *Cynodon dactylon* constantly exhibited a higher catalytic turnover rate at 16, 28, and 40°C than in *Zoysia japonica*, with analogous activation energy of 50.1-51.2 kJ mol^{-1} (Sage, 2002). However, it exceeded the photosynthetic capacity above 25°C, and was not a limiting factor at warm temperature.

Limited work has been done to examine and compare physiological responses in protein induction and degradation, as well as carbohydrate metabolism under heat stress in C_4 grasses. The objectives of the present research were (i) to evaluate the heat tolerance of bermudagrass and zoysiagrass standard cultivars using controlled environment and heating procedures, and (ii) to determine differences in stress responsive metabolite accumulation in C_4 standard cultivars to short- and long-term heat stress.

2. Materials and Methods

Experimental conditions

The research was carried out at the department of Agriculture, Food and Environment, University of Pisa, Italy. On 22 February 2011, plants of *Cynodon dactylon* × *C. transvaalensis* Burtt. Davy cv. 'Tifway' and *Zoysia japonica* Steud. cv. 'Meyer', selected as standard cultivars, were collected and clonally propagated as phytomers (1- to 2-cm segments of stolon obtained from mature plants, containing root tissue, crown, and shoot material) into a sphagnum moss peat-based growing medium, mixed with volcanic sand, into 160-hole seed trays, with a cell volume of 5 cm^3. Plants were established at 23°C (±4°C) day/night temperatures for 32 weeks in a greenhouse. During the active growing season a mineral solution (8N-7P-19.9 K + 4 Ca^{-2} Mg) at 1.3 g l^{-1} was supplied three times per week. The fertilization program was periodically adjusted according to the physiological age and state of the grass. Irrigation was applied as needed to prevent wilting and plant material was maintained at a cutting height of 2.5 cm throughout the entire pre-treatment phase. Mature plants were then acclimated in a growth chamber for four weeks before treatments were applied, and maintained at 22±1°C with a 12-h photoperiod and a light intensity of 90 µmol $m^{-2}s^{-1}$.

All treatments were performed in parallel. The control was maintained in a growth chamber, and stress was applied by exposing plants to 47±1°C with 45% relative humidity and 12-h photoperiod of 90 µmol $m^{-2}s^{-1}$ photosynthetically active radiation. Temperature was monitored with a datalogger (Campbell Scientific, Logan, UT, USA), and plants exposed to heat stress were sub-irrigated daily to avoid drought stress. For each species, five plants were removed from the heat chamber at target times (set at 6 h, 24 h, 48 h, 96 h, and 168 h), and finally transferred again to the growth chamber for assessment of vitality after three weeks. Biometric response to heat stress was evaluated as fresh weight (FW) (expressed in percentage compared to t0= 100%) regrowth in the growth chamber for three weeks.

Analysis of carbohydrates

Samples (0.5 g FW) were ground to a powder and extracted as described by Tobias *et al.* (1992) in order to quantify glucose, fructose, and sucrose (total soluble sugars, total soluble carbohydrates). Samples were assayed by coupled enzymatic assay methods (Guglielminetti *et al.*, 1999) measuring the increase in A_{340}. The accuracy of the method was tested using standards with known amounts of carbohydrates. Incubations of samples and standards were carried out at 37°C for 30 min. The reaction mixtures (1 ml) were as follows. Glucose: 150 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$, 2 mM ATP, 0.6 mM NADP, 1 unit Glc6P dehydrogenase, 1 unit hexokinase; fructose was assayed as described for glucose plus the addition of 2 units of phosphoglucose isomerase; the increase in A_{340} was recorded. Sucrose was first broken down using 85 units of invertase (in 50 mM Na-acetate, pH 4.6) and the resulting glucose was assayed as described above.

Recovery experiments evaluated losses taking place during the extraction procedures. Two tests were done for each metabolite by adding a known amount of authentic standards to the samples prior to the extraction. The concentration of the added standards were similar to those estimated to be present in the tissues in preliminary experiments. The percentage of recovery ranged between 94 and 107% depending on the sugar. Data were corrected on the basis of the recovery percentages obtained for

each sample, and expressed as µmoles hexoses equivalent g^{-1} FW.

Analysis of pigments

Pigments were extracted by incubating tissues (50-100 mg FW) in 1.5 ml 80% acetone for one week at 4°C in darkness. The absorbance of extracts was measured spectrophotometrically at 470.0, 663.2, and 646.8 nm. These absorbance values were used for calculation of chlorophyll *a*, chlorophyll *b*, and carotenoids contents in accordance with Pompeiano *et al.* (2013).

Protein extraction and separation

Leaves were collected at the experimental target times previously reported and extracted in 50 mM Tris-HCl buffer (pH 7.6 containing 10 mM DTT and 10% glycerol). Protein quantification was performed according to Guglielminetti *et al.* (1997). Equal amounts of protein (2 µg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% polyacrylamide gels followed by conventional silver nitrate staining.

Statistical analysis

The experiment was replicated for a total of four experimental replications. The statistical analyses of biometric and growth traits were performed using one-way analysis of variance (ANOVA) to determine whether significant differences among cultivars and groups existed. When significant differences were found, the means were compared using the Least Significant Difference (LSD) test. Significant differences for all statistical tests were evaluated at the level of p= 0.05. All computations were performed with R 2.15.0 (R Development Core Team, 2012) and R package *agricolae* (de Mendiburu, 2012). For the photosynthetic pigments and soluble carbohydrates data, the Student-Newman-Keuls (SNK) test was used for *a posteriori* multiple comparison of means.

3. Results

Heat tolerance

Both species exhibited an increasing susceptibility, expressed as percentage of fresh weight canopy regrowth relative to the control, following prolonged exposure to heat stress (Fig. 1). No significant difference between the two species was detected after 6 h of treatment. Bermudagrass showed significant (p< 0.001) higher resistance to heat stress than zoysiagrass at all target times after the first 6 h of imposed stress. Moreover, our data showed that, after 6 and 24 h of exposure at sub-lethal temperature, recovery of 'Tifway' was greater as compared to t0, although these differences were not significantly different *vs.* the control. No significant difference was observed until 96 h of heat stress for this species. A prolonged exposure to sub-lethal temperature revealed a significant decline, although canopy recovery at the last target time, 168 h, still displayed 49.8% of regrowth as compared to t0. In contrast, a rapid, sharp decline was clearly evident in 'Meyer' after 6 h of treatment, with 36.3% of regrowth as canopy recovery. No vitality was detected following 96 h of exposure.

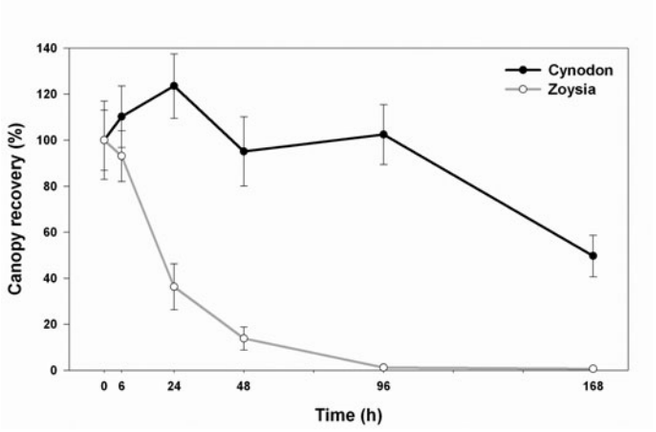

Fig. 1 - Changes in canopy recovery after heat treatments as percentage of living fresh tissues at control time. Error bars represent standard error of the mean (n=4).

Photosynthetic pigments

Clear differences in photosynthetic pigments were noticed between control and heat shock-stressed plants (Fig. 2). Under heat stress conditions, significant (p < 0.05) changes in chlorophyll *a, b* and carotenoid contents were observed, although the species had different behaviors. In bermudagrass, a progressive decline was detected from the initial hours of stress, particularly evident in chlorophyll *b* levels. Chlorophylls were completely degraded after 168 h of exposure, while the degradation of carotenoids was less severe at the end of the treatment (-64.7% compared to the control). In contrast, zoysiagrass pigments showed a sharp increase and attained a peak level at 48 h of heat exposure, resulting in a significant (p< 0.001) difference between treatments. Thereby indicating a significant decline under increasing heat stress as compared with the control. In bermudagrass, changes in photosynthetic pigments did

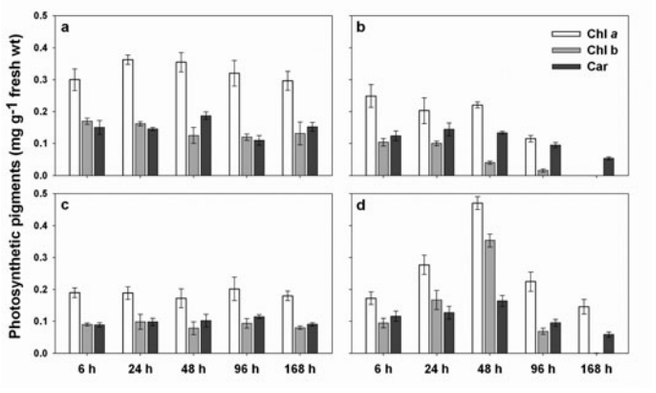

Fig. 2 - Leaf photosynthetic pigments in (a) bermudagrass control, (b) bermudagrass heat shock, (c) zoysiagrass control, and (d) zoysiagrass heat shock observed over time. Error bars represent standard error of the mean (n=4).

not lead to any significant difference in the carotenoids-to-chlorophylls ratio at 48 h exposure, whereas in zoysiagrass the ratio significantly ($p< 0.01$) increased under heat stress.

Soluble carbohydrates

Analysis of total soluble sugars data showed differential responses to heat stress in the two warm season grasses (Figs. 3, 4). With few exceptions, total soluble sugars (TSS) levels in the controls remained constant throughout the experiment. Under control and heat stress conditions, sucrose comprised the majority of the total sugar concentration in all the tissues analyzed.

In bermudagrass leaves, heat stress stimulated a significantly ($p< 0.001$) greater TSS production in the initial 48 h as compared with the control (Fig. 3). Although a pronounced peak after 24 h of stress was observed (with a concentration of 49.8 µmol g^{-1} FW), TSS declined gradually thereafter till 16.0 µmol g^{-1} FW, 42.2% lower than the control (Fig. 3B). Glucose and fructose concentrations generally remained constant throughout the investigation, and no significant differences were detected between the control and treated samples. Zoysiagrass leaves reduced significantly their TSS content soon after the beginning of the heat stress, with a sudden contraction (-52.4%) found at the 6 h sampling. A gradual decrease in TSS was observed when treatment was prolonged; the minimum concentration of 9.8 µmol g^{-1} FW at 96 h (-74.7% compared to the control) was reached, and a plateau level was attained at the last target time.

Bermudagrass roots contained significantly ($p< 0.001$) higher TSS than zoysiagrass under both control and heat stress conditions. Overall, TSS content decreased considerably in plants exposed to heat stress. The averages of all the independent observations were 64.6 and 32.7% lower than those of the control plants in bermudagrass and zoysiagrass, respectively (Fig. 4). Under heat conditions, sucrose levels showed a pronounced decline (-79.0% *vs.* the control) 6 h after the beginning of treatment. This metabolite significantly increased during the first 24 h to 36.8 µmol g^{-1} FW, but later decreased constantly with the treatment (Fig. 4 B). Significant decreases in glucose and fructose contents were recorded in bermudagrass roots exposed to stress. In 'Meyer', TSS content decreased considerably in both treatments compared to bermudagrass. Moreover, levels of TSS showed a sharp decline after 48 h of exposure to heat stress.

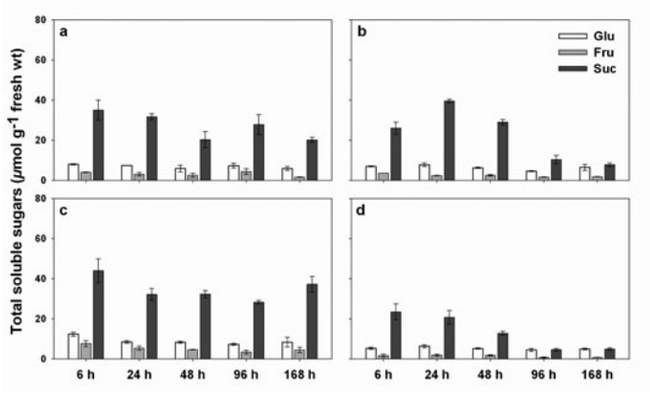

Fig. 3 - Glucose, fructose, and sucrose (as hexose equivalents) leaf contents in (a) bermudagrass control, (b) bermudagrass heat shock, (c) zoysiagrass control, and (d) zoysiagrass heat shock over time. Error bars represent standard error of the mean (n=4).

Protein expression during heat stress

In both C_4 grasses, soluble protein expressions were significantly affected in response to heat stress. In bermudagrass leaves, protein synthesis mostly ceased after 96 h of heat shock exposure, whereas in Japanese lawn grass degradation occurred after 48 h of treatment (Fig. 5). However, a few bands of zoysiagrass leaf protein SDS-PAGE persisted till the end of the treatment period. Degradation of a large band, corresponding to the large subunit of RuBisCO (about 50 kD), occurred after 48 h of heat exposure in zoysiagrass, while in bermudagrass it was no longer detectable at 96 h of heat stress.

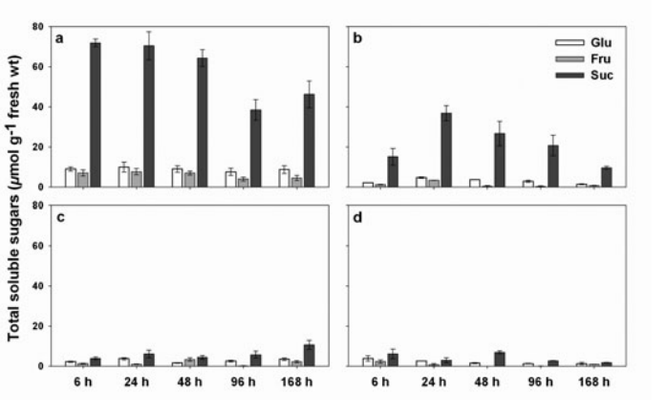

Fig. 4 - Glucose, fructose, and sucrose (as hexose equivalents) root contents in (a) bermudagrass control, (b) bermudagrass heat shock, (c) zoysiagrass control, and (d) zoysiagrass heat shock over time. Error bars represent standard error of the mean (n=4).

4. Discussion and Conclusions

In open fields usually plants are simultaneously exposed to combined drought and heat effects, two interacting abiotic stresses that limit growth and quality. Since initial reports, 'Meyer' has been identified as a heat and drought tolerant species (Forbes and Ferguson, 1947; Dunn, 1989). In the Midwest transition zone, it was observed to lose color in response to extreme midsummer heat, yet still leaving a playable surface (Dunn, 1998). However, a lower dehydration rate and drought resistance were attributed to zoysiagrass when compared to *Cynodon* spp., due to a limited root system, higher ET rate, and slower rate of epicuticular wax production under stress conditions (Beard and Sifers, 1997). The present study indicates that zoysiagrass has a moderate tolerance to prolonged heat stress compared to bermudagrass. After the initial 6 h period of stress, zoysiagrass canopy recovery exhibited a sharp decline, while bermudagrass maintained an unaltered plant regrowth response until 96 h of exposure.

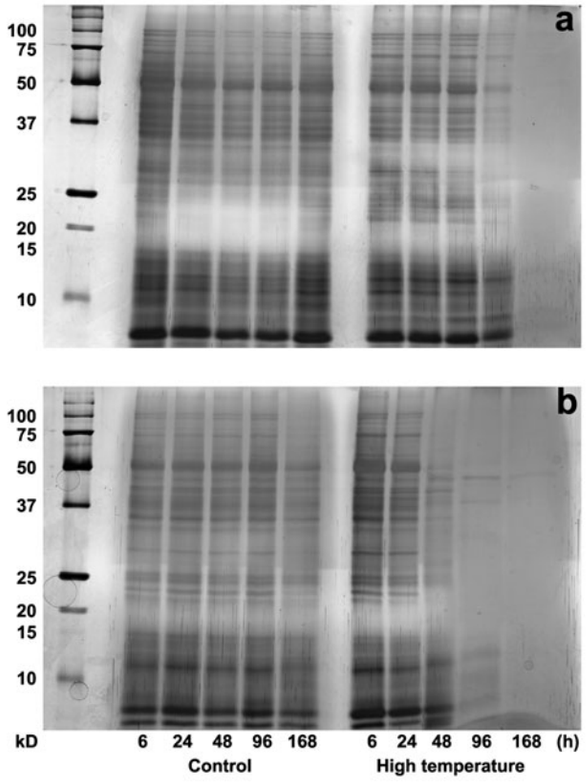

Fig. 5 - Representative SDS-PAGE profiles of leaf soluble proteins for (a) bermudagrass and (b) zoysiagrass, respectively with control (left) and exposure to high temperature (right) for 168 h. Equal amounts of protein were loaded in each lane. Standard molecular weights in kD are reported.

Physiological characterization of C_4 plants subjected to prolonged heat stress revealed significant differences between the species. While in bermudagrass a gradual net decline of photosynthetic pigments was observed throughout the experimental period, zoysiagrass enhanced chlorophyll synthesis during the first 48 h of heat stress, followed by a severe leaf senescence induced by a prolonged exposure. The increasing chlorophylls and carotenoids contents might reflect an adaptive physiologic response to the abiotic stress. It may be associated with a higher photosynthetic performance, as well as with a better dehydration tolerance of 'Meyer' in comparison to 'Tifway' (Kim, 1987). Moreover, under heat stress conditions, carotenoids resulted more stable than chlorophylls in both species, as shown by the chlorophyll *a, b*:carotenoids ratios in the treated plants, in agreement with Wahid (2007) observations.

Changes in TSS content exhibited consistent differences in the timing and severity of plant organ senescence induced by the heat treatment. Compared with bermudagrass, zoysiagrass had greater damage. Moreover, bermudagrass root tissues were more affected than leaves, which had a peak of soluble sugars after 24 h of exposure. Previous studies showed similar results in soybean (Djanaguiraman and Prasad, 2010; Djanaguiraman *et al.*, 2011) and, as observed in our study, this effect occurred despite a concomitant loss of chlorophyll pigments, usually attributed to membrane damage. Causes of this significant accumulation of soluble sugars are unknown, although degradation of starch (Geigenberger *et al.*, 1998) could be involved.

Examination of the pattern of leaf proteins subjected to SDS-PAGE showed an early degradation in zoysiagrass as thermal exposure proceeded. A significant net decline was observed after 48 h of exposure, while in bermudagrass an analogous decline in soluble protein was not detected until 96 h of treatment. Overall, bermudagrass showed a greater ability to cope with high-temperature stress, as indicated by the persistence of the band, corresponding to the large subunit of RuBisCO. In accordance with previous reports (Hashimoto *et al.*, 1989; Veerasamy *et al.*, 2007), our results showed that chlorophyll breakdown was related to protein degradation, as a progressive decline of both occurred under prolonged stress conditions.

In summary, although heat stress is not a detrimental factor for C_4 grass species, which usually respond positively to complex and simultaneous environmental conditions occurring in the field, the two species showed significant differences in their physiological response to continuous exposure to high temperature. Compared to bermudagrass, zoysiagrass showed a greater susceptibility to heat stress: differences in chlorophyll breakdown, TSS content and proteins expression revealed a different ability to species-specifically modulate its response to supraoptimal temperatures. Considering all the observed physiological parameters, bermudagrass provided a less marked response to heat stress, manifesting an enhanced thermotolerance not detected in zoysiagrass. For this species, the time-course experiment of metabolite changes during heat shock showed, in contrast, a sudden response, associated with a significant decline.

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