UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
Departamento de Botânica
Programa de Pós-Graduação em Biologia Vegetal

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ANA PAULA DE FARIA

ECOPHYSIOLOGICAL RESPONSES OF BRAZILIAN CERRADO INVASIVE GRASSES TO INCREASES IN CO_2 CONCENTRATION AND TEMPERATURE

Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Departamento de Botânica do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Biologia Vegetal.

Área de Concentração Fisiologia Vegetal

BELO HORIZONTE – MG

2015

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“There is freedom waiting for you,
On the breezes of the sky,
And you ask ‘What if I fall?’
Oh but my darling,
What if you fly?”

Erin Hanson

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FIGURES LIST

Chapter 1

TABLES LIST

Chapter 1

Chapter 4

INDEX

ABBREVIATIONS LIST

∅ – diameter

Ψ_w – water potential

A – net photosynthetic assimilation

AGR – absolute growth rate

AMG – amyloglucosidase

ANOVA – analysis of variance

Carot – carotenoids

Chl – chlorophyll

CO_2 – carbon dioxide

Co – control treatment in Chapter 1 (current CO_2 concentration and room temperature)

Ctrl – control treatment in Chapters 2 and 3 (current CO_2 concentration and room temperature)

DAG – days after germination

DF– degrees of freedom

DM – dry mass

E – rate of transpiration

EC – electrical conductivity

EC – elevated CO_2 treatment (doble CO_2 concentration and room temperature)

EC_0 – electrical conductivity measurements of water taken before the rehydration time interval whereupon kinetic curves start to stabilize

EC_f – electrical conductivity measurements of water taken after autoclaving

EC_i – electrical conductivity measurements of water taken at the beginning of the rehydration period

EC_t – electrical conductivity measurements of water taken at different rehydration times ranging from

0.5 to 22.5 h

EC_t – electrical conductivity measurements of water taken after the rehydration time interval whereupon kinetic curves start to stabilize

ECT – elevated CO_2 + temperature treatment (doubled CO_2 concentration and room temperature + 3 ºC)

EL – electrolyte leakage

ESRL – Earth System Research Laboratory

ET – elevated temperature treatment (current CO_2 concentration and room temperature + 3 ºC)

FACE – Free Air CO_2 Enrichment

F_v/F_m – maximum quantum yield

GLM – general linear model

g_s – stomatal conductance

HPAEC/PAD – high performance anion-exchange chromatography with pulsed amperometric detection

IPCC – Intergornmental Panel on Climate Change

LA – leaf area

MGT – mean germination time

MII – membrane injury index

MS – mean square

$NaBH_4$ – sodium borohydride

NaOH – sodium hydroxide

NPQ – non-photochemical quenching

NSC – non-structural carbohydrates

PEG – polyetilene glycol

PEP – phosphoenolpyruvate

PS – photosystem

R_c – electrolyte leakage calculated for control-treated tissues

RG – rate of germination

RGR – relative growth rate

RH – relative humidity

ROS – reactive oxygen species

R_s – electrolyte leakage calculated for PEG-treated tissues

RuBisCO – Ribulose-1,5-bisphosphate carboxylase/oxygenase

SC – structural carbohydrates

SEM – Standard Error Mean

SLA – specific leaf area

SLM – specific leaf mass

SS – sum of squares

T_{15} – temperature which promotes reduction in 15% of initial F_v/F_m values

T_{50} – temperature which promotes reduction in 50% of initial F_v/F_m values

USA – United States of America

WUE – water use efficiency

GENERAL ABSTRACT

Global climate changes and biological invasions are environmental disturbances that may interact synergistically, causing loss of biodiversity. The Brazilian Cerrado is a fragile environment which is greatly affected by anthropic actions. Much of its natural biodiversity has been lost because of spreading African grasses that have gradually replaced its landscapes and this problem is likely to be compounded by climate change. Since most invasive plant species have C4 photosynthetic pathway, many studies focused on understanding how these species respond to elevated CO_2 and temperature. This study aimed to evaluate ecophysiological responses of three invasive African grasses that are more threatening to Brazilian Cerrado biodiversity: *Urochloa brizantha*, *Urochloa decumbens* and *Megathyrsus maximus*. For this purpose, the early stages of development, growth, photosynthetic and biochemical responses to increasing CO_2 and temperature, and the protoplasmatic tolerance of plants grown under these conditions to acute thermal and water stress were investigated. The results obtained indicate that climate changes affect these species in all developmental stages investigated and in all levels, from protoplasm to whole plant. *U. brizantha* and *U. decumbens* were affected from the moment of emergence but only *M. maximus* was affected after seedlings establishment. All the three species had improved water use efficiency under elevated CO_2 and this increase also enhanced photosynthetic assimilation of *U. brizantha* and *M. maximus* and growth of *M. maximus*. Carbohydrate content of *M. maximus* plants was also affected, non-structural carbohydrates being more sensitive to climate changes than cellulose. Lignin content was affected by all environmental treatments, but only for *U. brizantha* plants. Despite the little positive responses during growth, increase in CO_2 and temperature improved *U. decumbens* protoplasmic responses to water deficit, and increase in CO_2 improved its acute heat shock tolerance. Taken together, the results indicate that the effects of increased CO_2 and temperature are species-specific and highlight that all of the three species could benefit in some way by the climate changes foreseen for 2100. It is imperative to investigate native species' responses as well as other invasive species co-occurring in the same environment to assess whether the invasive potential of these species could increase, and to what extent this could be an even greater threat to the biodiversity of the Cerrado.

General Introduction

One of the biggest challenges of the twenty-first century is to find solutions to the problems caused by global climate change. Currently it is widely accepted that carbon dioxide (CO_2) concentrations in the atmosphere have increased steadily over the past two centuries, mainly because of the increase in emissions associated with burning fossil fuels and changes in land use (IPCC 2013). In late 2014, mean CO_2 concentration was 398 μmol mol^{-1} in Mauna Loa, Hawaii (USA) (ESRL 2015) and is expected to further increase, reaching 936 μmol mol^{-1} at the end of the 21st century (IPCC 2013). Increases in CO_2 and other greenhouse gases concentrations may increase the radiant energy entering the earth, also causing a rise in global temperatures (Soon et al. 1999). Each of the last three decades has been successively warmer at the Earth's surface than any preceding decade since 1850 and it is predicted that global temperatures continue to rise, reaching increases between 1.1 and 4.8 ºC by 2100 (IPCC 2013). These increases in CO_2 atmospheric concentration and global mean temperatures are leading towards profound changes in rainfall patterns and could increase the severity and duration of periods with exceptionally high temperatures, commonly known as 'heat waves' (IPCC 2013).

Thereby, concerns about how plants and natural ecosystems will respond to such changes have increased, since climate change is already causing changes in species distribution (Lenoir et al. 2008). Global climate change can affect the productivity and composition of ecosystems directly and can also interact synergistically with other factors of disturbance provoked by any natural or human-caused event, contributing to the decline of native biodiversity in fragile environments (Baruch and Jackson 2005; Barbosa et al. 2010). One of the most important disturbances is facilitating the increase of non-native invasive species in adjacent plant communities. The presence of non-native invasive species in these ecosystems could threaten the existence of native plants and their associated organisms (Barbosa et al. 2010). Among plants, grasses are especially threatening invaders, as they can spread very easily, they are very competitive against native plants in many circumstances, and most of them tolerate fire and they are able to modify the environment severely (D'Antonio and Vitousek 1992). Many African grass species are invasive in other parts of the world, where they are reducing the biodiversity of indigenous communities, changing ecosystem processes and retarding ecosystem restoration (Milton 2004). Alien plants are known to have occurred in

Brazil since the 18th century, when African grasses started to be recorded in pastures near Rio de Janeiro (Zenni and Ziller 2011).

Climate change and the invasion of exotic species are two main factors that have contributed to the decline of native biodiversity in fragile environments like Brazilian Cerrado (Pivello et al. 1999; Barbosa et al. 2010). The Brazilian Cerrado is Brazil's second largest phytogeographical domain in area, surpassed only by the Amazon rainforest, and one of the richest savanna biomes of the world, with high levels of endemism, being considered an extremely important area for conservation (Myers et al. 2000). This biome is becoming dominated by invasive C4 African grasses that have been introduced to improve pasture productivity, but which escaped cultivation and invaded native areas (Pivello et al. 1999). In more open Cerrado areas, African grasses have spread in such magnitude that they are present today in practically every Cerrado fragment, dominating patches of the environment and outcompeting native herbs (Pivello et al. 1999). Among the C4 African grasses that are replacing Cerrado landscape are *Urochloa* spp. (palisade grasses) and *Megathyrsus maximus* (Tanzania grass). These species have rapid reproductive cycles, high dispersal ability and high rates of growth, regrowth and regeneration, herbivory tolerance, greater photosynthesis rates and nutrient use efficiency (D'Antonio and Vitousek 1992; Pivello et al. 1999). Furthermore, they are able to survive on acid and oligotrophic soils and in the presence of aluminum toxicity (Ramos et al. 2012), characteristics that may contribute to their prevalence in relation to native species (Pivello et al. 1999).

Predicting the future functioning of an ecosystem requires mechanistic understanding of how plants deal with different factors under future climate conditions such as high CO_2 concentrations and warmer temperatures (Naudts et al. 2014). Temperature and atmospheric CO_2 are important environmental parameters affecting plant growth, development and function (Eller et al. 2012) and have leading to both beneficial and negative impacts on plant species (Houghton et al. 2001). Such responses of different species can affect population dynamics (Raizada et al. 2009) and have been used to predict which groups of plants will have a competitive advantage in a particular region as a result of climate changes (Collatz et al. 1998). Since the effects of CO_2 and temperature on plants metabolism may counteract each other, the combined effects can be different from any factor separately (Morison and Lawlor, 1999). As these climatic factors will change simultaneously, to understand how plants have responded and will respond to climate change, along with the knowledge of their ability to adapt is an essential first step to understand the full impact that

multiple climate change factors will have on terrestrial ecosystems (Leakey et al. 2009; Eller et al. 2012). So, the effect of increasing atmospheric CO_2 and temperature, together or as separate factors, in plants has been extensively studied over the last decades, especially regarding seed germination and seedlings establishment (Ziska and Bunce 1993; Edwards et al. 2001), growth and photosynthetic performance (Colemam et al. 1991; Morgan et al 2001; Ainsworth et al. 2002; Leakey et al, 2004; Long et al. 2004; Hamilton et al. 2008; Allen et al, 2011; Farfan-Vignolo and Asard 2012), photosynthetic thermotolerance (Colemam et al. 1991; Kakani and Reddy 2007; Hamilton et al. 2008; Mishra et al. 2008; Wang et al. 2008), tolerance to water deficit (Sgherri et al. 1998; Baruch and Jackson 2005; Erice et al. 2007; Vu and Allen Jr. 2009) and carbon metabolism (Lafta and Lorenzen 1995; Blaschke et al. 2002; Souza et al. 2008; Oliveira et al. 2010; Schädel et al. 2010; Ibrahim and Jaafar, 2012; Jie et al. 2012; Richet et al. 2012; Grombone-Guaratini et al. 2013; Song et al. 2014).

Hence, the present study aimed to investigate the effects of increased CO_2 and temperature predicted by the Intergovernmental Panel on Climate Change (IPCC, 2013) for 2100 in ecophysiological responses of three of the most common C4 invasive grass species in Cerrado: *Urochloa brizantha*, *Urochloa decumbens* and *Megathyrsus maximus*, and to assess whether these effects could be a possible indication of an increase in invasive potential of these species in fragile environments, such as the Brazilian Cerrado and similar areas. To access these ecophysiological responses, initially the germination, reserve mobilization efficiency, autotrophy acquisition and early development responses of these species was evaluated, to understand how climate change could affect early stages of plant development. Growth, photosynthetic responses and water relations were subsequently investigated at a whole plant level to evaluate if these C4 species could benefit from CO_2 enriched atmosphere and elevated temperature. Afterward, the effects of climate changes at leaf level were analyzed to assess how leaf carbon metabolism (carbon assimilation and carbohydrates and lignin content) of these species would be influenced. And finally, we investigated if growth under the forecast climate change would influence the protoplasmic tolerance of these species to induced water deficit and acute heat shock.

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Chapter 1[1]

Predicting the impact of increasing carbon dioxide concentration and temperature on seed germination and seedling establishment of African grasses in Brazilian Cerrado

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Abstract Global climate changes and biological invasions are environmental disturbances that may interact synergistically, causing loss of biodiversity. As the early stages of development are the most sensitive and easily affected by these constraints, this study investigated the effects of increased carbon dioxide (CO_2) and temperature, as forecasted for 2100, on seed germination and early development of three species of invasive African grasses that have gradually replaced landscapes of the Brazilian Cerrado biome. It was observed that these parameters affected percentage and rate of germination in *Urochloa brizantha*, rate of germination and mean germination time in *Urochloa decumbens* and accelerated autotrophy acquisition in *U. brizantha*, *U. decumbens* and *Megathyrsus maximus*. Regarding root elongation, all species showed changes in total length, absolute and relative growth rate, but at different stages of development or time intervals, with increased temperature being more significant than increased CO_2, probably due to seed reserves still being the main carbon sources at this stage. Taken together, the results indicate that the effects of CO_2 and increased temperature are species specific and highlight the greatest potential of *U. brizantha* to germinate, and of *U. decumbens* for seedling establishment under these environmental changes.

Key words: *Brachiaria*, CO_2 and temperature, greenhouse gas, IPCC prediction, kernel mobilization, root growth, seed germination.

INTRODUCTION

Currently, it is widely accepted that atmospheric concentrations of carbon dioxide (CO_2) have been increasing steadily over the past two centuries, mainly because of the increase in emissions associated with the burning of fossil fuels and deforestation (Souza *et al.* 2008). Increases in CO_2 and other greenhouse gas concentrations may increase the input of radiant energy on earth, also causing a rise in global temperature (Soon *et al.* 1999). So, in the future, some plants will likely face situations that can cause increased negative impact on growth and development, thus reducing ecosystem productivity (Ciais *et al.* 2005) and biodiversity (Thomas *et al.* 2004).

Global climate change can affect the productivity and composition of ecosystems directly and can also interact synergistically with other factors of disturbance provoked by any natural or human-caused event, contributing to the decline of native biodiversity in fragile environments (Baruch & Jackson 2005; Barbosa *et al.* 2010). One of the most important disturbances is facilitating the increase of non-native invasive species in adjacent plant communities. The presence of invasive species in these ecosystems could threaten the existence of native plants and their associated organisms (Barbosa *et al.* 2010). Among plants, grasses are especially threatening invaders, as they can spread very easily, they are very competitive against native plants in many circumstances and most of them tolerate fire and are able to modify the environment severely (D'Antonio & Vitousek 1992). Many African grass species are invasive in other parts of the world, where they are reducing the biodiversity of indigenous communities, changing ecosystem processes and retarding ecosystem restoration (Milton 2004). Alien plants are known to have occurred in Brazil since the 18th century, when African grasses started to be recorded in pastures near Rio de Janeiro (Zenni & Ziller 2011). Although they have very aggressive behaviour against native grasses, the planting of such exotic grasses continues to be encouraged by

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agricultural agencies because of their high productivity as forage, and there is no control concerning the adverse effects they can bring (Pivello *et al.* 1999).

The Brazilian Cerrado is the second largest biome in area in the country, surpassed only by the Amazon Forest. It is a savanna-like biome that has some of the greatest biodiversity in the world, with high levels of endemism, being considered an area of extreme importance for conservation (Myers *et al.* 2000). This biome is becoming dominated by invasive C4 African grasses that have been introduced to improve pasture productivity, but which have escaped cultivation and invaded native areas (Pivello *et al.* 1999). Three main species of invasive African grasses that are increasingly in competition with the native Cerrado vegetation are *Urochloa brizantha*, *Urochloa decumbens* (known as palisade grasses) and *Megathyrsus maximus*. These species have rapid reproductive cycles, high dispersal ability and high rates of growth, regrowth and regeneration, characteristics that may contribute to their prevalence in relation to native species (Pivello *et al.* 1999). In more open Cerrado areas, African grasses have spread in such magnitude that they are present today in practically every Cerrado fragment, dominating patches of the environment and outcompeting native herbs (Pivello *et al.* 1999).

According to Ziska and Bunce (1993), the early stages of plant development, like seed germination and seedling establishment, are the most sensitive and easily affected by climate change. Such alterations can enhance germination percentage and/or anticipated germination and establishment (Ziska & Bunce 1993), but the first plant developmental stage affected is seed dormancy (Huang *et al.* 2010). Thus, a small growth advantage due to the time of germination or early seedling vigour can affect the growth characteristics of this plant years later (Hussain *et al.* 2001). Because climate change has effects on various plant physiological processes, it could certainly also influence these initial stages of plant development from seed germination to autotrophy acquisition (Ziska & Bunce 1993; Edwards *et al.* 2001). The transition from heterotrophic to autotrophic phase is crucial in the establishment and survival of plants (Escobar-Gutiérrez *et al.* 1998), besides being crucial for the recruitment of species at new and/or aggressive environments (Ribeiro & Borghetti 2014). As this passage is strongly dependent on both the kernel reserves and recently produced photosynthates (Santos & Buckeridge 2004; Lehmeier *et al.* 2005), a more efficient mobilization of intrinsic carbon stocks of seed could ensure greater competitiveness and survival through an early establishment of seedlings (McPeek & Wang 2007). Hence, the present study aimed to investigate the effects of increased CO_2 and temperature predicted by the Intergovernmental Panel on Climate Change (IPCC 2013) for 2100 in germination, reserve mobilization efficiency, autotrophy acquisition and early development of three related species of invasive African grasses: *U. brizantha*, *U. decumbens* and *M. maximus*, and to assess whether these effects could be a possible indication of an increase in invasive potential of these species in fragile environments, such as the Brazilian Cerrado and similar areas.

METHODS

Sampling and experimental treatments

Seeds of three grass species (Appendix 1), *Urochloa brizantha* (Hochst. ex A. Rich.) RD Webster cv Marandu, *U. decumbens* (Stapf) RD Webster cv Basilisk and *Megathyrsus maximus* (Jacq.) BK Simon & S.W.L. Jacobs cv Tanzania, from the same batch (2011–2012 harvest) were donated by Sementes Faria Co. (Belo Horizonte, Brazil). Seed germination and early seedling development was conducted in open-top chambers (1.53 m^3 each) placed inside a glasshouse located at the Institute of Biological Sciences of Minas Gerais, Belo Horizonte, Brazil (19°52′08.67″S 43°57′59.63″W 822-m elevation). Seeds and seedlings were subjected to four environmental treatments based on IPCC (2013) predictions for 2100: All data presented indicate minimum, mean and maximum values, respectively; (i) control – current CO_2 concentration (314, 360 and 388 μmol mol^{-1}) and room temperature (18.4, 23.9 and 44.7°C); (ii) elevated temperature – current CO_2 concentration (316, 360 and 384 μmol mol^{-1}) and 3°C above room temperature (20.0, 27.0 and 49.0°C); (iii) elevated CO_2 – doubled CO_2 concentration (590, 727.5 and 789 μmol mol^{-1}) and room temperature (18.6, 24.1 and 44.9°C); and (iv) Elevated CO_2 + temperature – double CO_2 concentration (586, 727.49 and 786 μmol mol^{-1}) and 3°C above room temperature (20.0, 27.2 and 48.0°C). All environmental treatments were conducted under natural photoperiod and air relative humidity (RH) in December 2011. The environmental conditions (CO_2 concentration, temperature, RH and light intensity) were monitored automatically throughout the experimental growth period.

Seed germination parameters

Seeds of each species were germinated in Gerboxes, transparent plastic boxes 11 cm × 11 cm × 3.5 cm height, lined with two layers of filter paper moistened with 5 mL of nystatin solution (0.2%), a standard fungicide used in germination studies to avoid fungal growth (Gomes *et al.* 2001) and without any known artefactual effects on germination. The germination boxes were arranged in a completely randomized order, and each treatment involved six replicates of 100 seeds per germination box. The seeds were observed each day for signs of germination. Seeds were considered germinated when the radicle had emerged about 2 mm. The tests were ended when there was no further germination after five consecutive days. Percentage of germination, rate of germination (RG) and mean germination time (MGT) were determined. Rate of germination was estimated according to

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Maguire (1962) as $RG = \Sigma(n/t_i)$, where (n) is the number of germinated seeds and (t_i) is the time when these seeds germinate in days, counting from the beginning of germination. Mean germination time was estimated according to Ellis and Roberts (1981) as $MGT = \Sigma dn/\Sigma n$, where (n) is the number of germinated seeds in days (d) counted from the beginning of germination.

Mobilization and utilization of seed reserves

On the first day of germination, a set of germinated seeds were randomly taken from germination boxes, placed in rolls of moistened filter paper (15 × 15 cm 80 g) and used for evaluating the mobilization and utilization of seed reserves during 13 days. For this evaluation, the experiment had four replications: one roll with 20 germinated seeds (composite samples) each, due to the small size of seeds and seedling mass. The samples were collected every 2 days from the first day of germination in a destructive sampling, then separated into kernel and seedling, and oven-dried at 80°C until constant mass. Twenty non-germinated seeds were also dried in the same way as described above. After drying, all samples were weighed using a precision balance (Shimadzu AY220). Kernel dry mass (DM) loss was calculated as the difference between the kernel DM at each harvest point and the initial DM of non-germinated seeds.

Early growth parameters

Another set of 30 germinated seeds were randomly taken from germination boxes and placed in rolls of moistened filter paper (15 × 15 cm 80 g) so that the roots could grow straight and be easily measured daily during 7 days. The total radicle length was determined daily using a digital caliper (Mitutoyo) and the radicle absolute growth rate (AGR) and relative growth rate (RGR) were calculated according to (Hunt 1978). Absolute growth rate was obtained from $AGR = (L_2 - L_1) / (T_2 - T_1)$ and RGR was obtained from $RGR = (\ln L_2 - \ln L_1) / (T_2 - T_1)$, where (L) is radicle length measured daily and (T) is the days when these measurements were taken.

Statistical analyses

Data were previously submitted to parametric normality and homoscedastic tests, following ANOVA for parametric data and general linear model (GLM) analysis for non-parametric data, and means were compared using contrast tests at 5% probability. For germination and reserve mobilization experiments, the environmental treatments were used as explanatory variables. In germination parameter experiments, percentage, mean time and RG were used as response variables. Germination percentage data were submitted to arcsine of the square root transformation before ANOVA. For reserve mobilization experiments, difference between DM of seedling and kernel and kernel DM loss were used as response variables. In early growth parameter analyses, AGR and RGR were used as response variables and the harvest point (DAG – days after germination) was used as an explanatory variable. For root elongation, a linear regression was set to root length measurements, and the slopes were used as the response variable and environmental treatments as explanatory variables in ANOVA analysis. ANOVA and GLM analyses were performed with R3.0.0 (free software) and linear regression analyses were performed with GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Seed germination parameters

The percentage of germination of *U. brizantha* seeds was significantly higher in elevated CO_2 treatments. However, the percentage of germination of other species (*U. decumbens* and *M. maximus*) was not affected by any of the environmental treatments (CO_2 and/or temperature) (Fig. 1).

Regarding the other germination parameters (Table 1), the increase in CO_2 and temperature together increased the rate of germination (RG) of *U. brizantha* seeds, and the increase in temperature increased the RG of *U. decumbens* seeds. The MGT of *U. decumbens* seeds was also significantly lower in all environmental treatments. *Megathyrsus maximus* seeds had none of these germination parameters changed in any of the environmental treatments, nor was the MGT of *U. brizantha* seeds changed.

Mobilization and utilization of seed reserves

The time in days when seedling DM equalled kernel DM occurred earlier in all environmental treatments

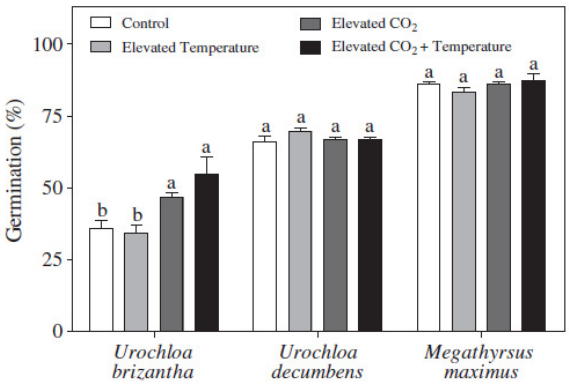

Fig. 1. Percentage seed germination of three invasive grasses species under increased carbon dioxide (CO_2) concentration and temperature. Values represent mean ± SEM. Comparisons were made between environmental treatments. Different letters indicate statistical differences according to ANOVA and post-hoc contrast test with $F = 7.1571$, $^{**}P < 0.01$.

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Table 1. Rate of germination and MGT of seeds of three invasive grass species under increased CO_2 concentration and temperature

	Urochloa brizantha		*Urochloa decumbens*		*Megathyrsus maximus*	
	RG	MGT (days)	RG	MGT (days)	RG	MGT (days)
Control	6.39 ± 0.51b	6.83 ± 0.33a	17.85 ± 0.62b	4.30 ± 0.14a	22.85 ± 0.37a	4.37 ± 0.10a
Elevated temperature	6.74 ± 0.60b	6.23 ± 0.19a	21.54 ± 0.62a	3.72 ± 0.10b	21.89 ± 0.49a	3.82 ± 0.44a
Elevated CO_2	8.88 ± 0.48b	6.38 ± 0.22a	19.23 ± 0.55b	4.04 ± 0.15b	22.70 ± 0.43a	4.16 ± 0.09a
Elevated CO_2 + temperature	11.84 ± 1.95a	5.80 ± 0.42a	20.19 ± 0.66a	3.85 ± 0.16b	23.00 ± 0.76a	4.24 ± 0.14a

Different letters indicate statistical differences. *F* and *P* values are presented in Appendix 3. Values represent mean ± SEM. Comparisons were made between treatments. CO_2, carbon dioxide; MGT, mean germination time; RG, rate of germination.

in relation to the control treatment (current environmental conditions) for *U. brizantha* (Fig. 2A–D) and *U. decumbens* (Fig. 2E–H); however, this did not change for *M. maximus* (Fig. 2I–L). For *U. brizantha*, until the end of the experiment, this time was not observed in the control seedlings (Fig. 2A), whereas in seedlings exposed to all environmental treatments, the DM of seedling and kernel equalized on the 13th day after germination (DAG – dashed lines in Fig. 2B–D). For *U. decumbens* seedlings subjected to the control treatment, seedling DM and kernel DM were equal on the 11th DAG (Fig. 2E), whereas for seedlings subjected to other environmental treatments (elevated CO_2 and/or elevated temperature) seedling DM and kernel DM became equal 2 days before, on the ninth DAG (dashed lines in Fig. 2F–H). For *M. maximus*, the moment when the DW of seedling and kernel became equal occurred on the seventh DAG for all environmental treatments (dashed lines in Fig. 2I–L).

Evaluating the difference in DM between seedling and kernel, it was observed that *U. brizantha* presented positive values only from 13th DAG (Fig. 3A). *Urochloa decumbens* showed positive values for this difference from the ninth DAG (Fig. 3B) and *M. maximus*, from seventh DAG (Fig. 3C). These positive values indicate that the seedling DM exceeded the kernel DM, as can also be seen in Fig. 2, at the points after dashed lines, where the values of seedling DM were higher than the values of kernel DM.

Statistically, some environmental treatment effects on values of the difference between DM of seedling and kernel were only observed on the 13th DAG. It was observed that for *U. brizantha*, only on the 13th DAG, seedlings exposed to elevated temperature and elevated CO_2 + temperature treatments showed positive values, and only those exposed to elevated temperature showed a significant difference (Fig. 3A). For *U. decumbens*, it was observed that from the ninth DAG, only seedlings exposed to elevated CO_2, and elevated CO_2 + temperature showed positive values, and that the seedlings exposed to elevated temperature presented positive values only on 11th DAG (Fig. 3B). Statistical differences in the values were observed only on 13th DAG, and only for seedlings exposed to elevated CO_2 + temperature (Fig. 3B). *Megathyrsus maximus* started to show positive values of difference between DM of seedling and kernel from the seventh DAG, for the seedlings exposed to control and elevated CO_2 treatments (Fig. 3C); but until the 13th DAG, none of the environmental treatments showed significant effects on these values (Fig. 3C).

The onset of autotrophy, marked by stabilization of kernel DM loss, occurred at different times for each species. For *U. brizantha* and *U. decumbens*, autotrophy acquisition was anticipated in 2 days in all environmental treatments, because this loss stabilized on the 11th and the 9th DAG, respectively, whereas under control treatment, this loss stabilized on the 11th DAG for *U. decumbens* kernel. Until the end of the experiment, stabilization of kernel DM loss was not observed for *U. brizantha* control plants. For *M. maximus*, this stabilization varied with treatments, also occurring on the ninth DAG in control and elevated temperature and on the seventh DAG in elevated CO_2 and elevated CO_2 + temperature treatments (Table 2).

Early growth parameters

The main factor affecting root elongation (Fig. 4) was temperature. For *U. brizantha*, roots exposed to elevated temperature and elevated CO_2 + temperature, and for *M. maximus*, roots exposed to elevated temperature treatment, were significantly longer than in other environmental treatments. In *U. decumbens*, no effect of environmental treatments on root growth was observed.

Related to AGR (Table 3), temperature was also the main factor affecting this parameter in *U. brizantha* and *M. maximus* roots. For *U. brizantha*, elevated temperature treatments promoted higher AGR from the second to seventh DAG, except for the third and the seventh days when all environmental treatments promoted AGR higher than control. For *M. maximus*, elevated temperature promoted higher AGR on the second, third, fourth and sixth DAG. The AGR of

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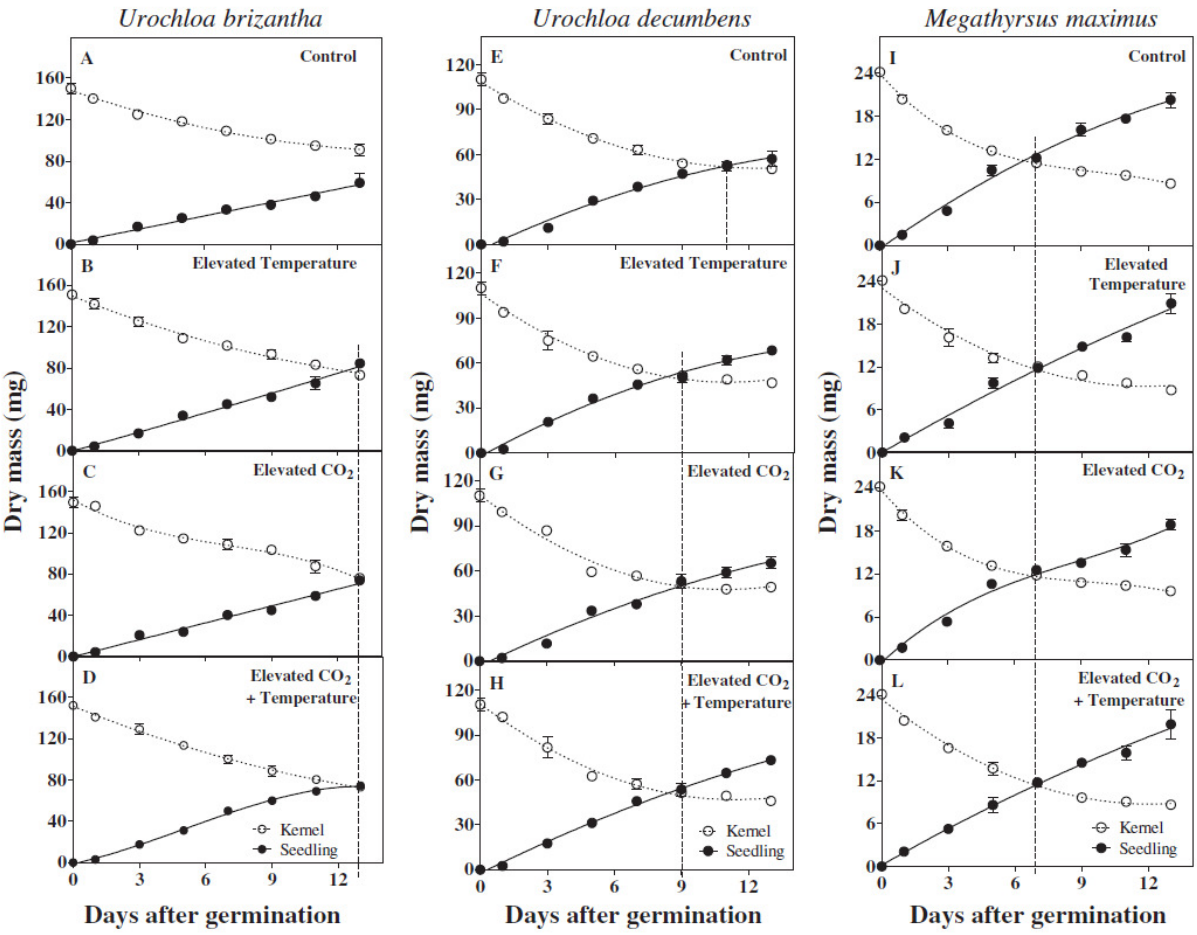

Fig. 2. Reserves utilization during seed germination and establishment of three invasive grasses species under increased carbon dioxide (CO_2) concentration and temperature (A - *Urochloa brizantha* seeds under Control treatment; B - *Urochloa brizantha* seeds under Elevated Temperature treatment; C - *Urochloa brizantha* seeds under Elevated CO_2 treatment; D - *Urochloa brizantha* seeds under Elevated CO_2 + Temperature treatment; E - *Urochloa decumbens* seeds under Control treatment; F - *Urochloa decumbens* seeds under Elevated Temperature treatment; G - *Urochloa decumbens* seeds under Elevated CO_2 treatment; H - *Urochloa decumbens* seeds under Elevated CO_2 + Temperature treatment; I - *Megathyrsus maximus* seeds under Control treatment; J - *Megathyrsus maximus* seeds under Elevated Temperature treatment; K - *Megathyrsus maximus* seeds under Elevated CO_2 treatment; L - *Megathyrsus maximus* seeds under Elevated CO_2 + Temperature treatment). White circles and dotted lines represent the loss of kernels dry mass, and black circles and solid lines represent growing seedlings. Dashed lines indicate the time when dry masses are equal. Values represent mean ± SEM.

U. decumbens was the least affected by environmental treatments, showing higher AGR only on the first, second and fourth DAG, and the main factor affecting this parameter was also temperature, despite all environmental treatments having promoted increased AGR on the second DAG. Regarding RGR (Table 3), it was observed that environmental treatments influenced this parameter in *U. brizantha* and *U. decumbens* roots only at the beginning of growth (first 3 days for *U. brizantha* and first 2 days for *U. decumbens*). From the third day (*U. decumbens*) and fourth day (*U. brizantha*), no significant effect was observed in RGR of roots and, sometimes, a negative effect of treatments in RGR was observed. For *M. maximus*, a significant effect of environmental treatments on RGR of roots on alternate days of observation (second, fourth and sixth days) was observed, and increased temperature influenced this growth.

DISCUSSION

Seed germination parameters

Preliminary germination tests were made (data not shown) and *U. brizantha* mean percentage germination was found to be similar to that presented here in

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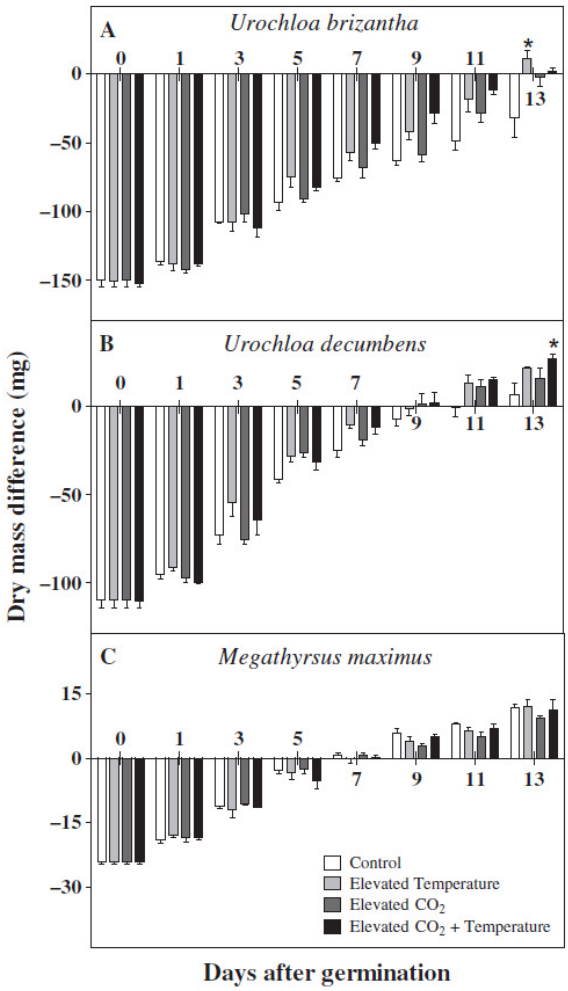

Fig. 3. Dry mass difference between seedling and kernel during seed reserve mobilization of three invasive grasses species (A - *Urochloa brizantha*; B - *Urochloa decumbens*; C - *Megathyrsus maximus*) submitted to increased carbon dioxide (CO_2) concentration and temperature. Values represent mean ± SEM. Comparisons were made between environmental treatments in each day. Asterisks (*) indicate statistical differences $F = 4.714$, $P < 0.05$ (*U. brizantha* 13th day); $F = 3.4893$, $P = 0.0500$ (*U. decumbens* 13th day).

control treatment values. These results are normal for the genus *Urochloa* and especially for *U. brizantha*, which has low germination due to the phenomenon of dormancy and thus produces poor uniformity in germination thereof (Garcia & Cícero 1992; Vieira *et al.* 1998; Martins & Silva 2001; Cavalcante-Filho & Usberti 2008). The most common causes of seed dormancy in this species are the physiological immaturity of the embryo and wrap impermeability to water (Garcia & Cícero 1992). The results obtained indicate that elevated CO_2 acted to increase seed germination percentage in *U. brizantha*. Most studies that found a positive effect of CO_2 on germination cannot explain the reasons for such a response. Ziska and Bunce (1993) and Andalo *et al.* (1998) speculate that CO_2 enrichment can indirectly regulate the primary carbon metabolism via ethylene, which is enhanced under elevated CO_2 and is a regulator of internal growth (Grodzinski 1992). Currently, we are not aware of any studies that demonstrate the effect of small increases in CO_2 altering ethylene production. Andalo *et al.* (1998) suggest that the elevated CO_2 could increase the activity of the enzyme phosphoenolpyruvate carboxylase (PEP) carboxylase in the radicle, and that this enzyme could have a critical role in the build-up of metabolic alterations needed to start a significant growth during the first phase of germination (Sangwan *et al.* 1992).

Although most studies focusing on the effects of climate change in the germination stage evaluate only the final percentage, it is also important to assess the RG and MGT, because rapid germination is an advantage for seedling establishment under field conditions (Parsons 2012). The results showed that the major factor increasing RG for *U. decumbens* was the increase in temperature. Similar results were observed for dandelion (McPeek & Wang 2007) and *Trichilia emetica* seeds (Sershen *et al.* 2014). Contrastingly, for *U. brizantha* seeds, elevated CO_2 acted synergistically with elevated temperature to enhance SG, whereas the same effect was not observed with the increase of each factor alone.

It is widely recognized that an increase in temperature can affect several aspects of seed biology, including viability (Garcia-Nuñes *et al.* 2001), dormancy (Ooi *et al.* 2006; Ribeiro *et al.* 2013) and patterns of germination (Ooi *et al.* 2009). Provided that sufficient water is available and that the optimal range for germination is not exceeded, high temperatures may even accelerate germinative development and improve the competitive ability of seedlings produced later (Sershen *et al.* 2014). In the specific case of *U. brizantha*, Vieira *et al.* (1998) showed that storage temperature affects seed dormancy behaviour of this species and that a temperature of 28°C accelerated overcoming seed dormancy in relation to other temperatures tested. Also, elevated storage temperatures such as 65 and 70°C before germinating could overcome dormancy and enhance seed germination (Vieira *et al.* 1998; Martins & Silva 2001; Cavalcante-Filho & Usberti 2008) and germination rate of *U. brizantha* (Martins & Silva 2001). All seeds used in this work were stored at room temperature before germination tests, but as shown by germination percentage presented by *U. brizantha* under control treatment, room temperature did not reach 28°C.

However, the effect of increased CO_2 and increased CO_2 + temperature together in early developmental stages is still controversial. Some studies consider that warming and elevated CO_2 concentrations would

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Table 2. Kernels DM loss due to reserve mobilization during seeds germination and seedlings establishment of three invasive grasses under increased CO_2 concentration and temperature

	DAG	*Urochloa brizantha* Kernels DM loss (mg)	*Urochloa decumbens* Kernels DM loss (mg)	*Megathyrsus maximus* Kernels DM loss (mg)
Control	1	9.8 ± 2.6c	12.6 ± 3.9e	3.8 ± 0.6d
	3	25.2 ± 2.0c	26.3 ± 4.5d	8.1 ± 0.4c
	5	31.8 ± 1.5c	39.3 ± 3.9c	10.9 ± 0.9b
	7	40.9 ± 3.3b	46.8 ± 4.8c	12.7 ± 0.5b
	9	48.9 ± 4.6b	53.9 ± 2.7b	13.8 ± 0.7a
	11	55.1 ± 1.6b	57.2 ± 3.1a	14.4 ± 0.7a
	13	59.1 ± 1.7a	59.6 ± 3.6a	15.5 ± 0.8a
Elevated temperature	1	9.1 ± 3.8c	16.1 ± 2.3c	4.0 ± 1.1c
	3	26.1 ± 3.5c	35.0 ± 7.9b	8.0 ± 1.7b
	5	42.1 ± 7.0c	45.6 ± 4.9b	10.9 ± 1.2b
	7	49.2 ± 5.6b	54.0 ± 5.0b	12.1 ± 0.4b
	9	57.4 ± 6.5b	58.4 ± 4.8a	13.3 ± 0.4a
	11	67.7 ± 6.4a	60.8 ± 5.6a	14.4 ± 0.6a
	13	77.7 ± 5.6a	63.3 ± 5.7a	15.4 ± 0.5a
Elevated CO_2	1	12.8 ± 2.9c	10.7 ± 2.2d	3.9 ± 0.8d
	3	32.0 ± 4.4b	23.1 ± 3.6c	8.3 ± 0.6c
	5	39.7 ± 2.9b	50.5 ± 4.5b	10.0 ± 0.9b
	7	45.5 ± 5.2b	52.2 ± 3.9b	12.3 ± 0.8a
	9	50.8 ± 4.3b	56.7 ± 2.2a	13.4 ± 0.6a
	11	66.5 ± 7.6a	61.2 ± 3.6a	13.8 ± 0.6a
	13	78.1 ± 5.4a	64.6 ± 4.2a	14.5 ± 0.7a
Elevated CO_2 + temperature	1	11.5 ± 2.1d	8.2 ± 3.1d	3.7 ± 0.5c
	3	23.0 ± 6.2d	28.6 ± 7.2d	7.5 ± 0.4c
	5	38.7 ± 3.2c	47.8 ± 2.6c	10.1 ± 0.6b
	7	51.9 ± 5.4b	53.1 ± 0.8b	12.7 ± 0.6a
	9	63.5 ± 5.3b	57.6 ± 2.8a	14.5 ± 0.5a
	11	71.6 ± 3.9a	58.8 ± 2.8a	15.1 ± 0.6a
	13	79.1 ± 4.2a	63.3 ± 2.9a	15.5 ± 0.5a

Different letters indicate statistical differences. *F* and *P* values are presented in Appendix 5. Values represent means ± SEM. Comparisons were made between days (DAG) in each environmental treatment. CO_2, carbon dioxide; DAG, days after germination; DM, dry mass.

affect seedling emergence via their indirect impacts on soil moisture and the cover of the surrounding plant community (Engel *et al.* 2009; Kardol *et al.* 2010) as well as the interaction between grasses and symbiotic endophytes (Brosi *et al.* 2011). In contrast, Classen *et al.* (2010) found that climatic changes both directly and indirectly alter seedling emergence and establishment of the studied species. Germination and establishment have an important ecological significance, besides being considered the most critical stage of a plant's life cycle, because of its high vulnerability to environmental stresses (Li *et al.* 2012). Because seedling emergence is synchronized with changes in the environment (Baskin & Baskin 1988), any environmental factor that alters the characteristic speed and/or percentage of germination of a species influences the potential for persistence of this species in natural environments (Parsons 2012). Climate change may influence germination and seedling recruitment, which may influence the population dynamics and thus the species composition and diversity in communities (Walck *et al.* 2011). The results here suggest that elevated CO_2 can enhance germination percentage of *U. brizantha*, that elevated temperature can increase RG of *U. decumbens* seeds and that elevated CO_2 + temperature can increase RG of *U. brizantha* seeds. All this improvement may place such species in ecological advantage, depending on environmental conditions faced during seedling establishment after radicle emergence. It is important to highlight that elevated CO_2 and warming interacted to affect the RG of *U. brizantha* seeds, a result that would have remained unknown if the experimental treatments were applied in isolation.

Mobilization and utilization of seed reserves

The patterns of reduction in kernel and increase in seedling DM observed may be explained by the fact that the transition from dependency on seed reserves to external resources occurs gradually and the length

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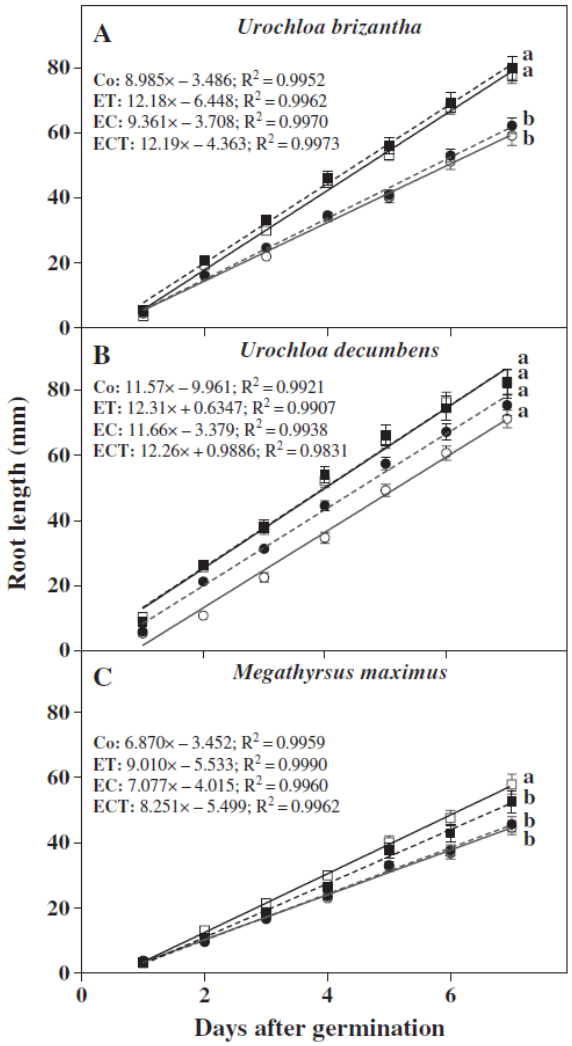

Fig. 4. Seedling root length (mm) of three invasive grasses species (A - *Urochloa brizantha*; B - *Urochloa decumbens*; C - *Megathyrsus maximus*) submitted to increased carbon dioxide (CO_2) concentration and temperature. Equations of straight lines and R^2 values are shown in the figure. (○) Co – Control, (□) ET – elevated temperature, (●) EC – elevated CO_2, (■) ECT – elevated CO_2 + temperature. Values represent mean ± SEM. Comparisons were made between linear regression slopes means. Different letters indicate statistical differences according to ANOVA and post-hoc contrast test of linear regression slopes with $F = 9.2203$, $^{***}P < 0.001$ (*U. brizantha*); $F = 4.3$, $^{**}P < 0.01$ (*U. decumbens*).

of the passage is determined by species and environment (Kitajima 1994). The sensitive seedling stage can be strongly influenced by available reserves (Santos & Buckeridge 2004), and therefore, one of the most studied processes in seedling development is the mobilization of complex polymers such as starch, proteins and lipids stored in tissues such as endosperm or cotyledons (Sánchez-Linares *et al.* 2012). The content of storage substances in seeds has profound effects on germination and establishment because it is used both as substrate and as an energy source for plant growth (Lehmeier *et al.* 2005). For the same reasons, the reserve content of seeds can also affect seedling responses to atmospheric CO_2 concentrations, which can interact with internal factors, such as nitrogen or carbon stocks of seeds (Hussain *et al.* 2001). Temperature rises, when the ideal range is not exceeded, can also increase growth rates, leaf area and biomass allocation to shoots of seedlings (Sershen *et al.* 2014). Thus, internal and external factors that regulate the early growth phase can act collectively to regulate subsequent seedling growth (Escobar-Gutiérrez *et al.* 1998), as was observed for *U. decumbens* seedlings, where all treatments accelerated the moment when seedling DM equalled kernel DM compared with control. Although we had not identified the moment when seedling and kernel DM equalled those of *U. brizantha* seedlings exposed to the control treatment, the fact that equivalence occurred on the 13th day in all other treatments indicates a trend that climate change promotes the same response in this species.

Seedling DM becoming greater than kernel DM indicated kernel reserves mobilization efficiency (Asch *et al.* 1999). The efficiency of reserve mobilization values disclosed herein may be somewhat overestimated because we assume that seedling photosynthesis had little effect on growth before they become autotrophic, although they had presented green leaves even before the overall growth rate become positive.

The transition from heterotrophy to autotrophy is crucial in the establishment and survival of plants. According to Asch *et al.* (1999), the time where a reduction in kernel DM stabilizes marks the beginning of autotrophic seedling stage, because the contribution of photosynthates becomes greater. In this study, it was observed that this transition occurred at different times depending on the treatment and varied among the species. For *U. decumbens*, the onset of autotrophic phase was accelerated by 2 days in all environmental treatments compared with the control. For *M. maximus*, only the increase in CO_2 concentration accelerated the onset of autotrophy by 2 days in relation to the control and elevated temperature treatments. Among these species, *M. maximus* has the smallest seeds (see Appendix 1). Small seeds may have higher surface to volume ratio and consequently a greater capacity for CO_2 diffusion (Ziska & Bunce 1993). This probably explains why the CO_2 concentration accelerated autotrophy acquisition in this species.

Due to space limitation, which could cause an effect on roots, it was not practical to continue the experiments until it was possible to observe the stabilization of kernel DW loss of *U. brizantha* in the control

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Table 3. Absolute growth rate and RGR of three invasive grasses species under increased CO_2 concentration and temperature

		AGR (mm)				RGR (mm)			
	DAG	Control	Elevated temperature	Elevated CO_2	Elevated CO_2 + temperature	Control	Elevated temperature	Elevated CO_2	Elevated CO_2 + temperature
Urochloa brizantha	1	4.55 ± 0.20a	3.76 ± 0.19b	4.86 ± 0.36a	5.72 ± 0.43a	1.46 ± 0.07a	1.29 ± 0.05b	1.51 ± 0.07a	1.67 ± 0.07a
	2	12.28 ± 0.46b	15.73 ± 0.51a	11.12 ± 0.34c	15.22 ± 0.61a	1.34 ± 0.04b	1.67 ± 0.04a	1.25 ± 0.05b	1.34 ± 0.05b
	3	6.11 ± 0.38d	10.37 ± 0.47b	8.66 ± 0.43c	12.20 ± 0.82a	0.32 ± 0.02b	0.43 ± 0.02a	0.43 ± 0.02a	0.46 ± 0.03a
	4	12.08 ± 0.70c	14.71 ± 0.57a	10.0 ± 0.64c	12.96 ± 1.04b	0.44 ± 0.03a	0.40 ± 0.01a	0.34 ± 0.02b	0.32 ± 0.02b
	5	6.05 ± 0.58b	8.49 ± 0.46a	6.3 ± 0.56b	9.89 ± 0.79a	0.16 ± 0.02a	0.18 ± 0.01a	0.16 ± 0.01a	0.19 ± 0.01a
	6	11.01 ± 0.97b	14.51 ± 0.73a	12.03 ± 0.79b	13.33 ± 0.90b	0.23 ± 0.02a	0.24 ± 0.01a	0.26 ± 0.01a	0.21 ± 0.01a
	7	7.93 ± 0.74b	10.11 ± 0.83a	9.18 ± 0.60a	10.34 ± 0.88a	0.14 ± 0.01a	0.13 ± 0.01a	0.16 ± 0.01a	0.14 ± 0.01a
Urochloa decumbens	1	5.28 ± 0.47b	10.35 ± 0.53a	5.87 ± 0.47b	8.99 ± 0.51a	1.58 ± 0.11b	2.30 ± 0.05a	1.68 ± 0.08b	2.15 ± 0.06a
	2	9.12 ± 0.76b	15.29 ± 0.89a	15.36 ± 0.56a	17.39 ± 1.04a	1.06 ± 0.10b	0.90 ± 0.04b	1.35 ± 0.05a	1.06 ± 0.05b
	3	11.72 ± 0.69a	11.96 ± 0.79a	10.07 ± 0.46a	11.74 ± 0.74a	0.83 ± 0.07a	0.37 ± 0.02b	0.39 ± 0.02b	0.37 ± 0.01b
	4	12.18 ± 0.47b	14.48 ± 0.90b	13.21 ± 0.55b	15.97 ± 0.83a	0.48 ± 0.03a	0.36 ± 0.05b	0.36 ± 0.01b	0.37 ± 0.04b
	5	14.51 ± 0.75a	12.43 ± 1.11a	12.78 ± 0.75a	11.77 ± 1.04a	0.37 ± 0.02a	0.22 ± 0.02c	0.25 ± 0.01b	0.19 ± 0.01c
	6	11.36 ± 0.80a	10.63 ± 1.27a	9.85 ± 0.90a	8.32 ± 1.03a	0.21 ± 0.01a	0.15 ± 0.02b	0.15 ± 0.01b	0.12 ± 0.02b
	7	10.69 ± 0.92a	8.61 ± 1.47a	8.42 ± 0.78a	8.27 ± 1.38a	0.16 ± 0.01a	0.10 ± 0.02a	0.11 ± 0.01a	0.11 ± 0.02a
Megathyrsus maximus	1	3.27 ± 0.22a	1.57 ± 0.35a	1.23 ± 0.60a	1.93 ± 0.36a	1.12 ± 0.07a	0.85 ± 0.12a	0.96 ± 0.19a	1.14 ± 0.11a
	2	7.31 ± 0.33b	11.52 ± 0.45a	8.70 ± 0.41b	8.92 ± 0.46b	1.22 ± 0.06b	1.44 ± 0.09a	1.11 ± 0.19b	1.26 ± 0.08b
	3	6.50 ± 0.50b	8.39 ± 0.47a	6.98 ± 0.29b	7.76 ± 0.51b	0.47 ± 0.02a	0.50 ± 0.03a	0.56 ± 0.02a	0.54 ± 0.03a
	4	5.89 ± 0.42b	8.55 ± 0.46a	7.00 ± 0.29b	7.75 ± 0.51a	0.29 ± 0.01b	0.33 ± 0.01a	0.35 ± 0.01a	0.34 ± 0.02a
	5	9.77 ± 0.61a	10.17 ± 0.93a	9.59 ± 0.75a	11.36 ± 1.10a	0.35 ± 0.01a	0.28 ± 0.02a	0.33 ± 0.02a	0.33 ± 0.02a
	6	4.12 ± 0.46b	7.43 ± 0.67a	4.50 ± 0.45b	5.22 ± 0.55b	0.12 ± 0.01b	0.16 ± 0.01a	0.12 ± 0.01b	0.13 ± 0.01b
	7	7.80 ± 0.74a	10.35 ± 1.22a	7.99 ± 0.85a	9.71 ± 0.97a	0.20 ± 0.02a	0.19 ± 0.02a	0.18 ± 0.02a	0.19 ± 0.02a

Different letters indicate statistical differences. *F* and *P* values are presented in Appendix 7. Comparisons were made between environmental treatments for each harvest point (DAG). Values represent means ± SEM. AGR, absolute growth rate; CO_2, carbon dioxide; DAG, days after germination; RGR, relative growth rate. *Ub*, *Urochloa brizantha*; *Ud*, *Urochloa decumbens*; *Mm*, *Megathyrsus maximus*.

treatment. Therefore, for this species, it was impossible to identify any trend in acceleration of autotrophy acquisition by environmental treatments. It can be said, at least, that climate changes preceded autotrophy acquisition, because seedlings exposed to all environmental treatments become autotrophic at the 11th day. Among these species, *U. brizantha* presented the largest seeds (larger pool reserves – see Appendix 1), which could explain the relatively late appearance of autotrophic growth (Asch *et al.* 1999). This delay could also be explained by genetic factors (Asch *et al.* 1999), or by other factors, because reserves use and transport are regulated by plant hormones such as abscisic acid and gibberellic acid (Pritchard *et al.* 2002; Eastmond 2006). Seeds reserves directly influence seedling growth only for a very short period of time, after which growth is based on new photosynthates (Hussain *et al.* 2001). Typically, the amount of seed resources is important for the development of leaf area and other organs necessary for the mobilization of external resources that ultimately allow the seedlings to maintain autotrophic growth (Paz & Martínez-Ramos 2003). In this context, for *U. brizantha*, the contribution of the intrinsic carbon reserves of the seeds to sustain the discrete but required embryo metabolic activity that occurs prior to, and at the point of, radicle emergence has been emphasized (Sánchez-Linares *et al.* 2012). The effect of growth is particularly important during the early stages of development, because the competition is greater between species during establishment and small plants are most affected by environmental constraints (Asch *et al.* 1999). The current largest amount of reserves in *U. brizantha* seeds may represent the strategy adopted by this species to ensure greater efficiency in competition with other species, delaying the acquisition of autotrophy.

Early growth parameters

Results obtained indicate that all environmental treatments had an influence in some way on the initial root growth of all species studied. Root elongation was affected by elevated temperature, but taking ARG and RGR into account, the effect of increased CO_2 was also observed for a few moments for *U. brizantha* and *U. decumbens*. However, temperature seems to be the main active factor on root growth, as elevated temperature improved root growth parameters of all species studied.

At the seedling stage, it is difficult to make predictions of plant establishment and how seedlings will forage for minerals and water without the prior knowledge of how root morphogenesis responds to climate change. Although it is generally recognized that

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increased temperature results in a higher germination rate and a faster early growth rate, the influence of elevated CO_2 in the first stages of root growth is poorly understood (Andalo *et al.* 1998). There are few systematic studies on the effects of elevated CO_2 at this critical stage of development. Most studies regarding subterranean plant responses are conducted with adult or already established plants (Rogers *et al.* 1993; Day *et al.* 1996). Andalo *et al.* (1998) found no significant effect of elevated CO_2 in *Arabidopsis thaliana* seedlings 6 and 9 days after germination. In contrast, Prior *et al.* (1994) observed a 19% increase in total root length of cotton in early growth under elevated CO_2, and Ferris and Taylor (1994) observed an increase in the extent of fine roots and cell elongation under elevated CO_2 in all species studied.

Results obtained by Ferris and Taylor (1994), using a biophysical analysis of root cell elongation as influenced by elevated CO_2, suggest that root growth is stimulated after increased cell expansion, and that increased phosphorus and cell wall tensiometric extensibility are probably crucial for root growth reinforced under elevated CO_2. For plants already established, the general conclusion is that CO_2 enrichment generally increases root growth (Rogers *et al.* 1992a,b), which has always been related to increased photosynthesis of above-ground structures. It must be remembered that the process studied here is less dependent on photosynthesis than the experiments of classical growth: while the first leaves appeared before the 7 days of the experiment, the use of kernel reserves continued until the 7th, 9th and 13th days in *M. maximus*, *U. decumbens* and *U. brizantha*, respectively, in our experiment. It is likely, for *U. decumbens*, which showed an increase in root growth under elevated CO_2, that this gas increased PEP carboxylase activity, which operates in the initial radicle growth (Sangwan *et al.* 1992) or promoted greater cell wall plasticity in radicle cells (Ferris & Taylor 1994).

For AGRs and RGRs of roots, the results indicate that root growth in the first days after germination is primarily responsible for the final root length after 7 days, increasing temperature being the main factor responsible for this early growth. The ability to germinate and establish at high temperatures is crucial for survival and recruitment of species in harsh environments (Ribeiro & Borghetti 2014). This faster root growth observed in all species can ensure a deeper exploration of soil volume and thus enhance absorption of nutrients and water available for plant establishment. This improved soil exploitation capacity has important implications for natural ecosystems where nutrients and water limitations can be extreme (Prior *et al.* 1994), as in the Brazilian Cerrado. Changes in rooting patterns also raise the possibility of changes in plant competition in ecosystems. And as plant roots are an interface with soil, the factors that affect them are important for the function of natural communities (Prior *et al.* 1994).

Taken together, the results presented here indicate that the effects of global climate change will be demonstrated in a species-specific manner in the early stages of development and tend to be beneficial for all three species studied. As competition is greater at the stage of establishment, any advance in this process can lead to competitive advantage. Palisade grass species (*U. brizantha* and *U. decumbens*) showed positive responses earlier from the moment of germination. Both benefited by reducing the time required for emergence and by an acceleration of the onset of autotrophy. *Urochloa brizantha* also benefited by increasing the percentage of germination. The effects of climate change were more evident in the responses of *M. maximus* only after the plant had been established. And it was notable that the increase in CO_2 concentration was important to ensure earlier establishment of this species. The acceleration in germination and in acquisition of autotrophy of palisade grasses confer significant competitive advantages and this increase in rooting observed for all species may allow a more efficient use of soil resources with climate change. All these possible competitively acquired advantages through climate change could intensify the invasive potential of these species, especially palisade grasses, in the near future, if native plants do not show the same responses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Seeds of the three species studied.
Appendix S2. ANOVA table of percentage germination.
Appendix S3. ANOVA table of rate of germination (RG) and mean germination time (MGT).
Appendix S4. Analysis of deviance table of dry mass difference.
Appendix S5. Analysis of deviance table of Kernel dry mass (DM) loss.
Appendix S6. ANOVA of linear regression slopes of root length.
Appendix S7. Analysis of deviance table of absolute growth rate (AGR) and relative growth rate (RGR).

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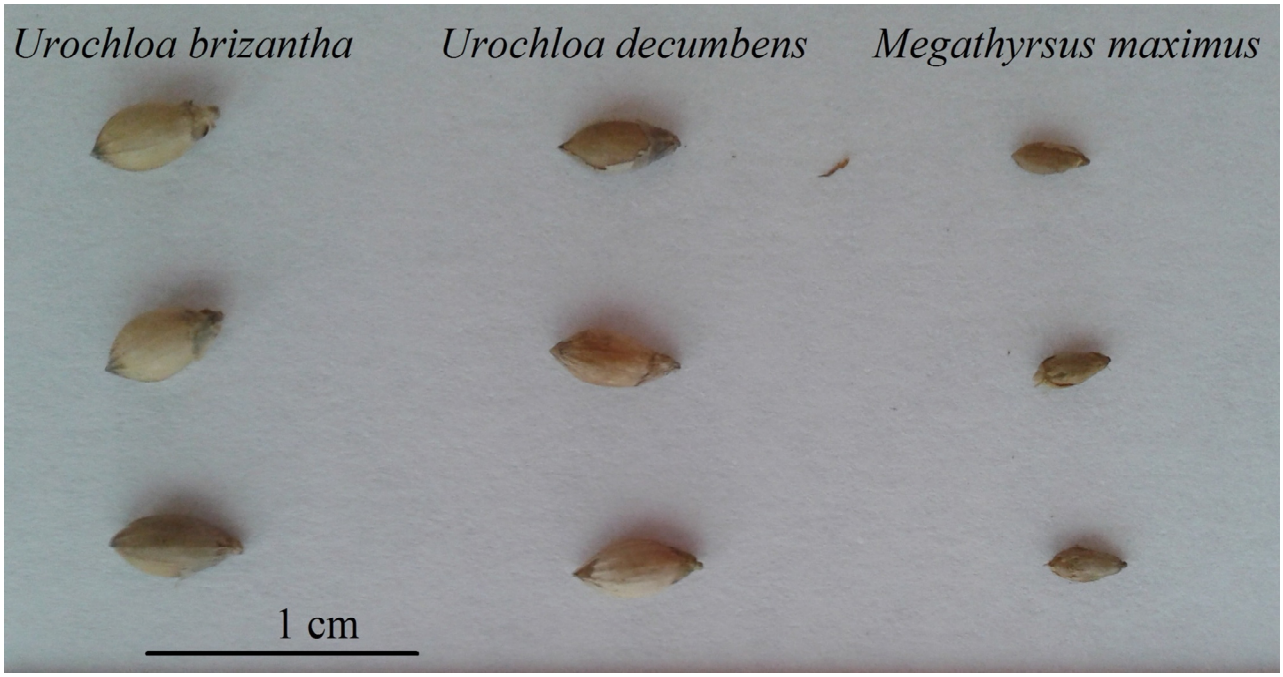

Appendix S1. Seeds of three invasive grasses species: *Urochloa brizantha* (Hochst. ex A. Rich.) RD Webster cv Marandu, *U. decumbens* (Stapf) RD Webster cv Basilisk and *Megathyrsus maximus* (Jacq.) BK Simon & S.W.L. Jacobs cv Tanzania.

Appendix 2. ANOVA table of Percentage of Germination - Fig. 1

Urochloa brizantha

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)	
Treatments	3	0.17932	0.059774	7.1571	0.00189	**
Residuals	20	0.16703	0.008352			

Urochloa decumbens

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)
Treatments	3	0.0051387	0.0017129	1.5194	0.2401
Residuals	20	0.0225466	0.0011273		

Megathyrsus maximus

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)
Treatments	3	0.011599	0.0038664	1.3979	0.2726
Residuals	20	0.055315	0.0027657		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 3. ANOVA table of Rate of Germination (RG) and Mean Germination Time (MGT) - Table 1

Rate of Germination (RG)

Urochloa brizantha

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)	
Treatments	3	113.50	37.833	5.3933	0.00695	**
Residuals	20	140.29	7.015			

Urochloa decumbens

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)	
Treatments	3	43.539	14.5130	6.4195	0.00319	**
Residuals	20	45.216	2.2608			

Megathyrsus maximus

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)
Treatments	3	4.461	1.4869	0.8642	0.4759
Residuals	20	34.410	1.7205		

Mean Germination Time (MGT)

Urochloa brizantha

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)
Treatments	3	2.7792	0.92640	1.7734	0.1846
Residuals	20	10.4476	0.52238		

Urochloa decumbens

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)	
Treatments	3	1.1097	0.36988	3.2337	0.04406	*
Residuals	20	2.2877	0.11438			

Megathyrsus maximus

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)
Treatments	3	0.9924	0.33081	0.958	0.4317
Residuals	20	6.9060	0.34530		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 4. (a) Analysis of Deviance table of Dry Mass Difference - Fig. 3A

Urochloa brizantha

13^{th} Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			15	7673.5			
Treatments	3	4151.1	12	3522.4	4.714	0.02135	*

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 4. (b) Analysis of Deviance table of Dry Mass Difference - Fig. 3B

Urochloa decumbens

9^{th} Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			15	1421.0			
Treatments	3	210.35	12	1210.7	0.695	0.5727	

11^{th} Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			15	1461.9			
Treatments	3	578.31	12	883.59	2.618	0.09897	.

13^{th} Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			15	1963.2			
Treatments	3	914.67	12	1048.5	3.4893	0.0500	.

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 4. (c) Analysis of Deviance table of Dry Mass Difference - Fig. 3C

Megathyrsus maximus

7^{th} Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)
NULL			15	25.858		
Treatments	3	2.6625	12	23.195	0.4592	0.7159

9^{th} Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)
NULL			15	53.034		
Treatments	3	19.927	12	33.108	2.4075	0.118

11^{th} Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)
NULL			15	59.694		
Treatments	3	17.362	12	42.333	1.6405	0.2322

13^{th} Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)
NULL			15	121.53		
Treatments	3	19.625	12	101.91	0.7703	0.5325

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 5. (a) Analysis of Deviance table of Kernel Dry Mass (DM) loss - Table 2

Urochloa brizantha

Control

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	9510.0			
Time	6	8861.5	21	648.5	47.823	3.591E-11	***

Elevated Temperature

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	16198.9			
Time	6	13535	21	2663.6	17.786	3.042E-07	***

Elevated CO_2

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	13164.5			
Time	6	11254	21	1910.1	20.623	8.521E-08	***

Elevated CO_2 + Temperature

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	17020.2			
Time	6	15290	21	1730.5	30.923	2.227E-09	***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 5. (b) Analysis of Deviance table of Kernel Dry Mass (DM) loss - Table 2

Urochloa decumbens

Control

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	8745.8			
Time	6	7505.7	21	1240.2	21.183	6.743E-08	***

Elevated Temperature

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	9323.8			
Time	6	6928.5	21	2445.3	9.9167	3.112E-05	***

Elevated CO_2

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	10385			
Time	6	9482.8	21	902	36.795	4.39E-10	***

Elevated CO_2 + Temperature

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	17020.2			
Time	6	15290	21	1730.5	30.923	2.23E-09	***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 5. (c) Analysis of Deviance table of Kernel Dry Mass (DM) loss - Table 2

Megathyrsus maximus

Control

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	448.98			
Time	6	409.23	21	39.75	36.033	5.346E-10	***

Elevated Temperature

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	469.64			
Time	6	380.43	21	89.21	14.925	1.31E-06	***

Elevated CO_2

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	380.73			
Time	6	338.72	21	42.01	28.22	5.165E-09	***

Elevated CO_2 + Temperature

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			27	17020.2			
Time	6	15290	21	1730.5	30.923	2.227E-09	***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 6. ANOVA of Linear Regression Slopes of Root Lenght - Fig. 4

Urochloa brizantha

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)	
Treatments	3	236.56	78.852	9.2203	1.61E-05	***
Residuals	116	992.04	8.552			

Urochloa decumbens

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)	
Treatments	3	7.72	2.5745	0.2449	0.8648	
Residuals	116	1219.51	10.513			

Megathyrsus maximus

	Df	Sum Sq.	Mean Sq.	F value	Pr(>F)	
Treatments	3	93.21	31.069	4.3	0.00647	**
Residuals	116	838.15	7.225			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 7. (a) Analysis of Deviance table of Absolute Growth Rate (AGR) and Relative Growth Rate (RGR) - Table 3

Urochloa brizantha

AGR

1st Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	433.11			
Treatments	3	58.084	116	375.02	5.9887	0.000786	***

2nd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1232.97			
Treatments	3	437.01	116	795.96	21.229	4.95E-11	***

3rd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1668.7			
Treatments	3	604.34	116	1064.3	21.956	2.49E-11	***

4th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	2350.2			
Treatments	3	345.53	116	2004.7	6.6647	0.0003428	***

5th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1595.9			
Treatments	3	302.86	116	1293.0	9.0565	1.95E-05	***

6th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	2724.0			
Treatments	3	208.92	116	2515.1	3.2119	0.02561	*

7th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	2583.2			
Treatments	3	290.28	116	2292.9	4.8952	0.003064	**

RGR

1st Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	16.259			
Treatments	3	2.1705	116	14.088	5.9571	0.000818	***

2nd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	10.1253			
Treatments	3	3.0819	116	7.0435	16.919	3.51E-09	***

3rd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	2.2133			
Treatments	3	0.3242	116	1.8891	6.6356	0.0003552	***

4th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1.8908			
Treatments	3	0.26821	116	1.6225	6.3916	0.0004789	***

5th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	0.58503			
Treatments	3	0.01382	116	0.57121	0.9357	0.4259	

6th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	0.84024			
Treatments	3	0.03054	116	0.80970	1.4583	0.2296	

7th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	0.3476			
Treatments	3	0.00937	116	0.33823	1.0711	0.3642	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 7. (b) Analysis of Deviance table of Absolute Growth Rate (AGR) and Relative Growth Rate (RGR) - Table 3

Urochloa decumbens

AGR

1st Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1252.6			
Treatments	3	484.26	116	768.3	24.371	2.66E-12	***

2nd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	3309.0			
Treatments	3	1086.8	116	2222.2	18.911	4.71E-10	***

3rd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1690.3			
Treatments	3	68.936	116	1621.4	1.644	0.1831	

4th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	2012.4			
Treatments	3	240.86	116	1771.5	5.2572	0.001949	**

5th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	3120.6			
Treatments	3	122.63	116	2997.9	1.5817	0.1976	

6th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	3732.3			
Treatments	3	152.77	116	3579.5	1.6503	0.1817	

7th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	4932.2			
Treatments	3	116	116	4816.2	0.9313	0.428	

RGR

1st Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	25.099			
Treatments	3	9.3419	116	15.757	22.925	1.004E-11	***

2nd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	14.896			
Treatments	3	3.3273	116	11.569	11.121	1.805E-06	***

3rd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	9.1259			
Treatments	3	4.5541	116	4.5718	38.516	< 2.2E-16	***

4th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	4.2277			
Treatments	3	0.29629	116	3.9315	2.914	0.03733	*

5th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1.6209			
Treatments	3	0.54231	116	1.0786	19.441	2.80E-10	***

6th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1.00736			
Treatments	3	0.13232	116	0.87504	5.8471	0.0009367	***

7th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1.02692			
Treatments	3	0.06117	116	0.96575	2.449	0.06715	.

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Appendix 7. (c) Analysis of Deviance table of Absolute Growth Rate (AGR) and Relative Growth Rate (RGR) - Table 3

Megathyrsus maximus

AGR

1st Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	224.77			
Treatments	3	11.323	116	213.45	2.0512	0.110600	

2nd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	881.34			
Treatments	3	276.52	116	604.83	17.678	1.62E-09	***

3rd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	776.59			
Treatments	3	62.663	116	713.92	3.3939	0.02034	*

4th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	752.10			
Treatments	3	116.14	116	635.96	7.0614	0.0002114	***

5th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	2675.1			
Treatments	3	57.224	116	2617.9	0.8452	0.4719	

6th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1213.9			
Treatments	3	197.49	116	1016.5	7.5126	0.0001225	***

7th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	3372.5			
Treatments	3	143.94	116	3228.6	1.7238	0.1659	

RGR

1st Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	22.365			
Treatments	3	0.94975	116	21.415	1.7149	0.167800	

2nd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	17.527			
Treatments	3	2.6502	116	14.877	6.888	0.0002611	***

3rd Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	2.6479			
Treatments	3	0.13706	116	2.5109	2.1107	0.1026	

4th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	0.61980			
Treatments	3	0.05773	116	0.56207	3.9714	0.009796	**

5th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1.6568			
Treatments	3	0.10195	116	1.5549	2.5353	0.06023	.

6th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	0.47896			
Treatments	3	0.04792	116	0.43104	4.2989	0.00648	**

7th Day

	Df	Deviance	Resid. Df	Resid. Dev.	F value	Pr(>F)	
NULL			119	1.2035			
Treatments	3	0.00453	116	1.1989	0.146	0.9321	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Chapter 2

Physiological traits related to growth of invasive African grasses on the Brazilian Cerrado in the scenario of climate change

Abstract Biological invasions are a threat to natural biodiversity and since most invasive plant species have C4 photosynthetic pathway, many studies focused on understanding how these species respond to elevated CO_2 and temperature. The present study evaluated physiological traits related to growth in three of the most common C4 invasive grass species in Brazilian Cerrado under elevated CO_2 concentration and temperature during 55 days. Results obtained show that increasing temperature has very little effect on the parameters assessed, affecting only *Urochloa decumbens* water relation responses. Apparently, the temperature rise acted to reduce the positive effects of elevated CO_2 when the two factors were applied together, affecting growth and photosynthetic assimilation of *Megathyrsus maximus* plants and water relations in all species studied. The only positive response of *U. decumbens* was improved water use efficiency under elevated CO_2. Only *M. maximus* showed improvement in growth under elevated CO_2, although this factor had caused changes in root/shoot ratio of *U. decumbens*. *Megathyrsus maximus* and *Urochloa brizantha* showed increased CO_2 assimilation under elevated CO_2, but in different growth periods. The improvement in growth responses of *M. maximus* can be attributed to improvements in water use efficiency, also observed for the other two species under elevated CO_2. Results show that positive responses of growth and photosynthesis of C4 species are species-specific and such adaptations can be a part of important strategies to compete with native species. In a climate change scenario, such physiological responses could indicate alterations in the local biodiversity of Brazilian Cerrado.

Keywords: C4 grasses, ecophysiological adaptations, gas exchange, photosynthesis, water relations.

Introduction

There is an increasing concern about global climate changes predicted for the next 85 years, with a parallel increase in atmospheric CO_2 concentration and global temperature, including issues of how this may affect ecosystems (Maroco et al. 1999). Important considerations are whether these climate changes will affect growth and metabolism of plants, and to what extent plant species have the potential to acclimate, maintaining an optimal balance (Maroco et al. 1999), since climate changes are already causing alterations in species distribution (Lenoir et al. 2008).

Predicting the future functioning of an ecosystem requires mechanistic understanding of how plants deal with different factors under future climate conditions such as high CO_2 concentrations and warmer temperatures (Naudts et al. 2014). Climate changes over the past two centuries, for example, had both beneficial and negative impacts on plant species (Houghton et al. 2001). Such responses of different species can affect population dynamics (Raizada et al. 2009) and have been used to predict which groups of plants will have a competitive advantage in a particular region as a result of climate changes (Collatz et al. 1998). For this reason, the effect of increasing atmospheric CO_2 and temperature, together or as separate factors, in the growth and photosynthetic performance of plants has been extensively studied over the last decades (Colemam et al. 1991; Morgan et al 2001; Ainsworth et al. 2002; Leakey et al, 2004; Long et al. 2004; Hamilton et al. 2008; Allen et al, 2011; Farfan-Vignolo and Asard 2012).

The negative effects of increasing temperature on plants are caused, to a great extent, by deleterious effects on photosynthesis, which is one of the most thermosensitive processes of plant physiology, since both light (electron transfer) and carbon reactions (Calvin-Benson cycle) of photosynthesis have thermolabile components (Hamilton et al. 2008). Plants with C4 photosynthetic pathway have an internal mechanism for CO_2 concentration around RuBisCO, which eliminates photorespiratory losses and almost saturates the Calvin-Benson cycle at the current atmospheric CO_2 concentration (Barnaby and Ziska 2012). Therefore, their stomatal conductance is lower than that of C3 plants at any CO_2 level, resulting in increased leaf temperature, which may increase the heat-related damage in C4 plants compared to C3 plants in the same habitat under elevated CO_2 concentrations (Hamilton et al. 2008). Contrastingly, as C4 species originated in warmer climates, on average, than C3 species (Sage and Monson 1999), these species are more tolerant to

increasing temperature than C3, therefore, C4 species may be less affected by heat stress in a future world of elevated CO_2 (Hamilton et al. 2008; Wang et al. 2008).

The two basic responses of plants to increased atmospheric CO_2 concentration are enhanced photosynthesis and reduced stomatal conductance. All other high-CO_2 effects on plants and ecosystems are derivatives of these changes (Long et al. 2004). Stomatal response to elevated CO_2 appears to be ubiquitous among photosynthetic different sub-types (C3, C4 and CAM). In general, increasing CO_2 reduces stomatal conductance and transpiration water loss with a subsequent increase in water use efficiency (WUE) (Barnaby and Ziska 2012). As C4 plants have an internal mechanism that concentrates CO_2 near RuBisCO, it is to be theoretically expected that the increase in CO_2 concentration will have minimal effect on their photosynthesis (Ziska and Bunce 1997; LeCain and Morgan 1998; Barnaby and Ziska 2012). Many of the studies reporting improvements in photosynthesis and/or growth of C4 species under elevated CO_2 attribute these improvements to the beneficial effect of CO_2 on water relations in water-limited environments (Maroco et al. 1999; Morgan et al 2001; Leakey et al 2004; Allen et al 2011). However, some studies have shown that some C4 species, such as blue grama (*Bouteloua gracilis*), maize (*Zea mays*), amaranth (*Amaranthus hypochondriacus*), pigweed (*Amaranthus retroflexus*), big bluestem (*Andropogon gerardii*), Indian grass (*Sorghastrum nutans*), sugarcane (*Saccharum officinarum*) and *Miscanthus giganteus*, grew better and/or had higher photosynthetic assimilation when grown under elevated CO_2 in an environment where the water was not limited (Read and Morgan 1996; Ziska and Bunce 1997; LeCain and Morgan 1998; Souza et al. 2008; Souza et al. 2013).

Despite the divergences between experimental studies, taken together, these results show that C4 plants have the potential to respond to elevated CO_2, although there may be differences due to species, cultivars, duration of exposure, light intensity, temperature, nutritional status, water stress and even pot size (Sage 1994; Drake et al. 1997). The basis for the observed improvement in growth of C4 plants under elevated CO_2 is not as clear as in C3 plants, but it seems unlikely that improved water relations represent, in all cases, the response of the C4 species under elevated CO_2 (Ziska and Bunce 1997).

Nevertheless, the investigation on the effect of CO_2 enrichment in C4 plants is limited in comparison with C3 plants (see, for example, Barnaby and Ziska 2012). Although C4 plants represent a small percentage (about 3%) of the total angiosperm species, they make a substantial contribution to productivity on a global

scale (see Sage and Monson 1999). In addition, many of these species are important invaders in various parts of the world where they are reducing biodiversity of natural communities and slowing ecosystem restoration (Milton 2004). This is the case of African grasses used as forage that are spreading rapidly in the Cerrado (Brazilian savanna) fragments, probably displacing native species and, therefore, constituting a threat to the local natural biodiversity (Pivello et al. 1999).

Thus, the present study aimed to investigate growth, photosynthetic assimilation and water relations of three of the most common C4 invasive grass species in Cerrado: *Urochloa brizantha*, *Urochloa decumbens* and *Megathyrsus maximus*. These plants were grown under CO_2 enriched atmosphere and temperature elevated to 3 ºC above ambient over 55 days to assess whether global climate changes forecast for 2100 by Intergovernmental Panel on Climate Changes (IPCC 2013) have the potential to alter physiological responses throughout the growth period.

Materials and Methods

Plant material, growth conditions and treatments

Seeds of the three species studied, *Urochloa brizantha* (Hochst. Ex A. Rich.) RD Webster, *Urochloa decumbens* (Stapf) RD Webster and *Megathyrsus maximus* (Jacq.) BK Simon & S.W.L. Jacobs, were germinated in germination chambers at 30 ºC and 12 h light + 12 h dark photoperiod. Seeds were considered germinated when the radicle had emerged about 2.0 mm. Three days after germination, 3 seedlings per pot were placed in 1.7 L plastic pots containing substrate composed of a mixture of sand and vermiculite (2:1) and irrigated with Hoagland and Arnon (1950) nutrient solution each three days, and with distillated water on the other days. Plants were grown for 55 days in open-top chambers (1.53 m^3 each), all designed according to Aidar et al. (2002), placed inside a glasshouse located at the Instituto de Botânica, São Paulo, Brazil (23º38'40"S, 46º36'38"W). Plants were subjected to four environmental treatments based on IPCC (2013) predictions for 2100, to the following specifications: (1) Ctrl [Control – current CO_2 concentration (minimum: 285.30 µmol mol^{-1}; mean: 345.29 µmol mol^{-1}; maximum: 418.30 µmol mol^{-1}) and room temperature (minimum: 11.17 ºC; mean: 23.92 ºC; maximum: 41.97 ºC)]; (2) ET [Elevated temperature –

current CO_2 concentration (minimum: 286.70 μmol mol^{-1}; mean: 345.40 μmol mol^{-1}; maximum: 418.70 μmol mol^{-1}) and 3 ºC above room temperature (minimum: 14.83 ºC; mean: 27.03 ºC; maximum: 44.97 ºC)]; (3) EC [Elevated CO_2 – double CO_2 concentration (minimum: 516.00 μmol mol^{-1}; mean: 712.26 μmol mol^{-1}; maximum: 892.60 μmol mol^{-1}) and room temperature (minimum: 11.62 ºC; mean: 24.10 ºC; maximum: 41.94 ºC)]; and (4) ECT [Elevated CO_2 + temperature – double CO_2 concentration (minimum: 519.30 μmol mol^{-1}; mean: 712.19 μmol mol^{-1}; maximum: 891.00 μmol mol^{-1}) and 3 ºC above room temperature (minimum: 14.95 ºC; mean: 27.26 ºC; maximum: 44.98 ºC)]. The experiments were conducted under natural photoperiod and relative air humidity (RH). The environmental conditions (CO_2 concentration, temperature and RH) were monitored throughout the entire experimental growth period and details are presented in Fig. 1.

Total biomass, root/shot ratio and leaf parameters analysis

Total biomass, root/shoot ratio and leaf parameters were evaluated through three destructive harvestings of plants. The first one at 15 days of growth and every 20 days (35 and 55 days) for those that followed. Four plants of each species and in each treatment were collected at each harvesting. For total biomass and root/shoot ratio analysis, green leaves, stems and roots were separated, oven-dried at 65 ºC until obtaining constant mass, and weighed on an analytical balance (Shimadzu AY220). Dry mass of individual organs were totaled in order to obtain total biomass. Root/shoot ratio was calculated as the ratio of roots dry mass to the sum of dry mass of leaves and stems. Leaf parameters consisted of leaf area (LA), specific leaf area (SLA) and specific leaf mass (SLM). To obtain LA, newly collected leaves were scanned before being oven-dried, and the area calculated by AxioVision 4.9.1 (Zeiss) program. For SLA and SLM, LA and leaves dry mass were used in calculations. SLA was calculated as LA/LDM and SLM was calculated as LDM/LA, where LA is leaf area and LDM is leaf dry mass.

Gas exchange and water use efficiency measurements

Measurements of instantaneous photosynthetic assimilation (*A*), stomatal conductance (g_s) and transpiration

rate (E) were performed before each harvesting, from 09:00 to 12:00 h under photosynthetic photon flux density of 1600 μmol m^{-2} s^{-1}, determined as light saturation condition for the tree species (Ziska et al. 1999; Dias-Filho, 2002; Gómez et al. 2013), and taken from six plants (four of which were used in biomass and leaf parameters analysis) of each species and in each environmental treatment from the middle portion of the first fully expanded leaf. Instantaneous water use efficiency was defined at the leaf level as the ratio of photosynthetic carbon gain (*A*) to transpirational water loss (E), *A*/E (Donovan and Ehleringer 1994). All measurements were performed using a portable photosynthesis system (LI-COR LI-6400XT). CO_2 concentrations during the measurements were maintained constant through the use of LI-6400XT CO_2 ampoules. For current CO_2 treatments, a CO_2 concentration of 360 μmol mol^{-1} was used and for elevated CO_2 treatments, a CO_2 concentration of 720 μmol mol^{-1} was used.

Extraction and quantification of chloroplastic pigments

Due to the small size of the plants after 15 days of growth, pigment quantification was done only on the second and third harvesting points (35 and 55 days). Leaf discs (∅ = 1 cm) of the same leaves used in gas exchange analysis were obtained and stored in 80% acetone for 48 h. They were then macerated in a mortar and pestle in liquid nitrogen and another 5 mL of acetone 80% was added to 10 mL. The extract was collected, centrifuged and analyzed spectrophotometrically by measuring absorbance at 649, 665 and 470 nm. Chlorophyll (chl) *a*, chl *b* and carotenoids (carot) were quantified according to Lichtenthaler and Wellburn (1983).

Statistical analysis

Data were analyzed by General Linear Model (GLM) and means were compared by contrast test at 5% probability using R 3.0.0 (free software). For all data, the environmental treatments were used as explanatory variables. The response variables used were total dry mass, root/shoot ratio for biomass analysis; LA, SLA and SLM for leaf parameters analysis; *A*, g_s, E and WUE for gas exchange parameters analysis and chl *a*+*b*, carot, chl *a*/*b* and chl/carot for pigment content analysis. Figures were made using GraphPad Prism 5

(GraphPad Software, Inc., San Diego, CA, USA).

Results

Total biomass and root/shoot ratio

Urochloa decumbens plants showed no significant difference between treatments for total biomass (Fig. 2B). For *U. brizantha* plants, differences between treatments were observed only after 35 and 55 days of treatments exposure. In both cases, all environmental treatments resulted in lower total biomass compared to plants grown under control treatment (Fig. 2A). Only *M. maximus* plants experienced significant environmental treatment effects during the whole growth period. After 15 days of treatment exposure, elevated temperature under current CO_2 concentration (ET) and elevated CO_2 under room temperature (EC) resulted in higher biomass, while the increase of both factors together did not induce biomass increase. On the 35^{th} and the 55^{th} days, plants grown under elevated CO_2 (EC) showed greater biomass than those grown under the other treatments (Fig. 2C).

For *U. brizantha* plants, reductions observed in total biomass under all environmental treatments on the 35^{th} and the 55^{th} days of treatment exposure were accompanied by reductions in root/shoot ratio. On the 35^{th} day, plants grown under elevated CO_2 treatments (EC and ECT) showed the lowest root/soot ratio values and on the 55^{th} day, plants grown under all environmental treatments showed equally reduced root/shoot ratio related to those grown under control treatment (Fig. 3A). Despite not having shown differences in total biomass, *U. decumbens* plants grown under elevated CO_2 (EC) showed greater root/shoot ratio compared to those grown under the other treatments on the 55^{th} day (Fig. 3B). And although *M. maximus* plants had presented differences in total biomass between treatments at all harvesting points, they did not show differences in root/shoot ratio at any of the harvesting points (Fig. 3C).

Leaf parameters

Only *M. maximus* plants showed differences between treatments for leaf parameters (Fig. 4C, F and I). For leaf area (LA), elevated temperature under current CO_2 concentration (ET) and elevated CO_2 under room

temperature (EC) promoted greater LA on the 15th day and elevated CO_2 under room temperature (EC) led to the same response on the 55th day (Fig. 4C). For specific leaf area (SLA), elevated temperature under current CO_2 concentration (ET) led to increased responses compared to the other treatments only on the 55th day (Fig. 4F). For specific leaf mass (SLM), differences were also observed only on the 55th day, with all environmental treatments providing a reduction in relation to control and elevated temperature treatments (ET and ECT) being responsible for the greatest reduction observed (Fig. 4I).

Gas exchange and water use efficiency

The three species studied showed different net photosynthetic assimilation (*A*) responses (Fig. 5). *Urochloa brizantha* plants showed differences between environmental treatments only on the 55th day, with elevated CO_2 under room temperature (EC) promoting enhanced *A* (Fig. 5A). *Urochloa decumbens* plants did not respond to treatments at any of the harvesting points (Fig. 5B). *Megathyrsus maximus* plants, in contrast, showed significant differences between treatments only on the 15th and 35th days. On the 15th day, plants grown under elevated CO_2 treatment (EC) presented greater *A*, while on the 35th day, plants grown under all environmental treatments showed lower *A* compared to those under control treatment (Fig. 5C).

Stomatal conductance (g_s) and rate of transpiration (E) was also differently affected by treatments in the three species, responses of these two parameters being quite similar (Fig. 6). *Urochloa brizantha* plants exposed to elevated temperature treatment (ET) showed higher g_s and E values on the 35th day and treatments with elevated CO_2 (EC and ECT) promoted lower g_s and E on the 55th day, with elevated CO_2 + temperature treatment (ECT) showing the lowest g_s and E values (Fig. 6A and D). For *U. decumbens* plants, elevated temperature treatment (ET) resulted in higher g_s and E on the 15th and 35th days, and elevated CO_2 treatments (EC and ECT) resulted in lower g_s and E on the 55th day (Fig. 6B and E). Differences in g_s and E responses for *M. maximus* plants occurred from the 35th day of treatment exposure. Elevated CO_2 treatments (EC and ECT) resulted in lower g_s and E on the 35th and 55th days (Fig. 6C and F), with elevated CO_2 + temperature treatment (ECT) showing the lowest E values on the 55th day (Fig. 6F).

Water use efficiency (WUE) was also differently affected by environmental treatments in the three species (Fig. 7). For *U. brizantha* plants, elevated CO_2 treatments (EC and ECT) promoted higher WUE on

the 35th and 55th days (Fig. 7A). For *U. decumbens* plants, elevated CO_2 treatments (EC and ECT) increased WUE on the 15th and 55th days, and elevated temperature treatment (ET) reduced WUE on the 35th day (Fig. 7B). *Megathyrsus maximus* plants grown under elevated CO_2 treatments (EC and ECT) showed increased WUE in all the harvesting points (Fig. 7C).

Chloroplastic pigment content

Since the plant leaves were still very small after 15 days of treatment, measurements of chloroplastic pigments were made from the second harvesting (35 and 55 days of treatment exposure – Fig. 8). *Urochloa brizantha* and *U. decumbens* showed differences between treatments in pigment content only on the 35th day (Fig. 8 A and B). For *U. brizantha*, all pigments analyzed were affected. Elevated CO_2 increased the total chlorophyll (chl) content (chl *a*+*b*), all environmental treatments increased carotenoids (carot) content and chl *a*/*b* ratio, elevated CO_2 treatments being responsible for the greatest increase in chl *a*/*b* ratio, and all environmental treatments decreased chl/carot ratio in relation to the control (Fig. 8A). For *U. decumbens*, elevated CO_2 led to an increase in in chl *a*/*b* ratio (Fig. 8B). *Megathyrsus maximus* was the only species to show differences between treatments in both harvestings. On the 35th day, elevated CO_2 + temperature led to a higher chl *a*/*b* ratio (Fig. 8C) and, on 55th day, elevated temperature increased chl *a*+*b* and carot content and chl *a*/*b* ratio (Fig. 8F).

Discussion

Growth responses observed were different among the studied species. *Megathyrsus maximus* was the only species to show positive growth responses with the increased CO_2 in the present study. These positive responses were observed for total biomass in all the three harvestings and for leaf area on the 15th and 55th days. Similar results were observed in maize plants grown for 30 days of growth in a CO_2 atmospheric concentration of 1100 ppm (Maroco et al. 1999). These maize plants exhibited a significant increase in leaf area (23%) and total biomass (20%), the increase in total biomass being related to increased biomass in all organs, while the increase in leaf area was mainly due to larger leaves (Maroco et al. 1999). For *M. maximus*, this greater leaf area was also due to larger leaves, since the number of leaves did not change between

environmental treatments (data not shown). Although these results suggest a slightly greater partitioning of assimilates to the photosynthetic tissues in plants grown in elevated CO_2 (Maroco et al. 1999), the differences for *M. maximus*' root/shoot ratio were not significant, indicating an increased biomass in all organs as well.

Increases in biomass of C4 plants with increasing CO_2 concentrations are generally attributed to changes in biomass partitioning, inflorescence development acceleration or delayed leaf senescence (Potvin and Strain, 1985; Knapp et al. 1993). But this is not true in all cases. Ziska and Bunce (1997), investigating 10 C4 species between crops and weeds, concluded that the increase in total biomass observed in four of these 10 species was not associated with a consistent increase in leaf area, changes in the partition between leaves, stems or roots or senescence under CO_2 treatments, but with a direct stimulation of increasing CO_2 on growth and photosynthetic rate. Some studies also report changes in biomass partitioning nevertheless without significant increase in biomass under elevated CO_2 (LeCain and Morgan 1998), similar to the results observed in this study for *U. decumbens*. This species showed an increase in root/shoo ratio under elevated CO_2 on the 35^{th} day, but this increase did not result in significant differences in total biomass between treatments. In barnyard grass (*Echinochloa crus-galli*), the increased CO_2 also enhanced root biomass (Potvin and Strain, 1985). For plants already established, the general conclusion is that CO_2 enrichment generally increases root growth (Rogers et al. 1992a, b), although different responses have been reported for closely related species. Root biomass of black grama (*Bouteloua eriopoda*) increased with high CO_2 while the same condition did not influence blue grama (*B. gracilis*) root growth (Hunt et al. 1996; Morgan et al. 1998). CO_2 effect on increase in root growth are generally smaller for C4 than for C3 plants (Hunt et al. 1996; Morgan et al. 1998; Wand et al. 1999), as well as above ground responses (Poorter 1993; Wand et al. 1999).

The reduction in total biomass and root/shoot ratio observed in *U. Brizantha* was due to the reduction in root biomass observed in all environmental treatments. Most experiments in high CO_2 have dealt with above-ground plant organs, but the below ground component can also be substantially influenced (Bowes 1993). Strain and Thomas (1991) also observed a reduction in total biomass of cotton plants due to reduction in root biomass under 270, 350 and 650 μbar of CO_2 concentration by root growth limitation in pots, but this reduction was rapidly reversed when root restriction was eliminated. If plants are grown in small pots or

close to each other, roots can suffer limitations due to pot size and nutrients and water supply can be lower than that necessary for growth (Poorter 1993). Root restriction does not seem to be the case in our study as the reduction in *U. brizantha*'s root biomass were observed from 35 days, when the plants were still relatively young and had full space to grow.

Instantaneous photosynthetic assimilation responses under elevated CO_2 also differed between the studied species. *Urochloa brizantha* and *M. maximus* plants showed improvement in carbon assimilation under elevated CO_2 compared to the control treatment, while *U. decumbens* photosynthesis was not affected by different environmental conditions. Ziska and Bunce (1997) also reported an increase in assimilation rates for eight of ten weedy and crop C4 species and LeCain and Morgan (1998) observed an improvement in assimilation response in two of the six species studied under elevated CO_2. These differences between photosynthetic rates in C4 species subjected to the same treatment conditions are probably due to specific differences (Sage 1994; Ziska and Bunce 1997), but taken together, indicate the potential of C4 species to enhance photosynthetic assimilation under high CO_2 (Maroco et al. 1999).

Growth response of *M. maximus* under elevated CO_2 may be attributed to improvements in water relations, since the positive responses were only observed in the first 15 days, while the increase in total biomass was also observed on the 55th day. According to Ziska and Bunce (1997), increasing water potential under elevated CO_2 could stimulate growth, even in moist soil, by increasing leaf area, without any increase in carbon exchange rate (Ziska and Bunce 1997). The results of water use efficiency presented by *M. maximus* corroborate this statement. Elevated CO_2 improved this species' WUE throughout the experimental period. This improvement was due to decreased stomatal conductance and transpiration after 35 days. A common effect of elevated CO_2 in leaves, regardless of photosynthetic pathway, is stomatal closure with consequent reduced transpiration (Barnaby and Ziska 2012). In general, increased CO_2 concentration reduces stomatal conductance and transpiration water loss, increasing WUE, usually defined as the ratio of carbon uptake by leaf water loss (Barnaby and Ziska 2012).

When photosynthetic rate under a certain CO_2 concentration differs from growth response under the same concentration, it can be said that there were photosynthetic acclimation or "down-regulation" (Ziska and Bunce 1997), i.e. the decrease of photosynthetic stimulation responses over few days to a few months (Barnaby and Ziska 2012). Acclimation responses are commonly observed in C3 species and have been

widely studied (Barnaby and Ziska 2012), but may also occur in C4 species such as blue panic (*Panicum antidotale*), blue grass (*B. gracilis*), maize (*Z. mays*) and sorghum (*Sorghum bicolor*) (Ghannoum et al. 1997; LeCain and Morgan 1998; Maroco et al. 1999; Watling et al. 2000; Leakey et al. 2004). It seems not to be the case for *M. maximus* plants. This species showed higher values of assimilation under elevated CO_2 only at 15 days, but such a short time should not have been sufficient to induce acclimation.

Photosynthetic acclimation to CO_2 seems to involve changes in plant metabolism through carbohydrates accumulation under CO_2 enrichment (Ainsworth et al. 2003; Ainsworth and Long 2005) and / or a reduction in nitrogen concentration in leaves and chlorophyll concentration (Grombone-Guaratini et al. 2013). Several studies have shown that atmospheric CO_2 enrichment can increase (Sgherri et al. 1998; Grombone-Guaratini et al. 2013), decrease (Maroco et al. 1999) or have no effect on the chlorophyll concentration (Ge et al. 2011). Related to carbohydrates accumulation, Moore et al. (1999) proposed a biochemical model in which increased levels of hexose caused inhibition of RuBisCO content. According to this model, as a consequence of increased CO_2, there is an increase in photosynthetic capacity and hence in the availability of assimilates more present in leaves, such as sucrose. The flux of hexose through hexokinase signals the source-sink imbalance and this imbalance is rectified through down-regulation of RuBisCO content (Moore et al. 1999; Long et al. 2004). We did not collect data to specifically investigate nitrogen content in leaves in this study. However, the chlorophyll content presented by *M. maximus* throughout the growth period, which was unchanged under elevated CO_2, is another indicative that the photosynthetic acclimation did not occurred in this species. However, enzyme activity assays and quantifications of carbohydrates in leaves are needed to verify the reason for the observed reduction in photosynthetic assimilation in *M. maximus*.

The improvement in CO_2 assimilation observed for *U. brizantha* under elevated CO_2 coincided with reductions in g_s and E with consequent improvement in WUE observed after 35 days. The improved water relation conditions are considered the primary basis for the increased assimilation under high CO_2 in C4 plants, but only under conditions of water restriction (Seneweera et al. 1998; Ghannoum et al. 2000). However, this should not have happened in our experimental conditions, since there was no water restriction (relative water content greater than 80% – data not shown). Our results show that *U. brizantha* has the potential to improve photosynthetic assimilation under elevated CO_2 even in well-watered conditions. One

possibility is that the assimilation of this species did not attain its maximum under ambient CO_2 concentration (Leakey et al. 2004). Several reports provide evidence contrary to the saturation of C4 photosynthesis at current CO_2 concentrations. LeCain and Morgan (1998) showed that although only two of the six C4 grasses studied had shown improvement in photosynthetic assimilation under elevated CO_2 concentration (700 μL L^{-1}), none of the six species had saturated photosynthesis under ambient CO_2 concentration of 350 μL L^{-1}, based on the results of A/C_i curves. Ziska and Bunce (1997) also reported the absence of photosynthetic saturation under ambient CO_2 concentration in eight of the ten C4 species investigated.

Increasing temperature (maximum ~45ºC) had little effect on physiological responses observed for the three species. At 35 days, elevated temperature increased g_s and E for *U. brizantha* plants, but without significant reductions in WUE. Similar increases in gs and E were observed for *U. decumbens* plants on the 35th day but for this species, WUE also reduced under elevated temperature treatment. The most interesting effect of increasing temperature was a reduction in the increased rates of WUE observed under elevated CO_2 (elevated CO_2 + temperature treatment) for all species. The heating effect reducing the positive effect of elevated CO_2 has been reported for various C3 and C4 plants (Colemam et al. 1991; Hamilton et al. 2008; Wang et al. 2008; Farfan-Vignolo and Asard 2012; Naudts et al. 2014). It is likely that the benefits of increased CO_2 have been neutralized by the negative effects of increasing temperature and *vice-versa*, as observed in other studies (Hamilton et al. 2008; Wang et al. 2008; Farfan-Vignolo and Asard 2012). The responses of whole plants for the combination of CO_2 and temperature increase may vary in different species and different growing conditions, and the magnitude or even the direction of plant responses to elevated CO_2 is dependent on the relationship between imposed temperatures and optimal temperature for growth (Hamilton et al. 2008).

In conclusion, the set of results presented here indicates that plants with C4 pathway can benefit from increasing CO_2 concentration through improvements in water relations, related or not to improvements in photosynthetic assimilation and / or improvements in growth. It seems that C4 responses to elevated CO_2 are species-specific. In any case, the interspecific variation in plant ecophysiological responses may have consequences that can alter ecosystems composition and productivity in a world of climate change. As plant responses to competition are closely related to the availability of water and nutrients, these species can gain a

competitive advantage over other species in a scenario of future climate change. *Megathyrsus maximus* showed improvement in growth, while *U. brizantha* showed improvement in photosynthetic assimilation. Such adaptations can configure important strategies to explore new environments, as these are species of great invasive potential and have an increasing occurrence in Brazilian Cerrado. In a global change scenario, such physiological responses could promote alterations in local biodiversity if native species do not show positive responses to climate change. From an ecological perspective, it will be interesting to investigate the responses of native species co-occurring in the same environment that these invasive, as well as to determine how changes in stressful environmental factors such as humidity, nutrition, light and temperature, in particular, may compromise the beneficial effects of elevated CO_2.

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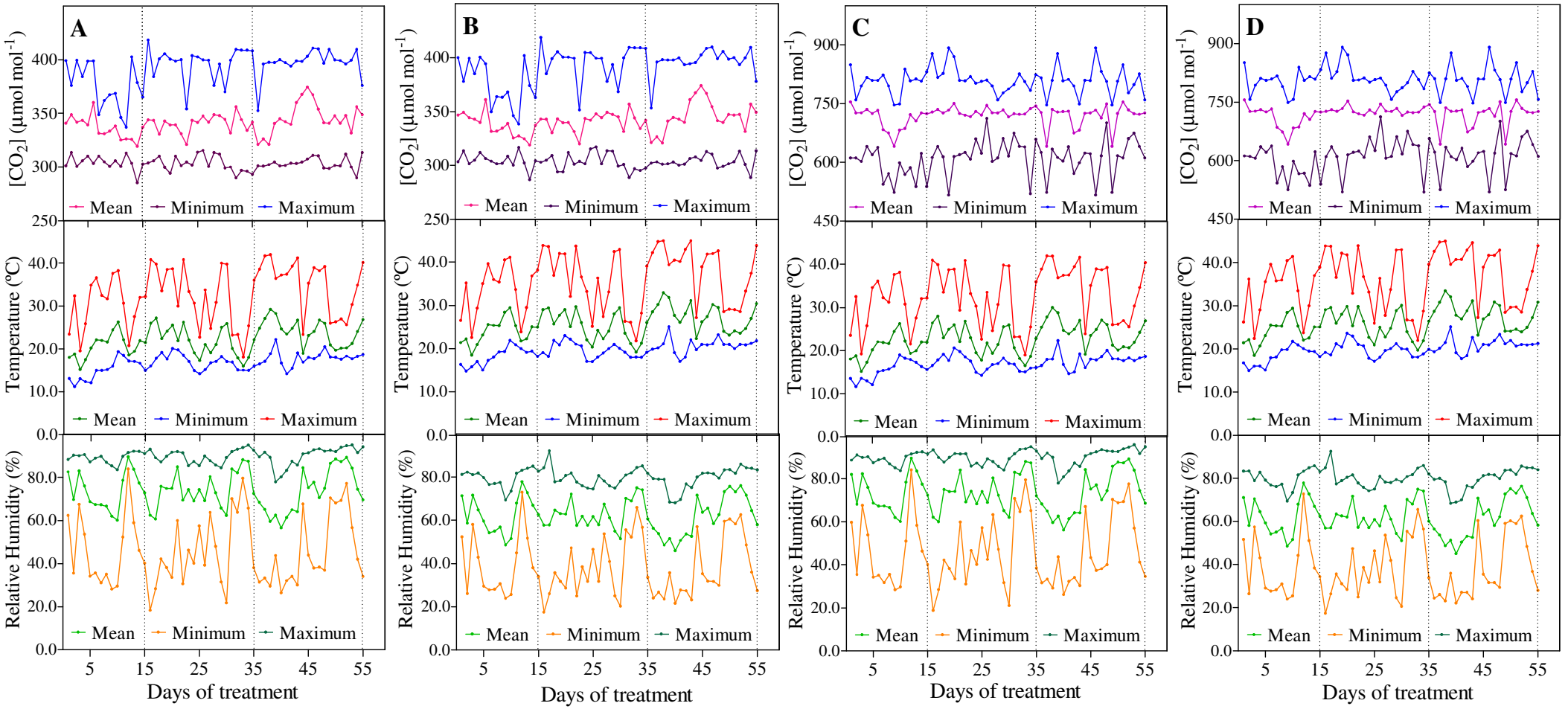

Fig. 1. Environmental conditions (CO_2 concentration, temperature and relative humidity) during 55 days of exposure to treatments. Each environmental treatment is identified as a conjunct of graphics: A – Ctrl (Control: current CO_2 concentration and room temperature); B – ET (Elevated temperature: current CO_2 concentration and room temperature + 3ºC); C – EC (Elevated CO_2: doubled CO_2 concentration and room temperature) and D – ECT (Elevated CO_2 + temperature: doubled CO_2 concentration and room temperature + 3ºC), respectively.

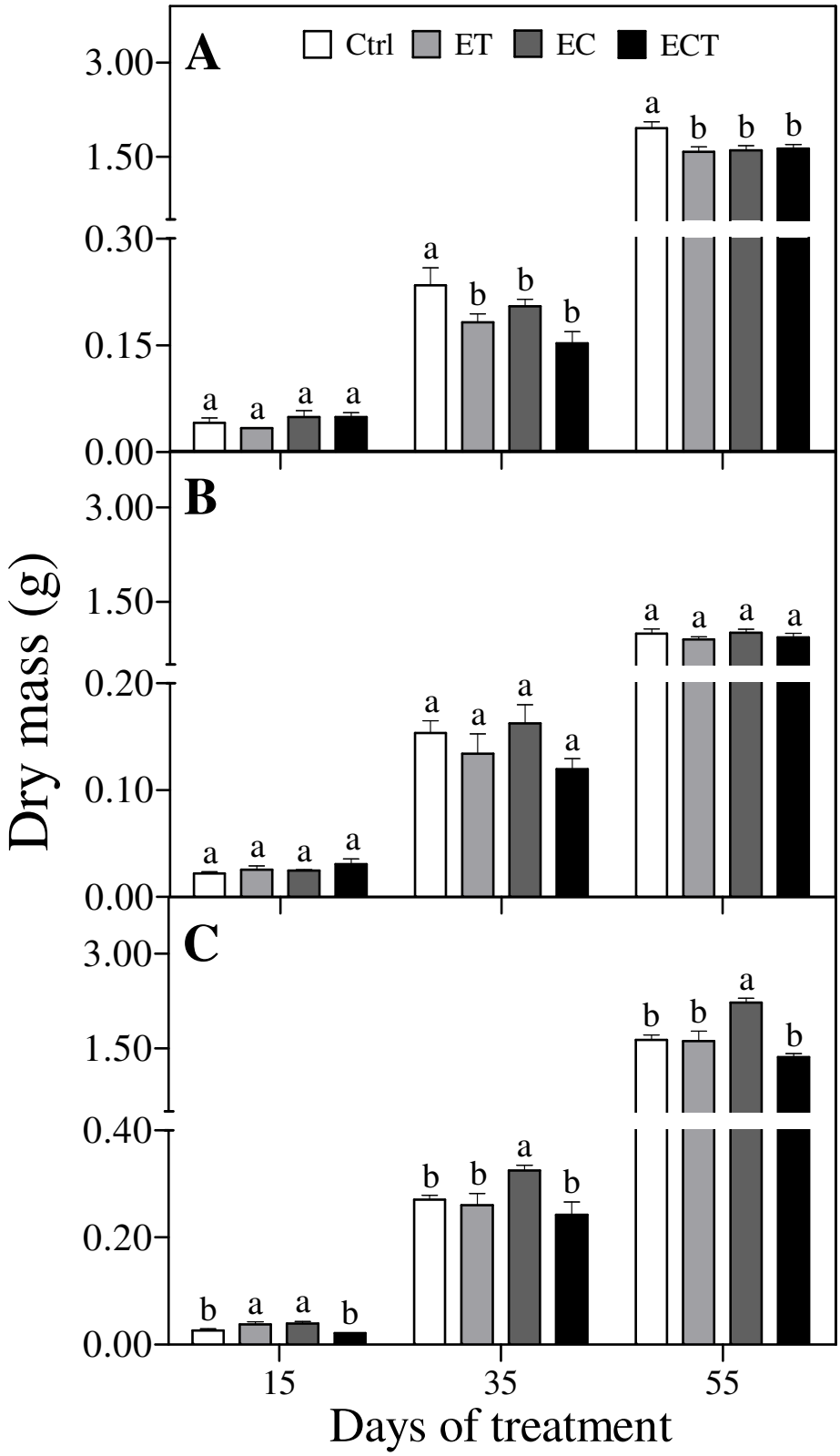

Fig. 2. Total dry mass of three invasive grass species (A – *U. brizantha*, B – *U. decumbens* and C – *M. maximus*) grown under elevated CO_2 concentration and/or elevated temperature treatments in three different harvesting points (15, 35 and 55 days of treatment exposure). Values represent mean ± SEM (n = 4). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature.

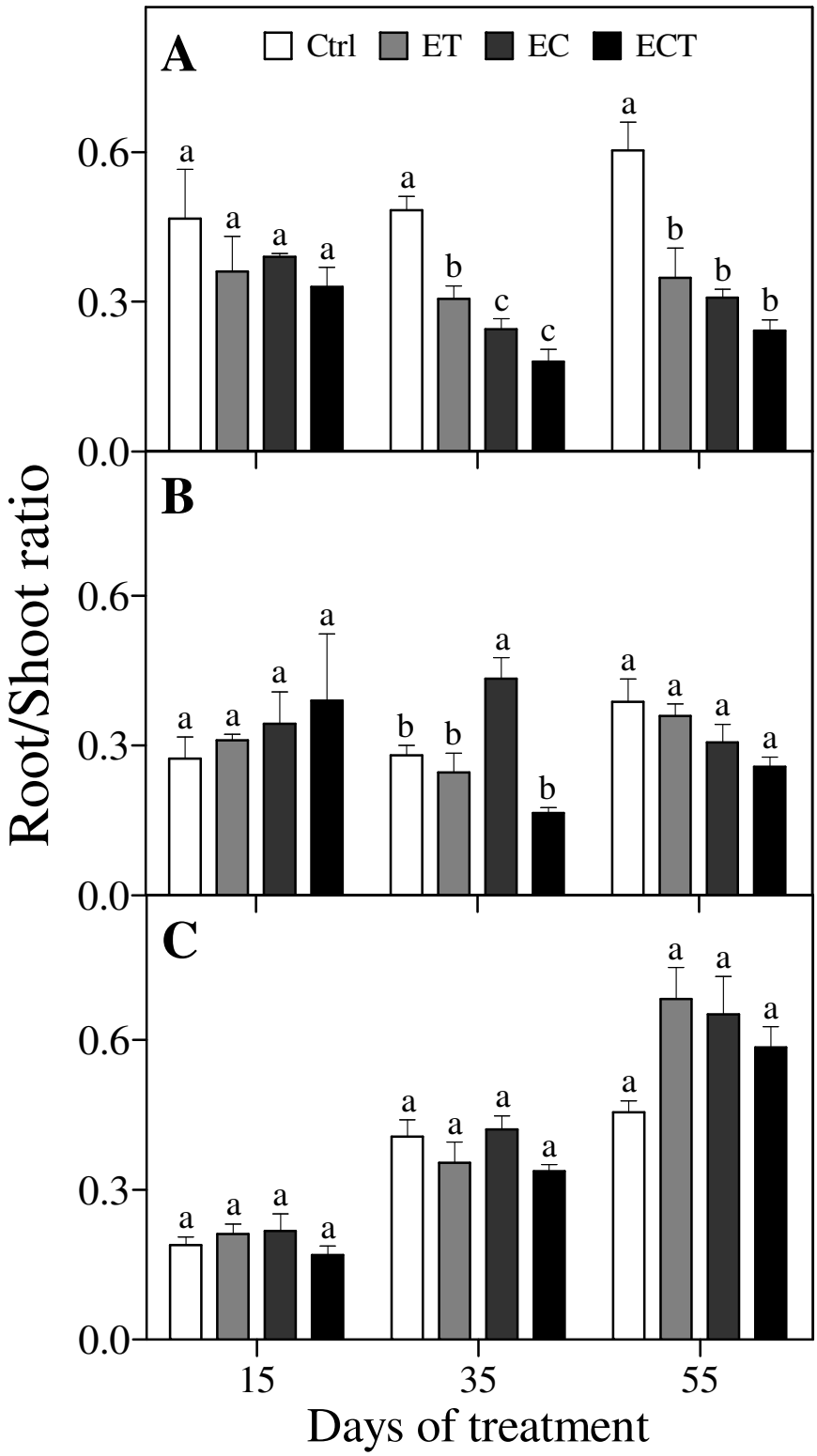

Fig. 3. Root/Shoot ratio of three invasive grass species (A – *U. brizantha*, B – *U. decumbens* and C – *M. maximus*) grown under elevated CO_2 concentration and/or elevated temperature treatments in three different harvesting points (15, 35 and 55 days of treatment exposure). Values represent mean ± SEM (n = 4). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature.

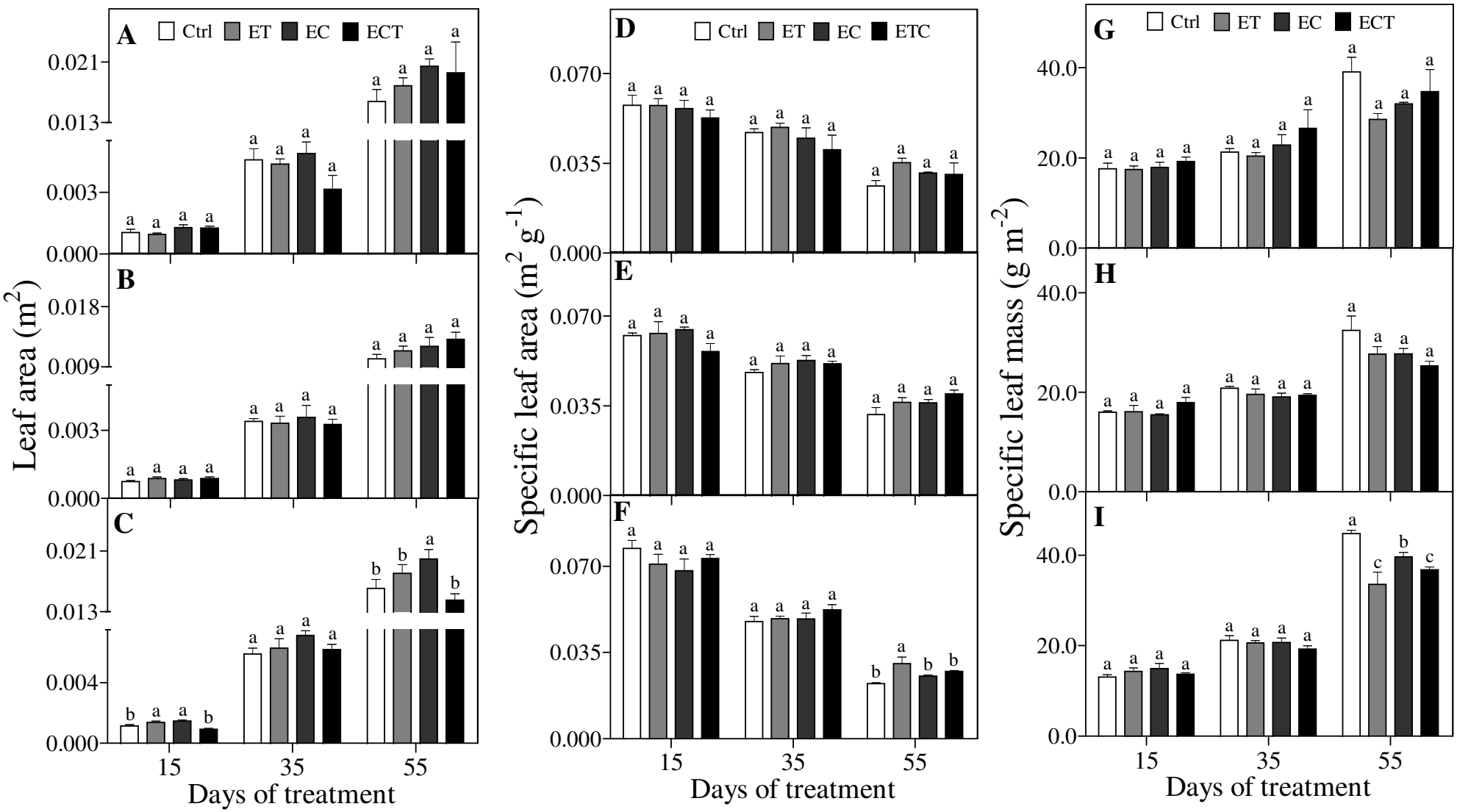

Fig. 4. Leaf parameters of three invasive grass species (A, D and G – *U. brizantha*, B, E and H – *U. decumbens* and C, F and I – *M. maximus*) grown under elevated CO_2 concentration and/or elevated temperature treatments in three different harvesting points (15, 35 and 55 days of treatment exposure). Values represent mean ± SEM (n = 4). Different letters represent statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature).

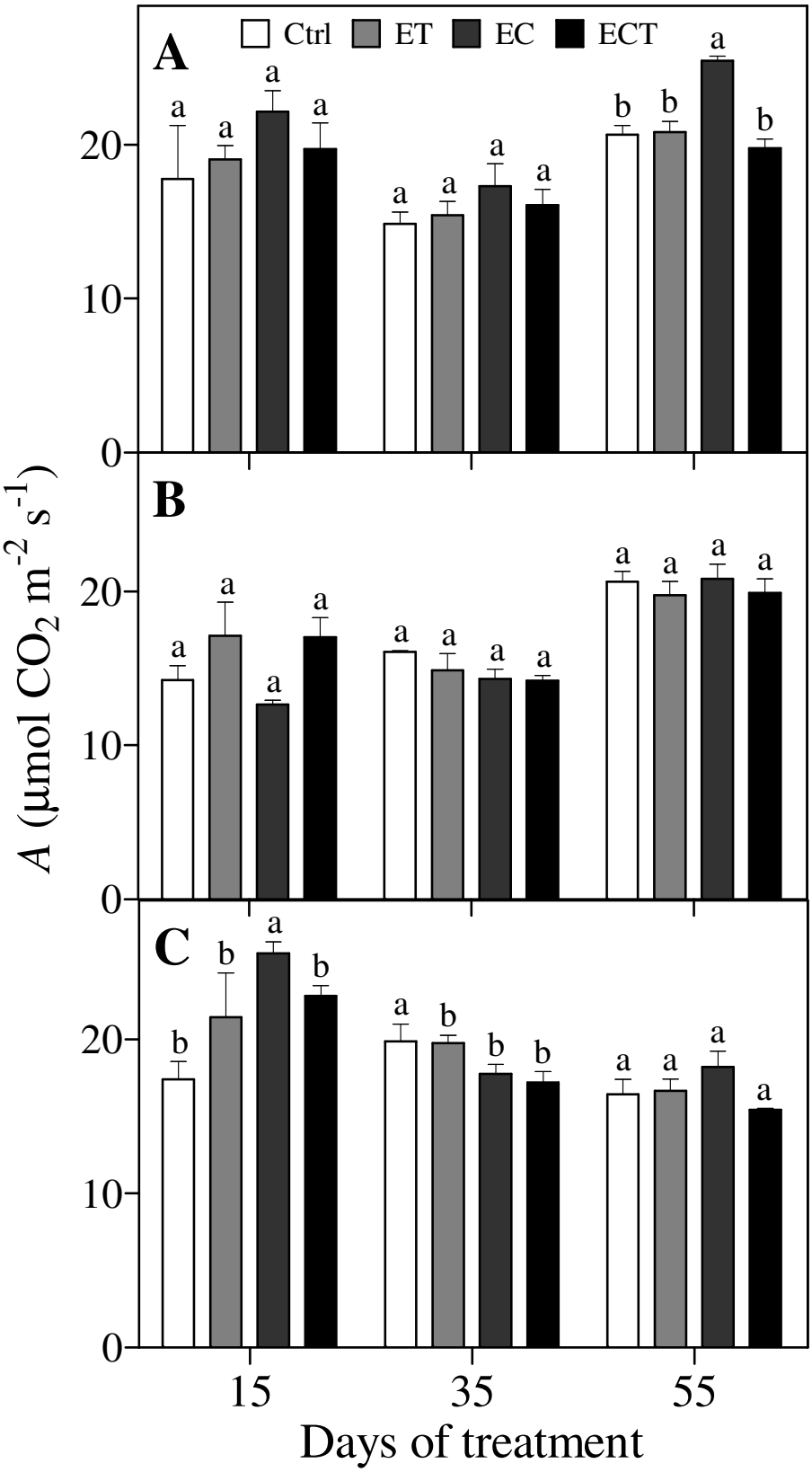

Fig. 5. Net photosynthetic assimilation (*A*) of three invasive grass species (A – *U. brizantha*, B – *U. decumbens* and C – *M. maximus*) grown under elevated CO_2 concentration and/or elevated temperature treatments in three different harvesting points (15, 35 and 55 days of treatment exposure). Values represent mean ± SEM (n = 6). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature.

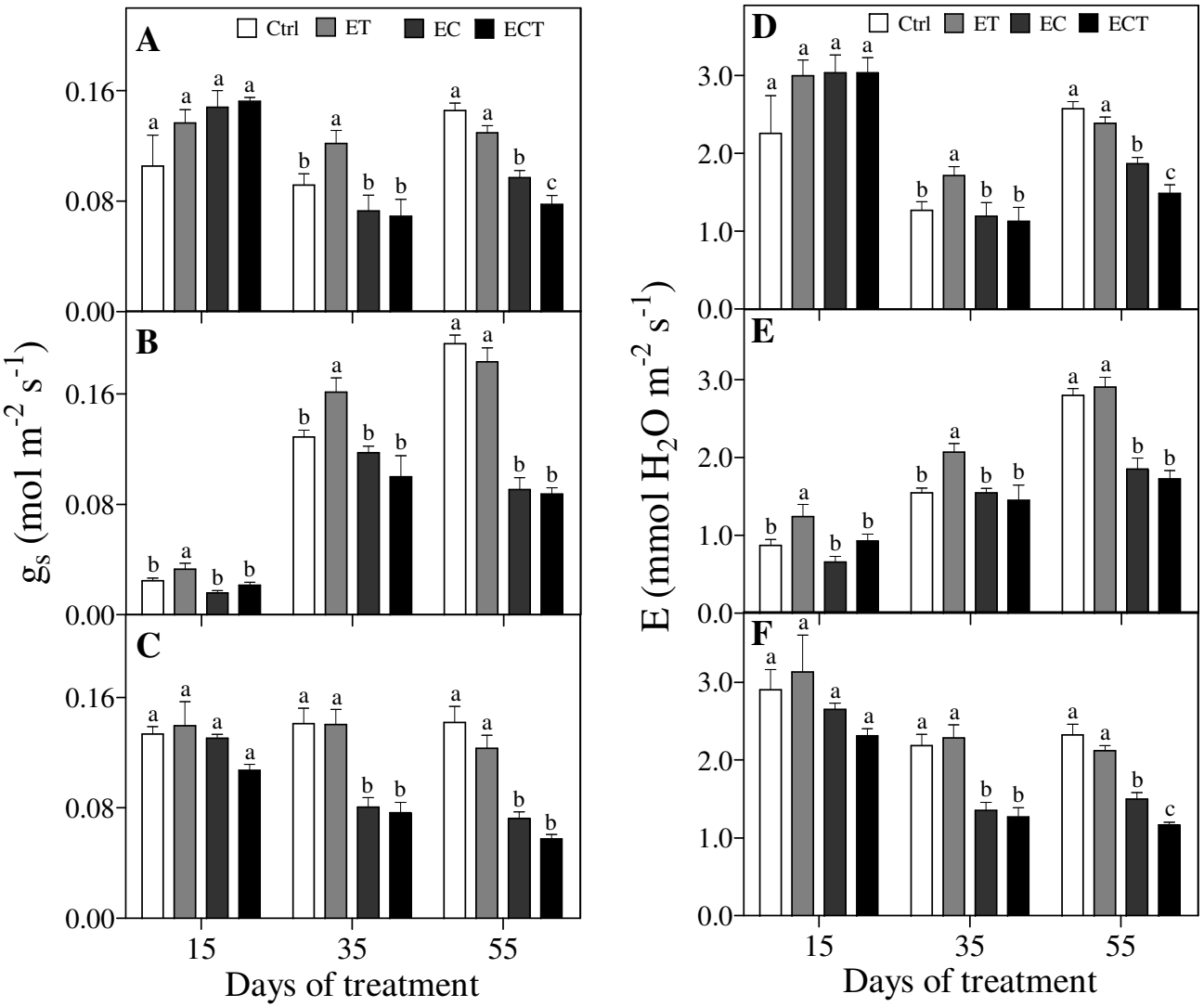

Fig. 6. Stomatal conductance (g_s) and rate of transpiration (E) of three invasive grass species (A and D – *U. brizantha*, B and E – *U. decumbens* and C and F – *M. maximus*) grown under elevated CO_2 concentration and/or elevated temperature treatments in three different harvesting points (15, 35 and 55 days of treatment exposure). Values represent mean ± SEM (n = 6). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature.

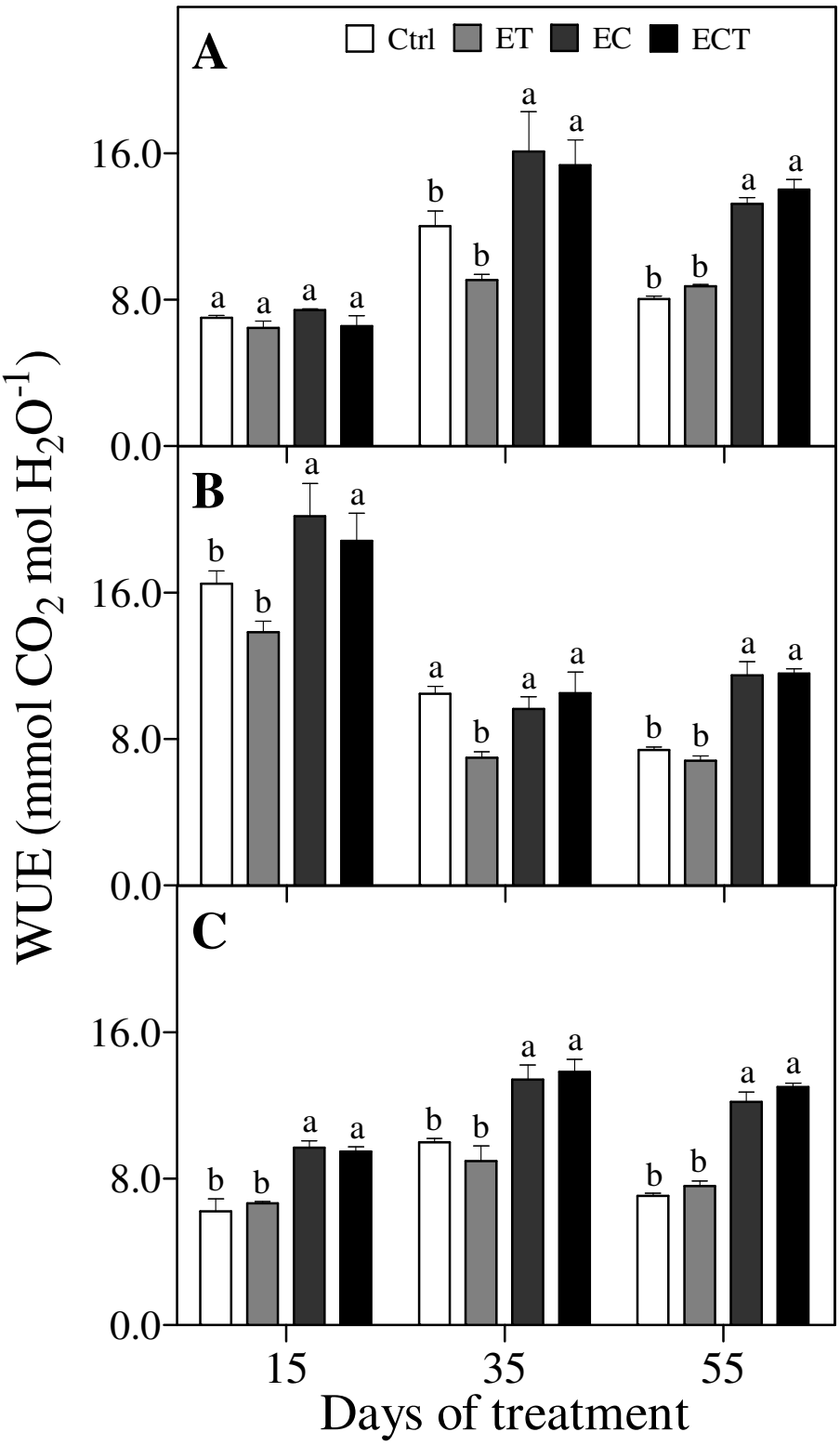

Fig. 7. Water use efficiency (WUE) of three invasive grass species (A – *U. brizantha*, B – *U. decumbens* and C – *M. maximus*) grown under elevated CO_2 concentration and/or elevated temperature treatments in three different harvesting points (15, 35 and 55 days of treatment exposure). Values represent mean ± SEM (n = 6). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature.

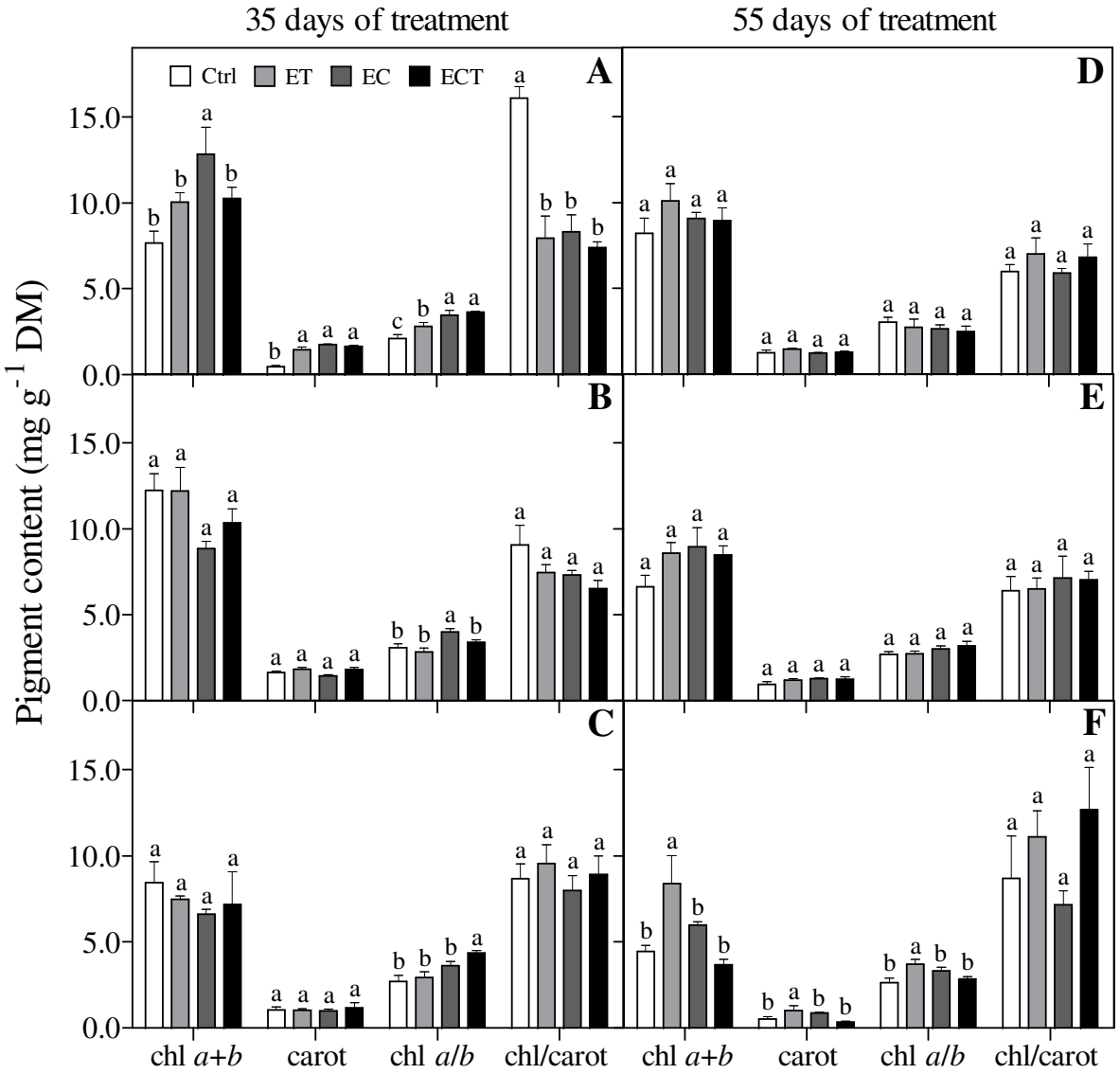

Fig. 8. Pigment content of three invasive grass species (A and D – *U. brizantha*, B and E – *U. decumbens* and C and F – *M. maximus*) grown under elevated CO_2 concentration and/or elevated temperature treatments in two different harvesting points (A, B and C – 35 days and D, E and F – 55 days of treatment exposure). Values represent mean ± SEM (n = 4). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature.

Chapter 3

Changes in growth, leaf carbohydrate content and gas exchanges of invasive African grasses affected by CO_2 and temperature increasing in the Brazilian Cerrado

Abstract Brazilian Cerrado has lost much of its biodiversity because of biological invasions, especially by African grasses. This problem is likely to be compounded by climate change with simultaneous increase in atmospheric CO_2 concentration and global mean temperature. Partition of photoassimilates directly influence growth responses in elevated CO_2 concentration, and can thus affect the invasiveness potential of certain species. The present study aimed to evaluate growth, leaf carbohydrate content and gas exchange of three invasive African grasses grown for 75 days under doubled CO_2 concentration and temperature elevated by 3 ºC in relation to room temperature. Results showed that although the three species presented C4 metabolism, all had some kind of positive response to increased CO_2. *Urochloa decumbens* showed only improvements in water use efficiency (WUE), while *Urochloa brizantha* showed an improvement in carbon assimilation and *Megathyrsus maximus* showed an improvement in growth under elevated CO_2. The most significant improvement of increased CO_2 in all three species appears to be the increase in WUE. This improvement in water relations probably explains the positive responses of photosynthesis and growth presented by *U. brizantha* and *M. maximus*, respectively. The increase in temperature affected leaf carbohydrate content of *M. maximus* plants by reducing sucrose, glucose and fructose content. These reductions were not related to thermal stress since photosynthesis and growth were not harmed. Cellulose content was not affected in any of the three species, just as lignin content in *U. decumbens* and *M. maximus*. All treatments promoted lignin content reduction in *U. brizantha*, suggesting a delay in leaf maturation of this species. Together, the results indicate that climate change may promote changes in growth, leaf carbohydrate content and/or gas exchange of the species studied and all of them could benefit in some way from these changes.

Keywords: climate change, C4 metabolism, photosynthesis, soluble sugars, water relations

Introduction

The Brazilian Cerrado is Brazil's second largest phytogeographical domain in area, surpassed only by the Amazon rainforest, and one of the richest savanna biomes of the world, with high levels of endemism, being considered an extremely important area for conservation (Myers et al. 2000). However, much of this biodiversity is being lost because of biological invasions. Species of African grasses used as forage are spreading rapidly in the Cerrado fragments, probably displacing native species and therefore constituting a threat to the local natural biodiversity (Pivello et al. 1999). And this problem is likely to be compounded by other factors, such as climate change with simultaneous increases in atmospheric carbon dioxide (CO_2) concentration and global mean temperature (Baruch and Jackson 2005).

Temperature and atmospheric CO_2 are important environmental parameters affecting plant growth, development and function, and both have changed in the recent past (Eller et al. 2012), primarily due to the burning of fossil fuels and secondarily due to change in land use (IPCC 2013). At the end of 2014, mean atmospheric CO_2 concentration was 398 μmol mol^{-1} in Mauna Loa, Hawaii (USA) (ESRL 2015) and 936 μmol mol^{-1} is expected at the end of the 21st century (IPCC 2013). At the same time, each of the last three decades has been successively warmer at the Earth's surface than any preceding decade since 1850 and it is predicted that global temperatures will continue to rise, reaching increases between 1.1 and 4.8 ºC by 2100 (IPCC 2013).

Therefore, concerns about how plants and natural ecosystems will respond to such changes have increased, since climate change is already responsible for changes in species distribution (Lenoir et al. 2008). Many groups have focused on studying the effects of increasing CO_2 and temperature, together or separately, in plants and ecosystems, especially regarding photosynthetic and growth performance (Eller et al. 2012; Farfan-Vignolo and Asard 2012; Grambone-Guaratine et al. 2013; Souza et al. 2008; Souza et al. 2013).

Climate change may affect the productivity of biota not only in relation to growth and resource allocation, but also changing the chemical composition of plant tissues (IPCC 2010). Most source-sink hypotheses assume that high CO_2 concentration promotes a relative increase in carbon availability which is accumulated on total non-structural carbohydrates and carbon-based secondary metabolites, provided

that carbon values exceed growth requirements (Peñuelas and Estiarte 1998). Carbon allocation for growth and differentiation should, by competition for internal resource of limited availability, reduce carbon allocation to secondary metabolism (Ibrahim and Jaafar 2012). Among the works that investigated the influence of climate change on carbon metabolism in plants, some of them found that higher CO_2 concentration increased production of non-structural carbohydrates as starch (Ibrahim and Jaafar, 2012), sucrose (Souza et al. 2008; Ibrahim and Jaafar 2012) and fructans (Oliveira et al. 2010). For structural carbohydrates, Schädel et al. (2010) found that the increased CO2 concentration had no significant effect on total hemicellulose concentrations in leaves and woody tissue in 14 of 16 species. Körner et al. (2005) have analyzed the litter composition of a temperate deciduous forest exposed to 530 ppm of CO_2 during 4 years in a FACE system (Free Air CO_2 Enrichment). They observed an increase of 21% in non-structural carbohydrate content and a decrease of 11% on lignin content. As for the increase of temperature, some studies report less accumulation of non-structural carbohydrates such as fructose and glucose in grasses (Naudts et al. 2014) and tomato (Jie et al. 2012) leaves.

Since the effects of CO_2 and temperature on plant metabolism may counteract each other, the combined effects can be different from any factor separately (Morison and Lawlor 1999). As these climatic factors will change simultaneously, to understand how plants will respond and adapt to a new environment is an essential first step to understand the full impact that multiple climate change factors will have on terrestrial ecosystems (Leakey et al. 2009; Eller et al. 2012). Thus, the present study aimed to investigate the effects of increases in CO_2 concentration and temperature predicted for 2100 by the Intergovernmental Panel on Climate Change (IPCC 2013) on growth, leaf carbohydrate content and gas exchange of three of the most common invasive species in the Brazilian Cerrado: *Urochloa brizantha*, *Urochloa decumbens* and *Megathyrsus maximus*, cultivated for 75 days in a CO_2 enriched atmosphere and temperature elevated in 3 ºC above room temperature.

Materials and Methods

Plant growth conditions and experimental treatments

Plants of three grass species, *Urochloa brizantha* (Hochst. ex A. Rich.) RD Webster cv Marandu, *U.*

decumbens (Stapf) RD Webster cv Basilisk and *Megathyrsus maximus* (Jacq.) BK Simon and S.W.L. Jacobs cv Tanzania were used in this study. Seeds of the three species were germinated in germination chambers at 30 ºC and 12 hours light, 12 hours dark photoperiod. Three days after germination, seedlings were placed in 1.7 L plastic pots containing a mixture of sand and vermiculite (2:1) as substrate and grown for 75 days in open-top chambers (1.53 m^3 each) designed according to Aidar et al. (2002). Plants were irrigated with Hoagland and Arnon (1950) nutrient solution every three days and with distillated water on the other days. Four environmental treatments based on IPCC (2013) predictions for 2100 were imposed to the growing plants: (1) Control – current CO_2 concentration (minimum: 285.30 μmol mol^{-1}; mean: 340.59 μmol mol^{-1} and maximum: 418.30 μmol mol^{-1}) and room temperature (minimum 11.17 ºC; mean: 22.91 ºC and maximum: 43.45 ºC); (2) Elevated temperature – current CO_2 concentration (minimum: 286.70 μmol mol^{-1}; mean: 340.99 μmol mol^{-1} and maximum: 418.70 μmol mol^{-1}) and 3 ºC above room temperature (minimum 14.83 ºC; mean: 26.23 ºC and maximum: 46.90 ºC); (3) Elevated CO_2 – doubled CO_2 concentration (minimum: 516.00 μmol mol^{-1}; mean: 721.40 μmol mol^{-1} and maximum: 892.70 μmol mol^{-1}) and room temperature (minimum 11.62 ºC; mean: 23.24 ºC and maximum: 43.91 ºC); and (4) Elevated CO_2 + temperature – double CO_2 concentration (minimum: 519.30 μmol mol^{-1}; mean: 721.40 μmol mol^{-1} and maximum: 891.00 μmol mol^{-1}) and 3 ºC above room temperature (minimum 14.95 ºC; mean: 26.56 ºC and maximum: 46.95 ºC). The experiments were conducted under natural photoperiod and relative air humidity (RH). The environmental conditions (CO_2 concentration, temperature, RH and light intensity) were monitored throughout all the experimental growth period and are presented in Fig. 1.

Biomass and leaf growth parameters analysis

Four plants of each species and in each environmental treatment were harvested at the 75th day of the experimental period. Biomass analysis consisted of accumulation of dry matter (total dry mass), and leaf growth parameters consisted of leaf dry mass, leaf area (LA) and specific leaf area (SLA). For biomass analysis, plant material (leaves, stems and roots) was oven-dried at 65 ºC temperature in a forced air circulation drying oven and weighed on an analytical balance (Shimadzu AY220) to obtain constant weight. Leaves dry mass was also estimated for leaf growth parameters analysis. To obtain LA, newly

collected leaves were scanned before being oven-dried, and the area calculated by AxioVision 4.9.1 (Zeiss) program. For SLA, LA and leaves dry mass were used in calculations. SLA was calculated as LA/LDM, where LA is leaf area and LDM is leaf dry mass.

Gas exchange parameters measurements

Instantaneous measurements of net carbon assimilation (*A*), stomatal conductance (g_s) and rate of transpiration (E) were performed using a portable photosynthesis system (LI-COR LI-6400XT) from 09:00 to 12:00 h. All measurements were performed under a photosynthetic photon flux density of 1600 μmol m^{-2} s^{-1}, determined as light saturation condition for the tree species (Ziska et al. 1999; Dias-Filho, 2002; Gómez et al. 2013), and taken from six plants of each species and in each environmental treatment from the middle portion of the first fully expanded leaf. CO_2 concentrations were maintained as constant during the measurements through the use of LI-6400XT CO_2 ampoules. For current CO_2 treatments CO_2 concentration of 360 μmol mol^{-1} was used and for elevated CO_2 treatments CO_2 concentration of 720 μmol mol^{-1} was used. Instantaneous water use efficiency was defined at the leaf level as the ratio of photosynthetic carbon gain (*A*) to transpirational water loss (E), *A*/E (Donovan and Ehleringer 1994).

Non-structural carbohydrates extraction and determination

Leaf samples were freeze-dried and ground in a ball mill. Three hundred milligrams of each sample were extracted five times in 3 mL of 80% ethanol at 80 ºC for 15 min, followed by centrifugation at 13,000 g for 10 min. The pooled supernatants were vacuum dried and re-suspended in water. Aliquots of ethanol extracts were purified through anion exchange columns (Dowex) and soluble sugars were analyzed by anion exchange chromatography coupled with pulsed amperometric detection (HPAEC/PAD) using an ICS 3000 Dionex system with a CarboPac PA-1 column (2 × 250 mm) using isocratic 12 mM NaOH. Different soluble sugars were identified by comparison with authentic standards (Sigma). Starch was quantified in the residue resulting from the extraction of soluble sugars according to the method described by Amaral et al. (2007). Starch was hydrolyzed by sequential digestion with thermostable α-amylase from *Bacillus licheniformis* and amyloglucosidase (AMG) from *Aspergillus niger* (Megazyme). The glucose

released was measured by mixing samples with glucose oxidase, peroxidase and 4-aminoantipyrine and phenol reagents and incubating at 30 ^{o}C for 15 min. (Glucose PAP Liquiform, CENTERLAB). This reaction was read at 490 nm, using glucose (Sigma) as standard. Glucose released was adjusted (-10%) to the mass of linked glucose that is present in starch.

Cellulose and lignin extraction and determination

Samples of 100 mg of freeze-dried and ground leaves, as described earlier, were used for cellulose and lignin dosage. Lignin content was determined by Klason's method (Hatfield et al., 1994 modified). Powdered material was resuspended in 100 µL of 72% sulfuric acid and incubated in a water bath at 30 ºC for 45 min. After incubation, the acid was diluted to 4% with distilled water and the samples were autoclaved for 1 h at 121 ºC. The hydrolyzate was centrifuged at 2000 g and the supernatant was discarded. The residue was washed three times in hot distilled water (~40 ºC), dried at 40 ºC and weighed on analytical balance (Shimadzu AY220). Dry mass corresponds to insoluble Klason's lignin. For cellulose content, samples were extracted with NaOH 8 M containing NaBH4 (0.4 mg mL^{-1}) for 1 h at room temperature. After extraction, the samples were centrifuged at 2000 g for 20 min and supernatant discarded. This process was repeated overnight, with new centrifugation and discarding supernatant steps. The residue was resuspended in water and neutralized with glacial acetic acid to pH between 6 and 8. After neutralization, samples were washed three times in distilled water with subsequent centrifugation at 2000 g for 20 min, discarding supernatant and drying the residue at 40 ºC. Insoluble material was digested in acetic-nitric acid at 100 ºC for 1 h. The cellulose was washed three times in water, dried at 40 ºC and weighed on analytical balance (Updegraff 1969).

Results

Biomass and leaf growth parameters

Urochloa brizantha and *U. decumbens* did not show changes in total dry mass when subjected to environmental treatments of increased CO_2 and/or temperature. For *M. maximus*, elevated CO_2 led to an

increase in total dry mass. *Megathyrsus maximus* plants grown under elevated CO_2 dry mass were 27.0% greater than those under control treatment, 20.5% greater than those under elevated temperature and 56.7% greater than those under elevated CO_2 + temperature (Fig 2). Regarding leaf growth parameters (Fig. 3), although they had no difference in the total dry mass, *U. brizantha* plants showed greater leaf dry mass in the treatments with increasing CO_2. Plants subjected to elevated CO_2 and elevated CO_2 + temperature had leaf dry mass 54.5% greater than those under control treatment and 21.4% greater than those subjected to elevated temperature. *U. decumbens* and *M. maximus* did not show differences among treatments for leaf dry mass (Fig. 3A). None of the species showed differences among treatments for leaf area (Fig. 3B) and specific leaf area (Fig. 3C) parameters.

Gas exchange parameters

Urochloa brizantha was the only species to show net carbon assimilation (*A*) enhanced by elevated CO_2 treatments (EC and ECT) in leaves. Elevated CO_2 + temperature were responsible for the best photosynthetic performance of this species over 75 days of growth (Fig. 4A). Elevated temperature promoted greater stomatal conductance (g_s) in leaves of all the three species and elevated CO_2 + temperature also increased g_s in *U. brizantha* leaves (Fig. 4B). Transpiration rate (E) also was increased under elevated temperature in *U. decumbens* and *M. maximus* leaves, while elevated CO_2 + temperature promoted the same response in *U. brizantha* leaves (Fig. 4C). Despite no differences being observed in g_s and E parameters, water use efficiency (WUE) was improved under elevated CO_2 in all the three species' leaves. Elevated CO_2 + temperature also promoted enhanced WUE in *U. decumbens* and *M. maximus* leaves, but to a less extended magnitude (Fig. 4D).

Non-structural carbohydrates content

The main soluble carbohydrates detected by high performance liquid chromatography analysis in leaves of all species studied were sucrose, glucose and fructose (Fig. 5A, B and C). *Urochloa brizantha* and *U. decumbens* did not show changes in these sugars contents. However, *M. maximus* plants grown under elevated temperature showed a decrease of 53.59% in sucrose (Fig. 5A), 50.6% in glucose (Fig. 5B) and

49.79% in fructose content (Fig. 5C) in relation to the other treatments. Also, *M. maximus* plants exposed to elevated CO_2 + temperature treatment showed an increase of 53.5% in leaf sucrose content related to control and elevated CO_2, and 157.89% related to elevated temperature treatment (Fig. 5A). Starch content in leaves of all species did not change with environmental treatments (Fig. 5D).

Cellulose and lignin contents

Leaf cellulose content was unchanged in all three species (Fig. 6A). For lignin content, *U. brizantha* plants showed a decrease when subjected to all environmental treatments compared to the control. Lignin content was 9.2% lower in plants grown under elevated temperature, 13.5% lower in those grown under elevated CO_2 and 9.8% lower under elevated CO_2 + temperature. *Urochloa decumbens* and *M. maximus* showed no changes in leaf lignin content for any of treatments.

Discussion

Elevated CO_2 promoted an increase in total biomass of *M. maximus* plants, yet this higher growth was not reflected in greater leaf dry matter nor in greater leaf area. Moreover, *U. brizantha* plants subjected to elevated CO_2 treatments (EC and ECT) exhibited higher dry leaf mass without, however, resulting in higher total biomass. Similar results were presented by common reed plants (*Phragmites australis*) (Eller et al. 2012). Higher growth under elevated CO_2 is commonly observed in C3 species (Ainsworth and Long 2005; Leakey et al. 2009; Schädel et al. 2010; Farfan-Vignolo and Asard 2012; Grombone-Guaratini et al. 2013; Ruiz-Vera et al. 2013), which have limited photosynthesis by photorespiration in current concentrations of atmospheric CO_2. However, some studies have also demonstrated that species with C4 photosynthetic pathway are also able to respond positively to increased CO_2 concentrations (Ziska and Bunce 1997; LeCain and Morgan 1998; Maroco et al. 1999; Souza et al. 2008; Souza et al. 2013). Results of large-scale experiments, however, show large variations and clearly demonstrated that the increase in CO_2 did not necessarily promote plant growth (Ainsworth and Long 2005; Leakey et al., 2009).

Urochloa brizantha was the only species to show improvement in carbon assimilation (*A*) under

elevated CO_2, and increased CO_2 and temperature jointly (ECT treatment) resulted in even more intense responses. This enhancement in *A* presented by *U. brizantha* plants was probably due to higher water use efficiency (WUE) under elevated CO_2 treatments. Improved WUE under elevated CO_2 were presented by all three species. One potential benefit of growth under elevated CO_2 is reduced stomatal conductance (g_s), reducing evapotranspiration and improving WUE (Ainsworth and Long 2005; Leakey et al. 2009). Although we did not observe a significant reduction in g_s or transpiration (E), there was a trend for such responses under elevated CO_2 which probably resulted in better WUE for all species and, consequently, to a greater carbon assimilation for *U. brizantha*.

Elevated temperature treatment (ET) promoted an increase in g_s and E of *U. decumbens* and *M. maximus* plants, but these increases did not result in reduced WUE nor *A*. Similar results were observed for potato (Lafta and Lorenzen 1995) and orange plants (Ribeiro et al. 2012). It might be expected that warm temperatures cause reduction in stomatal conductance due to increased evaporative demand and consequent imbalance of water relations in leaves (Jones, 1998). These lacks of reduction in WUE and carbon assimilation under elevated temperature indicate that the increase in g_s and E did not harm plant water status and that the increase in 3 ºC did not configure thermal stress. As plants were well hydrated, increased transpiration did not represent any imbalance in plants' water relations, similar to results observed for orange by Ribeiro et al. (2012).

The main soluble sugars identified by HPAEC/PAD in all the three species (glucose, fructose and sucrose) coincided with the profile obtained for tropical grasses by Moraes et al. (2012). These authors found that the fraction of neutral soluble carbohydrates analyzed for twenty-four grass species was composed of glucose, fructose, sucrose, maltose, raffinose and a series of linear oligosaccharides which co-eluted with the maltose-based compounds (Moraes et al., 2012). We also found raffinose and stachyose in this study, but in very low amounts (data not shown). In general, species that showed a positive growth and/or positive photosynthetic responses to elevated CO_2 had non-structural carbohydrate (NSC) content significantly higher in leaves (LeCain and Morgan 1998; Souza et al. 2008; Oliveira et al. 2010; Grombone-Guaratini et al. 2013). NSC are the primary energy available for growth and dry matter yield in grasses and are also associated with tolerance to environmental stresses (Moraes et al. 2012). However, in this study, elevated CO_2 did not increase the amount of NSC, even in *M. maximus* which had

enhanced growth. This species only showed an increased sucrose content in elevated CO_2 + temperature. Environmental treatments did not promote differences in NSC content for *U. brizantha* and *U. decumbens*.

Elevated temperature promoted reductions in sucrose, glucose and fructose contents of *M. maximus* plants. NSC content and composition are influenced by several factors, including genetic traits, photosynthetic efficiency, source-sink partitioning, as well as various abiotic factors. When moisture is not limited, temperature is one of the most crucial environmental factors (Moraes et al. 2012). High temperature modifies carbon metabolism enzymes, starch accumulation, and sucrose synthesis through specific genes that down-regulate carbohydrates metabolism (Ruan et al. 2010). Reductions in NSC content were also observed for tomato (Jie et al. 2012) and potato plants (Lafta and Lorenzen 1995) under a temperature rise of 10 ºC. In both studies, the authors attribute this response to a decrease in photosynthesis by inhibition of RuBisCO genes expression and to an increase in sucrose phosphate synthase activity, which inhibited fructose and glucose formation and induced a slight increase in sucrose levels in leaves of stressed plants (Lafta and Lorenzen 1995; Jie et al. 2012). However, there is no evidence of changes in photosynthetic carbon assimilation (*A*) or reduction in the total or leaf biomass in *M. maximus* grown under elevated temperature that may be related to such stress responses.

Depending on the source-sink balance, NSC can be accumulated in leaves or easily mobilized to other parts of plant (Moraes et al. 2012). Sucrose is the most important form of carbon translocated to developing and / or storage organs (Fallahi et al., 2008). The higher sucrose content in *M. maximus* plants exposed to a 3 ºC increase in temperature (ECT treatment) could be understood as a consequence of the large carbon requirements of growing organs (stems) acting as sinks. Although the stems' carbohydrate content has not been quantified, there was a significant increase in this organ's biomass (data not shown), which corroborates our hypothesis. In addition, the decreased sucrose, glucose and fructose content in this species under elevated temperature may also be indicative of carbohydrates mobilization through phloem to meet the carbon demand of sink organs.

Studies suggest that the high availability of soluble sugars (e.g., glucose and sucrose) during heat stress is an important physiological characteristic associated with heat stress tolerance (Liu and Huang, 2000). Sucrose is the final photosynthesis product and along with its cleavage products, regulates plant

growth and response to stress through carbon allocation and sugar signaling (Roitsch and Gonzalez 2004). Additionally, sugars have also been shown to act as antioxidants in plants (Lang-Mladek et al. 2010). At low amounts, sucrose acts as signaling molecule (Amiard et al. 2003), but at high amounts it becomes a reactive oxygen species (ROS) scavenger (Sugio et al. 2009).

Environmental treatments did not promote change in starch content. All the three species accumulated more starch than reducing sugars in leaves, as shown by the amounts recorded by plants grown under control treatment. This seems to be an intrinsic characteristic common in tropical grasses, especially species of Paniceae tribe (Moraes et al. 2012), to which all the three species studied belong. The composition of reserve carbohydrates in grasses is also genetic, influenced equally by environmental factors. While sucrose and fructans are reserve constituents predominant in grasses of temperate climate zones, sucrose and starch are predominantly accumulated in grasses originated from tropical zones (White 1973).

Cellulose content was not affected by any of the environmental treatments. Unlike NSC, the overall response of total structural carbohydrates (SC) content to changes in carbon supply seems to be weaker. Schädel et al. (2007) found constant concentrations of hemicellulose with increasing CO_2 concentrations in two of four grass species studied. Poorter et al. (1997) found no alterations in SC concentrations under high CO_2 concentrations in leaves of herbaceous crops and wild species, as well as in leaves of woody species. Naudts et al. (2013) also found no differences in SC levels between the current and future climates in the grasslands communities studied. However, Poorter et al. (1997) and Naudts et al. (2013) do not differentiate between hemicellulose and cellulose within the polysaccharides of the cell wall fractions analyzed. The insoluble sugar concentration reflects the levels of structural carbohydrates and starch, and is an indicator of sugar storage and cell wall formation activities (Naudts et al. 2013).

Regarding lignin content, only *U. brizantha* showed reduction in this parameter under all treatments compared to the control. Studies addressing lignin content under elevated CO_2, report various types of responses. Elevated CO_2 increased the lignin content in young poplar (Richet et al. 2012), decreased lignin content in beech (Blaschke et al., 2002), *Medicago lupulina* and *Lotus corniculatus* (AbdElgawad et al. 2014) and did not change the lignin content of *Poa pratensis* and *Lolium perenne*

(AbdElgawad et al. 2014). Poorter et al. (1997) also did not observe changes in lignin content for any of the 27 species studied. Physiologically, the lignification process marks an important step in plant development: tissue maturation (Blaschke et al. 2002). For beech, the observed reduction in leaf lignin content suggests that elevated CO_2 delayed structural compounds, including lignin, accumulation in leaves and, thus, the juvenile stage was slightly longer (Blaschke et al. 2002). The observed reduction in lignin content in *U. brizantha* can also be related to delayed leaf maturation. Although no significant differences were observed, there was a trend in increased leaf area of this species in the same treatments that promoted reductions in lignin content. As this species also had higher rates of carbon assimilation (*A*) under elevated CO_2 treatments, it is likely that the assimilated carbon is being moved to the NSC production at the expense of lignin synthesis. Since lignin is a final metabolic product and is therefore not reused, plants may preferably invest the excess carbon in growth and renewable resources such as sugar and starch, particularly in foliar tissues (Blaschke et al. 2002).

Taken together, the results suggest that despite having C4 metabolism, all the three species had metabolic responses modified by elevated CO_2. *U. decumbens* was the species least benefited by climate change effects, improving only WUE under elevated CO_2 treatments. However, as this species currently has aggressive invader potential already, it seems likely the distribution of this species will not be affected by climate change. Moreover, the occurrence of *U. brizantha* and *M. maximus* may increase if native species do not exhibit similar responses. Comparative studies between these species and highly responsive native species to climate change, as well as other invasive species with C3 metabolism that can be more responsive are needed to better assess the future occupation of the Brazilian Cerrado.

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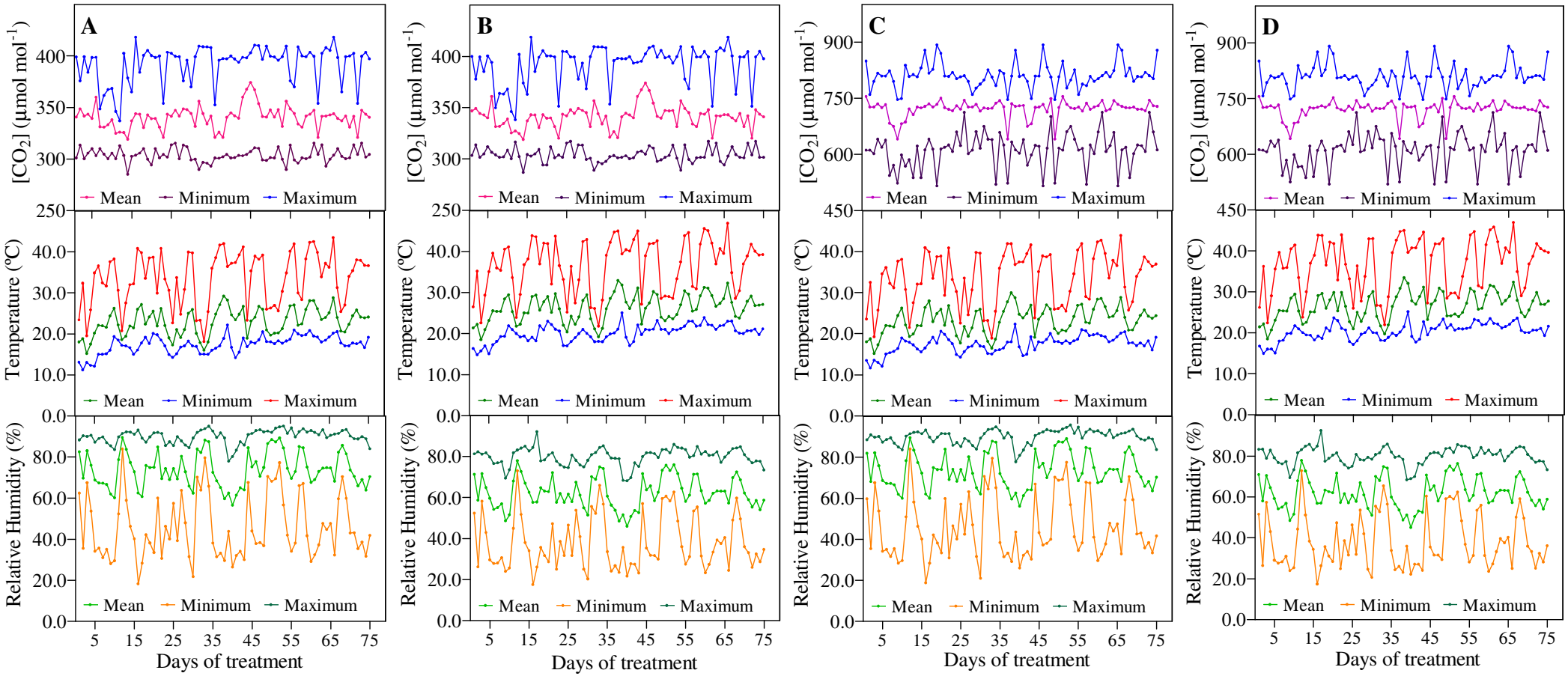

Fig. 1. Environmental conditions (CO_2 concentration, temperature and relative humidity) during 75 days of exposure to treatments. Each environmental treatment is identified as a conjunct of graphics: A – Ctrl (Control: current CO_2 concentration and room temperature); B – ET (Elevated temperature: current CO_2 concentration and room temperature + 3ºC); C – EC (Elevated CO_2: doubled CO_2 concentration and room temperature) and D – ECT (Elevated CO_2 + temperature: doubled CO_2 concentration and room temperature + 3ºC), respectively.

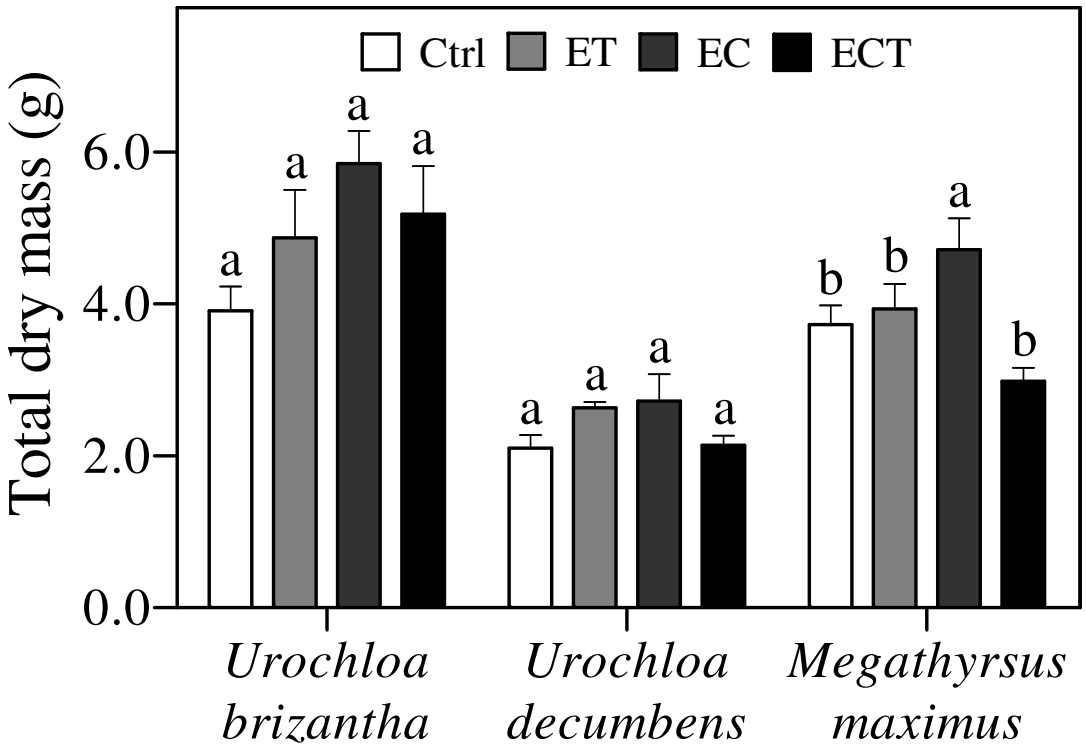

Fig. 2. Total dry mass of three invasive grasses species grown under elevated CO_2 concentration and/or elevated temperature treatments. Results represent mean ± SEM (n = 4). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature).

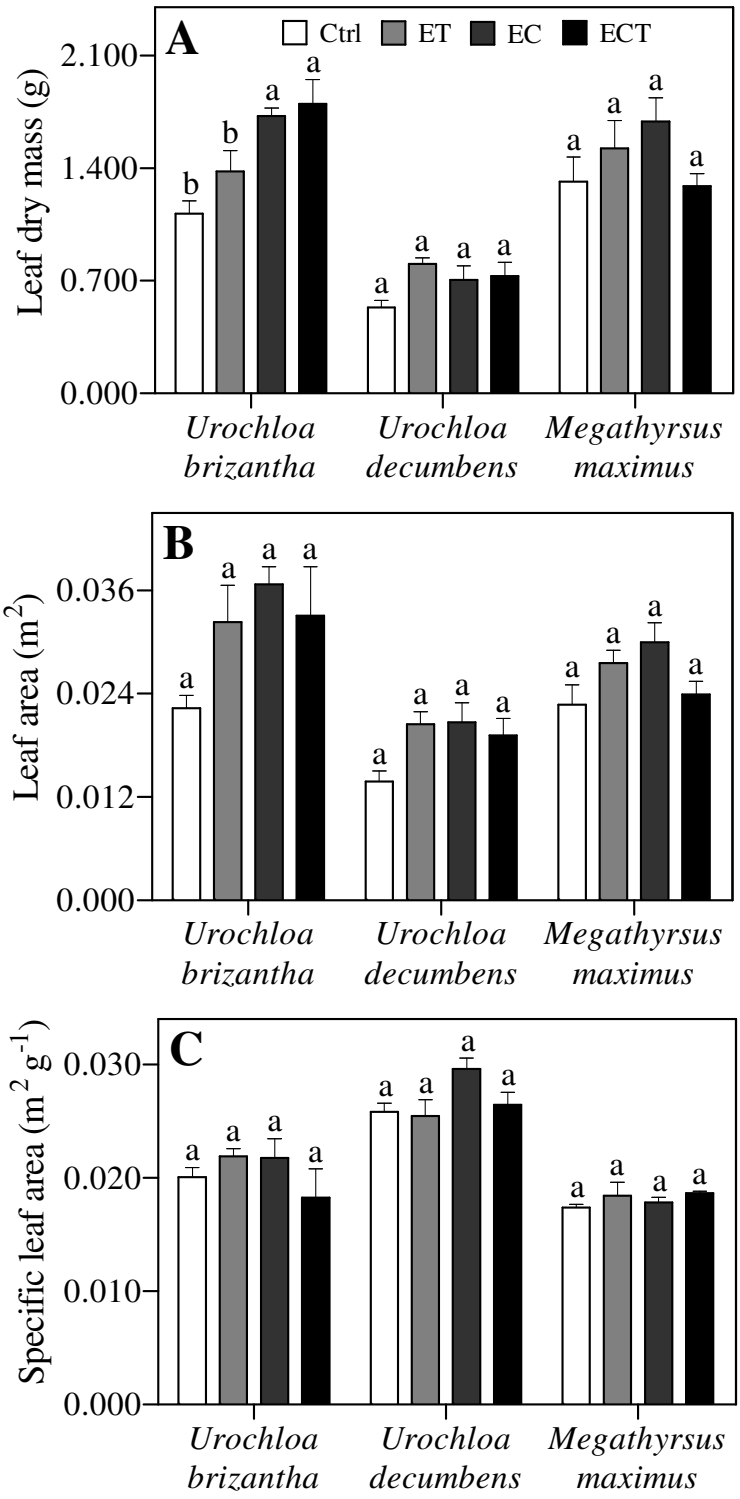

Fig. 3. Leaf growth parameters (A – Leaf dry mass, B – Leaf area and C – Specific leaf area) of three invasive grasses species grown under elevated CO_2 concentration and/or elevated temperature treatments. Results represent mean ± SEM (n = 4). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature).

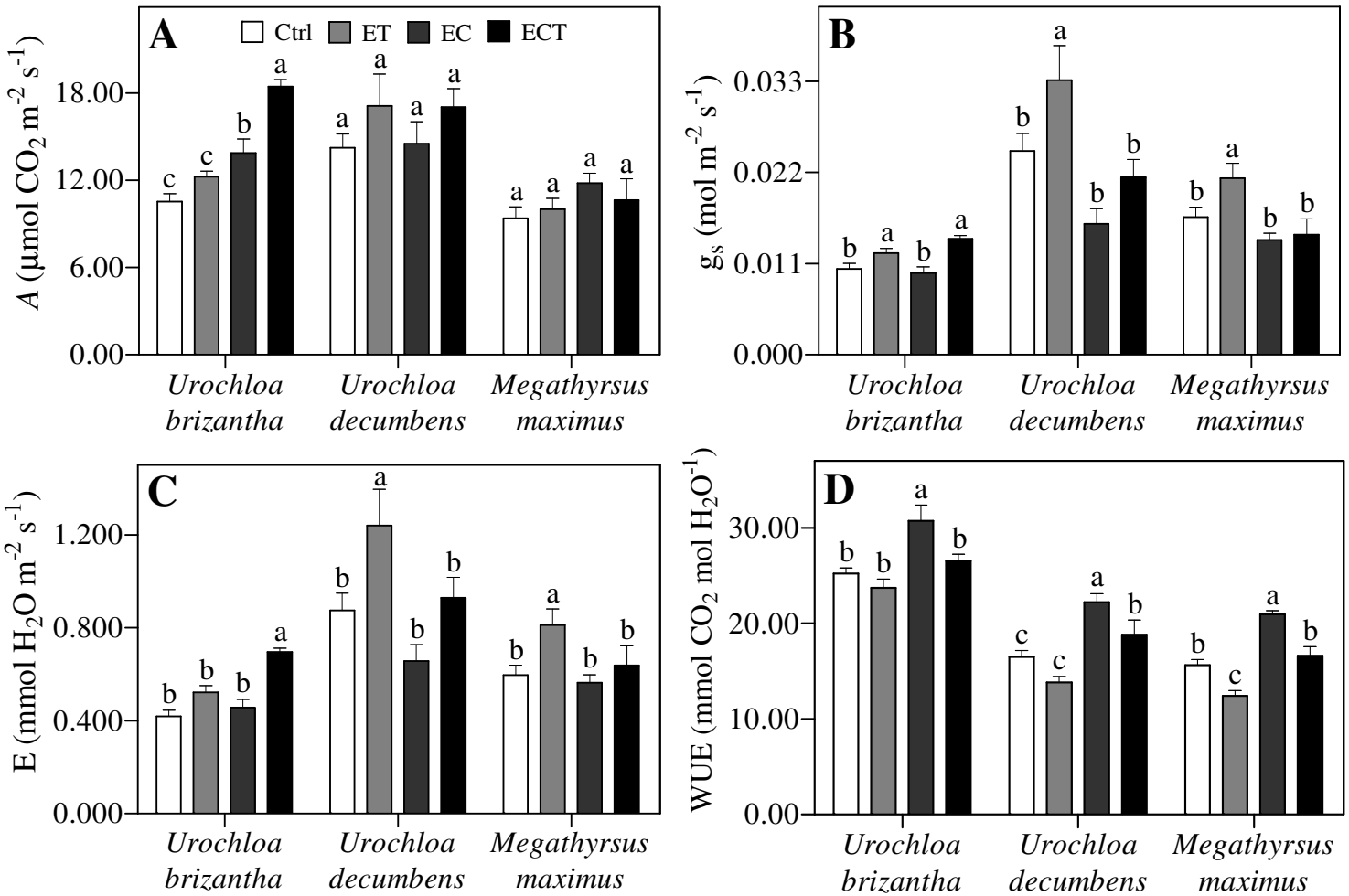

Fig. 4. Gas exchange parameters [A – Net photosynthetic assimilation (*A*), B – Stomatal conductance (g_s), C – Rate of transpiration (E) and D – Water use efficiency (WUE)] of three invasive grasses species grown under elevated CO_2 concentration and/or elevated temperature treatments. Results represent mean ± SEM (n = 6). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature).

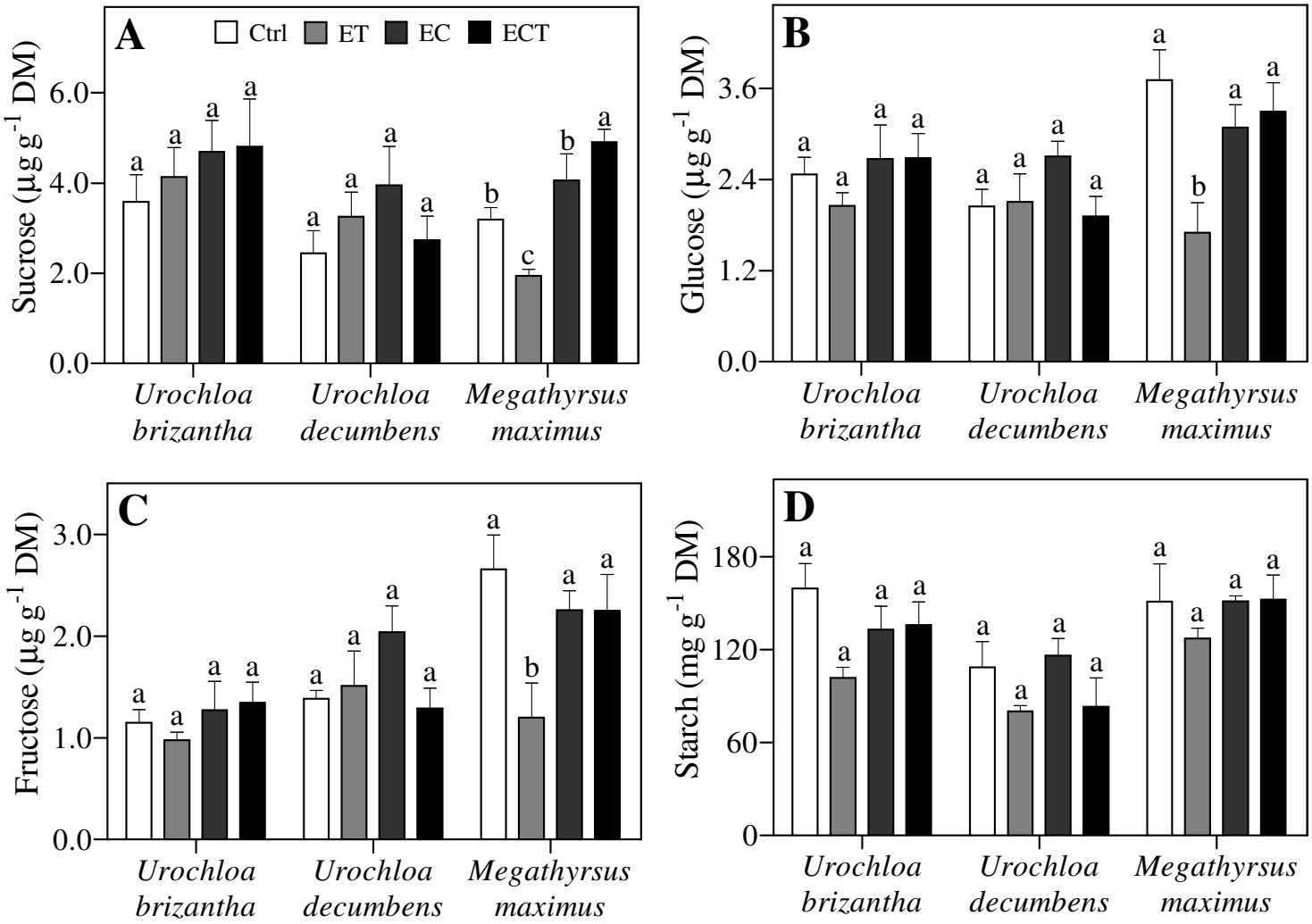

Fig. 5. Non-structural carbohydrates content (A – Sucrose, B – Glucose, C – Fructose and D – Starch) of three invasive grasses species grown under elevated CO_2 concentration and/or elevated temperature treatments. Results represent mean ± SEM (n = 4). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature).

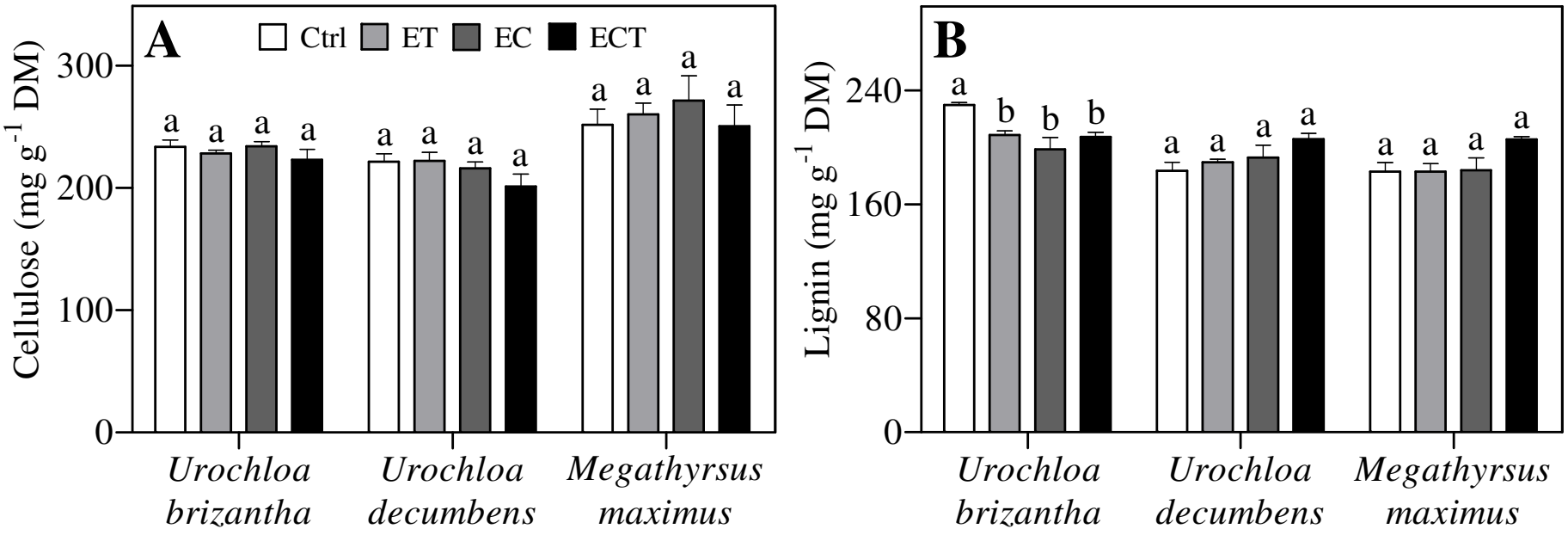

Fig. 6. Cellulose (A) and Lignin (B) content of three invasive grasses species grown under elevated CO_2 concentration and/or elevated temperature treatments. Results represent mean ± SEM (n = 4). Different letters indicate statistical differences ($P < 0.05$) between environmental treatments (Ctrl: Control; ET: Elevated temperature; EC: Elevated CO_2; ECT: Elevated CO_2 + temperature).

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Chapter 4[2]

ORIGINAL ARTICLE

Physiological approaches to determine the impact of climate changes on invasive African grasses in the savanna ecoregion of Brazil

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Abstract According to IPCC predictions for 2100, increasing CO_2 concentrations and global mean temperature could lead to a future scenario of increased incidence and duration of periods with exceptionally high temperatures and duration of drier seasons in some locations like Central Brazil. This extreme weather can affect fragile environments which are already suffering from anthropogenic disturbance that eventually culminate in biological invasions. One of these environments is the Brazilian Savanna (Cerrado), a biome with high levels of endemism, being considered an extremely important area for conservation. The present study aimed to investigate whether elevated CO_2 and temperature could modify protoplasmic tolerance to induced water deficit and acute heat shock in three species of invasive African grasses that are gradually replacing the Cerrado landscape. Results obtained from leaf tissues showed that elevated CO_2 and temperature had no effect on protoplasmic tolerances of *Urochloa brizantha* and that *Megathyrsus maximus* showed decreased thermotolerance. *Urochloa decumbens* showed improved tolerance responses to both types of these constraints undergone in vitro. Such adaptations to climate changes would probably represent an advantage in competition with other species. The results indicate that elevated CO_2 and temperature could cause modifications to protoplasmic responses of invasive grasses. The effects caused, however, depend on the species investigated. This ability to adapt or not to a changing environment may affect species distribution in natural and anthropized environments, especially in a future with predicted extreme weather.

Keywords Biological invasions · Brachiaria · Climate change · Drought · Heat waves

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Introduction

One of the biggest challenges of the twenty-first century is to understand the biological impact caused by global climate change. The steady increase of CO_2 and other greenhouse gases is leading toward a future climate with higher temperatures and profound changes in rainfall patterns (IPCC 2013). These changes could increase the severity and duration of periods with exceptionally high temperatures, commonly known as 'heat waves' (IPCC 2013). All these global climate changes can have important, but not completely understood consequences on plant growth and development (Farfan-Vignolo and Asard 2012).

Brazilian Savanna (Cerrado), located in Central Brazil, is the second largest biome in area, surpassed only by the Amazon rainforest, and is one of the richest savanna biomes of the world, with high levels of endemism, being considered an extremely important area for conservation (Myers et al. 2000). However, much of this biodiversity is being lost because of biological invasions. In this biome, the main limitations to plant growth are the seasonality of rainfall and low fertility of soils (Franco et al. 2014). Despite the oligotrophic soils, this region has topographical features that favor intensive agriculture and livestock, activities that have replaced natural vegetation (Franco et al. 2014). Cerrado is becoming dominated by invasive C4 African grasses that were introduced to improve pasture productivity, but escaped cultivation and invaded native

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grasslands (Baruch and Jackson 2005). Among the C4 African grasses that are replacing Cerrado landscape are *Urochloa* spp. (palisade grasses or brachiarias) and *Megathyrsus maximus* (Tanzania grass). In open and sunny areas, these grasses have many advantages over native species due to their fast reproductive cycle and regrowth, herbivory tolerance, greater photosynthesis rates and nutrient use efficiency (D'Antonio and Vitousek 1992; Pivello et al. 1999). Furthermore, they are able to survive in acid and oligotrophic soils and in the presence of aluminum toxicity (Ramos et al. 2012).

Biological invasions of exotic species and climate change are two main factors that have contributed to the decline of native biodiversity in natural environments (Baruch and Jackson 2005; Barbosa et al. 2010). Climate changes, promoted by increased atmospheric CO_2 and temperature, may interact synergistically with the invasion of exotic species, since the invasion and spread of exotic plants could be promoted by climate change (Vitousek et al. 1996; Ziska 2003). This interaction is affected by the biophysical constraints of the regional environment and by land use changes (Baruch and Jackson 2005; Barbosa et al. 2010). Cerrado region in Central Brazil, which currently has a 5-month-long dry season, is predicted to have decreased precipitation in nearly all months outside of the dry season (Costa and Pires 2010). Less rain would contribute to make the dry season longer and to increase the frequency and duration of heat waves (Costa and Pires 2010; IPCC 2013). All these climate changes could have potentially severe ecological consequences, as temperature and water availability are the most important abiotic filters controlling plant distribution, including the spread of invasive species (Thuiller et al. 2005).

Despite their apparent diversity, all seed plants have the same basic body plan and all plant cells have the same basic eukaryotic organization (Taiz and Zeiger 2010). Since protoplast is the essential component of plant cells, responses expressed at this level can be a good indicator of stress tolerance at a whole plant level (Clavel et al. 2005). Individual plants can also respond to changes in the environment, by directly altering their physiology or morphology to allow them to better survive the new environment. Such responses are often referred to as phenotypic plasticity (Debat and David 2001). Frequently by identifying the mechanistic basis of plant performance, we can gain insight into controls and components of fluxes in ecosystems, the invasibility of species into ecosystems, sensitivity of individual species and plant communities to changes in their environment and biotic interactions among different species (Ehleringher et al. 2001). Linkages between ecophysiology and ecosystem ecology are important for understanding many of the global changes taking place today. Many ecophysiological responses are used to design models that help us to understand ecosystems functions in a climate change scenario (Ehleringher et al. 2001). Since there is a strong relationship between individual plants' physiological status and ecosystem productivity and composition, understanding how plants respond to an environmental change at protoplast level or at organelle level could be a good indicator of a species, a community or even an ecosystem adaptation to such environmental changes (Ehleringher et al. 2001).

Several studies have been conducted on the physiological and molecular mechanisms involved in response to water deficit and heat stress tolerances at the protoplast level (e.g., Clavel et al. 2005; Farfan-Vignolo and Asard 2012; Salazar-Parra et al. 2012). At this level, stress factors such as water deficit and high temperatures can increase the production of toxic species such as superoxide, singlet oxygen and peroxide, known as reactive oxygen species (ROS) (Apel and Hirt 2004). Excessive ROS production leads to cellular oxidative stress, where the main targets are biological membranes (Apel and Hirt 2004), because they serve as cells' outer boundary, separating the cytoplasm from the external environment (Taiz and Zeiger 2010). The maintenance of their physical–chemical integrity under stress can be considered one of the best physiological indicators of protoplasmic tolerance, especially to water deficit in plants (Kocheva et al. 2005; Faria et al. 2013). Cell membrane stability can be easily evaluated by the minimally invasive technique of electrolyte leakage (Bajji et al. 2002; Faria et al. 2013; Kocheva et al. 2005, 2014). Additionally, the detrimental effects of heat stress on plants are caused, to a large extent, by negative effects on photosynthesis, which is among the most thermosensitive physiological processes (Kim and Portis 2005). Photosynthetic systems face a special challenge. They are designed to absorb a large amount of light energy and process it into chemical energy. At the molecular level, the energy in a photon can be damaging, particularly under unfavorable conditions. In excess, light energy can lead to the production of ROS, and damage can occur if the light energy is not dissipated safely (Asada 1999; Li et al. 2009). As the light reactions of photosynthesis take place in the specialized internal membranes of the chloroplast—thylakoids—and are driven by the photochemical complexes photosystems I and II (PSI and PSII) (Taiz and Zeiger 2010), this excess energy can increase ROS production which may cause damage to PS if not scavenged (Percival and Sheriffs 2002).

As increases in temperature and atmospheric CO_2 can have interactive effects on various physiological processes, including protoplasmic tolerance to water deficit and photosynthetic tolerance to acute heat shock (thermotolerance) (Sgherri et al. 1998; Baruch and Jackson 2005; Hamilton et al. 2008), several studies have focused on responses in

photosynthetic thermotolerance (Colemam et al. 1991; Kakani and Reddy 2007; Hamilton et al. 2008; Mishra et al. 2008; Wang et al. 2008) and tolerance to water deficit (Sgherri et al. 1998; Baruch and Jackson 2005; Erice et al. 2007; Vu and Allen 2009) in plants grown under elevated CO_2 and/or elevated temperature conditions. While most studies agree that high CO_2 enhances responses to water deficit by improving water use efficiency and/or reducing osmotic potential (Vu and Allen 2009), the results obtained concerning elevated CO_2 and thermotolerance are variable and are not fully understood. For example, high CO_2 induced positive (Hamilton et al. 2008; Mishra et al. 2008; Wang et al. 2008), negative (Hamilton et al. 2008; Wang et al. 2008) and also no effect on plant photosynthetic tolerance to acute heat shock (Colemam et al. 1991). The lack of a trend could be caused by the different methods employed to measure the effects on photosynthesis, including net carbon assimilation (Wang et al. 2008) or measurements of quantum yield of PSII (Hamilton et al. 2008; Mishra et al. 2008).

Understanding how plant protoplasts respond to global climate change could give some insights into how individual plants and some species could tolerate and adapt to a changing environment (Ehleringher et al. 2001). These comprehensions are essential to predict how these changes will affect species' distribution in natural environments (Kakani and Reddy 2007). As the species may have different adaptations to abiotic factors, responses to CO_2 and temperature changes can also be different. Hence, the present study aimed to determine whether climate conditions foreseen by the IPCC (2013) for 2100 would influence the protoplasmic tolerance of three aggressive Cerrado invasive grasses, *Urochloa brizantha*, *U. decumbens* and *Megathyrsus maximus*, to induced water deficit and acute heat shock. In this context, it would be possible to predict whether these changes may be indicative of alterations in their adaptations to growth in this region and similar sites.

Materials and methods

Plant material, growing conditions and experimental treatments

Plants of three grass species, *Urochloa brizantha* (Hochst. ex A. Rich.) RD Webster cv Marandu, *U. decumbens* (Stapf) RD Webster cv Basilisk and *Megathyrsus maximus* (Jacq.) BK Simon and S.W.L. Jacobs cv Tanzania, were propagated from seeds and grown hydroponically with Hoagland and Arnon (1950) nutrient solution for 45 days in open-top chambers (1.53 m^3 each—19°52′08.67″S 43°57′59.63″W 822 m elevation) (Fig. 1). Four environmental treatments were used based on the IPCC (2013) predictions, on the following specifications: (1) control—current CO_2 concentration (~360 µmol mol^{-1}) and room temperature; (2) elevated temperature—current CO_2 concentration and 3 °C above room temperature; (3) elevated CO_2—doubled CO_2 concentration (~720 µmol mol^{-1}) and room temperature; and (4) elevated CO_2 + temperature (~720 µmol mol^{-1} and 3 °C above room temperature). The experiments were conducted under natural photoperiod and air relative humidity (RH). The environmental conditions (CO_2 concentration, temperature, RH and light intensity) were monitored throughout the entire experimental growth period.

Protoplasmic tolerance evaluated after induced water deficit

The effect of water deficit on cell membrane stability was evaluated in vitro according to Bajji et al. (2002) modified by Faria et al. (2013), after cutting leaf discs (∅ = 1 cm) in three steps: (1) Determination of the best washing time required to remove most of the electrolytes derived from mechanical damage caused by cutting of tissues. (2) Measurement of kinetics of electrolyte leakage triggered by tissues at different water potentials, after spending 15 h in the dark in polyethylene glycol (PEG-6000) solutions providing the following water potentials (Ψ_w): without induced water deficit (deionized water), −1.8, −2.4 and −3.0 MPa. Electrolyte leakage (EL) was assessed as $EL = (EC_f - EC_i) - (EC_t - EC_i) \times 100$. For this calculation, electrical conductivity (EC) measurements of water were taken at the beginning of the rehydration period (EC_i), at different rehydration times ranging from 0.5 to 22.5 h (EC_f) and after autoclaving (1 atm, 121 °C in 20 min) and cooling samples at room temperature (EC_t). (3) Estimation of cell membrane stability by determining the membrane injury index (MII), expressed as: $MII = [(R_S - R_C)/(1 - R_C)] \times 100$, where R_C and R_S represent $(EC_f - EC_0) - (EC_t - EC_0)$ for control or PEG-treated tissues, respectively. For this calculation, EC was measured before (EC_0) and after (EC_f) the rehydration time interval, whereupon kinetic curves determined in step (2) became constant with respect to electrolyte leakage and then the samples were autoclaved before measurement (EC_t). An orbital shaker (Orbit Shaker, Lab-Line) was used in the washing step and electrical conductivity measurements were performed using a digital conductometer (Digimed DM-3, Digicron).

Protoplasmic tolerance evaluated after submitting leaf discs to heat shock

Protoplasmic tolerance to acute heat shock, related to the thermostability of PSII photochemistry, was evaluated by

Fig. 1 Open-top chambers (1.53 m^3 each) used for plant cultivation and imposition of environmental treatments

measurements of chlorophyll fluorescence determining the maximum quantum yield (F_v/F_m) of PSII using a modulated fluorometer (MINI PAM—Walz Mess und Regeltechnik). F_v/F_m provides an estimate of the photochemical efficiency of PSII, since it is inversely proportional to damage in the reaction centers (Baker and Rosenqvist 2004) and can serve as a good indicator of plant thermotolerance (Godoy et al. 2011). For each species and each environmental treatment, five leaf disks ($\varnothing = 1.5$ cm) were collected and dark acclimated. Acute heat shock was triggered by placing the samples in a thermostatic bath (Q–214 D2, Quimis) and increasing temperature of 2 by 2 °C. The initial fluorescence measurement was taken around 23–26 °C, and after each 2 °C increase, new F_v/F_m values were obtained until the highest temperature at which no fluorescence signal was observed. The F_v/F_m values obtained for each temperature were analyzed by a sigmoidal fit model and in each fitted curve the temperature which promotes reduction in 15 % (T_{15}) and 50 % (T_{50}) of initial F_v/F_m values were calculated according to Godoy et al. (2011).

Quantification of photosynthetic pigment content

The same leaf disks used in the evaluation of protoplasmic tolerance to heat shock, coupled with another disk cut from the same leaf and of equal size, were collected and stored in 5 mL of 80 % acetone for 48 h. They were then macerated in liquid N_2 using a mortar and pestle. After this, 5 mL more of 80 % acetone was added to the extract to a total volume of 10 mL. The extract was collected, centrifuged and analyzed by spectrophotometer for quantification of chlorophyll *a*, *b* and carotenoids according to Lichtenthaler and Wellburn (1983).

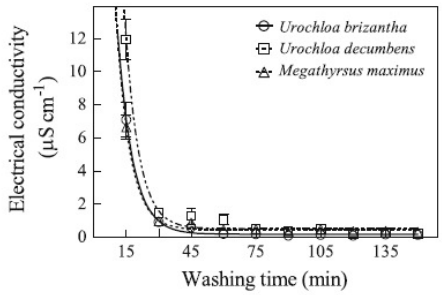

Fig. 2 Washing time test for complete removal of electrolytes due to mechanical damage caused by cutting leaf tissue disks of three invasive grass species. The time (in min) where electrical conductivity is greater is the washing time required to remove the electrolytes due to mechanical damage, avoiding overestimation of damage due to subsequent stress. Values represent mean ± SEM ($n = 8$)

Statistical analyses

The experimental design was completely randomized. Data were previously submitted to parametric normality and homoscedastic tests, following ANOVA analysis for parametric data and Kruskal–Wallis analysis for non-parametric data presented in Figs. 2, 4 and 5 and Tables 2 and 3. For these data, means were compared using the Tukey test at 5 % probability. Data presented in Fig. 3

were analyzed by general linear model (GLM) with contrasts' comparison at 5 % probability. Analyses of Figs. 2, 4 and 5 and Tables 2 and 3 were performed using BioEstat 5.0 (free software). Analyses of Fig. 3 to determine stabilization times of kinetic curve were performed using R 3.0.0 (free software) and are presented in Table 1.

Results

Protoplasmic tolerance evaluated after water deficit

For the three species studied, the washing time test determined that the best washing time was 15 min, since at this time interval there was an electrolyte leakage significantly greater than that observed at the other time intervals (Fig. 2). Also, a strong and stable pattern of electrolyte leakage kinetics was observed in all species grown in all four environmental treatments (CO_2 and/or temperature) and subjected to all water potentials induced by PEG solutions (−1.8, −2.4 and −3.0 MPa). Initially, there was a rapid phase of leakage in the first 8 h, followed by a stabilization plateau until the end of the rehydration period (Fig. 3 and Table 1).

U. brizantha did not change in response to water deficit tolerance for any of the environmental treatments (Fig. 3a–d; Table 2). For *U. decumbens*, environmental treatments promote alterations in tolerance to water deficit responses (Fig. 3e–h; Table 2). The increased atmospheric CO_2 concentration improved tolerance to water deficit in *U. decumbens* at the $\Psi_w = -3.0$ MPa (Fig. 3g; Table 2), as the electrolyte leakage was lower than that presented in the current environmental conditions (control—Fig. 3e; Table 2). Contrastingly, when both parameters, CO_2 and temperature, increased, the effect shown was not the same (Fig. 3h; Table 2). Tanzania grass (*M. maximus*) also did not change in response to water deficit tolerance for any of the environmental treatments (Fig. 3i–l; Table 2).

For *Urochloa* species, as water deficit increased, membrane injury also increased (Fig. 4a, b). Contrastingly, Tanzania grass showed no statistical differences among water potentials to which it had been submitted under any of the environmental treatments (Fig. 4c).

Comparing the environmental treatments, it was observed that none of them affected *U. brizantha* or *M. maximus* regarding membrane injury (Fig. 4a, c). For *U. decumbens*, increased CO_2 concentration decreased membrane injury at $\Psi_w = -2.4$ and −3.0 MPa and increased temperature increased membrane injury at $\Psi_w = -1.8$ MPa (Fig. 4b). However, in *U. decumbens*, at $\Psi_w = -2.4$ MPa, the increase in both CO_2 and temperature resulted in membrane injury similar to that of plants grown under high temperature, and at

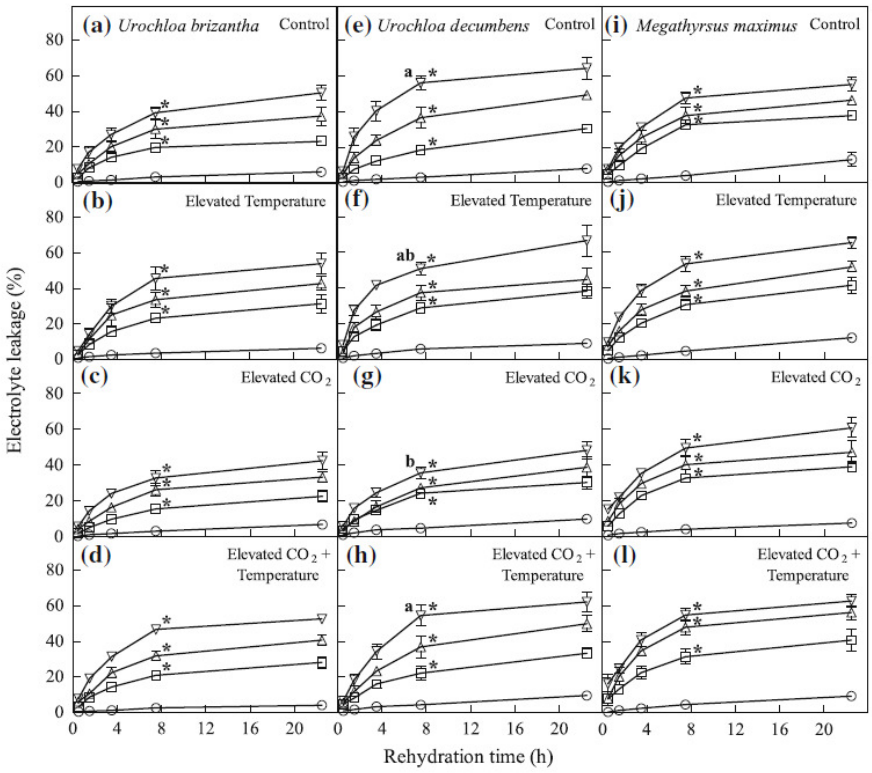

Fig. 3 Kinetics of electrolyte leakage from leaf tissues of three invasive grass species grown under different CO_2 concentrations (~360 or ~720 μmol mol^{-1}) and different temperatures (room temperature or room temperature +3 °C) subjected to water deficit induced by polyethylene glycol (PEG) 6000 solutions giving the following water potentials: *circle* 0, *square* −1.8, triangle −2.4 and *inverted triangle* −3.0 MPa. Values represent mean ± SEM ($n = 4$). *Asterisk* indicates the time when the kinetics stabilized (determined by comparison of contrasts after analysis of deviance presented in Table 1), and *letters* compare the electrolyte leakage at this time between environmental treatments (determined by comparison of means by Tukey test as in analysis presented in Table 2). *Different letters* represent statistical differences ($P < 0.05$)

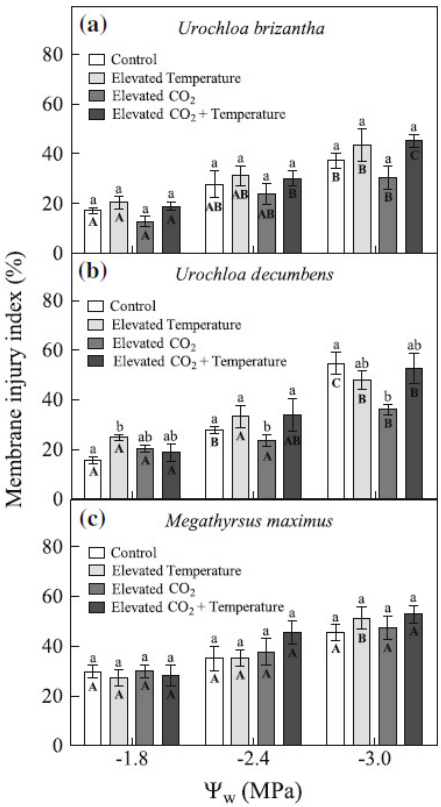

Fig. 4 Membrane injury index (*MII*) in leaf tissue from three invasive grass species grown under different CO_2 concentrations (~360 or ~720 µmol mol^{-1}) and different temperatures (room temperature or room temperature +3 °C) subjected to water deficit induced by polyethylene glycol (PEG) 6000 solutions providing the following water potentials: −1.8, −2.4 and −3.0 MPa. Values represent mean ± SEM ($n = 4$). *Lowercase letters* compare the environmental treatments with the same water potential. *Capital letters* compare different water potentials with the same environmental treatment. *Different letters* represent statistical differences ($P < 0.05$)

$\Psi_w = -1.8$ and -3.0 MPa, results presented were similar to those of plants grown under the current environmental conditions (Fig. 4b).

Protoplasmic tolerance to acute heat shock

None of the environmental treatments influenced T_{15} of any of the species studied, or T_{50} of *U. brizantha* (Table 3). However, it was observed that all treatments decreased T_{50} of *M. maximus* at approximately 2 °C (Table 3). For *U. decumbens*, increased CO_2 improved the protoplasmic tolerance to acute heat shock at about 0.6 °C and the increased temperature decreased such tolerance at about 0.7 °C (Table 3). The increase in both CO_2 and temperature conditions at the same time showed the same results as the current environmental conditions (control–Table 3).

Quantification of photosynthetic pigments' content

None of the environmental treatments had an effect on any pigments of *U. brizantha* analyzed (Fig. 5a). Increased CO_2 concentration decreased total chlorophyll and carotenoids content and chlorophyll *a*/*b* and chl/carot ratios of *U. decumbens* (Fig. 5b). Increasing temperature increased the content of all pigments of *U. decumbens* analyzed (Fig. 5b) and decreased chl/carot ratio of *M. maximus* (Fig. 5c). Increasing both CO_2 concentration and temperature produced similar results to those presented in the current environmental conditions (control) on all pigments of *U. decumbens* analyzed (Fig. 5b) and on chl/carot ratio of *M. maximus* (Fig. 5c). Total chlorophyll and carotenoids content and chlorophyll *a*/*b* ratio of *M. maximus* were not affected by any of the environmental treatments (Fig. 5c).

Discussion

This study corroborates the finding that the washing time duration of samples may interfere with electrolyte leakage, since very short intervals may not be sufficient to remove all ions from mechanical damage (Bajji et al. 2002) and very long intervals may result in a higher leakage due to an irreversible osmotic breakdown (Ehwald et al. 1984). The kinetics of electrolyte leakage for all environmental treatments imposed also followed a profile presented by wheat (Bajji et al. 2002), castor bean (Faria et al. 2013) and barley (Kocheva et al. 2005). According to Kocheva et al. (2005), this profile is due to the presence of the cell wall. The first part of the kinetics is due to the leakage of ions from the apoplast region, and it is only after the stabilization phase of the curve that cytoplasm begins to contribute to the process, due to a possible breakdown in the structure of the membrane.

The lower electrolyte leakage presented by *U. decumbens* grown at high CO_2 could be explained by the fact that these plants probably maintained cell turgor even in reduced water potential of PEG solutions. At the cellular level, a general stress response is the accumulation of ions and increase in the amount of metabolites that reduces the osmotic potential (Ming et al. 2012). Elevated CO_2 has been shown to cause a more negative osmotic potential in sugarcane leaves (Vu and Allen 2009). In this species, the level of soluble carbohydrates increased under high CO_2, indicating a possible osmotic adjustment that allowed photosynthetic activity to continue for at least one further day in plants submitted to water deficit (Vu and Allen 2009). It is likely that the increased CO_2 concentration had reduced the osmotic potential of *U. decumbens* cells, resulting in the lower electrolyte leakage that was presented.

Table 1 Analysis of deviance of electrolyte leakage kinetics from leaf tissues of three invasive grass species grown under different CO_2 concentrations (~360 or ~720 μmol mol^{-1}) and different temperatures (room temperature or room temperature +3 °C) subjected to water deficit induced by polyethylene glycol (PEG) 6000 solutions

		DF	Deviance	Res. DF	Res. Dev.	*F*	Pr (>*F*)†
Urochloa brizantha							
Control	Null			59	12,454.8		
	Ψ_W	2	2169.0	57	10,285.8	29.9010	5.48e−09***
	Reh. time	4	8028.5	53	2257.3	55.3380	<2.2e−16***
	Ψ_W × Reh. time	8	625.2	45	1632.2	2.1546	0.04974*
Elevated temperature	Null			59	9581.1		
	Ψ_W	2	1674.2	57	7906.8	34.9791	6.84e−10***
	Reh. time	4	6478.3	53	1428.5	67.6744	<2.2e−16***
	Ψ_W × Reh. time	8	351.6	45	1076.9	1.8364	0.09497
Elevated CO_2	Null			59	17,355.5		
	Ψ_W	2	1803.1	57	15,552.4	15.9746	5.72e−06***
	Reh. time	4	12,288.8	53	3263.5	54.4378	<2.2 e−16***
	Ψ_W × Reh. time	8	724.0	45	2539.6	1.6035	0.1507
Elevated CO_2 + temperature	Null			59	14,002.1		
	Ψ_W	2	2721.3	57	11,280.8	73.6402	6.44e−15***
	Reh. time	4	9760	53	1520.8	132.054	<2.2e−16***
	Ψ_W × Reh. time	8	689.3	45	831.5	4.6634	0.000336***
Urochloa decumbens							
Control	Null			59	23,397.4		
	Ψ_W	2	5928.9	57	17,468.5	66.2761	3.87e−14***
	Reh. time	4	13,928.1	53	3540.3	77.8479	<2.2e−16***
	Ψ_W × Reh. time	8	1527.6	45	2012.8	4.2689	0.000708***
Elevated temperature	Null			59	11,916.0		
	Ψ_W	2	993.7	57	10,922.2	16.0269	5.55e−06***
	Reh. time	4	9223.1	53	1699.1	74.3738	<2.2e−16***
	Ψ_W × Reh. time	8	304.0	45	1395.1	1.2259	0.3063
Elevated CO_2	Null			139	21,047.6		
	Ψ_W	2	3500.2	133	17,547.4	31.3050	3.024e−09***
	Reh. time	4	14,239.0	125	3308.4	63.6754	<2.2e−16***
	Ψ_W × Reh. time	8	792.7	105	2515.7	1.7724	0.108
Elevated CO_2 + temperature	Null			59	21,892.3		
	Ψ_W	2	3369.7	57	18,522.6	32.1114	2.16e−09***
	Reh. time	4	14,846.6	53	3676.0	70.7400	<2.2e−16***
	Ψ_W × Reh. time	8	1314.9	45	2361.1	3.1326	0.00669**
Megathyrsus maximus							
Control	Null			59	16,147.7		
	Ψ_W	2	1338.1	57	14,809.5	21.1175	3.40e−07***
	Reh. time	4	13,120.4	53	1689.1	103.5279	<2.2e−16***
	Ψ_W × Reh. time	8	263.4	45	1425.7	1.0392	0.4217
Elevated temperature	Null			59	20,443.4		
	Ψ_W	2	2682.9	57	17,760.5	44.6392	2.08e−11***
	Reh. time	4	15,845.3	53	1915.3	131.8195	<2.2e−16***
	Ψ_W × Reh. time	8	563.0	45	1352.3	2.3417	0.03387*

Table 1 continued

		DF	Deviance	Res. DF	Res. Dev.	*F*	Pr (>*F*)[†]
Elevated CO_2	Null			59	16,654.3		
	Ψ_w	2	1969.1	57	14,685.2	22.9071	1.38e−07***
	Reh. time	4	12,491.4	53	2193.8	72.6584	<2.2e−16***
	Ψ_w × Reh. time	8	259.7	45	1934.1	0.7554	0.643
Elevated CO_2 + temperature	Null			59	20,943.7		
	Ψ_w	2	2851.6	57	18,092.2	22.1655	1.99e−07***
	Reh. time	4	14,713.6	53	3378.6	57.1851	<2.2e−16***
	Ψ_w × Reh. time	8	484.0	45	2894.6	0.9406	0.4933***

Kinetics data are presented in Fig. 3

DF degrees of freedom

[†] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 2 Analysis of variance of stabilization time in kinetic curves of electrolyte leakage from leaf tissues of three invasive grass species grown under different CO_2 concentrations (~360 or ~720 μmol mol^{-1}) and different temperatures (room temperature or room temperature +3 °C) subjected to water deficit induced by polyethylene glycol (PEG) 6000 solutions

Sources of variation	−1.8 MPa				−2.4 MPa				−3.0 MPa			
	Treatments	Error	*F*	*P*	Treatments	Error	*F*	*P*	Treatments	Error	*F*	*P*
Urochloa brizantha												
DF	3	12	2.7978	0.0849	3	12	0.593	0.6344	3	12	2.2426	0.1352
SS	123.907	177.148			117.834	794.772			496.255	885.131		
MS	41.302	14.762			39.278	66.231			165.418	73.761		
Urochloa decumbens												
DF	3	12	3.2851	0.0578	3	12	1.069	0.4	3	12	4.6562	**0.022**
SS	221.79	270.052			289.049	10.8e+02			10.7e+02	91.9e+01		
MS	73.93	22.504			96.35	90.175			356.506	76.567		
Megathyrsus maximus												
DF	3	12	0.0978	0.9591	3	12	1.256	0.3332	3	12	0.7748	0.5323
SS	11.816	483.157			289.535	92.2e+01			143.065	738.581		
MS	3.939	40.263			96.512	76.821			47.688	61.548		

Kinetic curves and comparison of means by Tukey test are presented in Fig. 3 and analysis of deviance of kinetic data is presented in Table 1

DF degrees of freedom, *SS* sum of squares, *MS* mean squares

Bold value indicates statistical significance ($P < 0.05$)

Another possibility may be a protective effect of CO_2 to the membrane oxidative damage level (Farfan-Vignolo and Asard 2012; Salazar-Parra et al. 2012). One of the first consequences of water deficit is a change in cell membrane structure and function. Thus, the ability of an organism to maintain its membrane function and composition can indicate its resistance to stress (Kim and Portis 2005). In alfalfa plants, it was shown that the higher levels of unsaturated lipids in thylakoids of stressed plants grown at high CO_2 reduced the tendency of membranes to form non-lamellar configurations, helping to maintain a more fluid environment (Sgherri et al. 1998). The increase in tolerance in this plant was due not only to a lower reduction in water potential, but also to a different chemical composition of membranes that may have rendered them more functional in comparison with plants grown at an ambient CO_2 concentration (Sgherri et al. 1998). Elevated CO_2 could have a similar effect on *U. decumbens*, helping to explain the lower electrolyte leakage presented.

The lack of significant differences in membrane injury index for *U. brizantha* and *M. maximus* between environmental treatments in all Ψ_w imposed indicates that atmospheric climate conditions will not influence the ability of these species' protoplast to tolerate water deficit. Contrastingly, for *U. decumbens*, the results indicate that an increase in atmospheric CO_2 had a protective effect on membrane structure, since decreased membrane injury index in moderate ($\Psi_w = -2.4$ MPa) and severe ($\Psi_w =$

Table 3 Protoplasmic tolerance after acute heat shock in vitro assessed as the temperature (°C) drop in 15 (T_{15}) and 50 % (T_{50}) of photosystem (PS) II maximum quantum yield (F_v/F_m) of leaf tissue from three invasive grass species grown under different CO_2 concentrations (~380 or ~730 µmol mol^{-1}) and different temperatures (room temperature or room temperature +3 °C)

Protoplasmic tolerance after acute heat shock			
	Urochloa brizantha	*Urochloa decumbens*	*Megathyrsus maximus*
T_{15}—Temperature (°C) drop of 15 % in PSII maximum quantum yield (F_v/F_m)			
Control	38.89 ± 2.67	44.52 ± 0.97	43.89 ± 2.24
Elevated temperature	38.98 ± 3.52	43.72 ± 0.29	41.97 ± 2.45
Elevated CO_2	36.85 ± 4.75	44.98 ± 0.77	40.46 ± 1.72
Elevated CO_2 + temperature	39.34 ± 5.05	44.40 ± 1.16	41.58 ± 2.49
T_{50}—Temperature (°C) drop of 50 % in PSII maximum quantum yield (F_v/F_m)			
Control	45.50 ± 1.13	47.78 ± 0.36b	50.53 ± 0.64a
Elevated temperature	45.88 ± 2.73	47.06 ± 0.21c	47.50 ± 1.85b
Elevated CO_2	44.80 ± 3.46	48.36 ± 0.64a	46.41 ± 0.80b
Elevated CO_2 + temperature	46.10 ± 4.35	47.89 ± 0.39b	47.00 ± 2.69b

Values represent mean ± SD ($n = 5$)

Different letters indicate statistical differences ($P < 0.05$)

Absence of letters indicates no statistical differences

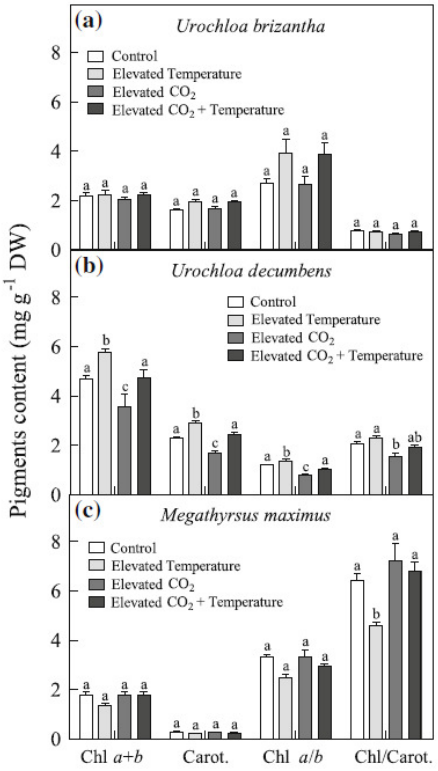

Fig. 5 Quantification of photosynthetic pigments of leaf tissue from three invasive grass species grown under different CO_2 concentrations (~360 or ~720 µmol mol^{-1}) and different temperatures (room temperature or room temperature +3 °C) after being subjected to in vitro acute heat shock. Values represent mean ± SEM ($n = 5$). *Different letters* indicate statistical differences ($P < 0.05$)

−3.0 MPa) water deficit rendered this species more tolerant to drought. Stress factors such as water deficit can increase ROS production, leading to changes in membrane permeability, composition and structure (Campos et al. 2003). Some authors argue that an increase in atmospheric CO_2 can potentially reduce the basal rate of oxygen activation and, consequently, ROS formation (Erice et al. 2007). Grapevine plants grown under high CO_2, high temperature and moderate water stress showed lower cellular oxidative damage than plants grown under current environmental conditions (Erice et al. 2007).

Elevated CO_2 could also have protected *U. decumbens* leaves against oxidative damage, mediated by water deficit, by increasing the activity of antioxidant enzymes. In alfalfa, the enhanced response to stress was due to the activation of advanced antioxidant systems that increase the ability of plants to prevent oxidative stress due to increased supply of photosynthates (Erice et al. 2007). In *Medicago lupulina* and *Lolium perenne*, the impact of water deficit on protein oxidation was also reduced in changed climate conditions (Farfan-Vignolo and Asard 2012). Furthermore, in *L. perenne* the effect of water deficit was increased by heating, but to a lesser extent, in high CO_2 conditions (Farfan-Vignolo and Asard 2012).

Regarding thermotolerance, the T_{50} responses of *U. decumbens* and *M. maximus* indicate a great influence from growth temperature. For *U. decumbens*, while elevated CO_2 promoted increased thermotolerance, elevated temperature reduced this response and elevated CO_2 and temperature showed similar response to the current climate conditions. It is likely that the possible beneficial effects of elevated CO_2 had been neutralized by the negative effects of increase in growth temperature and vice versa. *M. maximus* showed a significant reduction in thermotolerance for all environmental treatments related to the control. Probably for this species, growth temperature in all conditions exceeded its optimum, and elevated CO_2 could not ameliorate the situation. Hamilton et al. (2008) also found that the influence of CO_2 on photosynthetic

thermotolerance depends on the growth temperature. *Amaranthus retroflexus* and *Zea mays*, two C4 species, showed improvements in photosynthetic tolerance to heat shock when grown at high CO_2 and at temperatures close to its optimum for growth. When that growth temperature was exceeded, CO_2 did not have the same positive effect.

Virtually nothing is known about the mechanism(s) responsible for the increased thermotolerance of PSII in plants grown in elevated CO_2. Increasing CO_2 can affect plant responses to heat shock via a large number of metabolic processes (Colemam et al. 1991). One of the classic responses of plants grown in CO_2-enriched atmosphere is increased water use efficiency, in part by decreased stomatal conductance and transpiration (Vu and Allen 2009) which may increase acute heat shock tolerance by enhancing plant water status (Ainsworth et al. 2002). The improved tolerance of water deficit shown by *U. decumbens* grown at elevated CO_2 could be indicative of improved water use efficiency and this could explain the effect of elevated CO_2 on thermotolerance.

Despite having also improved the responses of water deficit tolerance in *U. brizantha*, elevated CO_2 did not have the same beneficial effect on thermotolerance response for this species. When plants are exposed to air temperatures above their optimal growth temperature, they can maintain tissue integrity, either changing the return radiation to maintain tissue temperature below or equal to the air temperature (Wahid et al. 2007) or increasing the expression of heat shock proteins (Colemam et al. 1991). Whether an increase in CO_2 affects the expression of heat shock proteins has been investigated by only a few studies, each showing different results as well as an absence of data for C4 species. The increase of CO_2 repressed the expression of genes related to the expression of heat shock proteins in rice (Fukayama et al. 2009) and *Populus* (Taylor et al. 2005), but increased the expression of these genes in *Arabidopsis thaliana* (Li et al. 2008). Thus, the factors responsible for PSII thermotolerance are probably a set of features and are likely associated with inter- and intra-specific variations (Colemam et al. 1991), since high temperature affects photosynthetic capacity of C3 plants more strongly than C4. Since they originated in regions of warmer climates, it is likely that C4 species are less affected by heat stress and may have evolved greater propensity for heat shock protein production during their evolution (Colemam et al. 1991), even if CO_2 concentration does not have a direct effect on the expression of genes encoding these proteins. *U. brizantha* may not have manifested changes in thermotolerance responses to any of the environmental treatments, probably due to a large production of heat shock proteins already constitutively present.

Photosynthetic pigment contents have also been associated with tolerance to heat stress (Haque et al. 2014). The results presented mainly for *U. decumbens* and *M. maximus* confirm the reports that changes in CO_2 concentration and increases in growth temperature can modify the content of photosynthetic pigments (Logan et al. 2009). Under elevated CO_2, *U. decumbens* showed decreased total chlorophyll and carotenoids contents. Similar results were found for alfalfa (Erice et al. 2007), *Phalaris arundinacea* (Ge et al. 2011) and wheat (Shanmugam et al. 2013). Elevated CO_2 also reduced chl *a*/*b* and chl/carot ratios in *U. decumbens*, agreeing with that found for wheat (Shanmugam et al. 2013), radish and soybean (Juknys et al. 2011).

When photosynthetic pigments like chlorophylls decreased, a significant fraction of absorbed light can induce ROS formation (Erice et al. 2007). Contrastingly, changes in electron transport rate may trigger alterations in size and composition of light harvesting complexes, leading to changes in absorbed light allocation to electron transport and alternative pathways (Logan et al. 2009). The fact that pigment content decreased in *U. decumbens* plants grown under elevated CO_2 while PSII function remained unchanged, as seen in T_{15} and T_{50} responses, is an indication of this species' thermotolerance (Mishra et al. 2008). Elevated CO_2 may have improved this species' antioxidant systems or have significantly decreased ROS formation, as reported for alfalfa (Aranjuelo et al. 2008). The lower membrane injury presented by *U. decumbens* under water deficit and elevated CO_2 is also an indication that increased CO_2 concentration reduces ROS formation in this species.

Elevated temperature promoted an increase in total chlorophyll and carotenoids contents, and reduced the chl *a*/*b* ratio in *U. decumbens*, similar to results found for alfalfa (Erice et al. 2007). This indicates that this species is trying to mitigate the effects of heat stress through the dissipation of excess energy to alternative pathways. In alfalfa, elevated CO_2 reduced the total electron flow in PSII and induced stimulation of non-photochemical quenching (NPQ), the dissipation of excess energy as heat (Aranjuelo et al. 2008). Thermal dissipation involves a pH gradient across the thylakoid membrane and xanthophyll (a type of carotenoid) cycle (Demmig-Adams and Adams 1996). Some specific metabolites such as α-tocopherol, carotenoids, flavonoids and simple phenolic compounds are antioxidants that may protect photosynthetic tissues by direct ROS detoxification (Pintó-Marijuan et al. 2013).

In *M. maximus*, the only pigment parameter changed by environmental treatments was the chl/carot ratio, which was decreased in plants grown under elevated temperature. This reduction was due to the slight decline in chlorophyll content, although this decrease was not significant. Similar responses were shown by *Phalaris arundinacea* (Ge et al. 2011). Increased pigment content in *U. decumbens* grown at high temperature and unchanged pigment contents in *M.*

maximus grown under all environmental treatments, despite the observed reduction in PSII thermotolerance, may indicate that those reductions were probably not lethal and these species could recover from acute heat shock. The absence of significant differences between environmental treatments for pigments content and T_{15} and T_{50} responses in *U. brizantha* plants reinforces the hypothesis of a higher constitutive thermotolerance in this species.

Taken together, the results presented in this study showed that increases in CO_2 concentration and temperature have different effects on three Brazilian Cerrado invasive African grasses. Protoplasmic tolerance of *U. brizantha* was not affected by water deficit or acute heat shock. In current environmental conditions, the species has a very aggressive invasive potential and is likely to remain a potential risk in fragile environments. *U. decumbens* could benefit from atmospheric changes predicted for 2100, since the increase in CO_2 and temperature improved its protoplasmic responses to water deficit, and increase in CO_2 improved its acute heat shock tolerance. For *M. maximus*, these changed parameters did not alter the protoplasmic tolerance to water deficit, but produced a decrease in PSII thermotolerance to acute heat shock. As there is a strong relationship between individual plants' physiological status and ecosystem productivity and composition, it is possible to indicate that changed atmospheric conditions (increase in CO_2 concentration and temperature) may affect the physiological responses of these plants at protoplast level and that this certainly could contribute to future changes in the composition of the species, especially in natural environments that already suffer from anthropogenic disturbance and biological invasions.

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Conflict of interest The authors declare that they have no conflict of interest.

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General Conclusions

Taken together, the set of results presented here indicates that the effects of global climate change will be demonstrated in a species-specific manner, as has already been reported in many other studies. Despite having C4 metabolism, all the three species studied had metabolic responses modified by elevated CO_2, in all of developmental stages investigated and at all levels, from protoplasm to whole plant. As competition is greater at the establishment stage, any advance in this process can lead to competitive advantage. Palisade grass species (*Urochloa brizantha* and *Urochloa decumbens*) showed positive responses earlier, from the moment of germination. Both benefited by reducing the time required for emergence and by an acceleration of the onset of autotrophy. *U. brizantha* also benefited by increasing the germination percentage. Contrastingly, only *Megathyrsus maximus* benefited from climate change effects after the plants had been established. During the growth period, the only positive response of *U. decumbens* was improved water use efficiency (WUE) under elevated CO_2, a response also presented by the other two species and commonly reported for plants grown under elevated CO_2. As plant responses to competition are closely related to the availability of water and nutrients, these enhanced water relations coupled with the increase in rooting observed for all species during establishment could configure a competitive advantage in a scenario of future climate change, especially in the Brazilian Cerrado, where the main limitations to plant growth are the seasonality of rainfall and low fertility of soils. The improvements in WUE were also responsible for the improvement in carbon assimilation shown by *U. brizantha* and for improvement in growth shown by *M. maximus* under elevated CO_2. The effects of elevated temperature were more significant in leaf carbohydrate content, by reducing soluble sugars (sucrose, glucose and fructose) content in *M. maximus*. However, these reductions were not related to thermal stress since photosynthesis and growth were not harmed. Elevated temperature also seems to act to reduce the positive effect of elevated CO_2 on many physiological responses. Structural carbohydrates (cellulose) and lignin content were less affected by climate changes. Cellulose content was not altered in any of the three species, and lignin content was reduced in *U. brizantha* in all environmental treatments, delaying leaf maturation of this species. During growth period, *U. decumbens* seemed to least benefit from climate change effects, since its only improvement was in WUE. Nevertheless, the results obtained in

protoplasmic tolerance under induced stresses showed that an increase in CO_2 and temperature improved its responses to water deficit, and an increase in CO_2 improved its acute heat shock tolerance. Adult plants of *M. maximus* seemed to be slightly sensitive to a temperature increase, since this environmental factor promoted changes in its carbohydrate content and the acute heat shock decreased its photosystem II thermotolerance. In conclusion, climate changes forecasted by IPCC for 2100 will certainly have effects in the ecophysiological responses of the three species studied. And these effects will be presented since the early developmental stages like germination. Palisade grass species (*U. brizantha* and *U. decumbens*) will apparently benefit more from climate change than *M. maximus*, since they presented a larger number of positive responses to increases in CO_2 concentration and temperature. Also, these species showed positive responses in the early developmental stages, such as germination and establishment, while *M. maximus* only begins to show positive responses after becoming established. Moreover, although it has few positive responses during growth, *U. decumbens* could benefit over other two species under conditions of drought and thermal stress. From an ecological perspective, it will be interesting to investigate the responses of native species co-occurring in the same environment as these, as well as responses of other invasive species to assess whether the invasive potential of these species will be enhanced in a scenario of climate change. Yet *U. decumbens* are the fourth most widespread invasive species over the Brazilian Cerrado (the most widely dispersed of the three studied species) according to a survey of invasive species, posing a threat to its biodiversity and will probably remain so for the next 85 years.