

Rothamsted Repository Download

A - Papers appearing in refereed journals

Carmo-Silva, A. E., Francisco, A., Powers, S. J., Keys, A. J., Ascensao, L., Parry, M. A. J. and Arrabaca, M. C. 2009. Grasses of different C4 subtypes reveal leaf traits related to drought tolerance in their natural habitats: changes in structure, water potential and amino acid content. *American Journal Of Botany*. 96, pp. 1222-1235.

The publisher's version can be accessed at:

- <https://dx.doi.org/10.3732/ajb.0800224>

The output can be accessed at: <https://repository.rothamsted.ac.uk/item/8q547/grasses-of-different-c4-subtypes-reveal-leaf-traits-related-to-drought-tolerance-in-their-natural-habitats-changes-in-structure-water-potential-and-amino-acid-content>.

© Please contact library@rothamsted.ac.uk for copyright queries.

GRASSES OF DIFFERENT C₄ SUBTYPES REVEAL LEAF TRAITS RELATED TO DROUGHT TOLERANCE IN THEIR NATURAL HABITATS: CHANGES IN STRUCTURE, WATER POTENTIAL, AND AMINO ACID CONTENT¹

ANA E. CARMO-SILVA,^{2,3,6} ANA FRANCISCO,⁴ STEPHEN J. POWERS,⁵ ALFRED J. KEYS,³
LIA ASCENSÃO,⁴ MARTIN A. J. PARRY,³ AND MARIA CELESTE ARRABAÇA²

²Centro de Engenharia Biológica (CEB) and Departamento de Biologia Vegetal, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal; ³Department of Plant Sciences,

Centre for Crop Genetic Improvement, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK;

⁴Centro de Biotecnologia Vegetal (CBV-IBB) and Departamento de Biologia Vegetal, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal; and ⁵Department of Biomathematics and Bioinformatics, Centre for Mathematical and Computational Biology, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK

Three grasses (Poaceae) of different C₄ subtypes, *Paspalum dilatatum* (NADP-malic enzyme [ME]), *Cynodon dactylon* (NAD-ME) and *Zoysia japonica* (phosphoenolpyruvate carboxykinase), occur in natural habitats that differ in annual rainfall. Their leaf characteristics were studied to identify traits related to drought tolerance. Plants were grown in pots, and water deficit was gradually induced by withholding water. Leaves of *Z. japonica* had the greatest and *P. dilatatum* the lowest relative dry matter content. Transverse sections of leaves that developed during the water deficit showed little change compared to control leaves, consistent with low phenotypic plasticity. Anatomical features distinguished the three species, with xeromorphic characteristics most strongly represented in *Z. japonica*. The leaf relative water content (RWC) decreased with the soil water content similarly for the three grasses. However, at 80% RWC, the leaf water potential was –3.1 MPa for *Z. japonica* and only –1.3 MPa for *P. dilatatum* and *C. dactylon*. Soluble amino acids, especially proline, increased as RWC decreased in leaves of *C. dactylon* and *Z. japonica*. Phenylalanine, valine, leucine, and isoleucine increased more in *Z. japonica* than in the other two species. The results provide evidence that *C. dactylon* and, especially, *Z. japonica* have evolved leaf traits better suited to arid habitats.

Key words: C₄ grasses; *Cynodon dactylon*; drought stress; leaf anatomy; *Paspalum dilatatum*; Poaceae; water deficit; *Zoysia japonica*.

Water is one of the most important factors limiting plant growth and agricultural productivity in many areas of the world. With the expected increase in aridity (Petit et al., 1999), an understanding of plant responses to drought conditions and the identification of traits appropriate to the expected climate changes are crucial to improve water use efficiency, plant production, and yields (Turner, 2004). C₄ grasses have higher water use efficiencies than their C₃ counterparts due to a greater affinity for CO₂ in combination with lower transpiration rates (Edwards et al., 1985). The presence of a CO₂-concentrating mechanism makes C₄ photosynthesis more competitive in conditions that promote carbon loss through photorespiration, such as high temperatures, high light intensities, and decreased water availability causing low intercellular CO₂ concentrations as a consequence of stomatal closure.

Natural selection pressures from changes in mean annual rainfall are likely to have influenced the diversification of the C₄ photosynthesis subtypes among the Poaceae (Hattersley and

Watson, 1992). Three classical subtypes of C₄ grasses are recognized by the main decarboxylating enzyme active in the photosynthetic pathway: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK). The geographical distribution of C₄ species (e.g., Hattersley, 1992; Cabido et al., 2008) shows that NADP-ME species are relatively more abundant in areas with higher annual rainfall, whereas NAD-ME species predominate in more arid zones and PEPCK species are less clearly associated with precipitation gradients. Brown (1999) observed that most of the cultivated C₄ species with agronomic importance are NADP-ME, possibly as a consequence of their occurrence in the wetter range of natural habitats, most appropriate for agriculture. Thus, a search for traits related to drought-resistance in NAD-ME (and/or PEPCK) species may be justified.

In addition to the specialized photosynthetic biochemistry, the leaves of most C₄ grasses have anatomical features associated with the CO₂-concentrating mechanism. These characteristics are known as Kranz anatomy (comprehensively reviewed by Dengler and Nelson, 1999). The term Kranz refers to a wreath of cells surrounding the vascular tissues and was first used by Haberlandt (1882). A suite of subtype-specific anatomical characteristics has subsequently been associated with each of the decarboxylation mechanisms (Prendergast and Hattersley, 1987; Dengler et al., 1994) and, although some variations occur (e.g., Prendergast et al., 1987), most C₄ grasses fit into one of the classical biochemical-anatomical subtypes (Hattersley and Watson, 1992). Hattersley (1992) suggested that differences in leaf anatomical structure could be associated with

¹ Manuscript received 3 July 2008; revision accepted 3 March 2009.

The authors thank Dr. S. Dufour, Rothamsted Research, for help and advice on analytical chemistry, and C. Oliveira, Olive Design, for help on quantitative analysis of leaf anatomy. A.E.C.-S. acknowledges Fundação para a Ciência e a Tecnologia for financial support (Grant SFRH/BD/13730/2003). Rothamsted Research is a grant-aided Institute of The Biotechnology and Biological Sciences Research Council.

⁶ Author for correspondence (e-mail: elizabete.carmosilva@gmail.com)

differential ability of grasses to cope with decreased water availability.

Three species of C₄ grasses of different photosynthetic subtypes were chosen for this study of drought tolerance on the basis that each not only has wild populations but also has varieties used as forage or for amenity turfgrass (Brown, 1999). The species were *Paspalum dilatatum* Poir. (subfamily Panicoideae, NADP-ME), *Cynodon dactylon* (L.) Pers (subfamily Chloridoideae, NAD-ME) and *Zoysia japonica* Steudel (subfamily Chloridoideae, PEPCK). Previous studies of the same three C₄ grasses under rapidly imposed water deficit (Carmo-Silva et al., 2007) and under slowly imposed drought stress (Carmo-Silva et al., 2008b) showed some differences in the response of photosynthesis and stomatal conductance by each of the three C₄ grasses to water deficit and suggested better drought tolerance in *C. dactylon* and *Z. japonica* than in *P. dilatatum*. However, the underlying metabolic and anatomical characteristics responsible for the differences remained unclear.

A number of morphological, physiological, biochemical, and molecular traits may increase the capacity of plants to resist unfavorable environments. Features associated with drought resistance can be adaptive, developing upon exposure to water deficit, or constitutive, resulting from selection of traits conferring better fitness to arid environments (Chaves et al., 2003). Drought resistance strategies include minimizing and tolerating tissue dehydration, which allow the maintenance of active physiological and biochemical processes. Internal regulation involving root–shoot signals and structural adjustments in the leaves are likely to play a determinant role in plant responses to water deficit (Lösch and Schulze, 1995).

Decreased rates of shoot growth constitute an early event in the response to water deficit (Saab and Sharp, 1989) and seem to be mediated by ethylene (Hussain et al., 1999). Controlled changes in leaf structure and/or anatomy in response to gradually induced drought conditions may increase plant resistance by favoring CO₂ assimilation with minimal water loss. However, severe dehydration often results in impaired membrane function and distorted organelles (Kramer and Boyer, 1995) and, ultimately, folding of the cell walls may be observed (e.g., Utrillas and Alegre, 1997). Some plants develop adaptive strategies to resist cell shrinkage in conditions of dehydration, for instance, through enhanced deposition of rigid cell wall constituents such as lignin.

Leaf dehydration can be minimized by decreasing evapotranspiration or by increasing water absorption from the drying soil. Processes that decrease water loss include closing stomata, rolling or folding leaves, and decreasing leaf water potentials (Chaves et al., 2003). Decreased leaf water potential may involve changes in turgor pressure, which depend on cell wall elasticity, or changes in the osmotic potential, which depend on the concentration of solutes in the cell (Kramer and Boyer, 1995; Lambers et al., 1998). Various roles have been ascribed to osmolytes and their metabolism in the cells, including protecting membranes and proteins, scavenging free radicals, and buffering the cellular redox potential (Hare et al., 1998). The capacity for osmotic adjustment, involving either roles in osmoprotection or in the antioxidant defense system, has been associated with drought tolerance in maize (Nayyar, 2003), sugarcane (Molinari et al., 2007), and pearl millet (Kusaka et al., 2005). Amino acids, especially proline, can increase markedly in C₄ grasses under water deficit and are likely to play a role in drought resistance (Jones, 1985). In addition to the possible contribution to osmoregulation, increased content of some

amino acids such as methionine and phenylalanine, may be also associated with increased synthesis of compounds involved in plant stress defense.

We propose that differences in the leaf structure of C₄ grasses, photosynthetic mechanism, and tissue contents may be determinants of differential rates of water loss by the leaves under conditions of decreased water availability. Therefore, shoot growth and leaf characteristics, including structure, anatomy, water relations, and amino acid content, were studied to identify traits related to differences in drought tolerance.

MATERIALS AND METHODS

Plant material and drought stress—The C₄ grasses *Paspalum dilatatum* Poir. cv. Raki (provided by AgResearch, Margot Forde Forage Germplasm Centre, New Zealand), *Cynodon dactylon* (L.) Pers var. Shangri-Lá (provided by Alípio Dias & Irmão Lda, Portugal), and *Zoysia japonica* Steudel 'Jacklin Sunrise Brand' (produced by Jacklin Seed Co., Post Falls, Idaho, USA; provided by Alípio Dias & Irmão Lda, Portugal) were grown from seeds and transferred to 1-L (15 cm deep) pots with peat-free compost in a glasshouse, as previously described (Carmo-Silva et al., 2008b). Artificial light (ca. 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was provided whenever the natural light fell below a photosynthetic photon flux density (PPFD) of 500 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ during a 16-h photoperiod. Temperature was maintained at ca. 25°C during the day and 18°C during the night. Each pot contained five plants and corresponded to an independent sample. All plants were well-watered until the beginning of the drought stress treatment.

Pots were placed according to a split-plot design, where each column of pots was a main plot of a particular species and sampling days (when applicable) and treatments (control vs. drought stress) were randomized in the split plots. Preliminary experiments showed that the decrease in water available in the soil (measured by the soil water content) and the consequent leaf dehydration (measured by the leaf relative water content, RWC) occurred at slightly different rates in the three species, with the decline in RWC starting on different days after watering the pots had been stopped. Therefore, we imposed a water deficit on the three species on consecutive days, by ceasing to provide water to the stress pots of *C. dactylon* first, *Z. japonica* next, and *P. dilatatum* last, to get more similar degrees of leaf dehydration on the sampling days. The control pots were watered once per day. Both control and drought-stressed plants of all three species were analyzed simultaneously (i.e., on the same days). Leaf samples were either taken for 3–4 consecutive days at the end of the drought period or on the last days of the drought period, which corresponded to 10, 11, and 12 d without watering the plants of *C. dactylon*, *Z. japonica*, and *P. dilatatum*, respectively. The plants of *P. dilatatum* and *C. dactylon* were 5 weeks old and *Z. japonica* 9 weeks old at sampling.

Three experiments were undertaken to obtain samples (1) for growth analysis and leaf anatomy studies, (2) for the determination of water relations, and (3) for the measurement of soluble amino acids contained in the leaves. Taking into account the different leaf sizes, we used the most recent fully expanded leaf of each plant of *P. dilatatum* and the two most recently developed fully expanded leaves of each plant of *C. dactylon* and *Z. japonica*. Samples were collected in the growth environment 4–5 h after the beginning of the photoperiod. Preliminary experiments showed that, within each pot, leaves comprising the samples taken were similar in terms of development, physiological and biochemical properties, and leaf relative water content.

Growth variables—Three control and three nonwatered pots were used per species. The number of leaves and tillers were counted on the five control and five drought-stressed plants in each pot (15 plants from three pots per species per treatment for a total of 90 plants from 18 pots) at the start of the experiment before water was withheld. After 12 d, at the end of the drought treatment, leaves and tillers were again counted.

Leaf anatomy—Leaf samples from three control and three nonwatered pots of each species (the same as used for growth analysis) were taken at the end of the drought treatment. The most recent fully expanded leaf of each plant, which had developed entirely during the period of water deficit, was chosen. Because anatomical variation occurs along the leaf length (e.g., Miranda et al., 1981), segments were taken from the middle portion of the leaf blades, equidistant from both ends. The leaf segments were fixed with 2.5% glutaraldehyde in 0.1 M

sodium phosphate buffer (pH 7.2), dehydrated in a graded ethanol series, and infiltrated and embedded with Leica Histo-resin (Leica Microsystems, Wetzlar, Germany). Transverse sections (2 μm) were cut using a rotary microtome (RM2155, Leica), stained with 0.05% toluidine blue O (Gutmann, 1995), and observed with a BX60F5 Olympus light microscope (Olympus, Tokyo, Japan). Images were recorded digitally using a DP50 Olympus camera and the Viewfinder Lite software (Pixera Corp., San Jose, California, USA).

Quantitative anatomical data from the images of leaf transverse sections were gathered using the Image-Pro Express 6.0 software (Media Cybernetics, Bethesda, Maryland, USA). Three analytical replicates were analyzed for each biological sample (with three control and three drought-stressed per species). The interveinal distance (IVD) was measured for each pair of consecutive longitudinal veins present on a half-leaf section, and the leaf thickness was measured on the thickest part of each vein on the same half-leaf section. The transverse half-leaf sections were divided into four different zones, from the center to the margin of the leaf, to have a representative sampling of the leaf anatomy. These four zones corresponded to (1) the central longitudinal vein, (2) a large longitudinal vein, (3) two small longitudinal veins between the center and the margin of the leaf, and (4) the two small longitudinal veins closest to the margin. The cross-sectional areas occupied by mesophyll (M) cells, bundle sheath (BS) cells, intercellular spaces (ICS), vascular tissues (VT), bulliform water storage (WS) cells penetrating the mesophyll, lower and upper epidermis (LE and UE), and sclerenchyma (S) were measured and expressed as a percentage of the total area measured of the half-leaf. These percentages were subsequently used to calculate the ratio between the area occupied by mesophyll and bundle sheath cells (M/BS).

Water content in leaves and soil—Four control and six nonwatered pots were used per species per day, for the last 4 days of the drought stress, making a total of 40 samples per species (16 control and 24 drought-stressed). One sample, consisting of two leaves for *P. dilatatum* and five leaves for *C. dactylon* or *Z. japonica*, was collected from each pot to determine the leaf relative water content (RWC). Fresh (FM), turgid (TM), and dry (DM) masses were measured and used to calculate RWC (Catsky, 1960). Leaf area was determined by scanning the turgid leaves and analyzing each image using the software Paint Shop Pro 9 (Jasc Software, Eden Prairie, Minnesota, USA) and Image J 1.33u (National Institutes of Health, Bethesda, Maryland, USA). The leaf water potential (LWP) was measured on the middle part of a recently developed fully expanded leaf, taken from each pot 5 h after the beginning of the photoperiod, using a pressure chamber (Ritchie and Hinckley, 1975). The soil water content (SWC) was determined in the upper soil layer, to a depth of 7–8 cm, in three opposite locations in each pot, using an HH2 moisture meter with a Theta probe (type ML2 \times , AT Delta-t Devices Ltd., Cambridge, UK) calibrated in the growing medium.

HPLC analysis of amino acids—Amino acids contained in leaf samples were determined by high-performance liquid chromatography (HPLC) of *o*-phthalaldehyde (OPA) derivatives as described in Carmo-Silva et al. (2008b). Reverse-phase HPLC was performed using a Waters Alliance 2695 Separation Module and a 474 Scanning Fluorescence Detector operated by Millennium³² software (Waters, Milford, Massachusetts, USA) with a Waters Symmetry C₁₈ 4.6 \times 150 mm column (part no. WAT 054278) protected with a 4 \times 3 mm guard cartridge (Phenomenex, Torrance, California, USA). The eluent used for the amino acid separation was obtained by mixing solvents containing different proportions of methanol, sodium acetate solution (pH 5.9), and tetrahydrofuran. Three control and five nonwatered pots were used per species per day during the last three consecutive days of the water deficit treatment, making a total of 24 samples per species (nine control and 15 drought-stressed). Each sample consisted of one leaf for *P. dilatatum* and three leaves for *C. dactylon* or *Z. japonica*. Amino acids were extracted from the leaf samples that had been quickly frozen in liquid N₂ (LN₂) and stored at –80°C, using 0.1 M HCl. Standard solutions of α -amino-*n*-butyric acid (used as internal standard) and of each amino acid present in the leaf extracts were prepared in 0.1 M HCl and diluted to have increasing concentrations for the respective calibration curves (0–25 μM).

Proline estimation—The proline content in the described acid leaf extracts was determined after reaction with ninhydrin (Bates et al., 1973). Standard solutions of proline with concentrations rising from 0 to 0.9 mM in 0.1 M HCl were used for the calibration curve. A volume of 0.25 mL was taken from each sample or standard solution into a centrifuge tube (15 mL) and mixed with 0.75 mL of 3% sulfosalicylic acid, 1 mL of glacial acetic acid and 1 mL of acid-ninhydrin reagent (140 mM ninhydrin in a solution 2:3 of glacial acetic acid and 6 M orthophosphoric acid), added consecutively. The tubes were thoroughly

vortexed and incubated at 100°C for 1 h. The reaction was stopped by placing the tubes on ice for 5 min. Toluene (2 mL) was added to each tube, and after mixing thoroughly, the phases were allowed to separate, and the absorbance of the upper phase at 520 nm was measured (CARY 300 Bio UV-Visible Spectrophotometer, Varian Analytical Instruments, Varian, Surrey, UK) using toluene as background.

Statistical analyses—All data were analyzed using the software GenStat 9.2 (Lawes Agricultural Trust, Rothamsted Research, Harpenden, UK). Analysis of variance (ANOVA) was applied to the shoot growth and leaf structure parameters and the leaf anatomy measurements to check for statistically significant differences (*F*-tests, $P < 0.05$) between species, treatments (control vs. drought stress) and for the interaction between these two factors. The number of leaves and tillers in the control and drought-stressed plants at the beginning of the drought period was found to be not significantly different between the two treatments for each species (leaves: $F_{1,12} = 0.06$, tillers: $F_{1,12} = 0.11$; $P > 0.05$). A square-root transformation was applied to the number of leaves and tillers produced per plant (to ensure that the assumptions of the analysis were not violated), and the sets of five individual plants in each pot were taken as analytical replicates. A logit transformation, given by $\log_e[\text{DW}/(100 - \text{DW})]$, was applied to the relative dry matter content. A \log_e transformation was applied to the values of the interveinal distance, the leaf thickness and to the different types of cellular areas or the ratio M/BS. The number of veins in each half-leaf was analyzed through a generalized linear mixed model (GLMM) using the fitting method of Schall (1991), assuming a Poisson distribution for the data and with a \log_e link function for the model, which takes account of the analytical replicates within samples. This method was used because the data were not normally distributed, even after transformation. The least significant difference (LSD) at the 5% level of significance was used to test between relevant means.

The relationship between RWC and SWC and between LWP and RWC was assessed through the use of nonlinear regression, fitting empirical exponential models and accounting for differences between species by way of model parameters. Regression analysis was applied to model the variation of the amino acid content with RWC, considering a squared term in this variable to check for nonlinearity. Nested models were compared using *F*-tests and then the nonsignificantly different parameters (*t*-tests, $P > 0.05$) in the significant terms (*F*-tests, $P < 0.05$) of each model were amalgamated to attain parsimony. The residuals were checked and found to conform to the assumptions of the analysis.

RESULTS

The three C₄ grasses of different subtypes, *Paspalum dilatatum* (NADP-ME), *Cynodon dactylon* (NAD-ME), and *Zoysia japonica* (PEPCK), differed in their response to water deficit, confirming the presence of more efficient drought tolerance mechanisms in *C. dactylon* and *Z. japonica* than in *P. dilatatum*.

Shoot growth and leaf structure—The number of leaves and tillers produced during the water deficit treatment was lower in the drought-stressed relative to the control plants of all three species (Fig. 1). Plants had an average of ca. 3 (*P. dilatatum*), 5 (*C. dactylon*), or 15 (*Z. japonica*) fully expanded leaves at the beginning of the drought treatment. These numbers increased during the period when water was withheld, such that, at the end of the experiment, control and drought-stressed plants had, respectively, ca. 10 and 9 (*P. dilatatum*), 23 and 19 (*C. dactylon*), or 32 and 29 (*Z. japonica*) fully expanded leaves. There was a highly significant difference (leaves: $F_{2,12} = 72.6$, tillers: $F_{2,12} = 65.8$; $P < 0.001$) between species and a strong effect (leaves: $F_{2,12} = 8.6$, tillers: $F_{2,12} = 11.1$; $P < 0.01$) of drought stress on the square root of the number of leaves and tillers formed per plant during the period of stress, the stress effect being similar for the three species (Appendix 1).

The specific leaf area (SLA) decreased in the drought-stressed compared to the control plants of *P. dilatatum* and *C. dactylon* (Fig. 2A), essentially a result of increased dry matter content (Fig. 2B) because the leaf area per total turgid leaf mass was not

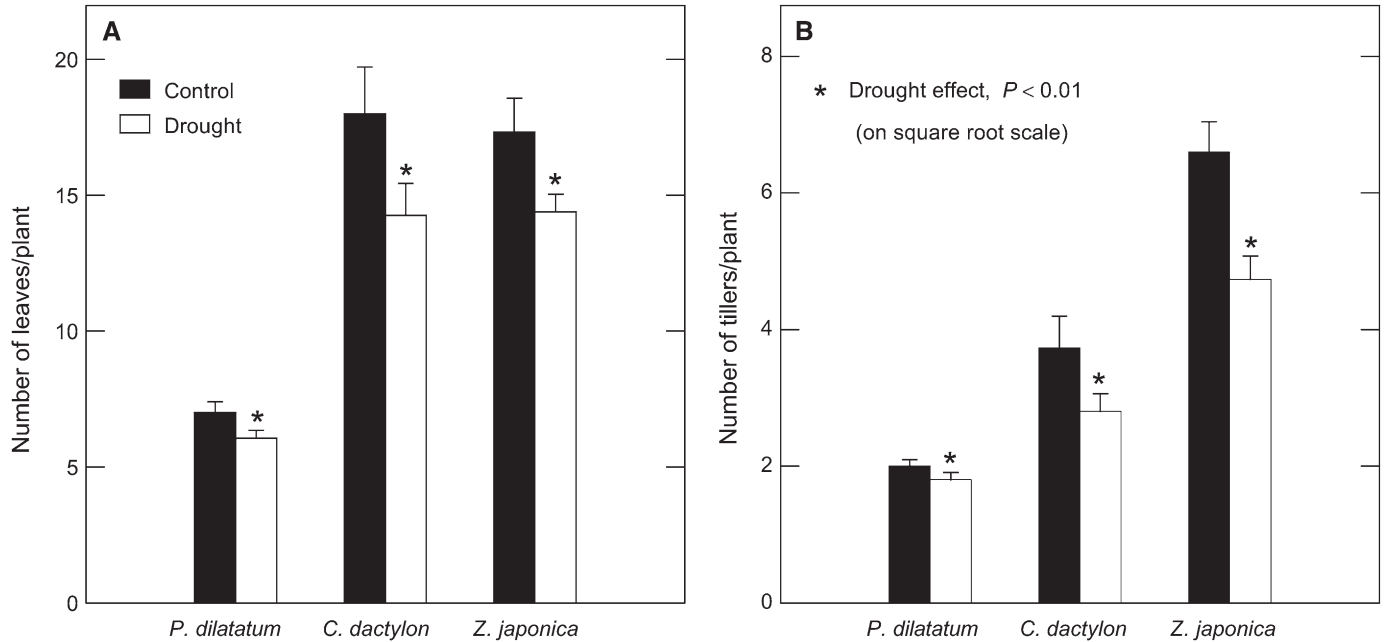


Fig. 1. Number of (A) leaves and (B) tillers produced in 12 d by the control (black bars) and drought-stressed (white bars) plants of *Paspalum dilatatum*, *Cynodon dactylon*, and *Zoysia japonica*. Values are means \pm SE of 15 plant samples. (See Appendix 1 for statistical analysis.)

affected by water deficit (results not shown). In *Z. japonica*, the percentage of leaf dry matter was greater, and the SLA smaller, than in *C. dactylon* and *P. dilatatum*, and these were not affected by drought stress (DM: $t = 1.5$, $df = 113$; SLA: $t = 0.6$, $df = 113$; $P > 0.05$).

C₄ leaf anatomy—The three C₄ grasses have typical Kranz anatomy for their C₄ subtype: the bundle sheath (BS) cells,

filled with numerous chloroplasts, surround the vascular tissues (VT) and are surrounded by the mesophyll (M) cells, with fewer and smaller chloroplasts (Fig. 3). In *C. dactylon* (NAD-ME) and *Z. japonica* (PEPCK), but not in *P. dilatatum* (NADP-ME), a mestome sheath is present between the BS cells and the VT. The BS chloroplasts are centripetal in *C. dactylon* and centrifugal or scattered around the cells in *P. dilatatum* and *Z. japonica*. The M arrangement is more compact and the intercellular

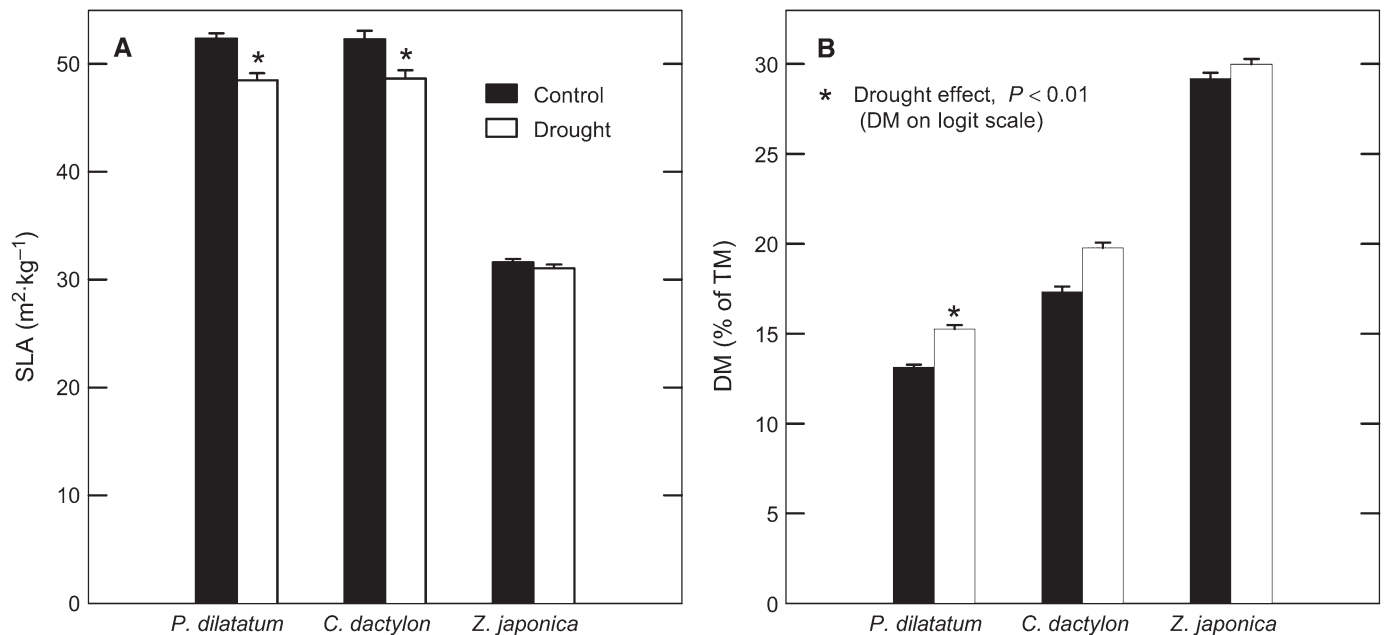


Fig. 2. (A) Specific leaf area (SLA) and (B) leaf dry mass as a percentage of turgid mass (DM/TM \times 100) for control (black bars) and drought-stressed (white bars) plants of *Paspalum dilatatum*, *Cynodon dactylon*, and *Zoysia japonica*. Values are means \pm SE of 16 (control) or 24 (drought-stressed) plant samples. (See Appendix 1 for statistical analysis.)

spaces (ICS) less abundant in *Z. japonica* than in *C. dactylon* and, especially, *P. dilatatum* (Figs. 3 and 4).

Girders of sclerenchyma were observed particularly below the large veins and in greatest proportion in *Z. japonica* (Fig. 3). This support tissue was also present at the leaf margins of *Z. japonica*, in extensive tracts, and of *P. dilatatum*, in smaller tracts, but not of *C. dactylon* (Fig. 4).

Water storage tissue, represented by colorless, highly vacuolated bulliform cells, was present in the three grasses but distributed differently. In *P. dilatatum*, water storage cells were spread across the adaxial surface of the leaf blade, constituting most of the upper epidermis (Figs. 3 and 4), and were very abundant above the prominent midrib region (Fig. 5). Conversely, in *C. dactylon* and *Z. japonica* bulliform water storage cells were present in grooves between each pair of consecutive veins, forming longitudinal rows along the leaves (Figs. 3 and 5).

The relative area occupied by the different tissues in the half-leaf transverse sections highlighted the greater relative area of BS in *C. dactylon* and *Z. japonica* than in *P. dilatatum* and the smaller relative area of M in *Z. japonica* (Fig. 6), so that the M/BS tissue ratio was greater in *P. dilatatum* than in *C. dactylon* and was lowest in *Z. japonica*. The gradual decrease in water availability did not cause changes in the relative leaf tissue areas, except for a slight variation of the M/BS tissue ratio (Fig. 6). Statistical analysis (on the \log_e scale) revealed an overall marginal effect ($F_{1,12} = 3.2$; $P < 0.1$) of drought stress, with an increase in the M/BS tissue ratio with water deficit in the leaves of *P. dilatatum* and, to a smaller extent, in *Z. japonica*, but not in *C. dactylon*.

Drought stress caused no significant changes in the leaf thickness, vein number, or interveinal distance (Fig. 7) (leaf thickness: $F_{1,12} = 0.3$, vein number: $F_{1,48} = 0.6$, interveinal distance: $F_{1,12} = 0.3$; $P > 0.05$). Leaf thickness was lowest in *Z. japonica*, and the number of veins was greatest in the leaves of *P. dilatatum*, which are wider and longer than in the other two grasses. These features also reflect the more compact cell arrangement in *Z. japonica*.

Water relations—The leaf relative water content (RWC) of *P. dilatatum*, *C. dactylon*, and *Z. japonica* started to decrease only when the amount of water in the soil decreased below a certain threshold (~10% SWC), so that the variation of RWC with SWC could be described by an asymptotic exponential model (Fig. 8A), with no significant difference between the three species ($F_{2,15} = 2.2$; $P > 0.05$; Appendix 2). In fully hydrated leaves of *P. dilatatum* and *C. dactylon*, the leaf water potential (LWP) was higher than in *Z. japonica* (Fig. 8B) and drought-stressed plants of the latter species showed a decrease of LWP to more negative values. An exponential model explained the variation of LWP with RWC in *P. dilatatum* and *C. dactylon*: the LWP decreased from -0.6 MPa in fully hydrated leaves to -1.3 MPa in nonwatered plants with 93% RWC and then remained constant for further decreased RWC. On the contrary, in *Z. japonica* the LWP decreased linearly with RWC, from a value of -1.2 MPa in fully hydrated leaves to -3.5 MPa in the most dehydrated leaves.

Amino acids—Despite the attempts to obtain leaf samples of the three species with similar levels of dehydration, in the amino acid experiment, the plants of *Z. japonica* attained lower RWC values (~40%) than those of *C. dactylon* (~60%) and *P. dilatatum* (~80%). Proline increased by about 2.5-fold in the three species

when the RWC decreased to 80% and rose steeply as the RWC decreased further (Fig. 9A; Appendix 2). The content of methionine was higher in *Z. japonica* and *C. dactylon* than in *P. dilatatum* and increased with the decrease of RWC in a nonlinear way, more accentuated as leaf dehydration increased (Fig. 9B). The amino acids phenylalanine, valine, isoleucine, and leucine were also much increased in the dehydrated leaves of *Z. japonica* (Fig. 9C–F). In *P. dilatatum* and *C. dactylon*, the content of leucine was negligible, and the other three amino acids increased with drought stress to a much lower extent in these species than in *Z. japonica*. Nevertheless, in *C. dactylon*, valine increased threefold when RWC decreased from 98 to 60%.

DISCUSSION

The response of the three different C_4 grasses to drought stress at the whole plant and leaf anatomical and biochemical levels reveal the relative differences in their strategies to cope with drought stress. Although some responses were common to all three species, specific adaptive or constitutive characteristics relate to the increased drought tolerance in *Cynodon dactylon* (NAD-ME) and *Zoysia japonica* (PEPCK) in comparison with *Paspalum dilatatum* (NADP-ME).

Drought-induced changes included decreased shoot growth and decreased leaf water content in the three species, increased leaf relative dry matter in *P. dilatatum* and *C. dactylon* (to values closer to those observed in *Z. japonica*), decreased leaf water potentials attaining lower values in *Z. japonica* than in *P. dilatatum* and *C. dactylon*, and increased contents of soluble amino acids in the leaves of the three species, but more notably in *C. dactylon* and especially *Z. japonica*. The leaf anatomy and tissue relative patterns showed no substantial changes with drought but revealed constitutive characteristics associated with better drought tolerance in *Z. japonica* and, to some extent, *C. dactylon*, than in *P. dilatatum*.

C_4 photosynthesis evolved from the C_3 pathway in response to decreased carbon dioxide and increased oxygen concentrations in the atmosphere, which can increase photorespiration. Conditions that favor this process, such as increases in temperature, aridity, and soil poverty, are driving forces to the evolution of C_4 traits (Sage, 2004). Our results support the hypothesis that aridity may also play a role in the evolution of the three different subtypes of C_4 grasses.

Drought stress induced decreased shoot growth but caused only minor changes in the leaf anatomical structure of the three grasses, revealing decreased plasticity with increased specialization toward better fitness to aridity—Plants of the three grass species produced fewer leaves and tillers in response to the drying soil. Decreased shoot elongation rates were also observed by Ghannoum et al. (2002) in several C_4 grasses in response to decreased water availability. The decreased shoot growth and decreased photosynthetic CO_2 assimilation rates (Carmo-Silva et al., 2008b) constitute an adaptive response to drought stress, and under such conditions, energy can be diverted to the synthesis of molecules involved in plant defense (Chaves and Oliveira, 2004).

The specific leaf area (SLA) decreased and the relative leaf dry matter increased with drought stress in *P. dilatatum* and *C. dactylon*, but did not change significantly ($P > 0.05$) in *Z. japonica*. The latter species is more xeromorphic, having constitutively higher relative leaf dry matter contents (and lower

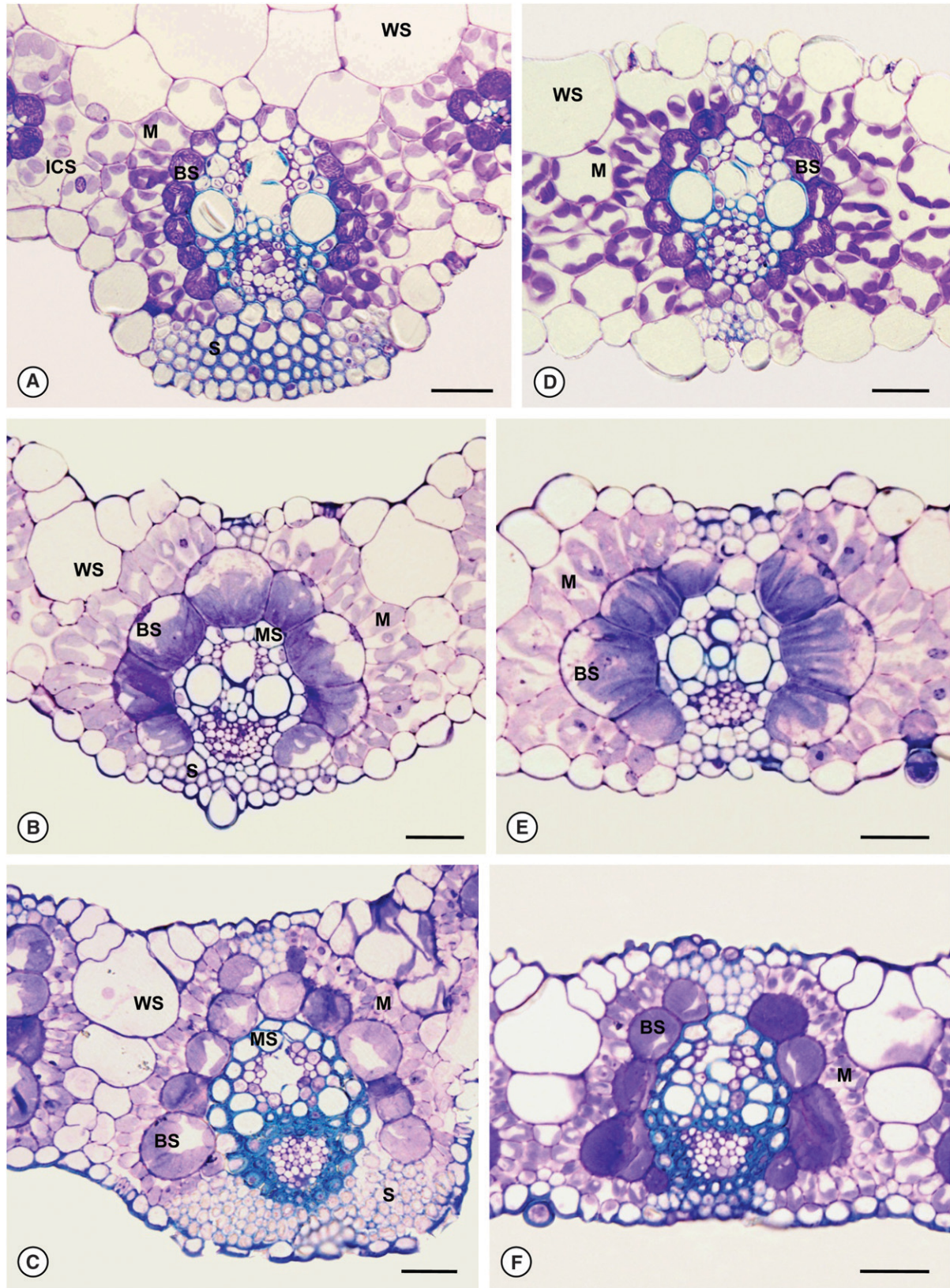


Fig. 3. Light micrographs of transverse sections of recently fully expanded leaves from well-watered plants of (A, D) *Paspalum dilatatum*, (B, E) *Cynodon dactylon*, and (C, F) *Zoysia japonica* stained with toluidine blue O. (A–C) Central longitudinal vein and (D–F) one large longitudinal vein of a half-leaf where cellular areas were measured are shown. BS, bundle sheath; ICS, intercellular spaces; M, mesophyll; MS, mestome sheath; S, sclerenchyma; WS, water storage cells. Scale bars = 30 μ m.

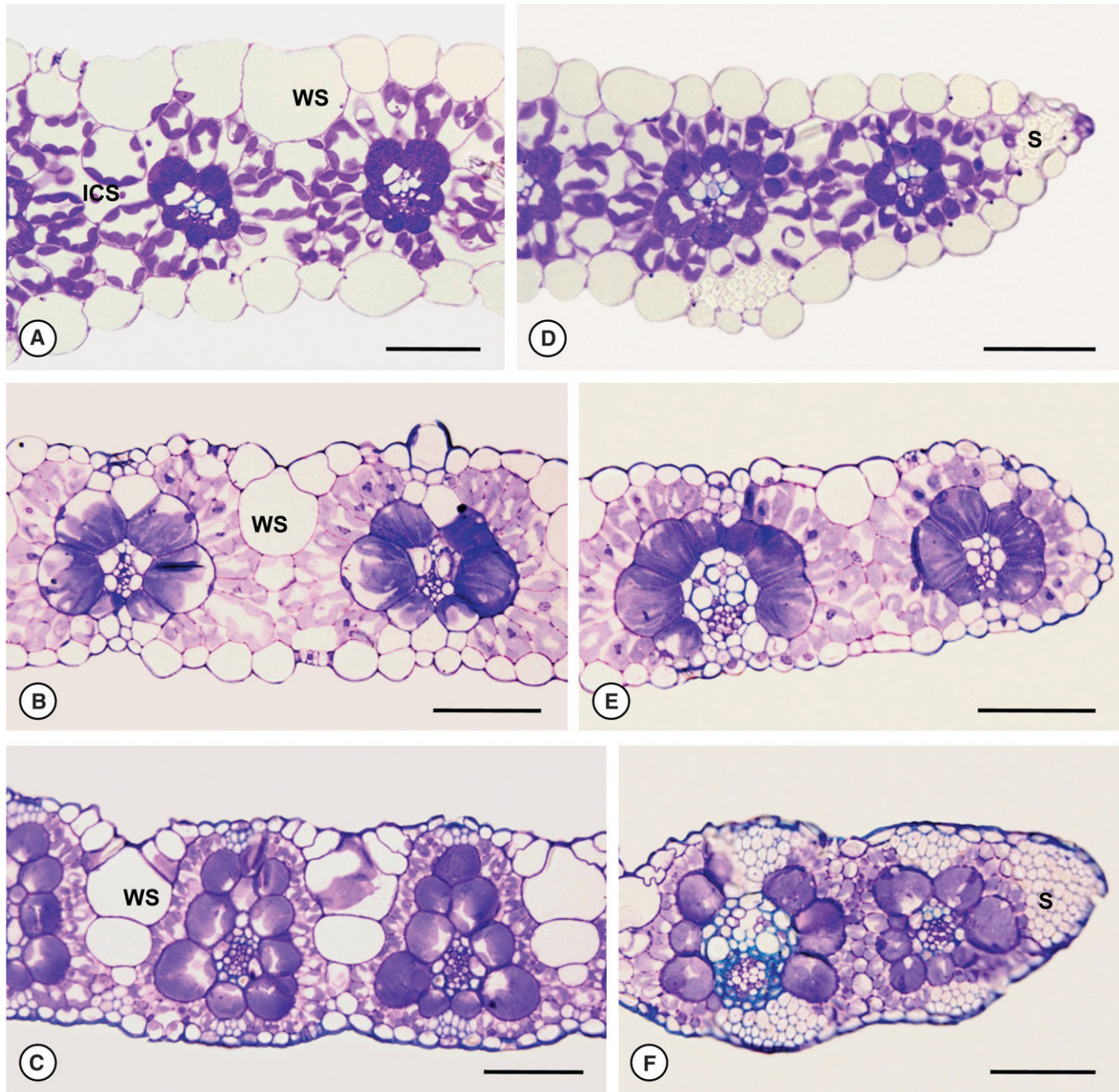


Fig. 4. Light micrographs of transverse sections of recently fully expanded leaves from well-watered plants of (A, D) *Paspalum dilatatum*, (B, E) *Cynodon dactylon*, and (C, F) *Zoysia japonica* stained with toluidine blue O. Two small longitudinal veins (A–C) between the center and the margin or (D–F) at the margin of one half-leaf where cellular areas were measured are shown. ICS, intercellular space; S, sclerenchyma; WS, water storage cells. Scale bars = 60 μm .

SLA) than *P. dilatatum* and *C. dactylon*. The increased leaf relative dry matter is an adaptive response to water deficit and may be associated with increased production of starch or soluble compounds. The lack of changes in *Z. japonica* indicates diminished plasticity in this PEPCK species compared to the NADP-ME (*P. dilatatum*) and NAD-ME species (*C. dactylon*), possibly as a consequence of the high degree of specialization already present in the well-watered leaves of this species. A

correlation between lower SLA and better ability to maintain leaf turgor under water deficit has previously been reported (Mitchell et al., 2008).

The slowdown in shoot growth provides evidence that the plants of the three C_4 grasses adjusted their development in response to the decreased soil water content. Nevertheless, moderate water deficit caused only minor changes in the leaf anatomy of the three C_4 grasses, supporting the idea that C_4 spe-

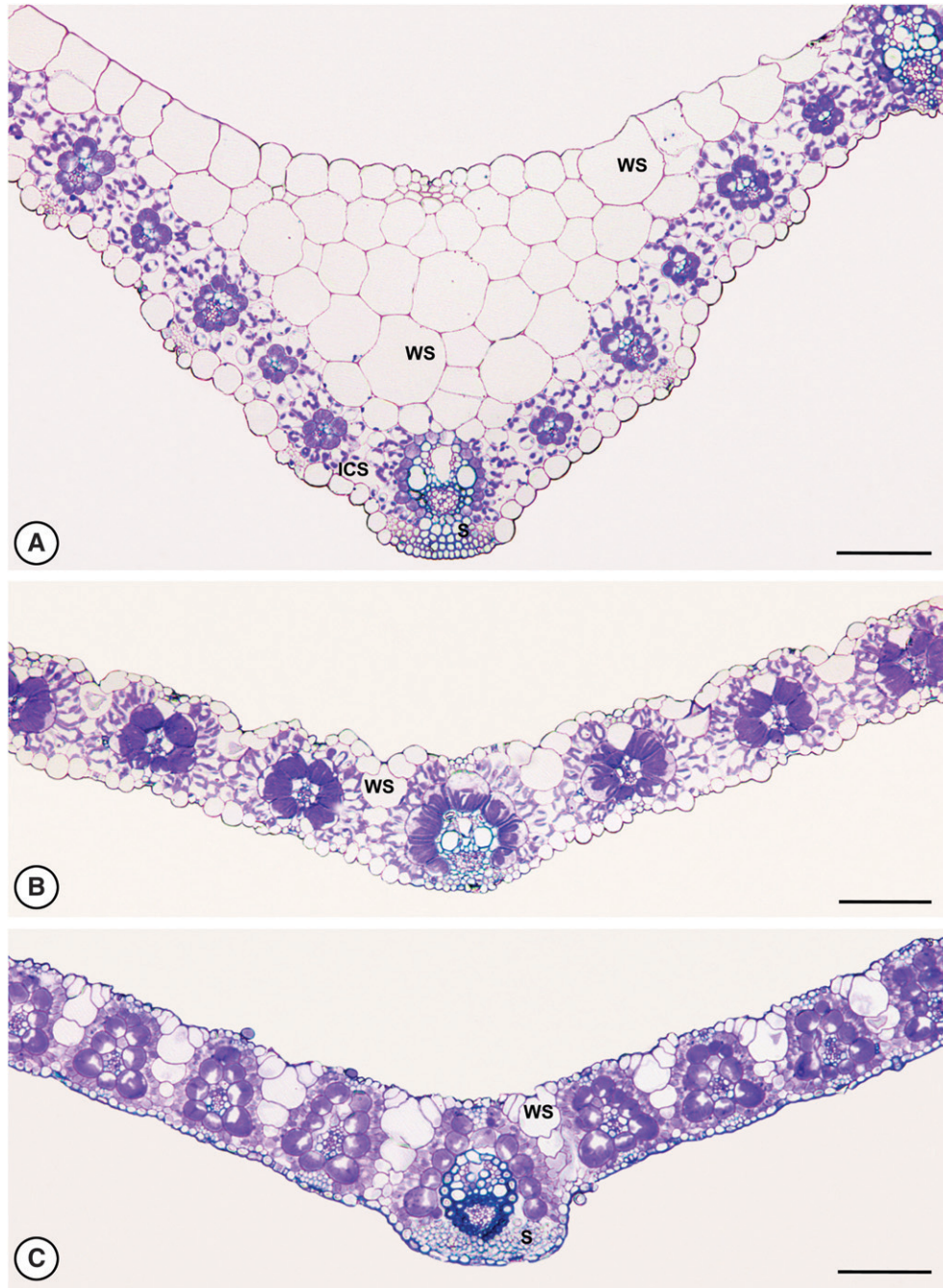


Fig. 5. Light micrographs of transverse sections of recently fully expanded leaves from well-watered plants of (A) *Paspalum dilatatum*, (B) *Cynodon dactylon*, and (C) *Zoysia japonica* stained with toluidine blue O. The central area of the leaf including the midrib is shown. ICS, intercellular space; S, sclerenchyma; WS, water storage cells. Scale bars = 100 μ m.

cies are relatively less plastic than C_3 species (Sage and McKown, 2006).

Water storage bulliform (WS) cells, present in the leaves of the three grasses, differed in their position relative to the vascular bundles. In the leaves of *P. dilatatum*, WS cells are spread over the surface and in several layers above the midrib. The loss of water from these cells results in the folding of the leaves when dehydration becomes severe. In *C. dactylon* and *Z. japonica*, WS cells are located in the central plane of the leaf, penetrating the mesophyll between each pair of consecutive veins,

more deeply in the PEPCK species where occasionally a “bridge” is formed between the upper and lower epidermis. The differential arrangement of these WS cells results in leaf shrinkage in *C. dactylon* and leaf curling in *Z. japonica* as dehydration becomes severe. The reduction of evaporative surface area decreases water loss and also light absorption, minimizing photo-inhibitory injury (Kramer, 1983).

The leaves of *Z. japonica* are characterized by a more compact arrangement of the cells and by the lowest M/BS tissue ratio in comparison with the leaves of the other two grasses.

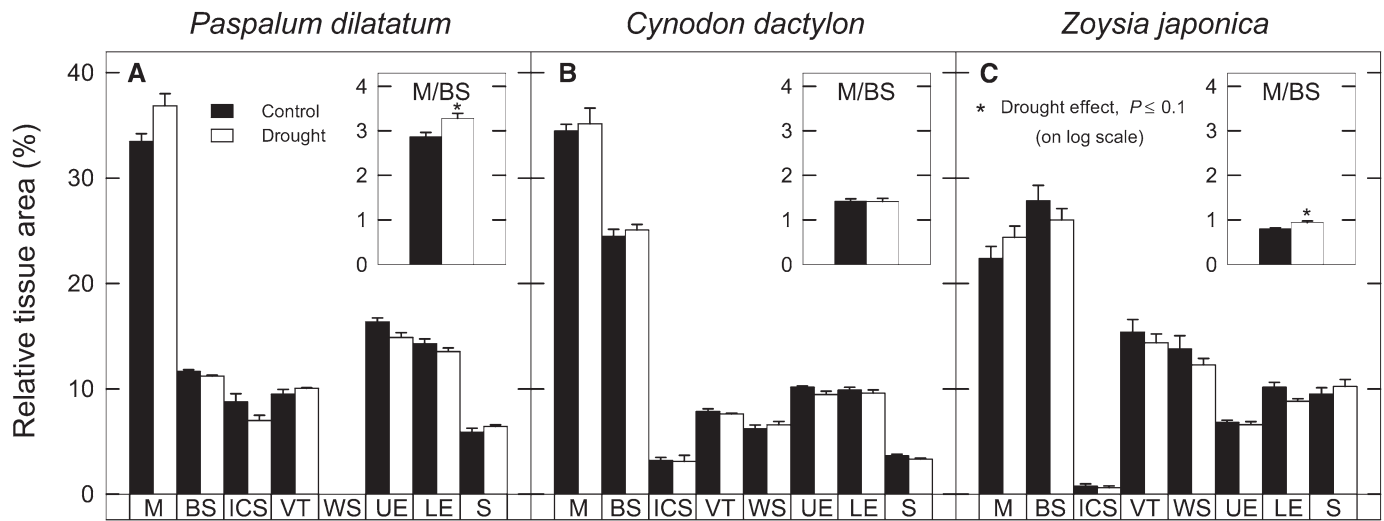


Fig. 6. Relative area occupied by each type of tissue in the half-leaf transverse sections: M, mesophyll cells; BS, bundle sheath cells; ICS, intercellular spaces; VT, vascular tissues; WS, bulliform water storage cells penetrating the mesophyll; UE, upper epidermis; LE, lower epidermis; S, sclerenchyma. The M/BS tissue ratio is also shown. Control (black bars) and drought-stressed (white bars) plants of *Paspalum dilatatum*, *Cynodon dactylon*, and *Zoysia japonica* were analyzed at the end of the drought period. Values are means \pm SE of three leaf sections from each of three leaf samples.

The high proportion of area occupied by BS cells, which are characterized by thicker cell walls (Dengler and Nelson, 1999), is consistent with the high relative leaf dry matter observed in *Z. japonica*. The higher abundance of sclerenchyma in *Z. japonica* is also likely to be associated with leaf stiffness and stronger xeromorphic features compared to the other two species. Additionally, the higher relative area occupied by vascular tissues in this species may reflect increased capacity for water uptake, as previously suggested for maize lines with differential drought resistances (Ristic and Cass, 1991). Anatomical features similar to those observed in *Z. japonica* were also reported in the C_4 grass *Sporobolus stapfianus*, a resurrection species (Vecchia et al., 1998).

In many C_4 grasses, an increased vein density is associated with a low M/BS tissue ratio and with lower vein-associated BS tissue per unit leaf surface (Hattersley, 1984; Dengler and Taylor, 2000). Studies of vein patterning were summarized by Dengler and Nelson (1999) and by Ogle (2003). The shift in vein density in C_4 grasses is related to increased minor and cross

veins, rather than major veins (Ueno et al., 2006). Vascular tissue is an expensive component for plants to make because lignin is one of the most energetically expensive molecules to synthesize. C_4 plants maintain a high vein density and a low interveinal distance (IVD) because of the requirement for close proximity of M and vein-associated BS cells, facilitating a rapid cycling of C_4 metabolites (Sage and McKown, 2006). The same authors report that the mean IVD in C_4 plants ($120 \mu\text{m}$) is less than half that observed in C_3 plants ($280 \mu\text{m}$), and that these lower IVD values are correlated to a decrease in M/BS tissue ratio.

The M/BS tissue ratio was greater in *P. dilatatum* than in the other two species, which agrees with previous descriptions for NADP-ME C_4 grasses (Hattersley, 1984; Dengler et al., 1994). The M/BS tissue ratio was increased by moderate drought in leaves of *P. dilatatum* and very slightly in *Z. japonica*, compared to well-watered plants. The ability of plants to alter their development and adjust, or acclimate, to changing environmental conditions may help to maintain photosynthetic performance, but must not disturb the functionality of the C_3 and C_4

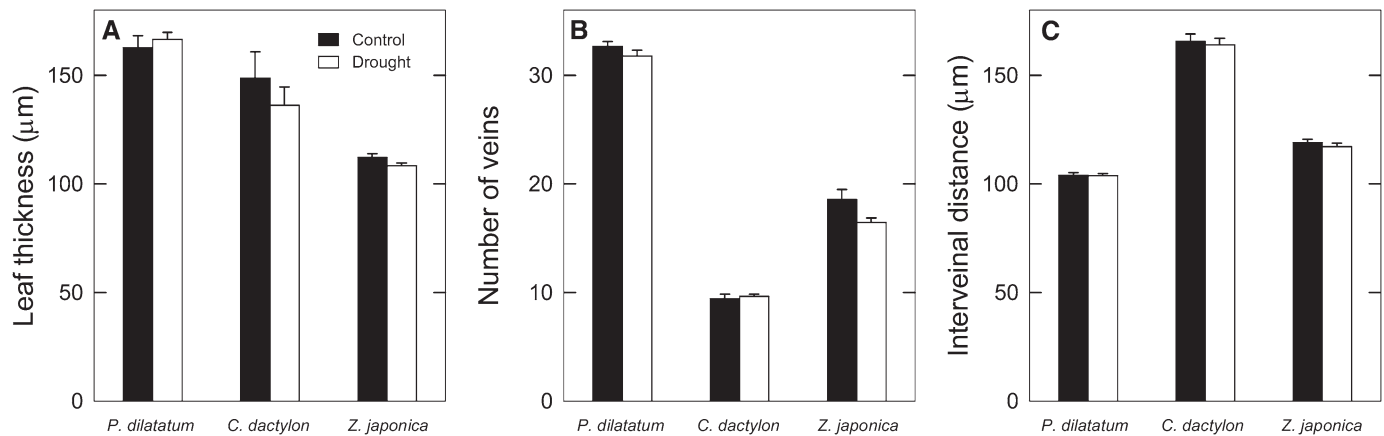


Fig. 7. (A) Leaf thickness, (B) number of veins per half-leaf, and (C) interveinal distance of control (black bars) and drought-stressed (white bars) plants of *Paspalum dilatatum*, *Cynodon dactylon*, and *Zoysia japonica*. Values are means \pm SE of three leaf sections from each of three leaf samples.

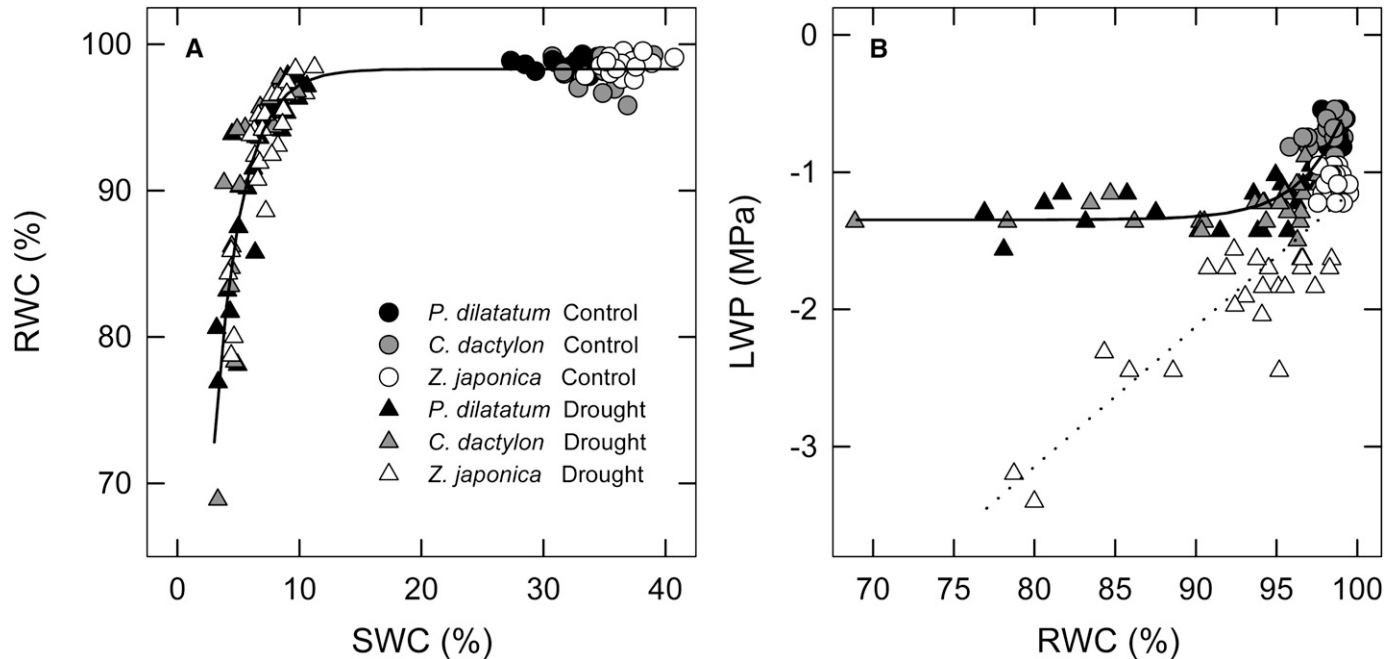


Fig. 8. Variation in water relations in the leaves of control (circles) and drought-stressed (triangles) plants of *Paspalum dilatatum* (black), *Cynodon dactylon* (gray), and *Zoysia japonica* (white). (A) Relative water content (RWC) plotted against soil water content (SWC) and (B) leaf water potential (LWP) in relation to RWC. Each point corresponds to one sample ($N = 120$). (See Appendix 2 for statistical analysis.)

cycles (Sage and McKown, 2006). Increases in the M/BS tissue ratio may increase the diffusion distances, interfere with diffusion pathways, or enhance pathways for CO₂ leakage from the BS cells, decreasing photosynthetic capacity and water use efficiency. In *C. dactylon*, there were no visible leaf anatomical adjustments in response to moderate water deficit, suggesting that the high photosynthetic performance, observed even with dehydrated leaves (Carmo-Silva et al., 2007, 2008b), may involve mostly biochemical adjustments. Impairment of structural functionality may, however, be observed in plants of this species under severe drought (Utrillas and Alegre, 1997).

Water loss from leaves is minimized more efficiently by *C. dactylon* and *Z. japonica* than by *P. dilatatum* through the use of different strategies—Decreased water availability in the soil resulted in decreased leaf relative water content (RWC) in the three C₄ grasses, but the leaf water potential (LWP) revealed a different strategy to avoid water loss in *Z. japonica* than in *P. dilatatum* or *C. dactylon*. The linear decrease of LWP with decreasing RWC is consistent with the presence of rigid cell walls in the leaves of *Z. japonica* (White et al., 2001), as indicated by the hardness, toughness, and stiffness of leaves of this grass species and by the higher relative dry matter than in the other two species. The presence of rigid cell walls promoting the decrease of LWP in response to water deficit avoids excessive leaf dehydration by favoring water absorption through the vascular system. The accumulation of compatible solutes, namely, amino acids such as proline, may also contribute to the decreased leaf water potential, through their involvement in osmotic adjustment (discussed later). Both the capacity for osmotic adjustment and the cell wall rigidity tend to be greater in C₄ than in C₃ grasses (Barker et al., 1993), and our results suggest that both features are greater in *Z. japonica* (PEPCK) than in *C. dactylon* (NAD-ME), and even more than in *P. dilatatum* (NADP-ME).

Stomatal conductance decreased earlier than photosynthesis under water deficit in the three C₄ grasses (Carmo-Silva et al., 2008b), slightly enhancing their water use efficiency under such conditions. The results obtained in that study suggested that stomatal closure is triggered early in response to decreased water availability, effectively minimizing water loss and contributing to the maintenance of high RWC values in the initial stages of water deficit. Importantly, in *C. dactylon*, the decrease in stomatal conductance was relatively more efficient than in the other two grasses. In *Z. japonica*, severe dehydration is impeded by leaf curling, which minimizes the surface for water evaporation, simultaneously with continuous lowering of the LWP. In *P. dilatatum*, a faster decrease in water availability in the soil resulted in the need to decrease the number of days without watering the plants of this species to attain similar degrees of dehydration for the three grasses. The faster drying of the soil reflects the high productivity of the NADP-ME species, at the expense of high water consumption. It is also a consequence of the different phenotype of *P. dilatatum*, producing bigger and fewer leaves than the other two grasses, and of the lower relative leaf dry matter (i.e., higher proportion of the leaf constituted by water) than in *C. dactylon* and *Z. japonica*.

Increased content of specific amino acids in the C₄ grass leaves provide evidence for biochemical adjustments in response to water deficit—The increased content of amino acids in the drought-stressed leaves of the three species is in accordance with previously reported data obtained for various C₄ grasses (Barnett and Naylor, 1966; Jones et al., 1980; Ford and Wilson, 1981; Kusaka et al., 2005). The accumulation of amino acids is likely to result mostly from their increased synthesis and may reflect the need for enhanced production of secondary metabolites with roles in plant stress defense. Additionally, the

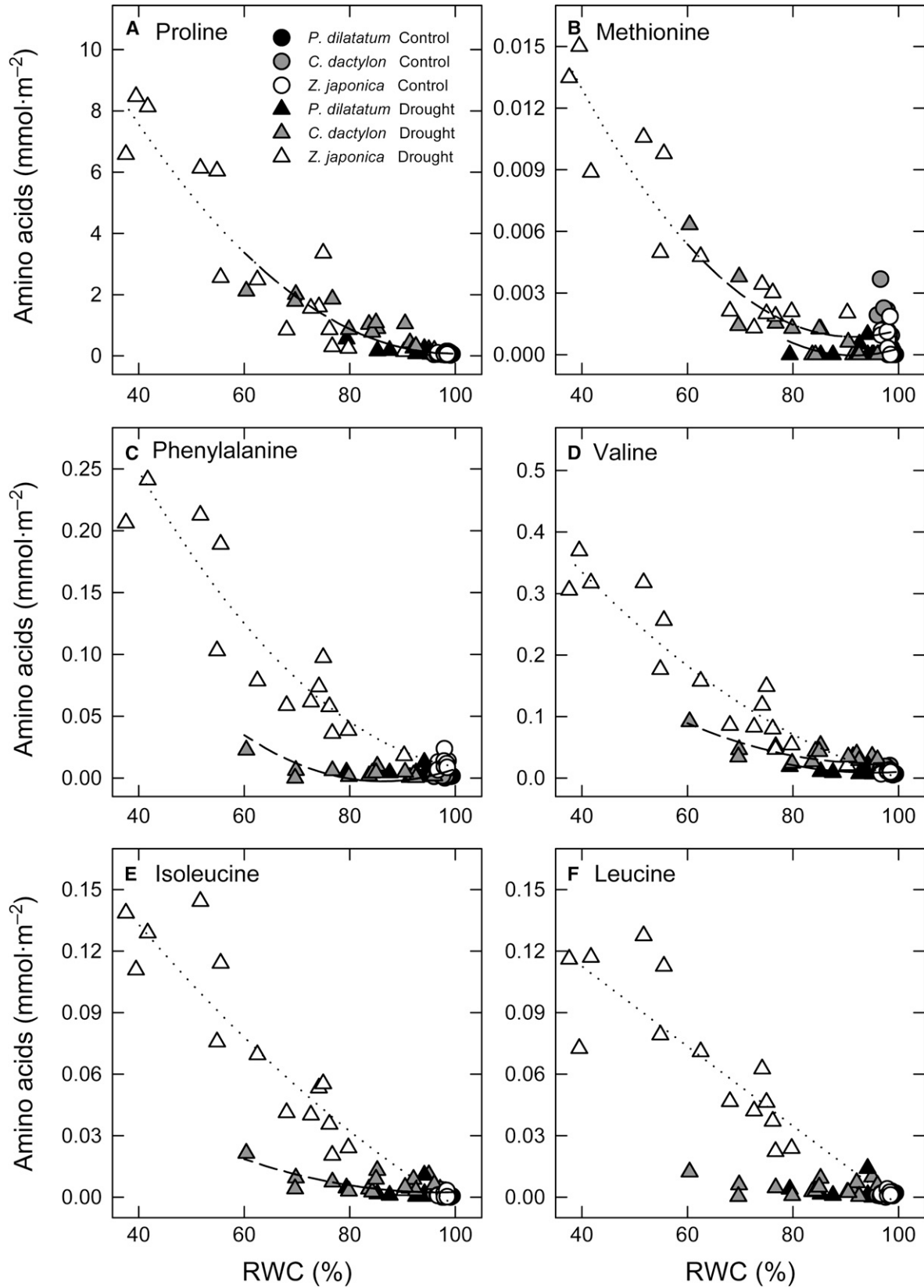


Fig. 9. Variation in amino acids content in relation to the relative water content (RWC) of control (circles) and drought-stressed (triangles) leaves of *Paspalum dilatatum* (black), *Cynodon dactylon* (gray), and *Zoysia japonica* (white). (A) proline, (B) valine, (C) methionine, (D) phenylalanine, (E) isoleucine, (F) leucine. Each point corresponds to one sample ($N = 72$). (See Appendix 2 for statistical analysis.)

active biosynthesis of some amino acids uses excess reducing power when photosynthetic carbon assimilation is decreased and confers protection against reactive oxygen species (e.g., Hare and Cress, 1997).

The proline content doubled when RWC decreased to ~80% in the leaves of the three species, but major accumulation occurred only under severe leaf dehydration, as previously described for C₄ grasses (Jones, 1985). Proline is one of the compatible solutes most widely accumulated in response to hyperosmotic stresses (Delauney and Verma, 1993). However, in several C₄ grasses, although proline accumulated in response to drought, its contribution to osmotic adjustment was negligible (Barker et al., 1993). Even though the role of proline in osmotic adjustment is a matter of controversy (see Hare and Cress, 1997), the capacity to accumulate this amino acid under drought conditions has been related to drought resilience in the C₄ crop sorghum (Blum and Ebercon, 1976). The protective role of proline in the adaptation of plants to drought is likely to involve a number of functions other than osmoregulation (Ashraf and Foolad, 2007).

Apart from proline, an increase in total amino acid content was observed in dehydrated leaves, and especially in those of *C. dactylon* and *Z. japonica* (Appendix S1, see Supplemental Data with the online version of this manuscript). The amino acids presented in Fig. 9 were the most drought-responsive in these C₄ grasses. Increased methionine, especially in *C. dactylon* and *Z. japonica*, with leaf dehydration is likely to reflect enhanced production of ethylene and/or polyamines under drought conditions. Ethylene is known to be involved in shoot growth restriction in response to the drying soil (Hussain et al., 1999), while polyamines are thought to provide the plant with increased drought tolerance, namely through antioxidant activity, although their mechanism of action remains unclear (Groppa and Benavides, 2008). Greater increases in phenylalanine, valine, isoleucine, and leucine were observed in *Z. japonica* than in the other two species, presumably related to an enhanced involvement of secondary metabolism. Phenylalanine is involved in the synthesis of lignin, which may contribute to the strengthening of cell walls, and of other secondary compounds that may play an important role in protection against reactive oxygen species under conditions that favor water loss (Grace and Logan, 2000).

Differential strategies of drought tolerance may be related to the evolution and geographical distribution of C₄ subtypes in grasses—The presence of more effective mechanisms of drought tolerance in *C. dactylon* and *Z. japonica* than in *P. dilatatum* agree with several reports on the distribution of C₄ grasses, which suggest that the dominance of NADP-ME species in comparison to the other C₄ subtypes is positively correlated with precipitation gradients (see Cabido et al., 2008). Hattersley (1992) suggested that the subtype of C₄ photosynthetic pathway could be appropriate to the ecology and management of desertified grasslands, with the persistent and drought-resistant NAD-ME grasses being potentially more fit for arid regions. The lower drought resistance of *P. dilatatum* is also in agreement with the distribution of the grass subfamily Panicoideae in areas with higher precipitation levels relative to the subfamily Chloridoideae (Taub, 2000). The hypothesis that the evolution and differential acquisition of adaptive traits under less favorable environments by each of the three C₄ grass species may be more relevant for their drought tolerance than their photosynthetic pathway or taxonomic background cannot be disregarded. Nevertheless, the results presented here show a

higher level of specialization of the C₄ Kranz anatomy traits in *C. dactylon* (NAD-ME) and, especially, *Z. japonica* (PEPCK), that is also related to better ability to cope with water deficit and the occupation of arid environments. Considering both the anatomical and biochemical characteristics of the PEPCK subtype of C₄ photosynthesis, it is conceivable that this subtype evolved from ancestral species with the NAD-ME photosynthetic pathway (Hattersley and Watson, 1992). The decarboxylation by PEPCK is a relatively simple addition to the C₄ photosynthetic pathway (Kellogg, 1999), and the enzyme may act as an alternative decarboxylase in species belonging to the NADP-ME and NAD-ME subtypes (see Carmo-Silva et al., 2008a). The association of C₄ grasses of the NAD-ME and PEPCK subtypes with better drought tolerances (relative to the NADP-ME subtype) should be further investigated using a more extensive number of species of each subtype and exploited to promote efficient use of water for agriculture and recreational purposes through the use of species better suited to arid environments.

Conclusion—The current study shows leaf traits that enhance the capabilities of C₄ grasses to tolerate drought, which may have evolved in ecosystems where water is in short supply. These traits include high relative dry matter, decreased mesophyll to bundle sheath tissue ratio, strategic deployment of bulliform cells to cause leaves to fold or roll during dehydration, the ability to establish a low LWP under drought stress, and an ability to accumulate proline and other free amino acids during leaf dehydration.

LITERATURE CITED

- ASHRAF, M., AND M. R. FOOLAD. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany* 59: 206–216.
- BARKER, D. J., C. Y. SULLIVAN, AND L. E. MOSER. 1993. Water deficit effects on osmotic potential, cell wall elasticity, and proline in five forage grasses. *Agronomy Journal* 85: 270–275.
- BARNETT, N. M., AND A. W. NAYLOR. 1966. Amino acid and protein metabolism in bermuda grass during water stress. *Plant Physiology* 41: 1222–1230.
- BATES, L. S., R. P. WALDREN, AND I. D. TEARE. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil* 39: 205–207.
- BLUM, A., AND A. EBERCON. 1976. Genotypic responses in sorghum to drought stress. III. Free proline accumulation and drought resistance. *Crop Science* 16: 428–431.
- BROWN, R. H. 1999. Agronomic implications of C₄ photosynthesis. In R. F. Sage and R. K. Monson [eds.], *C₄ plant biology*, 473–507. Academic Press, New York, New York, USA.
- CABIDO, M., E. PONS, J. J. CANTERO, J. P. LEWIS, AND A. ANTON. 2008. Photosynthetic pathway variation among C₄ grasses along a precipitation gradient in Argentina. *Journal of Biogeography* 35: 131–140.
- CARMO-SILVA, A. E., A. BERNARDES DA SILVA, A. J. KEYS, M. A. J. PARRY, AND M. C. ARRABAÇA. 2008a. The activities of PEP carboxylase and the C₄ acid decarboxylases are little changed by drought stress in three C₄ grasses of different subtypes. *Photosynthesis Research* 97: 223–233.
- CARMO-SILVA, A. E., S. J. POWERS, A. J. KEYS, M. C. ARRABAÇA, AND M. A. J. PARRY. 2008b. Photorespiration in C₄ grasses remains slow under drought conditions. *Plant, Cell & Environment* 31: 925–940.
- CARMO-SILVA, A. E., A. S. SOARES, J. MARQUES DA SILVA, A. BERNARDES DA SILVA, A. J. KEYS, AND M. C. ARRABAÇA. 2007. Photosynthetic responses of three C₄ grasses of different metabolic subtypes to water deficit. *Functional Plant Biology* 34: 204–213.

- CATSKY, J. 1960. Determination of water deficit in discs cut out from leaf blades. *Biologia Plantarum* 2: 76–77.
- CHAVES, M. M., J. P. MAROCO, AND J. S. PEREIRA. 2003. Understanding plant responses to drought—From genes to the whole plant. *Functional Plant Biology* 30: 239–264.
- CHAVES, M. M., AND M. M. OLIVEIRA. 2004. Mechanisms underlying plant resilience to water deficits: Prospects for water-saving agriculture. *Journal of Experimental Botany* 55: 2365–2384.
- DELAUNEY, A. J., AND D. P. S. VERMA. 1993. Proline biosynthesis and osmoregulation in plants. *Plant Journal* 4: 215–223.
- DENGLER, N. G., R. E. DENGLER, P. M. DONNELLY, AND P. W. HATTERSLEY. 1994. Quantitative leaf anatomy of C₃ and C₄ grasses (Poaceae): Bundle sheath and mesophyll surface area relationships. *Annals of Botany* 73: 241–255.
- DENGLER, N. G., AND T. NELSON. 1999. Leaf structure and development in C₄ plants. In R. F. Sage and R. K. Monson [eds.], *C₄ plant biology*, 133–172. Academic Press, New York, New York, USA.
- DENGLER, N. G., AND W. C. TAYLOR. 2000. Developmental aspects of C₄ photosynthesis. In R. C. Leegood, T. D. Sharkey, and S. von Caemmerer [eds.], *Photosynthesis: Physiology and metabolism*, 471–495. Kluwer, Dordrecht, Netherlands.
- EDWARDS, G. E., M. S. B. KU, AND R. K. MONSON. 1985. C₄ photosynthesis and its regulation. In J. Barber and N. R. Baker [eds.], *Photosynthetic mechanisms and the environment*, 287–327. Elsevier Science Publishers B.V. (Biomedical Division), Amsterdam, Netherlands.
- FORD, C. W., AND J. R. WILSON. 1981. Changes in levels of solutes during osmotic adjustment to water stress in leaves of four tropical pasture species. *Australian Journal of Plant Physiology* 8: 77–91.
- GHANNOUM, O., S. VON CAEMMERER, AND J. P. CONROY. 2002. The effect of drought on plant water use efficiency of nine NAD-ME and nine NADP-ME Australian C₄ grasses. *Functional Plant Biology* 29: 1337–1348.
- GRACE, S. C., AND B. A. LOGAN. 2000. Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 355: 1499–1510.
- GROPPA, M. D., AND M. P. BENAVIDES. 2008. Polyamines and abiotic stress: Recent advances. *Amino Acids* 34: 35–45.
- GUTMANN, M. 1995. Improved staining procedures for photographic documentation of phenolic deposits in semi-thin sections of plant tissue. *Journal of Microscopy* 179: 277–281.
- HABERLANDT, G. 1882. Vergleichende anatomie des assimilatorischen gewebesystems der pflanzen. *Jahrbücher für Wissenschaftliche Botanik* 13: 74–188.
- HARE, P. D., AND W. A. CRESS. 1997. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regulation* 21: 79–102.
- HARE, P. D., W. A. CRESS, AND J. VAN STADEN. 1998. Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell & Environment* 21: 535–553.
- HATTERSLEY, P. W. 1984. Characterization of C₄ type leaf anatomy in grasses (Poaceae). Mesophyll–bundle sheath area ratios. *Annals of Botany* 53: 163–179.
- HATTERSLEY, P. W. 1992. C₄ photosynthetic pathway variation in grasses (Poaceae): Its significance for arid and semi-arid lands. In G. P. Chapman [ed.], *Desertified grasslands: Their biology and management*. Linnean Society Symposium Series, no. 13, 181–212. Academic Press, London, UK.
- HATTERSLEY, P. W., AND L. WATSON. 1992. Diversification of photosynthesis. In G. P. Chapman [ed.], *Grass evolution and domestication*, 38–116. Cambridge University Press, Cambridge, UK.
- HUSSAIN, A., C. R. BLACK, L. B. TAYLOR, AND J. A. ROBERTS. 1999. Soil compaction. A role for ethylene in regulating leaf expansion and shoot growth in tomato? *Plant Physiology* 121: 1227–1237.
- JONES, C. A. 1985. *C₄ grasses and cereals: Growth, development and stress response*. John Wiley & Sons, New York, New York, USA.
- JONES, M. M., C. B. OSMOND, AND N. C. TURNER. 1980. Accumulation of solutes in leaves of sorghum and sunflower in response to water deficits. *Australian Journal of Plant Physiology* 7: 193–205.
- KELLOGG, E. A. 1999. Phylogenetic aspects of the evolution of C₄ photosynthesis. In R. F. Sage and R. K. Monson [eds.], *C₄ plant biology*, 411–444. Academic Press, New York, New York, USA.
- KRAMER, P. J. 1983. *Water relations in plants*. Academic Press, New York, New York, USA.
- KRAMER, P. J., AND J. S. BOYER. 1995. *Water relations of plants and soils*. Academic Press, San Diego, California, USA.
- KUSAKA, M., M. OHTA, AND T. FUJIMURA. 2005. Contribution of inorganic components to osmotic adjustment and leaf folding for drought tolerance in pearl millet. *Physiologia Plantarum* 125: 474–489.
- LAMBERS, H., F. S. CHAPIN III, AND T. L. PONS. 1998. *Plant physiological ecology*. Springer-Verlag, New York, New York, USA.
- LÖSCH, R., AND E.-D. SCHULZE. 1995. Internal coordination of plant responses to drought and evaporative demand. In E.-D. Schulze and M. M. Caldwell [eds.], *Ecophysiology of photosynthesis*, 185–204. Springer-Verlag, Berlin, Germany.
- MIRANDA, V., N. R. BAKER, AND S. P. LONG. 1981. Anatomical variation along the length of the *Zea mays* leaf in relation to photosynthesis. *New Phytologist* 88: 595–605.
- MITCHELL, P. J., E. J. VENEKLAAS, H. LAMBERS, AND S. J. BURGESS. 2008. Leaf water relations during summer water deficit: Differential responses in turgor maintenance and variation in leaf structure among different plant communities in south-western Australia. *Plant, Cell & Environment* 31: 1791–1802.
- MOLINARI, H. B. C., C. J. MARUR, E. DAROS, M. K. F. CAMPOS, J. F. R. P. CARVALHO, J. C. B. FILHO, L. F. P. PEREIRA, AND L. G. E. VIEIRA. 2007. Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.): Osmotic adjustment, chlorophyll fluorescence and oxidative stress. *Physiologia Plantarum* 130: 218–229.
- NAYYAR, H. 2003. Accumulation of osmolytes and osmotic adjustment in water-stressed wheat (*Triticum aestivum*) and maize (*Zea mays*) as affected by calcium and its antagonists. *Environmental and Experimental Botany* 50: 253–264.
- OGLE, K. 2003. Implications of interveinal distance for quantum yield in C₄ grasses: A modeling and meta-analysis. *Oecologia* 136: 532–542.
- PETTIT, J. R., J. JOUZEL, D. RAYNAUD, N. I. BARKOV, J. M. BARNOLA, I. BASILE, M. BENDER, ET AL. 1999. Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature* 399: 429–436.
- PRENDERGAST, H. D. V., AND P. W. HATTERSLEY. 1987. Australian C₄ grasses (Poaceae): Leaf blade anatomical features in relation to C₄ acid decarboxylation types. *Australian Journal of Botany* 35: 355–382.
- PRENDERGAST, H. D. V., P. W. HATTERSLEY, AND N. E. STONE. 1987. New structural biochemical associations in leaf blades of C₄ grasses (Poaceae). *Australian Journal of Plant Physiology* 14: 403–420.
- RISTIC, Z., AND D. D. CASS. 1991. Leaf anatomy of *Zea mays* L. in response to water shortage and high temperature: A comparison of drought-resistant and drought-sensitive lines. *Botanical Gazette* 152: 173–185.
- RITCHIE, G. A., AND T. M. HINCKLEY. 1975. The pressure chamber as an instrument for ecological research. *Advances in Ecological Research* 9: 165–254.
- SAAB, I. N., AND R. E. SHARP. 1989. Non-hydraulic signals from maize roots in drying soil: Inhibition of leaf elongation but not stomatal conductance. *Planta* 179: 466–474.
- SAGE, R. F. 2004. The evolution of C₄ photosynthesis. *New Phytologist* 161: 341–370.
- SAGE, R. F., AND A. D. MCKOWN. 2006. Is C₄ photosynthesis less phenotypically plastic than C₃ photosynthesis? *Journal of Experimental Botany* 57: 303–317.
- SCHALL, R. 1991. Estimation of generalized linear models with random effects. *Biometrika* 78: 719–727.
- TAUB, D. R. 2000. Climate and the US distribution of C₄ grass subfamilies and decarboxylation variants of C₄ photosynthesis. *American Journal of Botany* 87: 1211–1215.

TURNER, N. C. 2004. Sustainable production of crops and pastures under drought in a Mediterranean environment. *Annals of Applied Biology* 144: 139–147.

UENO, O., Y. KAWANO, M. WAKAYAMA, AND T. TAKEDA. 2006. Leaf vascular systems in C₃ and C₄ grasses: A two-dimensional analysis. *Annals of Botany* 97: 611–621.

UTRILLAS, M. J., AND L. ALEGRE. 1997. Impact of water stress on leaf anatomy and ultrastructure in *Cynodon dactylon* (L.) Pers. under natural conditions. *International Journal of Plant Sciences* 158: 313–324.

VECCHIA, F. D., T. EL ASMAR, R. CALAMASSI, N. RASCIO, AND C. VAZZANA. 1998. Morphological and ultrastructural aspects of dehydration and rehydration in leaves of *Sporobolus stapfianus*. *Plant Growth Regulation* 24: 219–228.

WHITE, R. H., M. C. ENGELKE, S. J. ANDERSON, B. A. RUEMMELE, K. B. MARCUM, AND G. R. TAYLOR. 2001. Zoysiagrass water relations. *Crop Science* 41: 133–138.

APPENDIX 1. Results of the analysis of variance (ANOVA) applied to the shoot growth (Fig. 1) and leaf structure variables (Fig. 2) for comparison between the three species of C₄ grasses (*Paspalum dilatatum* [P.], *Cynodon dactylon* [C.], and *Zoysia japonica* [Z.]) and the two treatments (control vs. drought stress) and the interaction species × treatment: mean values given by the ANOVA, standard error of the difference between means (SED), degrees of freedom (df), *F*-statistic (*F*), *P*-value (*P*), and least significant differences (LSD) at the 5% level.

Variables	Means	SED	df	<i>F</i>	<i>P</i>	LSD
No. of leaves (square root scale)	2.54 (P.)	0.14	12	72.6	<0.001	0.29
	3.95 (C.)					
	3.95 (Z.)					
	3.64 (control)	0.11	12	8.6	<0.01	0.24
	3.32 (drought)					
No. of tillers (square root scale)	1.37 (P.)	0.09	12	65.8	<0.001	0.19
	1.76 (C.)					
	2.35 (Z.)					
	1.94 (control)	0.07	12	11.1	<0.01	0.15
	1.71 (drought)					
SLA	52.4 (P. control)	0.9	113	4.6	<0.01	1.7
	48.5 (P. drought)					
	52.3 (C. control)					
	48.7 (C. drought)					
	31.6 (Z. control)					
31.1 (Z. drought)						
Leaf dry matter (logit scale)	-1.89 (P. control)	0.02	113	9.2	<0.001	0.05
	-1.72 (P. drought)					
	-1.56 (C. control)					
	-1.41 (C. drought)					
	-0.89 (Z. control)					
	-0.85 (Z. drought)					

Note: SLA, specific leaf area.

APPENDIX 2. Results of the regression analysis to assess the effects of drought stress on the water relations (Fig. 8) and amino acid content (Fig. 9) in the leaves of the three species of C₄ grasses (*Paspalum dilatatum* [P.], *Cynodon dactylon* [C.], and *Zoysia japonica* [Z.]): best models statistically significant, percentage of variance accounted for by the model (*R*²), residual mean square (*s*²), degrees of freedom (df) and *P*-value (*P*).

Analysis	Model	<i>R</i> ²	<i>s</i> ²	df	<i>P</i>
RWC vs. SWC	$y = 98.30(1 - e^{-0.4498x})$, SE 0.31, 0.0100 (P., C., and Z.)	81.5%	6.387	118	<0.001
LWP vs. RWC	$y = -1.347 + 3.47 \times 10^{-17} e^{0.3795x}$, SE 0.052, 2.54×10^{-16} , 0.0692 (P. and C.) $y = -11.315 + 0.1021x$, SE 0.309, 0.0006 (Z.)	84.1%	0.0408	115	<0.001
Proline vs. RWC	$y = 2106 - 42.22x + 0.2124x^2$, SE 134, 3.72, 0.0248 (P., C., and Z.)	90.5%	3255	69	<0.001
Methionine vs. RWC	$y = 3.81 - 0.0834x + 0.000456x^2$, SE 0.24, 0.0066, 0.000044 (P.) $y = 3.90 - 0.0834x + 0.000456x^2$, SE 0.24, 0.0066, 0.000044 (C. and Z.)	94.3%	3.792	66	<0.001
Phenylalanine vs. RWC	$y = 40.14 - 0.937x + 0.00543x^2$, SE 6.65, 0.152, 0.00089 (P. and C.) $y = 62.50 - 1.159x + 0.00543x^2$, SE 4.35, 0.128, 0.00089 (Z.)	89.1%	0.0102	68	<0.001
Valine vs. RWC	$y = 48.19 - 0.993x + 0.00521x^2$, SE 7.90, 0.178, 0.00104 (P.) $y = 49.73 - 0.993x + 0.00521x^2$, SE 7.83, 0.178, 0.00104 (C.) $y = 76.68 - 1.287x + 0.00521x^2$, SE 5.10, 0.150, 0.00104 (Z.)	92.7%	2.763	67	<0.001
Isoleucine vs. RWC	$y = 11.35 - 0.2290x + 0.00118x^2$, SE 3.47, 0.0791, 0.00046 (P. and C.) $y = 27.17 - 0.3938x + 0.00118x^2$, SE 2.27, 0.0667, 0.00046 (Z.)	93.7%	0.7513	67	<0.001
Leucine vs. RWC	$y = 19.14 - 0.1983x + 0.000030x^2$, SE 0.72, 0.0093, 0.000015 (Z.)	91.2%	0.8410	69	<0.001

Notes: LWP, leaf water potential, RWC, leaf relative water content; SWC, soil water content