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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

Ana E. Carmo-Silva, Ana Francisco, Stephen J. Powers, Alfred J. Keys, Lia Ascensão, Martin A. J. Parry, and Maria Celeste Arrabaça

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 NAD-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
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, PEPPC). 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 (Molnari 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 μmol m⁻² s⁻¹) was provided whenever the natural light fell below a photosynthetic photon flux density (PPFD) of 500 μmol m⁻² 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 recently 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 Historesin (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 BX60FS 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 transverse sectional areas occupied by mesophyll (M) cells, bundle sheath (BS) cells, intercellular spaces (ICS), vascular tissues (VT), bulliform water storage (WS) cells, and sclerenchyma (S) were measured and used to calculate RWC (Catsky, 1960). Leaf area was determined by scanning the leaf samples for the calibration curve. A volume of 0.25 mL was taken from each pot to determine the leaf relative water content (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 log transformation, given by \( \log([D/(100 – D)] \) was applied to the relative dry matter content. A log transformation was applied to the values of the transverse interval, 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 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 *Zosia 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 decreased 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 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 tolulone as background.
filled with numerous chloroplasts, surround the vascular tissues (VT) and are surrounded by the mesophyll (M) cells, with fewer and smaller chloroplasts. 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, 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; $P > 0.05$).

![Fig. 1](image1.png) **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 ± SE of 15 plant samples. (See Appendix 1 for statistical analysis.)

![Fig. 2](image2.png) **Fig. 2.** (A) Specific leaf area (SLA) and (B) leaf dry mass as a percentage of turgid mass (DM/TM × 100) for control (black bars) and drought-stressed (white bars) plants of *Paspalum dilatatum*, *Cynodon dactylon*, and *Zoysia japonica*. Values are means ± SE of 16 (control) or 24 (drought-stressed) plant samples. (See Appendix 1 for statistical analysis.)

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
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. scale) revealed an overall marginal effect (*F*<sub>1,12</sub> = 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*<sub>1,12</sub> = 0.3, vein number: *F*<sub>1,4,5</sub> = 0.6, interveinal distance: *F*<sub>1,12</sub> = 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 function. The relative leaf water potential (LWP) decreased from −0.6 MPa in fully hydrated leaves of *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*.

*C₄* photosynthesis evolved from the *C₃* 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 *C₄* traits (Sage, 2004). Our results support the hypothesis that aridity may also play a role in the evolution of the three different subtypes of *C₄* 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 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 *C₄* grasses in response to decreased water availability. The decrease in growth and increased photosynthetic CO₂ 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 μm.
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. 4](image-url)

**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-
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.
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 C₄ grass *Sporobolus stapfianus*, a resurrection species (Vecchia et al., 1998).

In many C₄ 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 C₄ 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. C₄ 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 C₄ metabolites (Sage and McKown, 2006). The same authors report that the mean IVD in C₄ plants (120 μm) is less than half that observed in C₃ plants (280 μm), 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 C₄ 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 C₃ and C₄
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**


Phenylalanine vs. RWC
RWC vs. SWC
Analysis Model $R^2$ $\sigma^2$ df $P$
---
RWC vs. SWC $y = 98.30 \left(1 - e^{-0.4988^x}\right)$, SE 0.31, 0.0100 (P, C, and Z) 81.5% 6.387 118 <0.001
LWP vs. RWC $y = -1.347 + 3.47 \times 10^{-17} e^{0.3795^x}$, SE 0.052, 2.54 \times 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 $y = 3.81 - 0.0834x + 0.00456x^2$, SE 0.24, 0.066, 0.00044 (P)
$y = 3.90 - 0.0834x + 0.00456x^2$, SE 0.24, 0.066, 0.00044 (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 $y = 19.14 - 0.1983x + 0.00030x^2$, 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

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.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Means</th>
<th>SED</th>
<th>df</th>
<th>F</th>
<th>P</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of leaves (square root scale)</td>
<td>2.54 (P) 3.95 (C) 3.95 (Z)</td>
<td>0.14 0.11 3.64 (control) 3.32 (drought)</td>
<td>12 12 10 12</td>
<td>72.6 8.6</td>
<td>&lt;0.001 0.01</td>
<td>0.29 0.24</td>
</tr>
<tr>
<td>No. of tillers (square root scale)</td>
<td>1.37 (P) 1.76 (C) 2.35 (Z)</td>
<td>0.09 0.07 1.94 (control) 1.71 (drought)</td>
<td>12 12 10 12</td>
<td>65.8 11.1</td>
<td>&lt;0.001 0.01</td>
<td>0.19 0.15</td>
</tr>
<tr>
<td>SLA</td>
<td>52.4 (P control) 48.5 (P drought) 52.3 (C control) 48.7 (C drought) 31.6 (Z control) 31.1 (Z drought)</td>
<td>0.9 0.71 1.94 (control) 1.71 (drought)</td>
<td>113 113 10 10</td>
<td>4.6 69</td>
<td>&lt;0.01 0.01</td>
<td>1.7 0.05</td>
</tr>
<tr>
<td>Leaf dry matter (logit scale)</td>
<td>−1.89 (P control) −1.72 (P drought) −1.56 (C control) −1.41 (C drought) −0.89 (Z control) −0.85 (Z drought)</td>
<td>−0.85 0.02 0.02 0.02 0.02 0.02</td>
<td>113 113 113 113</td>
<td>9 9 9 9</td>
<td>&lt;0.001 0.001 0.001 0.001 0.001 0.001</td>
<td></td>
</tr>
</tbody>
</table>

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 ($\sigma^2$), degrees of freedom (df) and P-value (P).