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RESEARCH PAPER

Why is it so difficult to identify a single indicator of water stress in plants? A proposal for a multivariate analysis to assess emergent properties

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ABSTRACT

Because of the complexity of plant responses to water deficit, researchers have attempted to identify simplified models to understand critical aspects of the problem by searching for single indicators that would enable evaluations of the effects of environmental changes on the entire plant. However, this reductionist approach, which is often used in plant sciences, makes it difficult to distinguish systemic emergent behaviours. Currently, a new class of models and epistemology have called attention to the fundamental properties of complex systems. These properties, termed 'emergent', are observed at a large scale of the system (top hierarchical level) but cannot be observed or inferred from smaller scales of observation in the same system. We propose that multivariate statistical analysis can provide a suitable tool to quantify global responses to water deficit, allowing a specific and partially quantitative assessment of emergent properties. Based on an experimental study, our results showed that the classical approach of the individual analysis of different data sets might provide different interpretations for the observed effects of water deficit. These results support the hypothesis that a cross-scale multivariate analysis is an appropriate method to establish models for systemic understanding of the interactions between plants and their changing environment.

INTRODUCTION

Water deficit is the main factor that limits crop yields worldwide. The effects of water deficit are perceived at all scales of organisation in plants, from changes in gene expression and physiological processes to effects on total biomass production (Pinheiro & Chaves 2011; Lisar *et al.* 2012; Souza *et al.* 2013). Because of the complexity of plant responses to water deficits, researchers are attempting to identify simplified models to understand the critical aspects of this problem in order to improve breeding programmes and crop cultivation techniques, thus allowing higher crop yields to be obtained in areas with water shortages (Chaves & Oliveira 2004; Valliyodan & Nguyen 2006; Lawlor & Tezara 2009; Flexas *et al.* 2012).

This general theoretical model and the corresponding scientific methods have been designed on the basis of a reductionist science that is founded on the belief that understanding complex phenomena lies in understanding their components (a Cartesian approach). We suggest that this epistemology underlies the strong tendency of some plant ecophysiologists and other scientists focused on the interactions between plants and their environment to search for single indicators that would enable accurate diagnosis of the effects of environmental changes on the whole plant. There are literally thousands of scientific reports that directly or indirectly consider the existence of such indicators.

However, a new class of models and epistemology have recently called attention to the fundamental properties of complex systems, properties that are generally observed at a larger scale in a system (a top hierarchical level) and that cannot be observed or inferred at smaller scales of observation in the same system (low hierarchical levels). Such properties are broadly referred to as 'emergent properties' (Mitchell 2009; Lüttge 2012). Within this scientific context, a new class of models that are focused on the analysis of network organisation in complex systems and their dynamic, emergent non-reductive properties have been proposed (Barabási 2003; Souza *et al.* 2009; Lucas *et al.* 2011; Lüttge 2012). In addition to the complex interactions among the components of complex networks generating emergent properties, one of the fundamental characteristics of biological organisms is the hierarchical organisation of their subsystems at different levels of the system (Schneider 1998; Ravasz *et al.* 2002). Although some properties of complex systems can be reproduced at different scales, showing fractal-like behaviour (Solé & Bascompte 2006), the pattern of networking, which exhibits nonlinearities between the different modules of a system, makes it difficult to obtain a scaling law that enables the inference of global behaviours from the observation of local modules. Indeed, scaling is one of the central problems in biology (Korn 1999; Brown & West 2000; Trewavas 2006).

From this general perspective, Souza & Cardoso (2003) and Vítolo *et al.* (2012) have argued that the search for indicators of stress, rather than following a systemic approach to understanding plant–environment interactions, may lead to the misinterpretation of experimental results or diagnoses under field conditions. Accordingly, Lawlor & Tezara (2009) have argued that, "finding such elusive *deus ex machina* (stress indicators) in very complex, partial and imperfect data require some faith (or dogmatism)." Therefore, the present study aims to provide theoretical and practical support for a systemic approach to ecophysiology (Souza *et al.* 2009; Lüttge 2012; Vítolo *et al.* 2012). Within this context, we propose that a multivariate statistical analysis conducted *via* principal components analysis (PCA) can be a suitable tool for quantifying global responses to environmental disturbances, allowing a specific and partially quantitative assessment of emergent properties to be conducted. According to McGarigal *et al.* (2000), the main purpose of PCA is to condense the information from a large number of original variables into a smaller set of new compound dimensions with a minimal loss of information. These new dimensions are defined based on linear combinations of the original variables, and these linear combinations are referred to as principal components (PCs). Based on analysing a complete set of experimental data *via* PCA, we show how observations made at specific scales can influence interpretation of the responses of plants to water deficit as a whole, providing empirical evidence of the limitations of a reductionist approach in ecophysiology.

MATERIAL AND METHODS

Plant material and experimental design

To improve generalisation of the results of this study, two well-known crops with different types of photosynthetic metabolism were compared: *Glycine max* (L.) Merrill cv. CD 202 (C_3 metabolism) and *Brachiaria brizantha* cv. Marandú (C_4 metabolism). Both species are extensively cultivated in tropical and subtropical regions of Brazil and worldwide. The seeds were germinated under greenhouse conditions in pots containing 12 kg of a 1:1 (v/v) mixture of a red–yellow Ultisol soil with an organic substratum, and were watered daily.

The plants were subjected to water deficit induced by the daily replacement of only 30% of the water lost by evapotranspiration during the previous day; the control group was maintained under 100% water replacement. Water replacement was performed gravimetrically based on daily weighing of the pots (Catuchi *et al.* 2012). The growth of the *B. brizantha* plants was made homogeneous at 35 days after germination by cutting to a height of 20 cm. The *G. max* plants were subjected to the water deficit when the fourth trifoliate leaf was fully expanded (phenological stage V4), whereas the *B. brizantha* plants were subjected to the water deficit approximately 50 days after germination. The plants were cultivated in a growth chamber with controlled light (600 μmol photons m^{-2} s^{-1}), day/night temperatures of 30/23 °C, 60% air humidity and a photoperiod of 16 h light.

The experiment was arranged in a fully randomised 2 × 2 factorial design (two irrigation regimes and two species), with seven replicates (one plant per pot) per treatment. Measurement of all the physiological parameters was performed after 30 days of cultivation of the plants under each irrigation regime. The physiological parameters investigated for the plants represented different levels of hierarchical organisation, from the quantum level (photochemical) to the whole-plant level (biomass), and included biochemical analyses of proline, sucrose, glucose and phenolic compounds and leaf gas exchange.

Measurement of physiological parameters

The relative water content (RWC) was determined according to Catsky (1960) by weighing the leaves using an analytical balance (0.0001 g precision). At the end of the treatments, the saturated mass of a leaf was obtained after immersion in distilled water in a dark room for 24 h, and the dry mass was obtained after drying in an oven at 60 °C until a constant mass was attained.

To estimate the integrity of biomembranes, the cellular electrolyte leakage per dry mass unit (EL_{DM}) was evaluated by measuring the electrical conductivity of an aqueous solution containing ten leaf discs collected from each replicate in all treatments using a portable electric conductivity meter (LTLutron, Taipei, Taiwan; model CD-4301; adapted from Matos *et al.* 2010).

Photosynthetic CO_2 response curves (*A/Ci* curves, where *A* is net assimilation of CO_2 and *Ci* is intercellular concentration of CO_2) were generated for the plants in each treatment according to the standard procedures described in Long & Bernacchi (2003). The *A/Ci* curves were adjusted using the models proposed by Monteiro & Prado (2006). All *A/Ci* curves were estimated using measurements obtained from healthy, fully expanded leaves of seven plants per treatment from 09:00 h to 13:00 h. The temperature inside the leaf chamber of an infrared gas analyser (Li-6400XTR; Li-Cor, Lincoln, NE, USA) was adjusted to 30 °C, and the vapour pressure deficit was maintained at 1.5 kPa with a dewpoint generator (model Li-610, Li-Cor) connected to the leaf chamber. Light was provided by LEDs emitting in the blue–red spectrum connected to the Li-6400XTR sampling chamber. The variables obtained from the *A/Ci* curves were photosynthetic potential (A_{maxCO2}), compensation point of photosynthesis in response to CO_2 (P_{comCO2}) and saturation point of photosynthesis in response to CO_2 (P_{satCO2}). The relative stomatal limitation to photosynthesis (L_S) was calculated according to Farquhar & Sharkey (1982). Photorespiration (*Pr*) and the maximum ratio of Rubisco carboxylation (V_{cmax}) for the plants with C_4 metabolism were calculated according to the model proposed by von Caemmerer (2000). The *Pr* and V_{cmax} values for C_3 plant metabolism were calculated according to the model proposed by Sharkey (1988) and Sharkey *et al.* (2007), which uses gas exchange values (for photosynthetic capacity and respiration in the dark) obtained at the saturation point of photosynthesis related to the photosynthetic photon flux density (PPFD). For this purpose, the PPFD curves of photosynthesis were produced through a preliminary test, according to Vítolo *et al.* (2012).

A fluorescence analysis of chlorophyll *a* was performed using a modulated light fluorometer (LI-6400-40) connected to the Li-6400XTR chamber. The estimated parameters were the potential (F_v/F_m) and effective ($\Delta F/F_m'$) quantum efficiency of photosystem II (PSII), antennal efficiency of PSII (F_v'/F_m'), photochemical (*qP*) and non-photochemical (*NPQ*) coefficients of fluorescence extinction and electron transport rate

(*ETR*) (Van Kooten & Snel 1990; Bilguer *et al.* 1995; Baker & Rosenqvist 2004).

The alternative electron sink (*AES*) was calculated as the ratio of the effective quantum efficiency of PSII at light saturation point ($\Delta F/F_m'$) to the quantum efficiency of CO_2 fixation (ϕCO_2), as proposed by Ribeiro *et al.* (2003). ϕCO_2 was calculated according to Edwards & Baker (1993).

The total chlorophyll content index (CCI) was determined with a portable chlorophyll meter (Model CCM 200; Opt-Sciences, Tyngsborough, Massachusetts, USA) using a fully expanded leaf from each plant in the study.

Plant growth analysis

At the time of collection, the total leaf area (*LA*) of each plant was determined with a portable leaf area integrator (model LI-3000A; Li-Cor, Lincoln, NE, USA). In all, two to three leaves per plant were reserved for biochemical analysis, and the dry mass of the remaining leaves was obtained after drying at 70 °C. The specific leaf mass (*SLM*) was estimated as the area/dry mass ratio. Stems and roots were also collected and dried separately. The total dry mass (DM_T) of each plant was obtained by summing leaf dry mass (DM_L), stem dry mass (DM_S), and root dry mass (DM_R).

Measurement of compatible osmolyte and soluble phenolic contents

At the end of the experiments, the leaves of the plants were collected, freeze-dried and ground into a powder using a mortar and pestle. For amino acid analysis, the extraction was performed with 70% ethanol (100 mg per 3 ml, 30 min) at room temperature in a shaker; for analysis of soluble sugars and phenolics, the extraction was performed with 70% ethanol (100 mg per 9 ml, 3 × 3 ml, 30 min) in a water bath at 60 °C, with occasional agitation. The proline and soluble amino acid contents were determined according to Ringel *et al.* (2003) and Cocking & Yemm (1954), respectively. The soluble sugars were determined according to Dubois *et al.* (1956), and phenolic acids were determined according to Ainsworth & Gillespie (2007).

Data analyses

The obtained data were analysed *via* a two-way ANOVA ($P = 0.05$), considering a 2 × 2 factorial design (two water regimes and two plant species), and the means were compared using a *post-hoc* Tukey's test ($P = 0.05$).

A multivariate analysis was performed *via* PCA to verify the grouping of the different observed plant responses to water deficit, taking into account the entire set of measured physiological parameters, and to simulate how the plant responses were grouped when different sets of data were analysed separately according to different scales of observation (Vítolo *et al.* 2012). Mathematically, PCA allows for possible redundancies in the data to be eliminated and definition of the most important variables separating the treatments (Mainly 2008). Because the measurement units differed between variables, the data in this study were log-transformed to reduce the effect of the numeric scale (McGarigal *et al.* 2000). A two-dimensional ordination graphic (with two major components), demarcated by two axes designated as the first principal component (PC1) and second principal component (PC2), was determined to be suitable for our analyses (Vítolo *et al.* 2012).

RESULTS

Experimental data

Relative water content and integrity of biomembranes

The induced water deficit reduced the RWC value in *G. max* and *B. brizantha* by 10% and 17%, respectively (Table 1). The EL_{DM} did not show significant variation when *G. max* plants were subjected to water deficit, whereas significant variation in this parameter was observed in *B. brizantha* plants, with a 60% increase in EL_{DM} compared to the well-watered controls. Remarkably, *B. brizantha* plants exhibited higher EL_{DM} values in relation to *G. max* plants, regardless of the irrigation regime (Table 1).

Effects of water deficiency on growth

The water deficit reduced the dry mass (DM_L, DM_S, DM_R and DM_T) and *LA* in both species. However, the *B. brizantha* shoot/root ratio (*S/R*) and *SLM* were not influenced by water deficiency (Table 2).

Photosynthetic responses

The stressed *G. max* plants presented 39% and 21% decreases in A_{maxCO2} and V_{cmax}, respectively, compared to well-watered

Table 1. Electrolyte leakage per unit dry mass (EL_{DM}) and relative water content (RWC) in leaves of *Glycine max* and *Brachiaria brizantha* plants under 100% and 30% water replacement. The capital letters indicate statistical differences ($P < 0.05$) between the water treatments, and different lowercase letters indicate statistical differences ($P < 0.05$) between the species.

water status and biomembrane integrity				
	G. max		*B. brizantha*	
	100%	30%	100%	30%
EL_{DM} ($mS\ g^{-1}$)	0.55^{Ab}	1.23^{Ab}	9.43^{Ba}	15.12^{Aa}
RWC (%)	87.3^{Ab}	79.3^{Ba}	94.9^{Aa}	78.6^{Ba}

Table 2. Root dry mass (DM_R), leaf dry mass (DM_L), stem dry mass (DM_S), total dry mass (DM_T), shoot/root ratio (*S/R*), total leaf area (*LA*) and specific leaf mass (*SLM*) of *Glycine max* and *Brachiaria brizantha* plants under 100% and 30% water replacement. The capital letters indicate statistical differences ($P < 0.05$) between water treatments, and different lowercase letters indicate statistical differences ($P < 0.05$) between species.

growth analysis				
	G. max		*B. brizantha*	
	100%	30%	100%	30%
DM_R (g)	59^{Ab}	11^{Bb}	454^{Aa}	153^{Ba}
DM_L (g)	59^{Aa}	16^{Ba}	35^{Ab}	16^{Ba}
DM_S (g)	62^{Aa}	13^{Ba}	52^{Aa}	13^{Ba}
DM_T (g)	180^{Ab}	41^{Bb}	543^{Aa}	183^{Ba}
S/R	1.95^{Ba}	2.47^{Aa}	0.20^{Ab}	0.20^{Ab}
LA (m^2)	0.21^{Ab}	0.08^{Bb}	0.78^{Aa}	0.32^{Ba}
SLM ($g\ m^{-2}$)	272^{Aa}	190^{Ba}	46^{Ab}	49^{Ab}

Table 3. Photosynthetic potential (A_{maxCO2}), CO_2 compensation point (P_{comCO2}), CO_2 saturation point (P_{satCO2}), relative stomatal limitation to photosynthesis (L_S), maximum ratio of Rubisco carboxylation (V_{cmax}) and photorespiration (*Pr*) of *Glycine max* and *Brachiaria brizantha* plants under 100% and 30% water replacement. The capital letters indicate statistical differences ($P < 0.05$) between the water treatments, and different lowercase letters indicate statistical differences ($P < 0.05$) between species.

A/Ci Curve

	G. max		*B. brizantha*	
	100	30	100	30
A_{maxCO2} ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	22.8^{Aa}	13.8^{Bb}	22.2^{Aa}	20.4^{Aa}
P_{comCO2} ($\mu mol\ mol^{-1}$)	81^{Aa}	78^{Aa}	16^{Bb}	43^{Ab}
P_{satCO2} ($\mu mol\ mol^{-1}$)	697^{Aa}	462^{Ba}	192^{Bb}	358^{Aa}
LS	23^{Aa}	30^{Ab}	19^{Ba}	46^{Aa}
V_{cmax} ($\mu mol\ m^{-2}\ s^{-1}$)	80^{Aa}	63^{Ba}	25^{Ab}	25^{Ab}
P_R ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	3.25^{Aa}	2.04^{Ba}	0.17^{Ab}	0.37^{Ab}

plants (Table 3). In contrast, these parameters remained unaltered in stressed *B. brizantha* plants. Furthermore, the values obtained for the P_{comCO2} and P_{satCO2} in stressed *B. brizantha* were increased by 168% and 86%, respectively, whereas P_{satCO2} and *Pr* in stressed *G. max* plants were reduced to 33% and 37%, respectively, compared to the control plants. The relative L_S in *G. max* plants showed no significant changes in response to the water deficit, whereas L_S in *B. brizantha* was increased by 59%, indicating increased resistance to the diffusion of CO_2 into the leaves.

The chlorophyll fluorescence parameters were not affected by water deficit in either species, except for *ETR* in *B. brizantha*, which was reduced by 22% (Table 4).

Content of compatible osmolytes and phenolic compounds

The proline, soluble amino acids and sucrose content increased significantly in response to water deficit in both species (Table 5). However, the content of phenolic compounds, which may have antioxidant activity, was reduced in *B. brizantha* plants (27%), but increased significantly in *G. max* (27%).

Table 4. Potential and effective quantum efficiency of PSII (F_v/F_m, $\Delta F/F_m{'}$, respectively), photochemical and non-photochemical quenching (*qP* and *NPQ*, respectively), electron transport rate (*ETR*), alternative electron sink (*AES*) and chlorophyll content index (*CCI*) of *Glycine max* and *Brachiaria brizantha* plants grown under 100% and 30% water replacement. The capital letters indicate statistical differences ($P < 0.05$) between water treatments, and different lowercase letters indicate statistical differences ($P < 0.05$) between species.

Cl_a Fluorescence

	G. max		*B. brizantha*	
	100%	30%	100%	30%
F_v/F_m	0.731^{Aa}	0.745^{Aa}	0.793^{Aa}	0.788^{Aa}
$F_v{'}/F_m{'}$	0.452^{Aa}	0.487^{Aa}	0.397^{Aa}	0.392^{Ab}
$\Delta F/F_m{'}$	0.302^{Ba}	0.388^{Aa}	0.177^{Ab}	0.185^{Ab}
qP	0.657^{Aa}	0.830^{Aa}	0.445^{Aa}	0.472^{Ab}
NPQ	1.65^{Ab}	1.63^{Aa}	2.26^{Aa}	2.40^{Aa}
ETR ($\mu molm^{-2}\ s^{-1}$)	63^{Ab}	70^{Aa}	76^{Aa}	59^{Ba}
AES	14.5^{Ba}	21.0^{Aa}	7.1^{Ab}	8.4^{Ab}
ICC	10.9^{Ab}	18.8^{Bb}	22.8^{Ba}	38.5^{Aa}

Principal components analysis

According to each set of parameters, the PCA showed different response patterns for each species and under each irrigation condition. The PCA performed with all of the parameters measured in the experiment accounted for 64.2% of total variation of the original data (Fig. 1). PC1 explained 39.2% of the variance, whereas PC2 accounted for 25.0%. The variables that contributed most to discriminate between groups had eigenvector values >0.27. The main components for PC1 were *LA*, DM_R, DM_T and *S/R*, and were *ICC*, sucrose, DM_S and DM_L for PC2. The spatial ordination of the parameters separated four groups, allowing for clear distinctions to be made between the two species (left and right quadrants) and the water treatments (upper and lower quadrants).

When only growth parameters were examined, PCA accounted for 92.7% of the total variation of the original data (Fig. 2A). PC1 explained 58.8% of the total variation and distinguished the species, whereas PC2 explained 33.9% of the total variation and allowed separation of the water treatments. The main variables for PC1 were DM_R, *LA* and DM_T; while DM_L, DM_S and *SLM* were important for PC2.

Only the two species could be separated (PC1, 49.4%) in the PCA performed with variables related to *A/Ci* curves, whereas PC2 showed no significant influence, based on the broken-stick criterion (Vítolo *et al.* 2012; Fig. 2B). The most important parameters for PC1 discrimination were P_{satCO2}, P_{comCO2} and *LS*. In this analysis, it was possible to distinguish a group consisting of *G. max* plants with 100% water replacement, although there was overlap of the other treatments.

Although chlorophyll fluorescence parameters are also photosynthetic variables, the results of the PCA performed with only the photochemical parameters was distinct from those obtained when examining *A/Ci*-related parameters (Fig. 2C). The PCA of the photochemical data set accounted for 77.8% of the total variance. PC1 explained 53.4% of the variation and allowed for separation of the species, whereas PC2 explained 24.4% of the total variance and did not allow the irrigation treatments to be distinguished. The main parameters in the PC1 analysis were $\Delta F/F_m{'}$, *qP* and *NPQ* (eigenvector values >0.39); $F_v{'}/F_m{'}$ and F_v/F_m were the main parameters for PC2 (eigenvector values >0.5).

Table 5. Content of proline, soluble amino acids (AA), sucrose (sugar) and phenolics (phenol) in *Glycine max* and *Brachiaria brizantha* plants grown under 100% and 30% water replacement. The capital letters indicate statistical differences ($P < 0.05$) between water treatments, and different lowercase letters indicate statistical differences ($P < 0.05$) between species.

compatible osmolytes and phenolic compounds

	G. max		*B. brizantha*	
	100%	30%	100%	30%
Proline ($umoles\ g^{-1}$)	0.2^{Ba}	13.4^{Aa}	0.5^{Ba}	8.9^{Aa}
AA ($nmoles\ g^{-1}$)	32.8^{Ba}	52.5^{Aa}	24.5^{Ba}	39.3^{Ab}
Sugar ($mg\ g^{-1}$)	25.7^{Bb}	38.5^{Ab}	41.9^{Ba}	68.2^{Aa}
Phenol ($mg\ g^{-1}$)	13.9^{Bb}	17.7^{Aa}	19.4^{Aa}	14.0^{Bb}

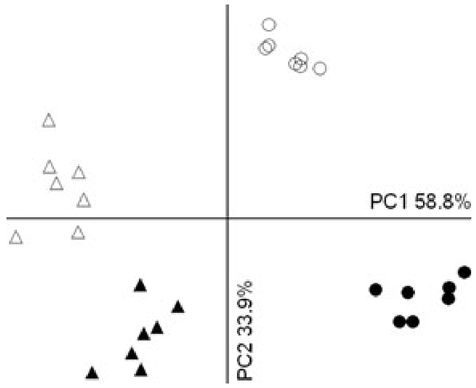

Fig. 1. Ordination generated *via* principal components analysis based on the entire data set obtained (Tables 1–5). Triangles represent *Glycine max*, and circles represent *Brachiaria brizantha*; open symbols represent plants grown under a water deficit, and closed symbols represent well-watered plants. The percentage of variation explained by each principal component (PC1 and PC2) is shown.

When the compatible osmolytes and phenolic compounds were analysed separately (biochemical level), PC1 explained 42.8% of variation in the data and allowed for separation of the two irrigation regimes (Fig. 2D). The main biochemical parameters that allowed a distinction between the groups were proline (eigenvector values >0.68) and total amino acid content (eigenvector values >0.62).

DISCUSSION

Physiological results

Plants with C_3 and C_4 metabolism share many fundamental physiological processes. However, there are also significant differences between these two types of photosynthesis that may result in different responses to water stress (Ghannoum 2009).

The imposed water deficit (30% replacement of water) in this study was sufficient to reduce RWC values in both species (Table 1), affecting their photosynthetic metabolism in different ways and reducing growth of the plants. Studies based on estimated data from *A/Ci* curves have suggested that the regeneration of ribulose-1,5-bisphosphate is affected by water deficit, as both the activity and content of Rubisco are reduced significantly under this condition (Martin & Ruiz-Torres 1992; Lal *et al.* 1996; Escalona *et al.* 1999; Bota *et al.* 2004). However, our results showed that V_{cmax} was maintained in *B. brizantha* plants under the induced water deficit and may have increased A_{maxCO2} homeostasis. Furthermore, the decrease of A_{maxCO2} in the water-stressed *G. max* plants can likely be explained by a decrease in V_{cmax}, indicating a biochemical limitation of photosynthesis. The reduction of *Pr* observed in *G. max* supports this hypothesis because when water deficit reduces the activity of Rubisco independent of *Ci*, indicating a biochemical constraint, both the carboxylation and oxygenation activities of Rubisco are reduced at similar rates (Ghannoum 2009).

According to previous reports (Godde 1999; Chaves & Oliveira 2004, the stress imposed in the present study should be sufficient to reduce the examined fluorescence parameters, interfering with the PSII electron transport chain and, therefore, with net photosynthesis. However, our results indicate that the photochemical apparatus of the two species were not affected by the induced water deficit, with the exception of a reduction in *ETR* observed for *B. brizantha* (Table 4). Accordingly, some authors have shown that the photochemical apparatus is less sensitive to water deficit than are biochemical processes (Flexas *et al.* 2009; Bertolli *et al.* 2012).

Among chemical changes observed in the plants under water deficit, the accumulation of compatible solutes (proline, total amino acids and soluble sugars) and phenolic compounds is related to mechanisms that allow plants to increase their tolerance to low water availability (Moussa & Abdel-Aziz 2008; Cia *et al.* 2012). Although compatible solutes enable plants to maintain cellular turgor pressure (Chaves 1991; Sanders & Arndt 2012), phenolic compounds are related to antioxidant activity, which is necessary to minimise the effects of excess energy caused by reduced ATP consumption *via* the Calvin cycle, a pathway that is impaired by water deficit (Smirnoff 2005; Petridis *et al.* 2012).

Our results indicate that the accumulation of osmolytes in the two species under the applied water deficit (Table 5) might have represented an attempt to maintain cell turgor, preventing RWC from reaching critical levels (below 50%) and possibly causing major and irreversible physiological damage (Lawlor & Cornic 2002). However, the changes in levels of phenolic compounds differed between the two species under water deficit, with the phenolic compound content increasing in *G. max* and decreasing in *B. brizantha* (Table 5). These results were consistent with the changes observed in A_{maxCO2} (Table 3) and in *AES* (Table 4). A_{maxCO2} decreased in *G. max* under water deficit, whereas *ETR* remained stable, likely resulting in the overproduction of reactive oxygen species, demanding higher antioxidant activity and improvement of alternative routes for excess electron production (Ribeiro *et al.* 2003; Miyake 2010); the higher amount of phenolic compounds and higher *AES* value, respectively, support this assumption. Furthermore, the water deficit did not affect A_{maxCO2} in *B. brizantha*, and the obtained *ETR* values were relatively low, indicating the avoidance of oxidative stress, as demonstrated by the lower amount of phenolic compounds (Table 5) and reduced *AES* value (Table 4).

Multivariate analysis: quantifying global responses and addressing the difficulty of finding indicators of stress

Although some of the results obtained for the examined physiological parameters in each species may be associated with the irrigation treatments, results of the PCA showed that interpretations of the observed plant responses depended on the specific group of data evaluated. For instance, the differentiation between the experimental factors (two species × two water regimes) was markedly different when all of the analysed parameters were considered together (Fig. 1) compared with the analysis performed only with biochemical parameters (Fig. 2D). Although four groups are clearly observed in Fig. 1, allowing the species and water treatments to be distinguished, such a distinction between the analysed factors is not found in Fig. 2D. The arrangement of the treatments provided in Fig. 2C (photochemical parameters) also show overlaps, hindering separation of the experimental factors. This result shows that, unlike assumptions of some authors (Demming-Adams *et al.* 1996; Komura *et al.* 2010; Ogaya *et al.* 2011), chlorophyll fluorescence often cannot be considered to represent a reliable indicator of stress in plants. Similarly, when *G. max*

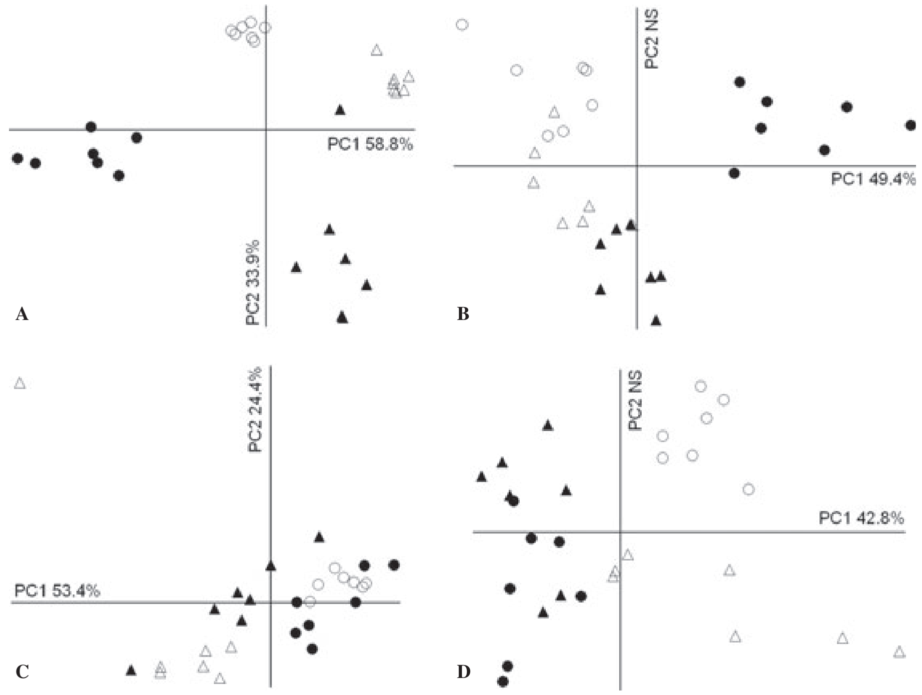

Fig. 2. Ordination generated *via* principal components analysis based on A: data for the growth parameters shown in Table 2, B: variables from the *A/Ci* curve presented in Table 3, C: variables from the chlorophyll fluorescence analysis shown in Table 4, and D: the compatible osmolyte and phenolic compound variables shown in Table 5. Triangles represent *Glycine max*, and circles represent *Brachiaria brizantha*; open symbols represent plants grown under a water deficit, and closed symbols represent well-watered plants. The percentage of variation explained by each principal component (PC1 and PC2) is shown; NS indicates that there was no significant principal component.

and *B. brizantha* were subjected to high temperatures, the photochemical changes detected were poorly correlated with the effects on plant development (Vítolo *et al.* 2012). Regarding gas exchange parameters, PCA was also fairly efficient in separating all of the experimental factors, whereas PC2 was not significant (Fig. 2B), and the separation between treatments based on growth parameters (Fig. 2A) was quite close to results of the PCA performed using the entire experimental data set (Fig. 1). These results suggest that there was an uncoupling between local changes (alterations in low-level physiological traits) and whole-plant performance summarised as biomass production.

Other studies have also shown that when different sets of data (or observation scales) from the same experiment are analysed separately, the results may suggest different responses to the same environmental disturbance. Souza & Cardoso (2003) observed that growth parameters were more sensitive to water stress than biochemical parameters (*i.e.* sugar, proline and protein contents) in *Eucalyptus grandis* cultivated *in vitro*. Fenta *et al.* (2012) reported that, in a soybean cultivar, gas exchange (CO_2 net assimilation and stomatal conductance) was reduced by water deficit, although electron transport in the photochemical apparatus and biomass remained stable. Furthermore, Levitan *et al.* (2010) studied the effects of temperature and CO_2 in *Trichodesmium* and observed an uncoupling between the transcription of genes related to the metabolism of carbon and nitrogen and the enzymatic activities of the corresponding proteins, concluding that the level of transcription cannot be directly extrapolated to metabolic activity.

According to Schlichting & Smith (2002), studies addressing phenotypic plasticity should consider the hierarchical levels of plant organisation because there is a continuity of responses at different levels. As an illustration of this rationale, the signals perceived at the cellular level result in physiological changes, which, in turn, may affect the morphological characteristics of the plant as a whole (Keurentjes *et al.* 2011). Our results suggest that there is no simple and linear rule for the transition between different levels of plant metabolic/morphological organisation. Therefore, studying plant responses to stress factors considering different levels of organisation independent of each other (as simulated here) may lead to different interpretations of the status of the plant as a whole, causing a misunderstanding of the 'real' condition of the capacity of the plants to tolerate or resist environmental disturbances. In contrast, cross-scale multivariate analysis allows for a more consistent interpretation of a complex set of parameters, enabling local effects to be distinguished from global (emergent) effects (Vítolo *et al.* 2012), supporting, for instance, a more consistent breeding programme.

Emergent properties and why it is difficult to identify indicators of stress

Reductionism, specialisation and modularity are considered basic requisites for understanding biological systems in modern science (Lüttge 2012). Nevertheless, modularity, or considering the whole as the sum of its parts, has proved to be insufficient for obtaining complete comprehension of living systems. According to de Kroon *et al.* (2005), the responses of plants to their living environment are the sum of all of their modular responses to local conditions plus all of the interaction effects resulting from the integration of individual modules. Indeed, the integration of modules allows the emergent properties of biological systems to be revealed, thus making them unique individual entities (Lüttge 2012).

Emergence is a process that characterises self-organising systems. Self-organisation is a process of pattern formation (*i.e.* the arrangement of elements organised in space and/or time) through interactions occurring between internal elements of the system, without determinant guidance by agents external to the system (Camazine *et al.* 2001). According to Lüttge (2012), the expression of genes and processes related to epigenetic effects is a clear example of self-organised processes in living systems, whereby the properties of the elements that are involved in these processes do not allow a direct understanding of the emergent patterns.

Therefore, the central question related to the thesis of the lack of consistency regarding the existence of specific indicators of plant stress is based on the theory that the global properties of complex biological systems are emergent (Camazine *et al.* 2000). Accordingly, changes in the specific elements of a system, *e.g.* changes in the concentration of a given molecule or in individual modules, such as in a metabolic reaction, may not represent or even allow for inference of the behaviour of the overall system. A typical example is the increase of proline observed in water-stressed plants, which, despite the well-known osmoprotection properties of this amino acid (Delauney & Verma 1993; Kishor *et al. 2005*), is not always a good indicator of drought tolerance (*e.g.* Mazzafera & Teixeira 1989). This fact is relatively easy to understand when considering that an eventual increase in the concentration of proline may be derived either from an increase in the efficiency of its biosynthetic pathway or from the degradation of proteins. In each case, the general state of the plants and the consequences for their survival are clearly quite different.

Although the environment may clearly trigger consistent responses in plants, the pattern of the response is determined by the internal dynamics of the system itself. These internal dynamics are integrated in a complex metabolic network that operates based on certain rules regarding interactions. The rules that specify the interactions between the system components are followed using only local information, without reference to a pre-existing global pattern (Camazine *et al.* 2000). In complex systems, such interactions are typically nonlinear processes based on negative and positive feedback: negative feedback plays a crucial role in maintaining homeostasis of the system, whereas positive feedback plays a role in pattern formation, propagating and amplifying signals in the system. The two processes work together in the formation and stabilisation of new patterns of organisation, making the prediction of their global behaviour quite difficult, which is one of the typical characteristics of emergent properties (Camazine *et al.* 2000; Souza *et al.* 2005; Lüttge 2012).

In conclusion, the results and arguments presented here show that the classical approach of individual analysis (modular) of different data sets might provide different interpretations regarding the effects of water deficit in plants. Such interpretations do not necessarily imply that the analysis of a particular data set will produce misinterpretations but, instead, suggest that the search for a single indicator to be used to determine the response of plants to environmental perturbations hampers a more accurate diagnosis and a deeper understanding of the interactions between plants and their environment. Therefore, our data support the hypothesis that a cross-scale multivariate analysis (Lüttge 2012; Vítolo *et al.* 2012) is an appropriate method for establishing models that will allow for a systemic understanding of the complex interactions between plants and their changing environment.

In other words, the search for unique indicators that can be used to determine or predict global plant behaviour in response to environmental factors may actually be a search for a type of 'holy grail' or a '*deus ex machina*', as argued by Vítolo *et al.* (2012) and Lawlor & Tezara (2009).

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