

UNIVERSIDADE FEDERAL DE SÃO CARLOS

**SIMULAÇÃO DO CLIMA DE 2050 EM CAMPO E SEUS
EFEITOS SOBRE O CRESCIMENTO DE FORRAGEIRAS**

- LÍVIA HAIK GUEDES DE CAMARGO BORTOLIN –

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SÃO CARLOS
ESTADO DE SÃO PAULO – BRASIL
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Tese apresentada à Universidade Federal de São Carlos, para a obtenção do título de Doutora em Ciências, na área de concentração de Ecologia e Recursos Naturais, sob orientação do professor Dr. Carlos Henrique Britto de Assis Prado.

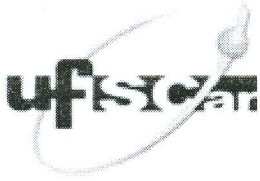
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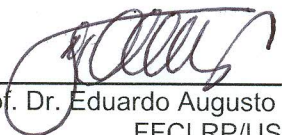
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“O rio atinge seus objetivos, porque aprendeu a contornar os obstáculos.”

(Lao Tsé)

- RESUMO E HIPÓTESE GERAL -

Essa pesquisa foi desenvolvida para entender as respostas climáticas de duas forrageiras tropicais a um cenário futuro previsto para 2050, independente da época do ano. Nós consideramos a hipótese do consórcio entre as duas forrageiras ser uma alternativa de pastagem em um clima futuro.

Para testar essa hipótese, nós estudamos as forrageiras sob controle de concentração de CO₂ e temperatura em condições de campo utilizando um sistema denominado Trop-T-FACE. Esse sistema nos deu competência para mimetizar condições atmosféricas previstas para 2050 (600 ppm de CO₂ na atmosfera e aquecimento de 2 °C na temperatura da cobertura vegetal). A gramínea C₄ *Panicum maximum* e a leguminosa arbustiva C₃ *Stylosanthes capitata* foram cultivadas como na prática agrícola vigente, em uma área de 2500 m² no campus da Universidade de São Paulo em Ribeirão Preto – SP, Brasil.

No inverno de 2013 e outono de 2014 as forrageiras cresceram em consórcio irrigado. No outono de 2015, *S. capitata* cresceu em plantio solteiro sem irrigação. Dezesesseis parcelas em forma de anéis com 2 m de diâmetro foram utilizadas para o acompanhamento do crescimento e do desenvolvimento das espécies C₃ e C₄ durante períodos de aproximadamente 35 dias de crescimento após poda inicial. Vários níveis de organização vegetal foram acompanhados em condições de campo nas parcelas Controle, nas parcelas com elevada concentração de CO₂ (eC), sob aquecimento (eT) e sob a combinação de tratamentos (eC+eT).

No primeiro capítulo está descrito o experimento realizado no inverno de 2013, com *P. maximum* em consórcio irrigado com *S. capitata*. As condições climáticas de temperatura foram sub-ótimas para o crescimento da gramínea C₄. Sendo assim, o aquecimento promoveu claramente o desenvolvimento da folhagem. A maior concentração de CO₂ atmosférico provocou uma “regulação para baixo” (*downregulation*) no acúmulo de biomassa foliar. As alterações provocadas pelas mudanças atmosféricas causaram também modificações na concentração de N na folha e na partição de biomassa no corpo da planta. Sob o tratamento combinado (eC+eT), os efeitos inibitórios do aumento de CO₂ na folhagem foram compensados pelo incremento resultante do aquecimento. Portanto, em condições climáticas futuras, durante o inverno na região Sudeste do Brasil, o aquecimento das folhas irá mitigar a

inibição por excesso de carbono, se o consórcio estiver livre de impedimentos hídricos e nutricionais.

No outono de 2014, um novo experimento foi realizado com *P. maximum* e *S. capitata* crescendo em consórcio irrigado. Esse experimento está descrito nos capítulos segundo e terceiro.

No segundo capítulo, estão os resultados de *P. maximum* no consórcio irrigado. O objetivo da realização desse experimento durante o outono foi principalmente comparar a influência do aquecimento nas folhas da gramínea em uma época mais quente do ano. Durante o outono, os tratamentos aceleraram os eventos fenológicos foliares da gramínea C₄, incluindo o início da senescência. O aumento isolado na concentração atmosférica de CO₂ (eC) ou em combinação com o aquecimento (eC+eT) condicionou folhas mais estreitas, provavelmente por alteração no processo de formação do meristema foliar. As alterações na largura da folha podem provocar mudanças na qualidade da forragem e afetar o consumo pelo gado. No entanto, a presença de folhas estreitas foi compensada por um maior número de folhas e a biomassa por perfilho se manteve.

Por outro lado, a leguminosa C₃ crescendo no consórcio irrigado durante o outono de 2014, apresentou várias alterações sem diferença estatística significativa para o crescimento vegetativo, apesar do aquecimento (eT) ter se mostrado prejudicial ao mesmo. Os resultados desse experimento estão descritos no terceiro capítulo. Por ser uma leguminosa adaptada a climas quentes, as principais alterações negativas observadas em *S. capitata* sob aquecimento (eT) foram atribuídas à competição com *P. maximum* no consórcio. O aquecimento isolado (eT) estimulou mais o crescimento da gramínea, que sombreou e atenuou a chegada de calor à leguminosa. No entanto, o aquecimento isolado (eT) estimulou significativamente o florescimento. No tratamento que simulou aquecimento e concentração de CO₂ no clima de 2050 (eC+eT) ocorreu mais ramificações devido ao intenso florescimento no ápice do ramo e a consequente interrupção da dominância apical. O cenário previsto em clima futuro não é favorável apesar da biomassa foliar para essa espécie de C₃ permanecer a mesma entre os regimes atmosféricos aplicados. Além disso, a irrigação de extensas áreas aquecidas de pastagem é inviável economicamente e ecologicamente e não aumentaria a disponibilidade de biomassa foliar de *S. capitata* crescendo em consórcio em 2050.

Para identificar a real influência da irrigação no crescimento de *S. capitata*, um novo experimento foi realizado durante o outono de 2015, com *S. capitata* em plantio solteiro e sem irrigação. O enriquecimento atmosférico com CO₂ não incrementou nem a biomassa e nem a área foliar. Por outro lado, ocorreu maior investimento em flores em detrimento dos compartimentos vegetativos no ramo. No entanto, o incremento do florescimento só foi possível com disponibilidade de água no solo superior a 0,3 m³ m⁻³. O aquecimento combinado com a reduzida disponibilidade de água no solo provocou elevada mortalidade dos ramos. A elevação da concentração de carbono atmosférico prevista para 2050 não será suficiente para compensar os efeitos negativos do aquecimento de cerca de 2 °C na produção de biomassa foliar, em condições de campo sem irrigação, nessa leguminosa arbustiva C₃.

Sendo assim, o consórcio, mesmo irrigado, não se mostrou como alternativa de pastagem no clima previsto para 2050. O plantio solteiro da leguminosa, sem irrigação, trouxe resultados ainda mais preocupantes. Os dados obtidos nesses estudos podem embasar o desenvolvimento de novas estratégias de manejo do pasto. Além disso, trazem informações relevantes para o desenvolvimento de políticas públicas para sustentar a cadeia produtiva de carne e leite, a maior do Brasil e uma das maiores do mundo.

- ABSTRACT AND GENERAL HYPOTHESIS -

This research was conducted to understand the climate responses of two tropical forages to a future scenario predicted for 2050, regardless of the season. We consider the hypothesis of the consortium between the two forages is a grazing alternative in a future climate.

To test this hypothesis, we studied two forages under controlled atmospheric CO₂ concentration and temperature in field conditions using a system named Trop-T-FACE. This system gave us the competence to mimic atmospheric conditions predicted for 2050 (600 ppm of CO₂ in the atmosphere (eC) and an increase of 2 °C in the canopy temperature). The C₄ grass *Panicum maximum* and the C₃ legume *Stylosanthes capitata* grew on current agricultural practice, in an area of 2500 m² on the campus of the University of São Paulo in Ribeirão Preto – SP. We tested the hypothesis about the consortium between the two forages being a grazing alternative in a future climate.

In the winter of 2013 and the autumn of 2014, the forage grew on irrigated consortium. In the autumn of 2015, *S. capitata* grew in monoculture without irrigation. Sixteen parcels in a ring form with 2 m of diameter were used for monitoring the growth and development of the species C₃ and C₄ species during growth periods of 35 days approximately after the initial cut. Several plant organization levels were accompanied under field conditions in Control plots, plots with elevated CO₂ concentration (eC), under heating (eT), and under high CO₂ concentration and heat (eC+eT).

In the first chapter is the experiment conducted during the winter of 2013, with *P. maximum* in irrigated consortium with *S. capitata*. The climatic conditions of temperature were suboptimal for the C₄ grass growth. Thus, the warming explicitly promoted the foliage development. The higher atmospheric CO₂ concentration caused downregulation in leaf biomass accumulation. The changes resultant of the atmospheric alterations also caused modifications of leaf N concentration and biomass partition in the plant. Under combined treatment (eC+eT), the inhibitory effects of the CO₂ increase were offset by the increment resultant of warming. Therefore, in the future climatic conditions, during the winter in the Brazilian Southeast region, the heating of the leaves will mitigate the inhibition by excess carbon, as long as the consortium is free of water and nutritional impediments.

In the autumn of 2014, a new experiment was performed with *P. maximum* and *S. capitata* growing in irrigated consortium. This experiment is described in the second and third chapters.

In the second chapter are the results of *P. maximum* in irrigated consortium. The purpose of conducting this experiment in the autumn was mainly to compare the influence of warming on the grass leaves in a warmer season. During the autumn, the treatments accelerated the leaf phenology of the C₄ grass, including leaf senescence. The isolated increase in the atmospheric CO₂ concentration (eC) or combined with warming (eC+eT) conditioned narrower leaves, probably by alterations in the leaf meristem formation process. Changes in leaf width may cause modifications in forage quality and affect the consumption by the cattle. However, the presence of narrower leaves was compensated by a greater number of leaves and the tiller biomass remained.

On the other hand, the C₃ legume growing in irrigated consortium during the autumn of 2014, presented several changes with no statistical differences in vegetative growth, despite the heat (eT) have been shown to be harmful to it. The results of this experiment are described in the third chapter. Being a legume adapted to warm climates, the main negative changes observed in *S. capitata* under warming (eT) were attributed to competition with *P. maximum* in the consortium. The separate heating (eT) stimulated further growth of the grass, which shadowed and softened the heat arrival in the C₃ species. However, the warming (eT) significantly stimulated the flowering. In the treatment that simulated warming and CO₂ concentration in the 2050 climate (eC+eT), there were more branches due intense flowering at the apex of the shoot and consequently interruption of apical dominance. The predicted future climate scenario is not favorable besides leaf biomass in this C₃ species remaining the same among applied atmospheric regimes. Furthermore, the irrigation of extensive grazing warmed areas is economically and ecologically unviable and did not increase the availability of leaf biomass of *S. capitata* in the consortium in the year 2050.

An experiment was conducted during the autumn of 2015, with the legume in monoculture without irrigation to identify the real influence of the irrigation on *S. capitata* growth. CO₂ atmospheric enrichment increased neither biomass nor the leaf area. On the other hand, it occurred greater investment on flowers at the expense of vegetative shoot compartments. Nonetheless, enhancing flowering was only possible with soil water content

greater than $0.3 \text{ m}^3 \text{ m}^{-3}$. Warming combined with soil water shortage caused higher mortality of shoots. The rise in atmospheric CO_2 concentration predicted for 2050 will not be enough to mitigate the damaging effects on leaf biomass production of the warming of about $2 \text{ }^\circ\text{C}$, in field conditions without irrigation, in this shrub C_3 legume.

Thus, the consortium, even irrigated, was not as an alternative pasture in climate predicted for 2050. The monoculture of the C_3 legume without irrigation brought results even more concerning. The data obtained in these studies can base the development of new pasture management strategies. Also, they provide relevant information for the development of public policies to support the productive chain of meat and milk, the largest in Brazil and one of the largest in the world.

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- LIST OF ABBREVIATIONS -

Treatments

- **eC** CO₂ atmospheric enrichment (600 ppm CO₂)
- **eT** Canopy warming (2 °C above current temperature)
- **eC+eT** CO₂ atmospheric enrichment (600 ppm CO₂) and canopy warming (2 °C above current temperature)

***Panicum maximum* parameters**

- **FLL** Final Leaf Length
- **LAR** Leaf Appearance Rate
- **LER** Leaf Elongation Rate
- **LLS** Leaf Lifespan
- **MLW** Maximum Leaf Width
- **NGL** Number of Green Leaves
- **PHY** Phyllochron (LAR⁻¹)
- **SL** Stem Length

Climate classification

- **CA** Camargo
- **KG** Köppen - Geiger
- **TH** Thornthwaite

Other abbreviations

- **FACE** Free Air Carbon Dioxide Enrichment
- **GHG** Greenhouse gas
- **IPCC** Intergovernmental Panel on Climate Changes
- **OTC** Open Top Chamber
- **T-FACE** Temperature Free Air Controlled Enhancement

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- CONTEXTUALIZATION OF RESEARCH AND FUNDING -

The research described in this thesis is part of the thematic project entitled "MiniFACE climate-change impact experiment to analyze the effects of elevated CO₂ and warming on photosynthesis, gene expression, biochemistry, growth, nutrient dynamics and yield of two tropical contrasting forage species" (FAPESP 2008/58075-8). The Foundation for Research of the São Paulo State (Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP) also granted doctoral scholarship to Livia H. G. de C. Bortolin (FAPESP 2012/20847-5).

The thematic project involved several research institutions and researchers, making possible the development of institutional research and training people with experience and knowledge in the use of the FACE system. Moreover, it allowed the improvement in the process of collecting and processing data. Also, the research provided theoretical material for the development of new techniques of pasture management and public policy for maintaining the production of meat and milk.

Teamwork was essential throughout the process. The members of all research groups of thematic project could cooperate with each other, allowing excellent results and productive research. Four scientific papers resulted from the research presented in this thesis, one for each chapter. Also, it will be produced and published a literature review gathering all these results to existing data in the literature, critically and at various organizational levels of the plants. The literature review is expected to be published after the publication of four scientific articles cited above.

The first chapter was published in *PLoS ONE* (DOI: <http://dx.doi.org/10.1371/journal.pone.0149620>) in the year 2016. The second chapter is being finalized to be submitted in *Planta*. The third chapter was accepted under major review in *PLoS ONE*. The fourth chapter was submitted in *Annals of Botany*, together with leaf anatomy data obtained by the research group guided by prof. Dr. Milton Groppo, from University of São Paulo (USP). This research group was also part of the thematic project. The master student Eduardo Habbermann worked directly with Dr. Groppo team and was primarily responsible for the collection and analysis of data of leaf anatomy.

- BASIC LITERATURE -

INTRODUCTION

At the beginning of the Industrial Revolution, the atmospheric concentration of CO₂ was about 280 ppm (1). Currently, it reaches values above 400 ppm. The average annual CO₂ concentration for the year 2015 was 401.85 ppm (2). It is a significant increasing since 1960 when 320 ppm of CO₂ was recorded at the Mauna Loa Observatory in Hawaii (2). Projections estimate a future atmospheric CO₂ concentration above 600 ppm for the year 2050 and may exceed 800 ppm by the end of the century (3,4). The higher CO₂ concentration may influence the energy balance of the Earth (5).

Several activities have contributed to rising global atmospheric concentration of carbon dioxide. These activities are, primarily, the increasing use of fossil fuels, industrial processes as the concrete production and land use change such as deforestation, forest burns, and agriculture. The cement industry is responsible for approximately 3% of global emissions of greenhouse gases (GHG) and for almost 5% of total CO₂ emissions (6). According to Brazilian Ministry of Technology, Science, and Innovation (MTCI), the contribution of energy production for emissions of GHG has increased from 16% in 2005 to 32% in 2010 in Brazil (7). GHG emissions from agriculture correspond to 35% of all Brazilian GHG emission mostly due to the use of nitrogen fertilizers and the methane resulting from the digestive processes of cattle (7).

Among the GHG, CO₂ is the main gas related to plant growth by participating directly in photosynthesis. The Brazilian Institute of Geography and Statistics (8,9), stated that forest burns and changes in land use contributed for more than 75% of the CO₂ emission in Brazil. Deforestation resultant from the woods burns to deploy pastures and agriculture further aggravates the situation by reducing the uptake of carbon from the atmosphere by trees and the amount of water transported daily to the atmosphere by plant transpiration (10).

The increase in GHG emissions causes global warming and climate change. Probably the CO₂ is no longer primarily responsible for the greenhouse effect, but it may have been the originator. CO₂ probably served as a trigger, i.e. after the rise of the CO₂ concentration, the temperature began to increase. This seemingly small increase drove up the amount of thermal energy in the atmosphere, which caused more water to evaporate. Water vapor is a

heteroatomic GHG and initiated a process of self-feeding temperature rise and changes in precipitation patterns. On the other hand, the highest concentration of clouds in the atmosphere reflects solar energy. The effect of increasing the air temperature is greater than the cooling augmentation by reflection. Currently, several heteroatomic gases like CO₂, CH₄, NO_x and mainly H₂O contribute to increased temperature in the atmosphere.

Predictions of global climate change indicate increases in temperature and dry season in some regions (11). Accordingly, the average surface temperature of the Earth has consistently increased since 1980 (12). The year 2015 was the warmest year on record (12). Fourth and fifth reports of Intergovernmental Panel on Climate Changes (IPCC) (1,3) indicated a high correlation between the increasing of GHG concentration and the average rising of Earth surface temperature. The increase in CO₂ atmospheric concentration and other GHG have lead to unprecedented changes in the Brazilian climate according to National Institute for Space Research (INPE) from Brazil and the UK Met Office-Hadley (13).

The IPCC (3) uses a range of future scenarios based on social, technological, economic, and environmental conditions for predicting the atmospheric CO₂ concentration and temperature, and projects an increase in the surface temperature of 2 °C by 2050 with very different stages of human development in a scenario named B1. This less pessimistic emission scenario designs a future CO₂ atmospheric concentration of 600 ppm for the year 2050 (3,4,14). Along with increased CO₂ atmospheric concentration and the rise of global average atmospheric temperature (1), there are changes in rainfall patterns in many regions of the Earth (11). These climate changes will have a significant economic and ecological impact on grassland and forests (15) because heating and the greater availability of CO₂ will affect plant growth, development, and yields (5,16). Since forages are directly exposed to atmosphere conditions in the field, climate change will significantly impact the management of pastures (17).

The cattle industry is one of the highlights of Brazilian agribusiness in the world scenario. Brazil owns the second largest effective herd in the world, with around 200 million heads and has been the world's largest meat exporter since 2004, with 20% of internationally traded meat and sales in over 180 countries (18). The value of the meat and milk production chains has been estimated at US\$ 17 billion (18). The tropical climate and the large size of Brazil contribute to this success since they allow the maintenance of the herd by forages growing in the field. However, despite the great importance to the food industry, the effects of climate change on forages and their acclimation to the combined effects of elevated CO₂

concentration and warming have not yet been evaluated under field conditions in Brazil. Studies on tropical forages are necessary and relevant for predict future impacts and provide adequate management. Heating and greater availability of CO₂ in the atmosphere will impact the managements of pastures significantly once the forages are directly exposed to atmosphere conditions in the field (17). In fact, there is a multi-biome gap in experimental data from the tropics and subtropics on plant responses to future climatic changes (19,20). As most of the Brazilian pastures are rain-fed, they probably will be impaired with the predicted change in rainfall patterns such as poorly and unevenly distributed rainfall with long periods of drought in the tropics (11). Physiological responses to drought include stomatal closure, decreased photosynthetic activity, altered cell wall elasticity, and even generation of toxic metabolites that causes plant death (21). Also, insect herbivory increases and plant biomass reduces on water-stressed plants (22). Therefore, studies that show the effects of water deficit on predicted future climate in tropical pastures become relevant.

C₃ AND C₄ PHOTOSYNTHESIS RESPONSES TO CLIMATE CHANGES

C₄ plants represents less than 4% of terrestrial plant species, while C₃ plants represents approximately 95%. However, C₄ crops such as sugarcane, maize, sorghum and some of forage grasses are responsible for about 20% of global net primary productivity (23–25). The changes in the availability of CO₂ concentration, water, and temperature will affect the whole trophic chain of the planet (26) and will have a significant economic and ecological impact on grassland and forests (27).

Carbon dioxide plays a major role in plant metabolism because it is the single carbon source for the photosynthetic production of carbohydrates. The current atmospheric concentration of CO₂ is lower for saturating photosynthesis especially for C₃ plants (28). Thus, C₃ species respond positively to increased atmospheric CO₂ concentrations (29). Stimulation of photosynthesis by increasing CO₂ concentration has been widely described (4,27,30–33). On the other hand, as a consequence of CO₂ increase, plant photorespiration may reduce. Recently, has been suggested that the reduction of photorespiration-photosynthesis ratio in response to the ~100 ppm CO₂ increase over the 20th century was *ca.* 25% (34). Mathematical models predict that CO₂ concentration at 600 ppm could raise C₃ photosynthesis up to 40% (4). *Panicum maximum* (C₄) and *Stylosanthes hamata* (C₃) produced 67 and 85% more fresh and dry biomass, respectively, under 600 ± 50 ppm CO₂ in open top chambers (OTC) (35). The increase in atmospheric CO₂ concentration caused a rise of 30% in the dry biomass production in a pasture based on two C₃ forages (*Lolium perenne* and *Trifolium repens*) growing in experimental rooms (36,37). Elevated CO₂ concentrations also caused 25% increase in yield of C₃ legume *Arachis glabrata* growing intercropped with C₄ grass *Paspalum notatum* in a temperature-gradient greenhouse (38). Besides physiological adaptations, this increment is also coupled with leaf anatomical alterations, such as increased mesophyll size and chloroplasts numbers per cell (39). On the other hand, the stimulation of photosynthesis in C₃ plants under 500-600 ppm CO₂ was significantly lower (14%) in field experiments conducted on free-air carbon dioxide enrichment, the FACE system (19,31,40). It happens because the concentration of CO₂ is lower in FACE than OTC (4). Also, in FACE there is a direct influence of external factors on plants (41), setting the results of FACE closer to the reality (31,41). Moreover, elevated atmospheric CO₂ concentration may partially offset the harmful effects of warming on C₃ plants by synergistically increasing carbon uptake via

photosynthetic capacity up-regulation and by better access to water (42). Nonetheless, for each 1.0 °C increment in seasonal temperature, a reduction between 3-16% in crop yield is expected (16,43) due to increased respiration and photorespiration in C₃ species.

Plants clearly respond to increased CO₂ concentrations. Even for some C₄ species, the current concentration of atmospheric CO₂ is below the CO₂ saturation point for net photosynthesis (44). Indeed, some results from OTC studies have found that an elevated CO₂ concentration can stimulate biomass production in C₄ species (35,45). However, the net photosynthesis and growth of C₄ species are less responsive than those of C₃ species under elevated CO₂ (31). In contrast to physiological responses, information on morphological alterations in the leaf blades of C₄ grasses under future climatic change is scarce. This is an important gap in knowledge because the anatomical and morphological traits of the leaf blades of *Panicum maximum* can affect animal preferences (46).

LEAF MORPHOLOGY, FLOWERING AND YIELD ALTERATIONS

Several studies have focused on physiological and yield attributes of plant species to global change conditions. However, a few studies assessed the morphological and leaf anatomical traits in combined conditions of elevated CO₂ and warming as well as flowering responses. Indeed, leaf anatomy and growth contribute significantly to the adaptation of plant species to environmental conditions (47).

Anatomical and morphological traits of leaf blade in *Panicum maximum* are related to animal preference and may influence consumption and digestibility, thus interfering in forage quality (46). Considering this, leaf width and length provide valuable complementary information on using this grass as fodder in the future (46). These leaf morphological parameters are closely related to leaf meristematic activity. The activity of meristems plays central roles in plant developmental processes (48). The investigation of the responses of meristems to the environment may contribute to understanding the plant growth since it depends on the supply of new cells produced by meristems (48) and it reflects on plant plasticity and adaptation to the environment. Two meristems control leaf growth: 1. shoot apical meristem, which gives rise to leaf meristem and is responsible for the leaf width; and 2. leaf meristem, responsible for the leaf growth in length (48).

In C₃ crops, the growth temperature above a finely tuned threshold can trigger flowering, bypassing the need for other inductive stimuli (49). High temperature during flowering may lower CO₂ effects by reducing grain number, size and quality (50). Increased temperatures may also reduce CO₂ effects indirectly, by increasing water demand (50). The combination of 450 ppm CO₂ concentration and 0.8 °C temperature increased yield by approximately 5.3% in rain-fed spring wheat (51). Nonetheless, the combination of 450 ppm CO₂ concentration and 1.8 °C warming reduced wheat yield by roughly 5.7% (51). As the increase in CO₂ atmospheric concentration also leads to a rise in temperature, forecast how C₃ forage plants in the field will respond to a heated and carbon enriched atmosphere is necessary for predicting foliage production.

THE CONSORTIUM WITH C₃ AND C₄ FORAGES

The prediction of less advantage of C₄ plants regarding C₃ species under high CO₂ concentration does not necessarily happen in ecosystems. C₄ species growth is as sensitive as C₃ species to changes in CO₂ concentration especially when the water supply constrains growth, a common condition in pastures containing C₄ species (52). Higher temperatures will likely increase the response to CO₂ in warm season grassland (52). Besides, environmental conditions in subtropical climate are favorable to C₄ species and should remain favorable under elevated CO₂ (52). C₄ forages must be affected by CO₂ concentration increase without losing the greatest relative biomass production capacity, and higher nutrients use efficiency (52). Also, availability of water in the systems is limited. A sudden improvement in the availability of soil moisture under elevated CO₂ may favor the C₄ species that were not as affected by water stress (52).

Threatened water resources, changes in precipitation pattern and global warming are factors that interact strongly with the C₄ plants growth responses to increasing atmospheric CO₂ (25). These conditions will influence competition between C₃/C₄ and among shrubs/grass (25). In a consortium between *Abutilon theophrasti* (C₃) e *Amaranthus retroflexus* (C₄), CO₂ increases the intensity of plant-plant interactions and increases the growth of survived individuals (5). Probably rapid growth leads to a fierce competition by other features that rapid growth requires, affecting the survival of plants.

Mortality and difference in the sizes of *Abutilon theophrasti* (C₃) e *Amaranthus retroflexus* (C₄) are increased with the rise of temperature and CO₂ concentration (53). The characters variation is higher under high CO₂ concentration and/or high temperature. This response may indicate significant events, such as acclimatization and change in floral phenology by changing the size of the reserves for flowering (53). As the size of the individual is often related to fertility, an increase in size in response to elevated CO₂, together with the decreased survival of individuals can result in a smaller population (53). The changes in response to elevated CO₂ may cause a reduction in the minimum size of the individual for the time of beginning of flowering (53). It may lead to a lower population as a result of accelerated growth and greater competition for resources, particularly nutrients (53).

The consortium between *Stylosanthes guianensis* cv. Mineirão (C₃ legume) and *Panicum maximum* cv. Mombaça (C₄ grass) may be an alternative pasture for fertile soils in a

vast area of Brazilian Cerrado vegetation (54). The major C₄ grasses used as forage for cattle in Brazil belong to the genera *Panicum* and *Brachiaria* (46). *Stylosanthes capitata* Vogel (C₃) is a forage legume of high quality and when grown in consortium with *Panicum maximum* Jacq (C₄) results in increased production of biomass and improves the nutritional quality of the forage (55). *Stylosanthes capitata* is a hybrid of *Stylosanthes macrocephala* and *Stylosanthes pilosa* (56). According to Brazilian Agricultural Research Corporation (EMBRAPA), this forage is well accepted by livestock, has no compounds that interfere with its health, and is widely used in pastures (57).

P. maximum cv. Mombaça produced approximately 3200 kg ha⁻¹ of dry matter in monoculture. In a consortium with *S. guianensis* cv. Mineirão, this production has increased to about 4500 kg ha⁻¹ (54). Also, *Panicum maximum* intercropped with *Stylosanthes hamata* in open top chambers at a 600 ± 50 ppm CO₂ increased the plant height and biomass production (35). Moreover, in that consortium, the assimilatory functions and chlorophyll accumulation was significantly influenced (35). Answers on how the consortium will respond in future climate will provide information to future pasture management.

- THE TROP-T-FACE SYSTEM -

The Trop-T-FACE system is a new combined Free-air Temperature and CO₂ Controlled Enhancement system to evaluate the performance of tropical pastures under future climate change scenarios. The FACE component for elevated CO₂ treatments under field conditions was a modified POPFACE sonic injection system of pure CO₂ designed by our colleagues (58). Sixteen plots were created inside rings with a diameter of 2 m. A small FACE system named miniFACE provided the treatment of elevated CO₂ on eight of these plots. The eight plots of miniFACE were placed randomly within the experimental area so that no ring was less than 10 m from another to minimize CO₂ cross-contamination (Fig. FACE 1).

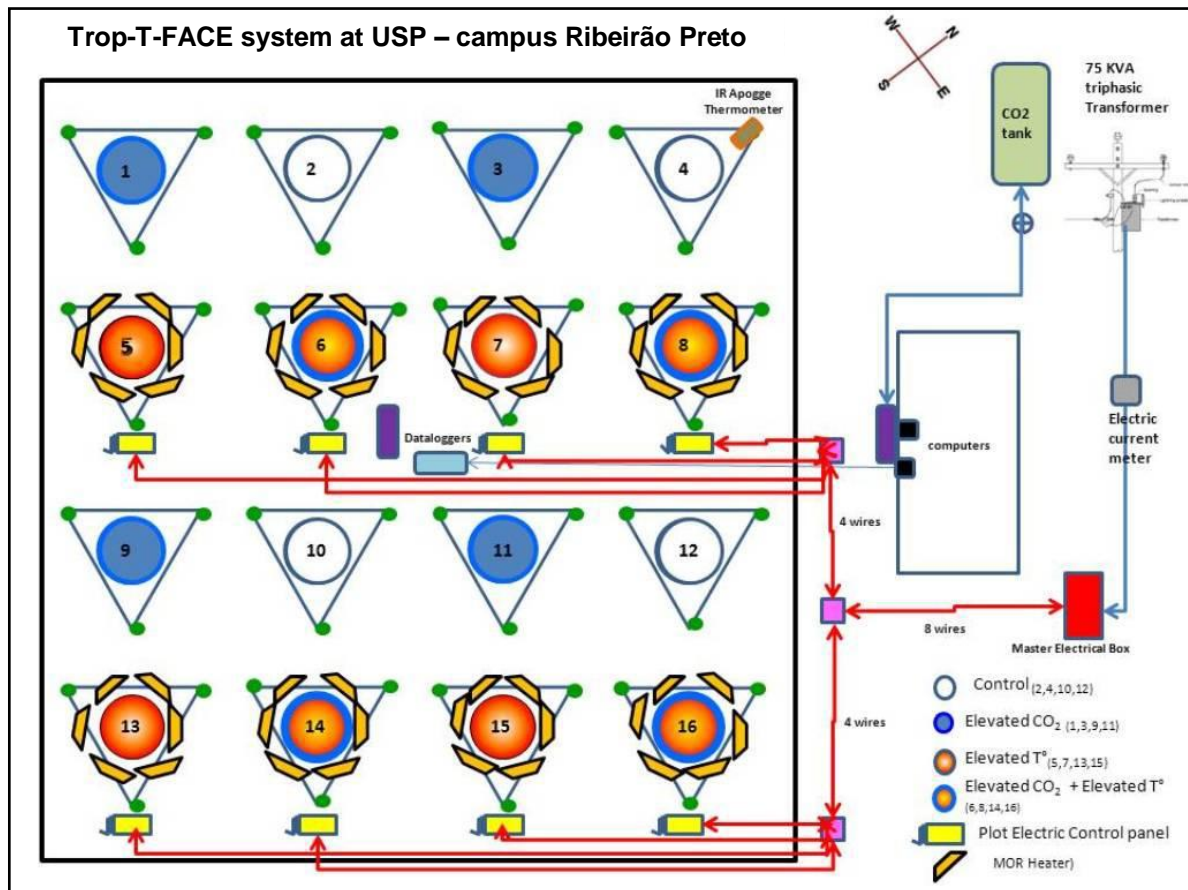


Fig. FACE 13. Experimental design of Trop-T-FACE system, located at University of São Paulo, in Ribeirão Preto campus.

The CO₂ was sampled at the center of each miniFACE ring by an air pump and monitored by a high-speed gas analyzers CARBOCAP infrared gas analyzer (IRGA) Model

GMP 343 (Vaisala, Finland (Fig. FACE 2)). Each IRGA monitored the CO₂ concentration for manipulating the supply of pure CO₂ gas in each plot. The amount of pure CO₂ was controlled by varying the pressure within the gas release pipes with an automatic pressure regulator (SMC Corporation, ITV series, Japan (Fig. FACE 3)). The flow of each valve was controlled by adjusting the valve tension that is determined by a programmable control system using the microprocessor-based Proportional Integration Device (PID algorithm).



Fig. FACE 14. CARBOCAP infrared gas analyzer (IRGA) Model GMP 343 (Vaisala, Finland).

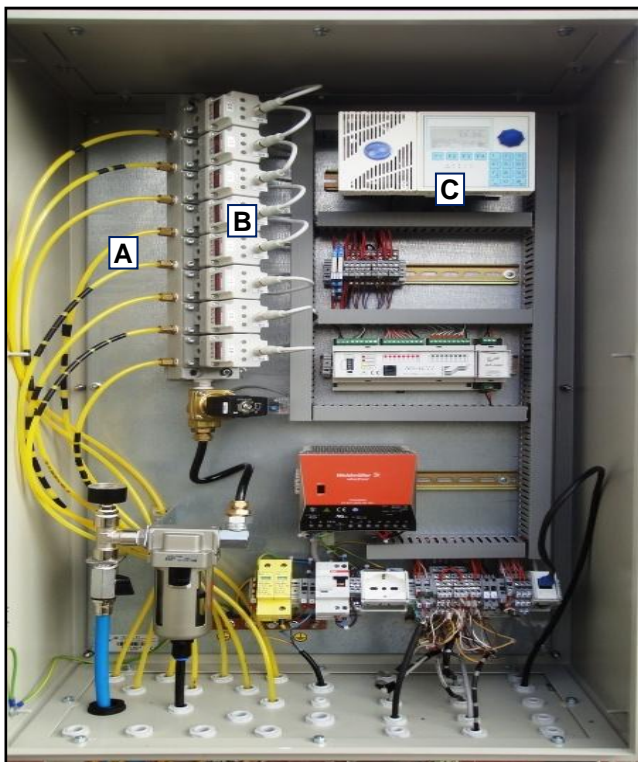


Fig. FACE 15. Gas release yellow pipes (A) with the automatic pressure regulator (SMC Corporation, ITV series, Japan) (B). The flow of each valve was controlled by a programmable control system using the microprocessor-based Proportional Integration Device (PID algorithm) (C).

The CO₂ concentration measured at the center of each miniFACE ring and the wind speed determined by an anemometer located 2 m above the ground in the center of the plantation area (Fig. FACE 4) were integrated with PID determining the voltage of the valve and thus the rate of release of pure CO₂ in each ring (Fig. FACE 5). CO₂ fumigation began at sunrise and ended at sunset each day. All variables (CO₂ concentration, valve voltage, and wind speed) were recorded every minute on a computer located in the central control of the miniFACE system inside a container near the plantation (Fig. FACE 6). The CO₂ treatments consisted of two concentrations of CO₂. In four separated plots out of miniFACE, there was the actual environmental CO₂ concentration at approximately 400 ppm (Fig. FACE 1). In eight miniFACE plots, then plants were under elevated CO₂ concentration in 50% of the environmental concentration at 600 ppm (Fig. FACE 1).



Fig. FACE 16. Anemometer.



Fig. FACE 17. CO₂ fumigation micro holes in outer ring side surrounding each plot.



Fig. FACE 18. Central control of the Trop-T-FACE system inside the container near the field.

Four of those eight plots of miniFACE were also under elevated canopy temperature treatment (Fig. FACE 1). The temperature free-air controlled enhancement (T-FACE) component of the Trop-T-FACE controls the canopy temperature by means of infrared ceramic heaters (model FTE-750-240 Salamander, Mor Electric, MI, USA (Fig. FACE 7)) controlled by a PID control system and suspended above the ground as described by our colleagues (59). The heaters were mounted on Salamander ALEX-F reflectors (Mor Electric,

MI, USA (Fig. FACE 7)) and stayed suspended from a triangular aluminum pole system. There were six heaters per plot, with a heater at each point of the hexagon. A similar array of dummy heaters consisting of aluminum reflectors without a heating element was installed in the reference plots to produce a consistent amount of shade between the warmed and reference plots.



Fig. FACE 19. Heaters model FTE-750-240 Salamander ceramic infrared heating elements, mounted on Salamander ALEX-F reflectors (Mor Electric, MI, USA) suspended at 0.8 m above the canopy.

Eight warmed plots and eight reference plots (Controls) were distributed in the plantation area (Fig. FACE 1). In the warmed plots, the heaters were maintained at 0.8 m above the canopy of the plants. Infrared thermometer (model SI-1H1-L20, Apogee Instruments, USA (Fig. FACE 8)) measured the temperature of the canopy. To control the voltage of the heaters, we used a PID control system installed in a model CR1000 data logger with AM25T multiplexors (Campbell Scientific, USA (Fig. FACE 9)) (59). To monitor and collect the data, we used the LoggerNet software (Campbell Scientific, USA) installed on a computer (Fig. FACE 6). For communication, an NL201 network link interface (Campbell, Scientific, USA) and a wired Ethernet network connection with the data logger. The T-FACE system provided elevated temperature to +2 °C above the ambient canopy temperature in the warmed plots. On reference plots remained the ambient temperature.



Fig. FACE 20. Infrared thermometer model SI-1H1-L20 (Apogee Instruments, USA).

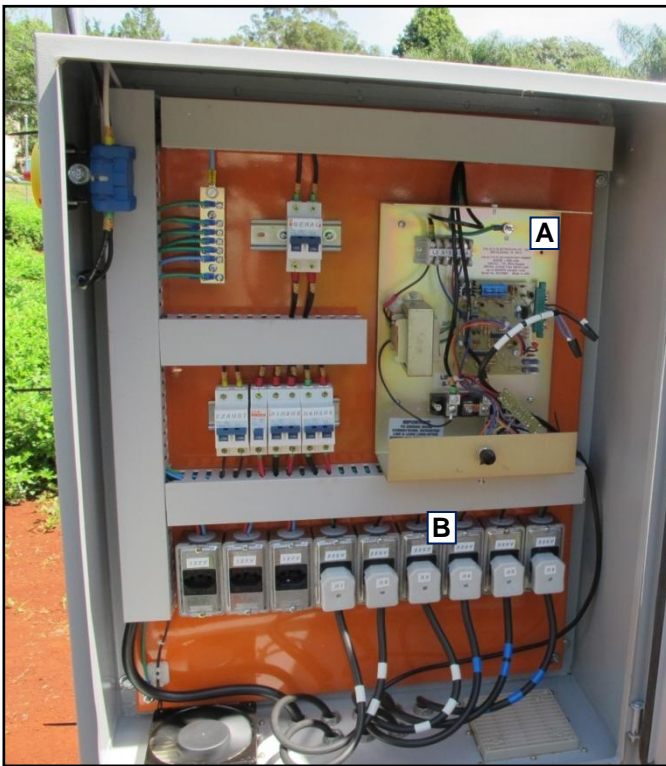


Fig. FACE 21. PID algorithm installed in a CR1000 data logger (A) with AM25T multiplexors (Campbell Scientific, USA) (B).

Theta Probe soil moisture (ML2x) and temperature (ST2) sensors (Fig. FACE 10) connected to a data logger DL2e (Delta-T Devices, UK) monitored, respectively, the soil water content and the soil temperature in each ring. An automatic microclimatic station (WS-HP1) monitored and continuously stored the climatic data (temperature, relative humidity, irradiance, and precipitation) using specific sensors (Fig. FACE 11). Once the plants were grown under field conditions, soil and air moisture were monitored continuously. There were water sprinklers for irrigating the entire area of all 16 plots during the period of the experiments with irrigation (Chapters 1-3) for maintaining the soil moisture near field

capacity (Fig. FACE 12). In the experiment without irrigation (Chapter 4), the sprinklers were used only during the implementation of the area.



Fig. FACE 22. Theta Probe soil moisture (ML2x) and temperature (ST2) sensors.

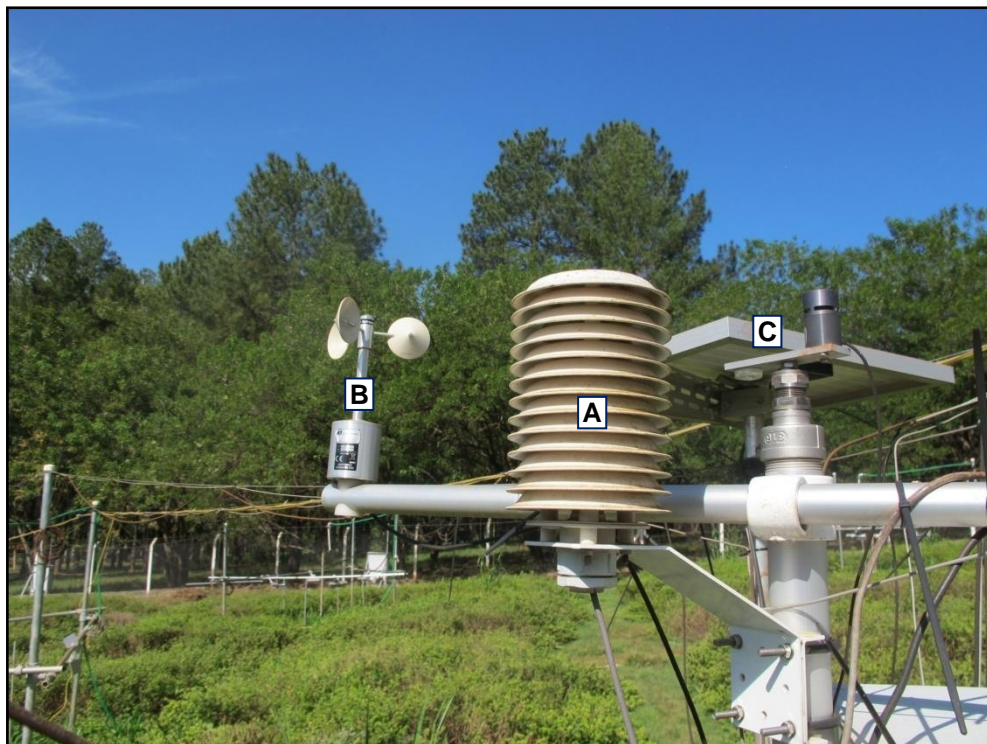


Fig. FACE 23. Automatic microclimatic station (WS-HP1) with a pluviometer (A), an anemometer (B) and a solar panel to capture solar irradiance (C).



Fig. FACE 24. Water sprinklers suspended at the center of the plot.

- 1 -

LEAF DYNAMICS OF *PANICUM MAXIMUM* UNDER FUTURE CLIMATIC CHANGES

ABSTRACT

Panicum maximum Jacq. ‘Mombaça’ (C₄) was grown in field conditions with sufficient water and nutrients to examine the effects of warming and elevated CO₂ concentrations during the winter. Plants were exposed to either the ambient temperature and regular atmospheric CO₂ (Control); elevated CO₂ (600 ppm, eC); canopy warming (+2 °C above regular canopy temperature, eT); or elevated CO₂ and canopy warming (eC+eT). The temperatures and CO₂ in the field were controlled by temperature free-air controlled enhancement (T-FACE) and mini free-air CO₂ enrichment (miniFACE) facilities. The most green, expanding, and expanded leaves and the highest leaf appearance rate (LAR, leaves day⁻¹) and leaf elongation rate (LER, cm day⁻¹) were observed under eT. Leaf area and leaf biomass were higher in the eT and eC+eT treatments. The higher LER and LAR without significant differences in the number of senescent leaves could explain why tillers had higher foliage area and leaf biomass in the eT treatment. The eC treatment had the lowest LER and the fewest expanded and green leaves, similar to Control. The inhibitory effect of eC on foliage development in winter was indicated by the fewer green, expanded, and expanding leaves under eC+eT than eT. The stimulatory and inhibitory effects of the eT and eC treatments, respectively, on foliage raised and lowered, respectively, the foliar nitrogen concentration. The inhibition of foliage by eC was confirmed by the eC treatment having the lowest leaf/stem biomass ratio and by the change in leaf biomass-area relationships from linear or exponential growth to rectangular hyperbolic growth under eC. Besides, eC+eT had a synergist effect, speeding up leaf maturation. Therefore, with sufficient water and nutrients in winter, the inhibitory effect of elevated CO₂ on foliage could be partially offset by elevated temperatures and relatively high *P. maximum* foliage production could be achieved under future climatic change.

Keywords – elevated CO₂ concentration, forage, leaf development, leaf phenology, global warming

1.1 INTRODUCTION

Currently, the atmospheric CO₂ concentration reaches above 403 ppm, a significant increasing since 1960, when 320 ppm was recorded at the Mauna Loa Observatory in Hawaii (2). This is highly important because CO₂ is a greenhouse gas and causes global warming and climate change. Accordingly, the average surface temperature of the Earth has consistently increased since 1980 (12). The Intergovernmental Panel on Climate Change (3) uses a range of future scenarios based on social, technological, economic, and environmental conditions to predict the atmospheric CO₂ concentration and the Earth's surface temperature. The IPCC projects an increase in the surface temperature of 2 °C by 2050 with very heterogeneous stages of human development in a scenario named A2. In this scenario, the concentration of atmospheric CO₂ will be around 600 ppm (28). Heating and the greater availability of CO₂ will affect plant growth, development, and yields (5,16). Since forages are directly exposed to atmosphere conditions in the field, climate change will significantly impact the management of pastures (17).

Brazil has been the world's largest meat exporter since 2008, with 200 million head of cattle, and the value of the meat and milk production chains has been estimated at US\$31 billion (18). The favorable climate and large size of Brazil contribute to this success. However, despite the great importance of ranching, the effects of climate change on forages and their acclimation to the combined effects of elevated CO₂ concentration and warming have not yet been evaluated under field conditions in Brazil. In fact, there is a multi-biome gap in experimental data from the tropics and subtropics on plant responses to future climatic changes (19,20).

Plants clearly respond to increased CO₂ concentrations, since even for some C₄ species, the current concentration of atmospheric CO₂ is below the CO₂ saturation point for net photosynthesis (44). Indeed, some results from open-top chamber studies have found that an elevated CO₂ concentration can stimulate biomass production in C₄ species (35,45). However, the net photosynthesis and growth of C₄ species are less responsive than those of C₃ species under elevated CO₂ (31). In contrast to physiological responses, information on morphological alterations in the leaf blades of C₄ grasses under future climatic change is scarce. This is an important gap in knowledge because the anatomical and morphological traits of the leaf blades of *Panicum maximum* can affect animal preferences (46).

The purpose of this work was to determine the effects of future climate change on foliar growth and the stages of leaf development in tillers of Guinea grass (*Panicum maximum* Jacq.) following the A2 scenario. The major C₄ grasses used as forage for cattle in Brazil belong to the genera *Panicum* and *Brachiaria* (46). Our results will support pasture management in farms in future climate changes since the experiment was carried out under field conditions using a mini Free-air Carbon Dioxide Enrichment (miniFACE) system and a Temperature Free-air Controlled Enhancement (T-FACE) system.

We hypothesized that a temperature increase of 2 °C will stimulate foliage production in *P. maximum*, especially if there is no water shortage. This would be possible, especially in winter, because *P. maximum* is a C₄ tropical forage with a high optimal leaf temperature for photosynthesis (30 °C –40 °C) (60,61). Heating probably stimulates the rate of leaf expansion, as found in C₄ buffel grass *Cenchrus ciliaris*, resulting in higher foliar area and biomass (45). On the other hand, an elevated CO₂ concentration will promote little or no change in leaf area and leaf biomass of *P. maximum* since the C₄ photosynthetic pathway already acts as a CO₂ concentration mechanism (62). Nonetheless, if leaf growth rate, leaf area, and leaf biomass will increase under warming in winter, the effect of elevated CO₂ on heated leaves of *P. maximum* in the field would be hard to predict. Therefore, we tested the leaf dynamics of *P. maximum* under current conditions and the predicted A2 atmospheric scenario for the year 2050 by controlling the temperature and CO₂ concentration in the field.

1.2 MATERIALS AND METHODS

1.2.1 EXPERIMENTAL AREA, PLANT MATERIAL, SOIL PREPARATION, AND SOWING

The study was conducted on a 2500 m² field at the campus of the University of São Paulo (USP) in Ribeirão Preto municipality, state of São Paulo, Brazil (21°10'S and 47°48'W, 800 m altitude). According to the Köppen-Geiger (KG), Ribeirão Preto shows the Aw classification, a tropical climate with rainy summer and dry winter (63,64). The KG classification is efficient in macroscale, so we also considered the Thornthwaite classification (TH) which is efficient in mesoscale (65). According to TH, Ribeirão Preto city shows the B_{2r}B_{4a}' classification (66), a humid climate, with little or no water deficiency in dry season from April to September (66,67). The Camargo climate classification (CA) combines the simplicity of KG with the accuracy of TH using an agroclimatic zoning (65). According to CA, Ribeirão Preto city shows the TR-SBi classification, a subhumid tropical climate with dry winter (65). Historical data from 1982 to 2012 present the average annual temperature and rainfall of 21.9 °C and 1508 mm, respectively (68). For the period August-September, the historical data (1982-2012) show an average temperature of 21.2 °C, with minimum and maximum temperatures of 14.2 °C and 28.2 °C, respectively (68).

According to the U.S. Department of Agriculture and "Embrapa Solos" in Brazil, the soil in the area is a dystrophic Red Latosol (Oxisol) (69). The area is fenced, and soil analysis, contouring, railing, and soil pH correction by liming had been performed previously. The value of the average initial pH was 4.0 to 4.5 and remained at 5.0 to 5.5 after liming. Chemical soil fertilization happened after pH correction according to the initial nutrient availability. Therefore, the soil was appropriate and homogeneous at the time of planting.

Seeds of Guinea grass, *Panicum maximum* Jacq. (Poaceae), were planted into holes 30 cm apart in 12 m × 12 m plots. NPK 4-14-8 fertilizer was used at a dose of 1 t ha⁻¹ applied into the holes during planting. Only three plants per hole were maintained after germination. Sixty days after planting, the tussocks were cut at a height of 30 cm from the ground. This trimming established a full, similar canopy among treatments as the standard practice for managing *P. maximum* in natural conditions.

1.2.2 TROP-T-FACE SYSTEM, WATERING, AND TREATMENTS

The Trop-T-FACE system was previously described (Section ‘The Trop-T-FACE system’, pages 22 to 29). The experiment occurred from 22nd August to 20th September 2013, during winter, with *Panicum maximum* in irrigated consortium with *Stylosanthes capitata*.

The design of this experiment to evaluate foliar growth and stages of leaf development in tillers of *P. maximum* included four treatments: ambient conditions (Control), an atmospheric CO₂ concentration elevated to 600 ppm (eC), a canopy temperature elevated 2°C above the ambient temperature (eT), and both an elevated CO₂ concentration and canopy temperature (eC+eT). Each treatment had four replicates, totaling 16 experimental units.

1.2.3 FOLIAGE MEASUREMENTS AND DATA ANALYSIS

Foliage growth and stages of leaf development were set in the context of the tiller, the basic unit of growth in grasses. Three randomly equidistant tussocks of *P. maximum* were selected in each plot. Five tillers were chosen per tussock, resulting in 15 tillers per plot for assessing foliage. Since the experiment was performed with four replicates per treatment, 60 tillers were measured per treatment. Data collection took place on August 22 and 29 and September 3, 9, 12, 19, and 20 in 2013.

Foliage traits recorded on each date included the number of expanded and expanding leaves (with and without visible ligule, respectively), the number of senescent leaves (with at least 50% of the area yellowing), and the length of the expanding leaves. From these measurements, average values per tiller were obtained in each treatment, and the following parameters were calculated.

The number of leaves that emerged in each period between data collection days was named the leaf appearance rate (LAR, day⁻¹). We obtained LAR as the number of leaves from the current data collection day minus the number of leaves from the previous data collection day, with only positive values considered. The final LAR in a corresponding treatment was obtained by summing all positive values from each measurement time divided by the number of days in the entire experiment (30 days). This parameter indicated the rate of the appearance of leaves per tiller. Among the original 60 tillers marked per treatment, 58, 56, 56, and 57 in the Control, eC, eT, and eC+eT treatments, respectively, were usable for obtaining the average value of LAR.

The leaf elongation rate (LER, cm day⁻¹) per tiller was calculated as the length of expanding leaves on the current data collection day minus the length of these leaves from the

previous data collection day, divided by the number of days between the two data collection times. Among the original 60 marked tillers per treatment, 59, 59, 60, and 58 in the Control, eC, eT, and eC+eT treatments, respectively, were usable for obtaining the average value of LER through the end of the experiment. An average value was calculated for the entire experimental period to show how fast the expanding leaves grew in length per tiller per day.

The combined number of expanded and expanding leaves (leaves with and without a ligule, respectively) resulted in the number of green leaves per tiller (NGL), disregarding senescent leaves. Senescent leaves were identified as those with more than 50% of their area without green color. The NGL was calculated as the total number of expanded and expanding leaves on each data collection day. An average NGL value was obtained for the entire experimental period to show how many functional leaves were maintained in each treatment. The total number of leaves considered was 349, 351, 358, and 349 in the Control, eC, eT, and eC+eT treatments, respectively. From the seven data collection days, it was possible to calculate the average number of green, expanded, expanding, and senescent leaves per tiller in each treatment.

From the data collection days, it was possible to compute the expanded, expanding, and senescent leaves accumulated per tiller. The accumulated values were obtained as the number of leaves on the first data collection day plus the number of leaves on subsequent data collection days. Trimming at the beginning of the experiment cut some expanding and expanded leaves, causing a permanent, typical scar on the leaf blade. In contrast, leaves that expanded after pruning had a typical intact apex. Therefore, it was possible to count the number of intact and cut green leaf blades among the expanded and expanding leaves per tiller. The leaf area of *P. maximum* was measured using the WinDIAS Leaf Image Analyses System (Delta-T, UK). The marked tillers of *P. maximum* in the field were collected intact by cutting their stem base at the ground level at the end of the experiment (21 September). The leaves were detached, and the area per tiller was measured. The foliage of each identified tiller was dried separately in a stove with forced air circulation at 60°C to obtain the leaf biomass. In addition, the foliar nitrogen content, N%, was determined in each ring using expanded and expanding leaves (the green leaves) by the semi-micro-Kjeldahl methodology (70). Overall, four measurements of foliar nitrogen were carried out, and the average N% was obtained for each treatment at the end of the experiment.

Statistical analyses were performed with the open software BioEstat, version 5.3 (Instituto Mamirauá, Brazil). A D'Agostino-Pearson test was used to verify whether data were

normally distributed. Since the datasets did not show a normal distribution, the non-parametric Mann-Whitney test was used to comparing the datasets among treatments. Significance was determined at $p < 0.1$ for LAR, LER, and foliar nitrogen content and $p < 0.05$ for the others foliage measurements in order to compare the datasets among treatments. Non-linear trend lines created in Microsoft Excel[™] were used to perform the correlation analysis between leaf area and leaf mass.

1.3 RESULTS

Days were usually cloud-free, with solar radiation reaching nearly 1 kW m^{-2} around midday during the experiment (Fig 1.1A). Nonetheless, cloudy days with solar radiation of around 0.5 kW m^{-2} occurred during September 3–4 and September 18–19 in 2013. Thus, air temperature was lower and relative air humidity was higher than on typical days (Fig 1.1B). On August 28, 2013, an unusually cold air temperature of $3 \text{ }^{\circ}\text{C}$ at predawn, when the soil temperature reached the lowest value (Fig 1.1B). The soil temperature varied between 15 and $25 \text{ }^{\circ}\text{C}$ in and the eT and Control treatments (Fig 1.1C). Only during the early morning, the soil temperature was almost the same in the two treatments. However, after 10:00 A.M., the soil temperature in eT was approximately $2 \text{ }^{\circ}\text{C}$ above the Control (Fig 1.1C).

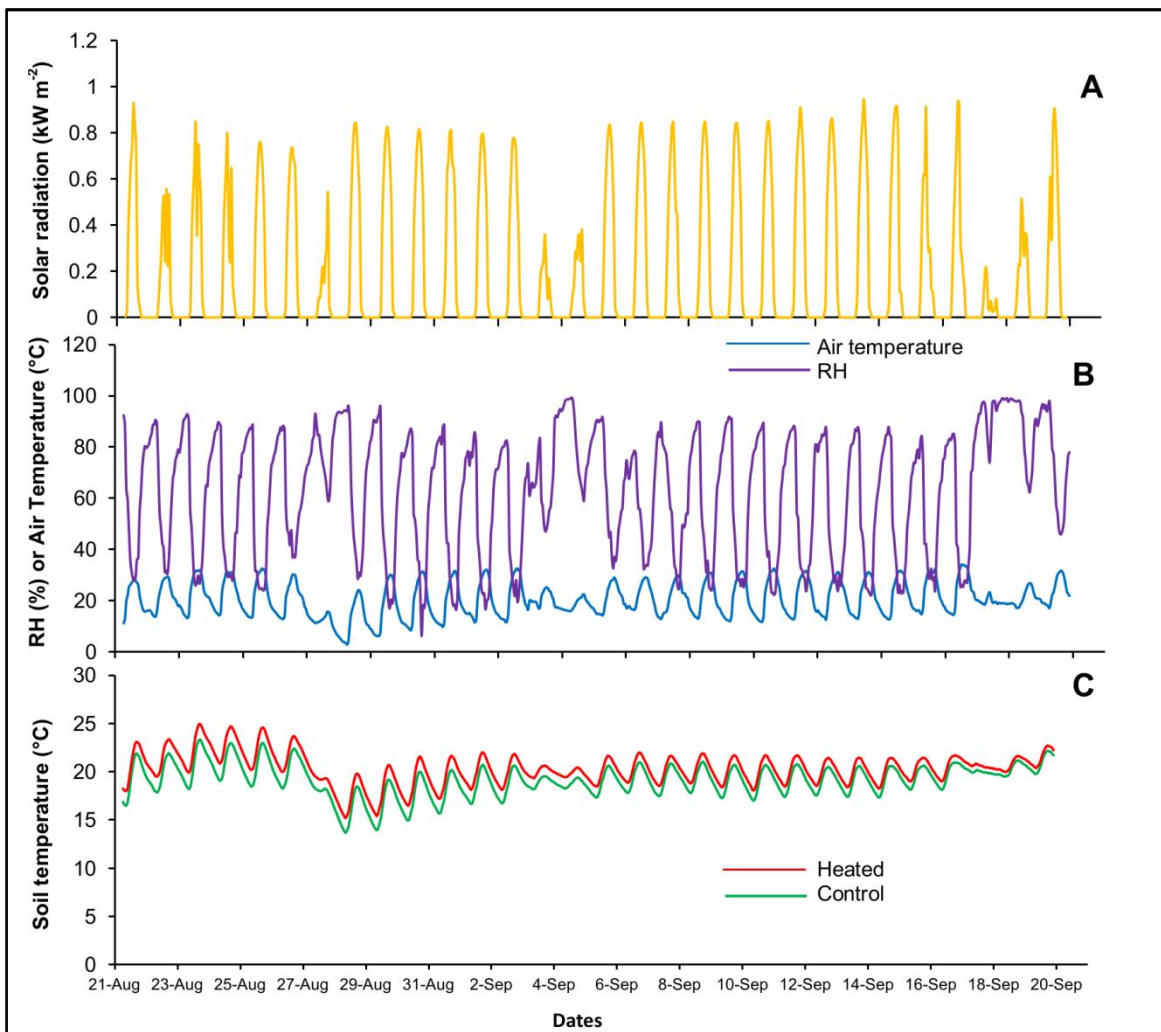


Fig 1.1 Daily courses of meteorological conditions and soil temperature during the period of the experiment, from August 22 to September 20, 2013. A - Total solar radiation. B - Air relative humidity (RH) and air temperature. C - Soil temperature in control and heated plots.

The canopy temperature in the Control and eT treatments was usually between 30 °C and 10 °C during the day (Fig 1.2A). Nonetheless, a colder period occurred on August 26–27, when the canopy temperature was as 3 °C at night (Fig 1.2A). Despite the 20 °C variation during a day, the target canopy temperature of +2 °C in the eT treatment was achieved throughout the experiment, especially during the night (Fig 1.2B). The differences in canopy temperature between the Control and eT treatments reached values below the set point temperature (+2 °C) for short periods because of leaf transpiration, particularly at midday (Fig 1.2B).

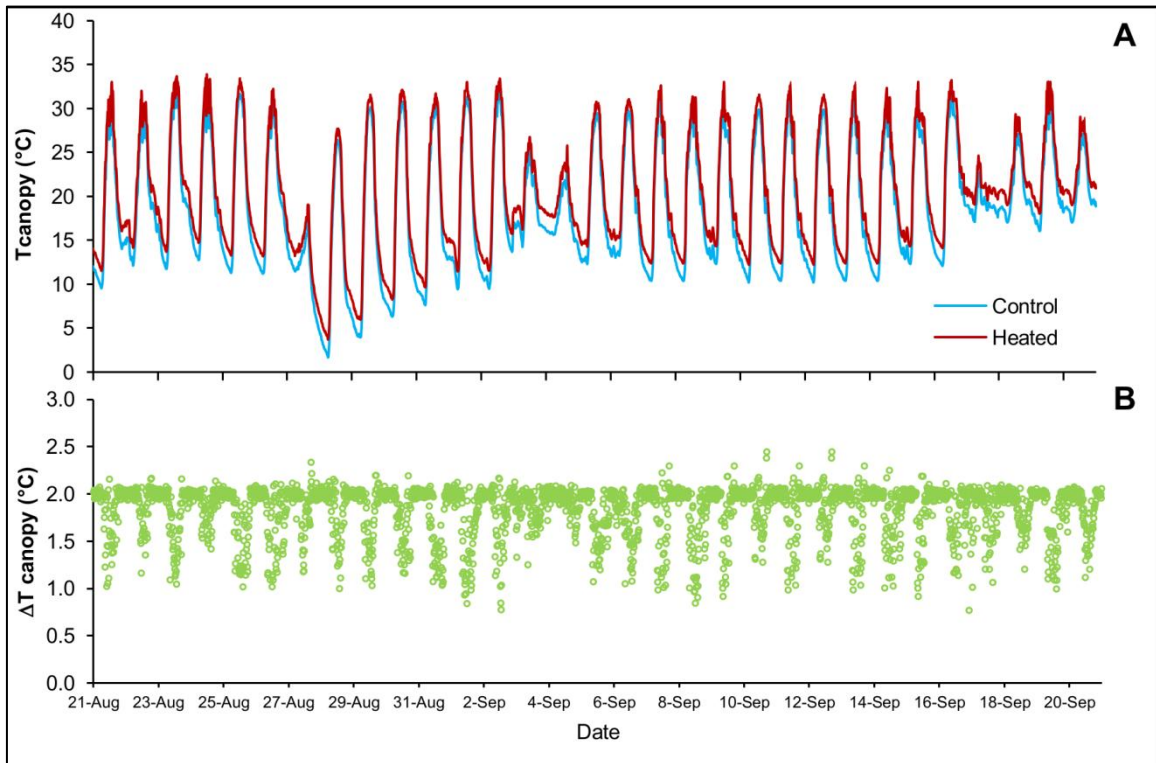


Fig 1.2 Canopy temperature during the period of the experiment, from August 22 to September 20, 2013. A - Canopy temperature of heated (solid line) and Control (dashed line) treatments. B - Difference (ΔT canopy) between heated and regular canopy temperatures indicating the deviations from the target elevated temperature of 2 °C above the Control.

Compared to the Control, the eT treatment showed a significant ($p < 0.05$) increase in green (Fig 1.3A) and expanded leaves (Fig 1.3B). There was an unanticipated inhibitory effect on foliage in the eC+eT treatment, resulting in the significantly lowest number of expanding leaves (Fig 1.3C). Even with these significant differences in foliar dynamics, the number of senescent leaves was not affected by any treatment (Fig 1.3D). It is worth noting that, despite the non-significant difference at $p < 0.05$, the number of green, expanded, and expanding leaves were lower in the eC than in the Control treatment (Fig 1.3A, B, and C, respectively).

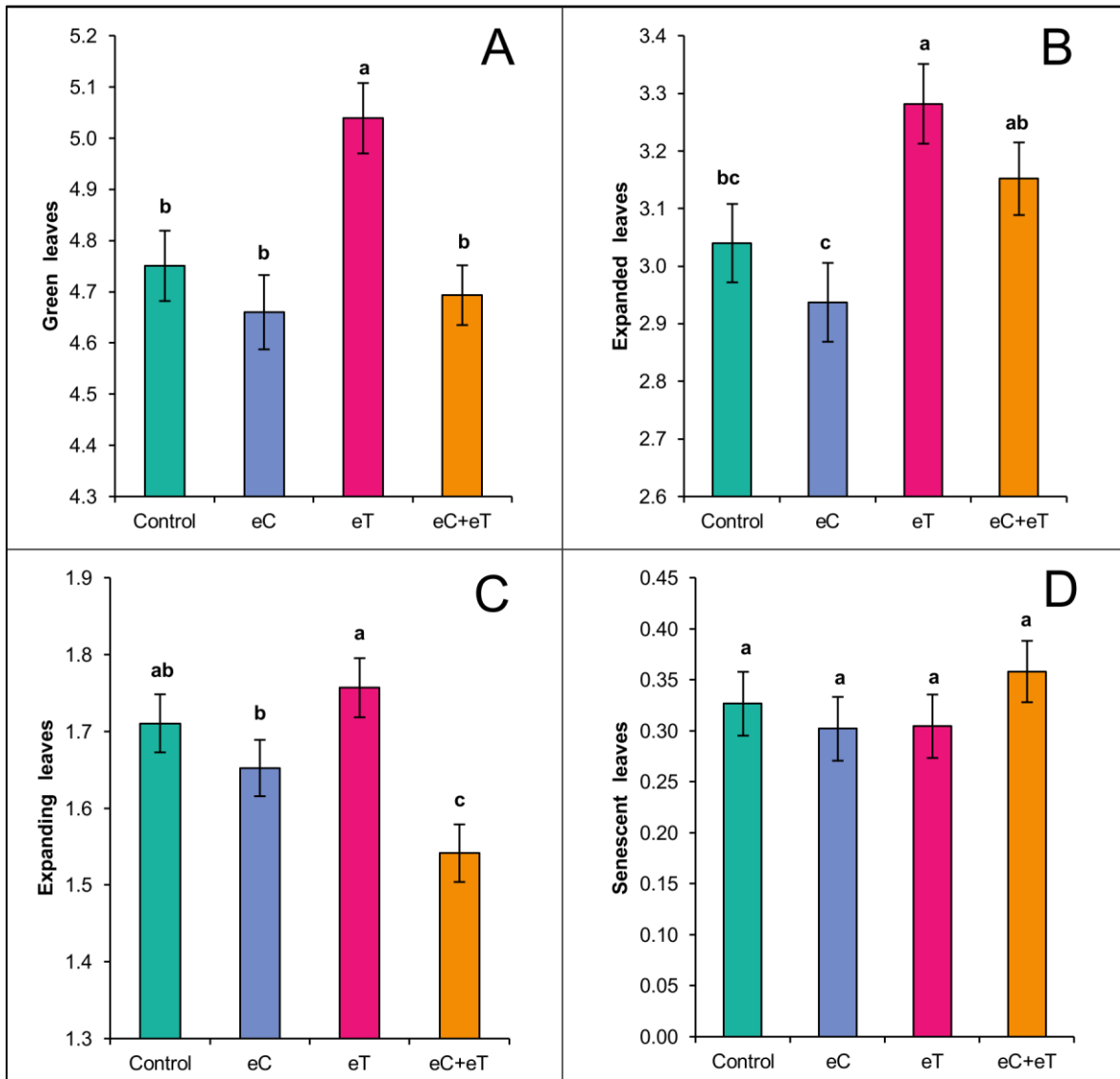


Fig 1.3 Number of green (A), expanded (B), expanding (C), and senescent (D) leaves per tiller of *Panicum maximum* under ambient CO₂ and canopy temperature (Control), under an elevated CO₂ concentration of 600 ppm (eC), under an elevated canopy temperature of +2 °C (eT), and under both treatments (eC+eT). Bars indicate average values and lines at the top of bars indicate the standard error. Different letters above bars indicate significant differences among datasets according to a Mann-Whitney test at $p < 0.05$.

The LAR was significantly higher ($p < 0.05$) in the eT and eC+eT treatments than the Control treatment (Fig 1.4A). The LER was also significantly higher ($p < 0.05$) in the eT and eC+eT treatments than in the control (Fig 1.4B). Therefore, leaves took more time to appear on tillers in the Control treatment and grew more slowly than in the eC treatment. This foliar dynamics explains the lower leaf area (Fig 1.4C) and leaf biomass (Fig 1.4D) in the Control and eC treatments than in the eT and eC+eT treatments. Leaf area was 17% higher in the eT and eC+eT treatments than the Control (Fig 1.4C). The leaf biomass was 22% and 15% higher in the eT and eC+eT treatments, respectively, than in the Control (Fig 1.4D).

Therefore, in relation to the Control, the eC treatment applied alone did not significantly change LAR and LER (Fig 1.4A and B) and the foliar area and leaf biomass (Fig 1.4C and D).

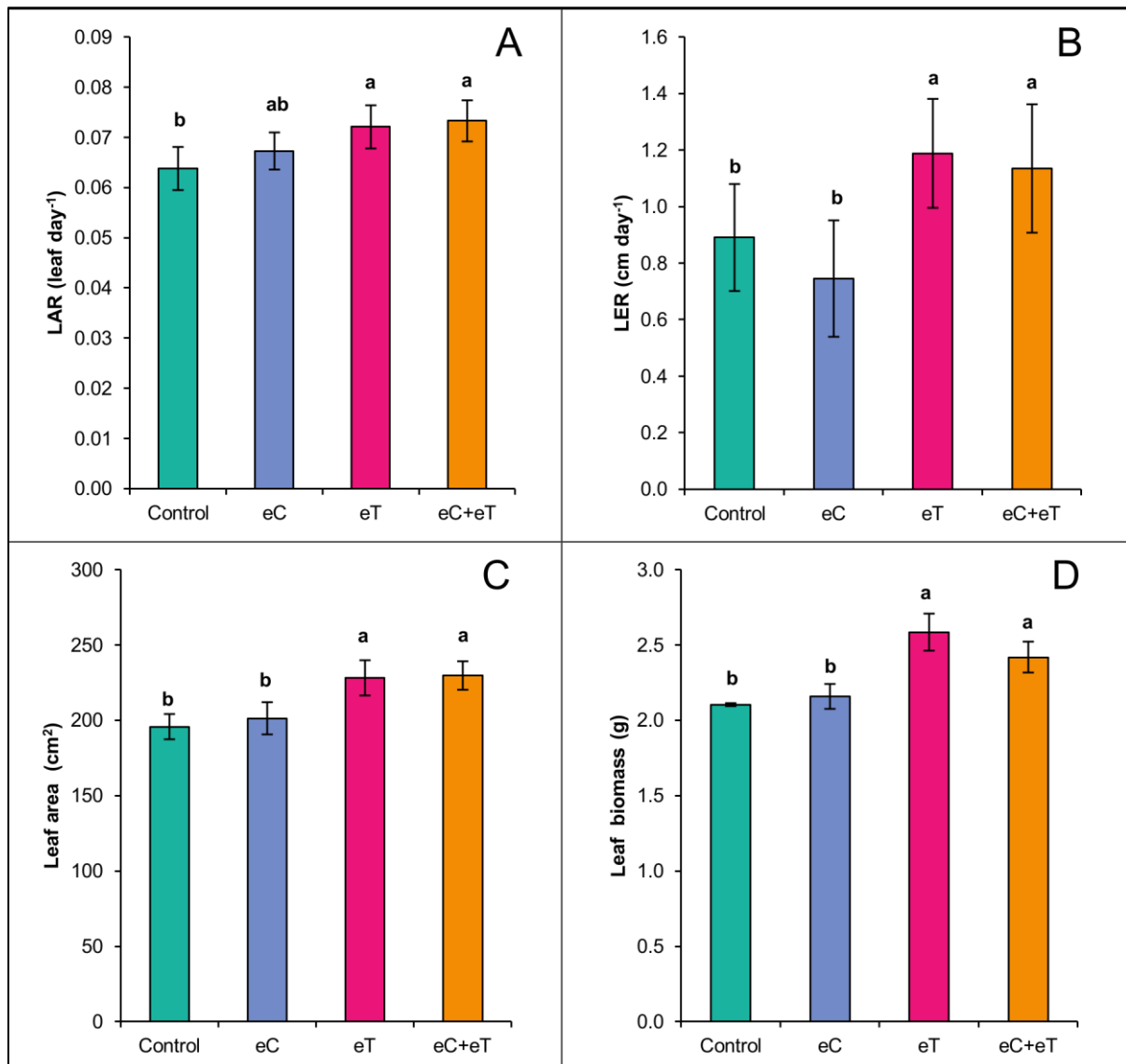


Fig 1.4 Leaf appearance rate (LAR, A), leaf elongation rate (LER, B), leaf area (C), and leaf biomass (D) per tiller of *Panicum maximum* under regular CO₂ and canopy temperature (Control), under elevated CO₂ concentration of 600 ppm (eC), under elevated canopy temperature of +2 °C (eT), and under both treatments (eC+eT). Bars show average values and lines at the top of the bars show the standard error. Different letters above bars indicate significant differences among datasets according to a Mann-Whitney test at $p < 0.05$.

Figure 1.5 shows that the eC treatment had the lowest leaf/stem ratio on a mass basis (Fig 1.5A). Therefore, it is clear that the eC treatment favored stems. In addition, the highest and lowest leaf nitrogen content were in the eC and eT treatments, respectively (Fig 1.5B). This contrasting leaf nitrogen content could be a result of the dilution and concentration in foliage caused by the highest and lowest LER under the eT and eC treatments, respectively

(Fig 1.4B). In the Control treatment, there was a nonlinear increase in leaf biomass as a function of leaf area (Fig 1.6A). However, in the eC treatment the leaf biomass-area relationship had a rectangular hyperbolic response with a higher degree of correlation (Fig 1.6B). Alternatively, leaf biomass and area had a linear relationship in the eT treatment (Fig 1.6C and D). This indicates that there were important differences in leaf blade formation among treatments.

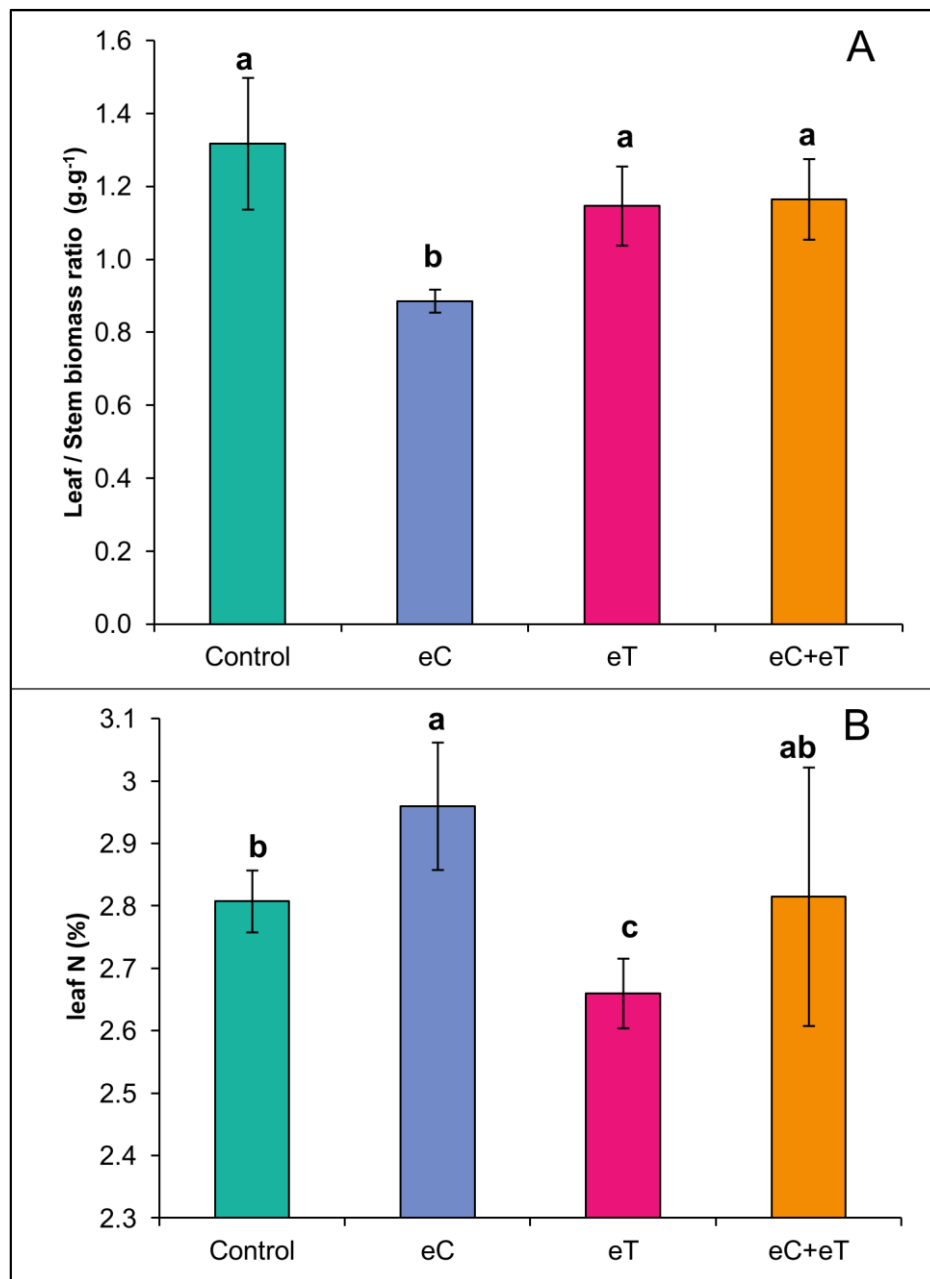


Fig 1.5 The leaf/stem biomass ratio (A) and leaf nitrogen content (B) of *Panicum maximum* under regular concentration of CO₂ and canopy temperature (Control), under elevated CO₂ concentration of 600 ppm (eC), under elevated canopy temperature of +2 °C (eT), and under both treatments (eC+eT). Bars show average values and lines at the top of the bars the standard error. Different letters above the bars indicate significant differences among the datasets after the Mann-Whitney test at p < 0.1.

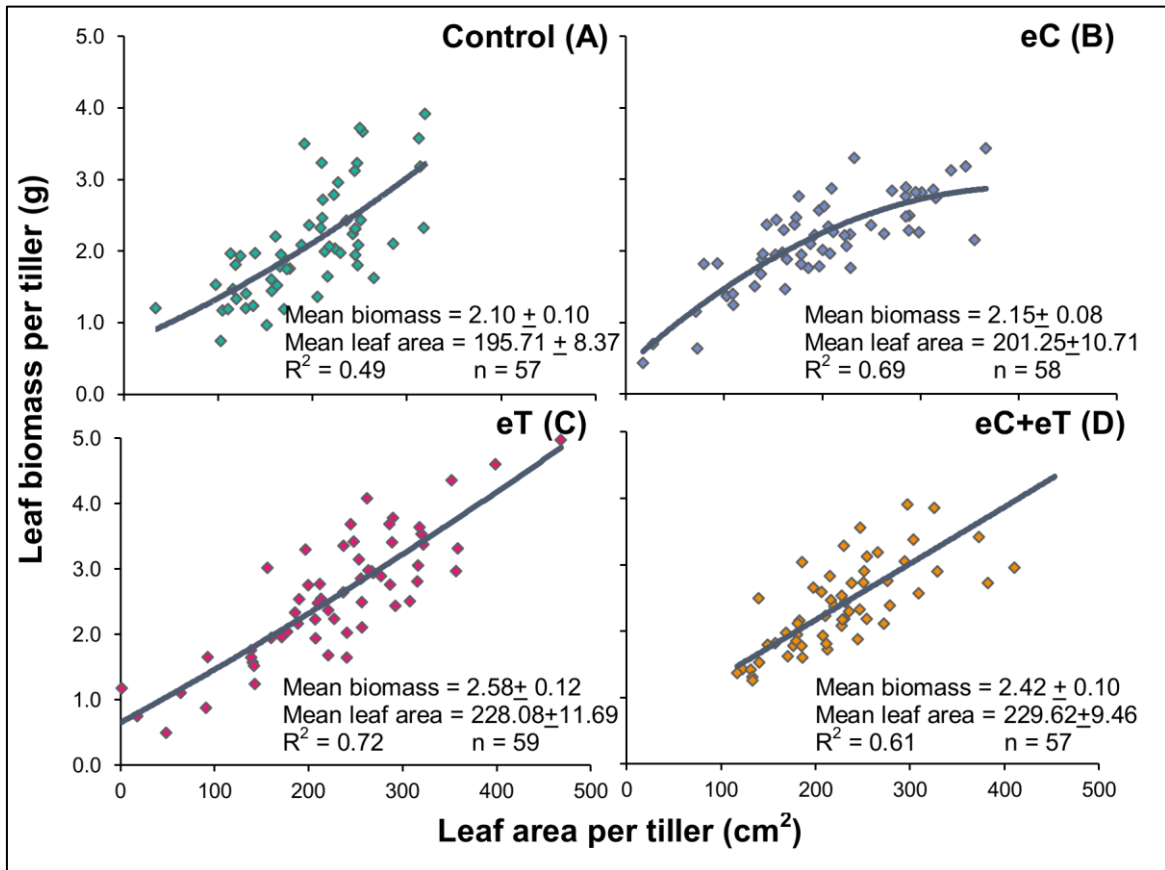


Fig 1.6 Average values of leaf biomass as a function of leaf area per tiller of *Panicum maximum* under regular atmospheric CO₂ concentration and canopy temperature (Control, A), under elevated atmospheric CO₂ concentration of 600 ppm (eC, B), under elevated canopy temperature of +2 °C (eT, C), and under both treatments (eC+eT, D).

At the end of the experiment, the eT treatment had the most expanded and expanding leaves (Fig 1.7A and B, respectively), and the eC treatment had the most senescent leaves. In addition, the eC treatment had the fewest cut-expanded leaves at the end of the experiment. Therefore, the variables that represented foliage gain (Fig 1.7A and B) were highest in the eT treatment. Besides, high foliage loss, represented by the many senescing leaves and few cut-expanded leaves, took place in the eC treatment (Fig 1.7C and D). However, it is notable that the eC treatment had the fewest expanding instead of expanded leaves from August 29 to September 20 (Fig 1.7A and B).

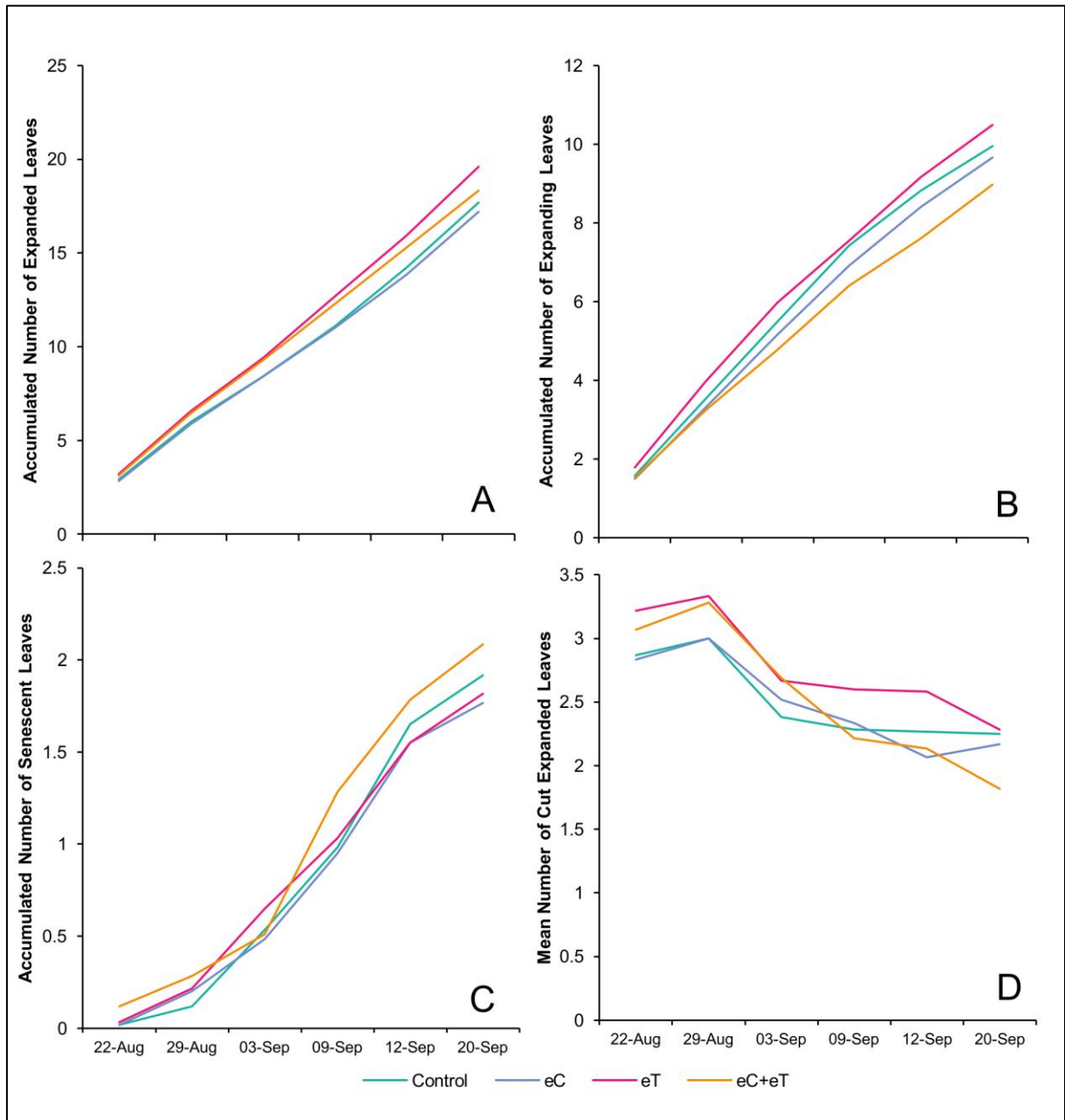


Fig 1.7 Accumulated number of expanded (A), expanding (B), and senescent (C) leaves; and the mean number of cut-expanded leaves (D) per tiller of *Panicum maximum* growing under different atmospheric conditions. A - Regular concentration of CO₂ and canopy temperature (Control). B - Elevated CO₂ concentration of 600 ppm (eC). C - Elevated canopy temperature of +2 °C (eT). D - Combination of treatments (eC+eT). The days of measurement were August 22 and 29, and September 3, 9, 12, and 20, 2013.

1.4 DISCUSSION

In C₄ species, net photosynthesis has a peak value between 30 °C–40 °C and is only half of the maximum at 20 °C (71). In fact, the optimum temperature values for *Panicum coloratum* were 36.1 °C and 38.1 °C under high (35/30 °C day/night) or moderate (25 °C /20 °C day/night) temperature regimes (60). Therefore, in this experiment *Panicum maximum* grew under suboptimal temperatures for photosynthesis during the day throughout the experiment, since the maximum temperature did not reach 35 °C even at midday. Thus, an increase of 1 °C –2 °C above the regular temperature during winter (Fig 1.2) probably led to a significant difference in the carbon net assimilation after 30 days in *Panicum maximum*. Further, the increase in leaf temperature towards the optimum for photosynthetic activity promoted foliage production (Figs. 1.3A, B, and C, Fig 1.4, and Fig 1.7A and B). Besides, eT did not favor leaf senescence or limit the survival of cut-expanded leaves. Consequently, high leaf area and leaf biomass was observed when eT was applied alone or in combination with eC. Indeed, since the eT treatment had the most expanded and expanding leaves, it had the highest number of green leaves per tiller.

The benefits of elevated CO₂ to C₄ crop species are controversial. C₄ crops benefit from elevated CO₂ only under drought that causes low stomatal conductance and an increased concentration of intercellular CO₂ and water use efficiency (72). However, the supply of C₄ acids may exceed the carboxylation capacity, resulting in the leaking of CO₂ from the bundle sheath under a high atmospheric CO₂ concentration (73). In this sense, the downregulation of the molecular components of C₄ carbon assimilation may occur under a high atmospheric CO₂ concentration. Actually, the carboxylation efficiency and CO₂ saturation rate of photosynthesis were lower in the C₄ crop *Sorghum bicolor* grown free of water stress at elevated CO₂ (700 ppm, growth chamber), accompanied by a 49% reduction in the phosphoenolpyruvate carboxylase content of leaves (74). In addition, the carboxylation efficiency and the activities of C₄ enzymes in *Zea mays* were reduced in a growth chamber with CO₂ enrichment at 750 ppm (75).

An increase in leaf area and leaf biomass allocation is not expected to be concomitant with the downregulation of photosynthetic enzymes under high CO₂ concentrations. Indeed, the leaf area and LAR did not change in C₄ *Zea mays* under 750 ppm in five different day/night temperature regimes in a growth chamber without water stress,

ranging from 19/13 to 38.5/32.5 °C (75). In our experiment using Trop-T-FACE, plants grown under elevated CO₂ also were not significantly different from the control in LAR, LER, leaf area, and leaf biomass (Fig 1.4). Elevated CO₂ could be beneficial at suboptimal temperatures or net photosynthesis by raising the leaf temperature through low stomatal conductance and transpiration (72). Even so, if leaf temperature rose under eC, it was not beneficial in terms of leaf area or biomass production (Fig 1.4C and D).

On the other hand, the C₄ *C. ciliaris* significantly increased biomass production and had a three-fold increase in the leaf area index after 120 days under 600 ppm of CO₂ in an open top chamber (35). In another experiment, three cultivars of *C. ciliaris* (Biloela, Aridus, and West Australian) were exposed to 370 and 550 ppm of CO₂ for 50 days in a growth chamber (45). The West Australian cultivar had significantly more forage mass and a higher shoot/root ratio in 550 ppm. In addition, there was an increase in the total dry mass under different N supplies in *Panicum coloratum* and, especially, *C. ciliaris* grown under 850 ppm CO₂ in a growth chamber (73).

Biomass gain results in studies with FACE systems are lower than those in CO₂ chamber studies (31,33). While FACE systems allow plant exposure to elevated CO₂ under field conditions, growth chambers (phytotrons) and open top chambers (OTC) eliminate natural factors, such as wind and a broader daily range of leaf temperature. In addition, phytotrons and OTC limit the volume of soil for root growth (31), altering an important source-sink relationship in carbon metabolism (33). Besides, OTC studies have had a mean CO₂ concentration of 700 ppm, while studies with FACE use 550-600 ppm (4).

There is evidence that the eC treatment inhibited foliage development even though it was not different from the Control in LAR, LER, leaf area, or leaf biomass. The eC treatment yielded a significant decrease in the leaf/stem biomass ratio (Fig 1.5A). Leaf biomass was exponentially related to leaf area in the Control; however, a rectangular hyperbolic response was observed in the eC treatment limiting the increment of leaf dry biomass to below 3.0 g per tiller (Fig 1.6A, B). Indeed, the fewest green (Fig 1.3A) and expanded (Fig 1.3A) leaves during the experiment and the fewest accumulated expanded leaves (Fig 1.7A) were found in the eC treatment.

Elevated CO₂ could affect leaf quality beyond leaf population dynamics and biomass partitioning in winter. On one hand, the impairments in leaf growth in eC resulted in a high leaf N concentration (Fig 1.5B), which could increase the nutritional value of such foliage. On the other hand, the eC treatment altered the biomass-area relationship in leaves, limiting the

amount of biomass for expanded leaves. This means that large leaves in the eC treatment tended to have a thinner mesophyll cross-section. The leaf morphological trait most associated with animal preference is leaf width, a finding attributed to the wider mesophyll cross section (46,76). Instead, the limitation in the biomass gain of large leaves imposed by the eC treatment did not appear in the eC+eT treatment (Fig 1.6 C, D).

Higher temperatures could compensate for the inhibitory effect of elevated CO₂ by increasing the number of expanded leaves (Fig 1.3B). However, the synergistic inhibitory effect of the two resulted in the eC+eT treatment having the fewest expanding leaves during the experiment (Fig 1.3C). In fact, the mean number of cut-expanded leaves decreased in all treatments owing to the senescence with no possibility of replacement of such leaves shortly after pruning (August 29). However, the decrease in the population of cut-expanded leaves was higher in the eC+eT treatment (Fig 1.7D). This indicates that future increases in temperature and CO₂ will speed up the senescing of leaves in *P. maximum* growing free of water and nutrient shortage in winter. This was confirmed by the highest number of accumulated senescent leaves in the eC+eT treatment between September 9–20 (Fig 1.7C). Indeed, the accumulated number of young leaves, such as expanding leaves, was lowest in the eC+eT treatment from September 3 through the end of the experiment (Fig 1.3B). Therefore, the influence of the eC+eT treatment on early ligule formation resulted in a high accumulated number of expanded and senescent leaves (Fig 1.7A, C, respectively).

In summary, the most green, expanding, and expanded leaves and the highest LAR and LER occurred in the eT treatment. In addition, heated plots had significantly higher leaf area and leaf biomass. The increase in LER and LAR without a change in the number of senescent leaves could explain how tillers were able to produce large foliage area and leaf biomass under elevated temperatures. Contrastingly, the eC treatment had the lowest LER and the fewest expanded and green leaves, with no substantial differences from the Control. The possible inhibitory effect of elevated CO₂ on foliage development in winter was noticeable when comparing the eT and eC+eT treatments, since there were fewer green, expanded, and expanding leaves in the eC+eT treatment. The corresponding stimulatory and inhibitory effects on foliage of the eT and eC treatments resulted, respectively, in the dilution and concentration of foliar nitrogen. Despite no difference in leaf area and leaf biomass between the Control and eC treatments, the inhibitory effects of elevated CO₂ on foliage could be confirmed by the eC treatment having the lowest leaf/stem biomass ratio. The change in leaf biomass-area relationships from linear or exponential growth to rectangular hyperbolic

growth in the eC treatment also showed the inhibitory effect of elevated CO₂ on foliage. Therefore, the relationship between eC and eT was not antagonistic, but at least once had a synergistic effect in accelerating leaf maturation.

1.5 CONCLUSIONS

Several levels of organization related to leaves will significantly affect *P. maximum* under future climatic change, at least in winter. Elevated temperatures and CO₂, or their combination, will alter leaf morphology, development, phenology, and autotrophic-heterotrophic biomass partitioning. Leaf area-mass relationships will become linear as temperatures rise or develop a clear hyperbolic relationship with a limit below 3.0 g of dry matter per tiller under elevated CO₂. With sufficient water and nutrients, elevated winter temperatures will result in higher leaf expansion and appearance rates. More green leaves and a higher leaf area and biomass per tiller will occur with rising temperatures if *P. maximum* grows free of drought and nutritional stresses in winter. Contrastingly, when combined with elevated temperatures, elevated CO₂ will inhibit foliage formation by favoring biomass partitioning to stems or by speeding up leaf maturation. Despite the CO₂-temperature synergism, elevated temperatures can partially offset the inhibitory effects of CO₂ on leaf development and biomass partitioning, leading to a higher foliage area and biomass than in current atmospheric conditions. The significant changes in the mass-area relationship and nitrogen concentration in leaves under elevated CO₂ will very likely alter, respectively, the leaf palatability and leaf functioning of *P. maximum*.

- 2 -

LEAF ONTOGENY IN A C₄ PASTURE UNDER A FUTURE CLIMATE SCENARIO

ABSTRACT

Panicum maximum Jacq. ‘Mombaça’ (C₄) grew under field conditions with sufficient water and nutrients to examine the effects of warming and elevated CO₂ concentration on Southeast of Brazil during autumn. We implemented four climatic regimes: control with current atmospheric conditions, high CO₂ concentration (600 ppm, eC), elevated canopy temperature (2 °C above canopy ambient temperature, eT) and the combination eC+eT. The Trop-T-FACE system provided free-air carbon dioxide enrichment and temperature controlled enhancement under field conditions. CO₂ enrichment provoked a downregulation in foliage area by narrowing the leaves. On the other hand, both warming and CO₂ enrichment increased the leaf appearance and elongation rates, accelerating foliar phenological events. Contrastingly, eC was responsible for shortening the stem probably by decreasing apical meristematic activity. Therefore, atmospheric regimes affected the formation (eC) or the activity (eC and eT) of foliar meristems. The total leaf area reduction per tiller by narrower leaves in eC could be partially counteracted by higher rates of leaf elongation and leaf appearance under eC or eT. The combined treatment (eC+eT) speeded up similarly the foliar phenology, but synergistic effect took place reducing the total leaf area per tiller. Thus, global climate change will modify foliar ontogeny and phenology causing significant space-time variations in *P. maximum* foliage at several levels of organization. Such changes as the reducing leaf area and width could hamper the use of *P. maximum* as pasture in the future by interfering in forage availability and quality.

Keywords – elevated CO₂ concentration, foliage growth, forage consortium, leaf development, leaf meristem, leaf phenology, global warming

2.1 INTRODUCTION

Carbon dioxide (CO₂) is the chief greenhouse gas that results from human activities and causes global warming and climate change. Approximately 40% of atmospheric CO₂ concentration comes from increasing use of fossil fuels and changes in land use (4). The 2015 average annual concentration of CO₂ in the atmosphere was approximately 400 ppm (2). For the past decade (2005-2015) the average annual increase was 2.1 ppm per year (2). The Intergovernmental Panel on Climate Change (IPCC) use a range of future scenarios based on social, technological, economic and environmental conditions for predicting the atmospheric CO₂ concentration and temperature. The A2 IPCC (3) scenario projects a future atmospheric CO₂ concentration of 600 ppm and an increasing by 2°C of air temperature for the year 2050 (3,4,14). The growth, the development, and the plant yield will be outlined by the future climate changes (5,16).

The cattle industry is one of the highlights of Brazilian agribusiness in the world scenario. The Brazil owns the second largest effective herd in the world, with around 200 million heads. According to Ministry of Agriculture, Livestock and Supply (MAPA), Brazil has taken the lead in exports since 2004, with 20% of internationally traded meat and sales in over 180 countries (18). This crowd provides the development of meat and milk productive chains estimated in US\$ 17 billion (18). The climate and territorial extension of Brazil contribute to this success since they allow the maintenance of the herd by forages growing in the field. However, despite the great importance of ranching, the effects of climate change on forages have been not evaluated yet under field conditions in Brazil. Studies on tropical forages are necessary and relevant for predict future impacts and provide adequate management. Heating and greater availability of CO₂ in the atmosphere will affect the managements of pastures significantly once the forages are directly exposed to atmosphere conditions in the field (17). In fact, there is a multi-biome gap in experimental data in the tropics and subtropics about the plant responses to future climatic changes (19,20).

The net photosynthesis and growth under elevated CO₂ are less responsive in C₄ than C₃ species (31). However, even some C₄ species respond to increased CO₂ concentration given that the current concentration of atmospheric CO₂ is under the CO₂ saturation point for net photosynthesis (44). Indeed, some results with open top chamber indicated that the elevated CO₂ concentration can stimulate the biomass production in C₄ species (35,45). In

contrast to physiological responses, the results of morphological alterations in leaf blade of C₄ grasses in future climatic change is scarce.

The major C₄ grasses used as forage for cattle in Brazil belong to the genus *Panicum* and *Brachiaria* (46). When compared to other forages, the C₄ tropical grass *Panicum maximum* cv Mombaça had the best growth performance when intercropped with C₃ legume *Stylosanthes guianensis* cv Mineirão (54). The consortium increased the capacity of forage production when compared at *Panicum maximum* monocrop (54). Nonetheless, anatomical and morphological traits of leaf blade in *P. maximum* are related to animal preference and may influence consumption and digestibility, thus interfering with the forage quality (46). Leaf width and length provide valuable complementary information on using this grass as fodder in the consortium in the future because morphological differences among *P. maximum* genotypes did not alter biomass accumulation (46). These leaf morphological parameters are closely related to leaf meristematic activity. The activity of meristems plays central roles in plant developmental processes (48). The investigation of the responses of meristems to the environment may contribute to understanding the plant growth since it depends on the supply of new cells produced by meristems (48) and it reflects on plant plasticity and adaptation to the environment. Two meristems control leaf growth: 1. shoot apical meristem, which gives rise to leaf meristem and is responsible for the leaf width; and 2. leaf meristem, responsible for the leaf growth in length (48).

Our purpose is to show possible alterations caused by climate change on growth, biomass production and leaf ontogeny of *P. maximum* intercropped with *S. capitata* during autumn. To do so, we considered the B1 scenario outlined by the IPCC (3) as a parameter to regulate the TROP-T-FACE system (Free-air temperature and CO₂ controlled enhancement system on tropical pastures) where the consortium was deployed.

P. maximum intercropped with *Stylosanthes hamata* in open top chambers at a 600±50 ppm CO₂ increased the plant height and biomass production (35). Moreover, in that consortium, the assimilatory functions and chlorophyll accumulation was significantly influenced (35). One hypothesis of our experiment is that elevated atmospheric CO₂ concentration on the TROP-T-FACE system will rise leaf photosynthetic rate (77) providing resources to *P. maximum* emit more leaves and to develop them more rapidly. As a consequence of the accelerated development, leaf lifespan should be reduced. We expect that elevated CO₂ concentration will inhibit leaf expansion reducing leaf area because the investment on carbon assimilation area will not be necessary for a high atmospheric CO₂

environment (14). Also, there should be no changes in the total biomass of the shoot due to the plant response plasticity to atmospheric conditions presented, since C₄ photosynthesis already have a CO₂ concentration mechanism and slightly respond to atmospheric CO₂ rising (25). In fact, plants growing on FACE systems presents less positive results than those growing in chambers because FACE system allows the study of the effects of elevated CO₂ concentration on plants and ecosystems grown under natural conditions without enclosure and submitted to other environmental variables (31).

Under warming, we did not expect significant changes in *P. maximum* growth. There will be modest changes in photosynthetic rates in response to warming, such as those expected for C₄ plants within or between seasons, or the warming anticipated as a result of global climate change (60). Moreover, C₄ plants are adapted to warm climates, and the Brazilian autumn carries suboptimal conditions to C₄ photosynthesis most of the day (71,78). In fact, sixteen separate studies all indicate that the current distributions of C₄ monocots are tightly correlated with temperature: elevated temperatures during the growing season favor C₄ monocots (79). Thus, we expect only modest growth stimulation at leaf level under warming.

Under the simulated future climatic scenario, our hypothesis is that carbon remains inhibiting leaf expansion since the temperature will not mitigate the limiting effects of carbon on leaf area. Moreover, it could appear a negative synergistic effect on leaves of both carbon and temperature, resulting in smaller leaves and longer stems. Thus, if the elevated CO₂ concentration and temperature combined effects act at leaf and stem meristems changing the leaf size patterns, the use of *P. maximum* as forage may be compromised (46).

2.2 MATERIAL AND METHODS

2.2.1 EXPERIMENTAL AREA, SPECIES, PLANTING, AND STANDARDIZATION

The experimental area was located on the campus of the University of São Paulo (USP) in Ribeirão Preto city, the state of São Paulo, Brazil (21°10'S and 47° 48'W, 500 to 800 m altitude). According to the Köppen-Geiger (KG), Ribeirão Preto shows the Aw classification, a tropical climate with rainy summer and dry winter (63,64). The KG classification is efficient in macroscale, so we also considered the Thornthwaite classification (TH) which is efficient in mesoscale (65). According to TH, Ribeirão Preto city shows the B_{2r}B'_{4a}' classification (66), a humid climate, with little or no water deficiency in the dry season from April to September (66,67). The Camargo climate classification (CA) combines the simplicity of KG with the accuracy of TH using an agroclimatic zoning (65). According to CA, Ribeirão Preto city shows the TR-SBi classification, a subhumid tropical climate with dry winter (65). Historical data from 1982 to 2012 show the average annual temperature of 21.9 °C, with minimum and annual maximum temperatures of 18.4 °C and 23.9 °C, respectively, and an average annual rainfall of 1508 mm at this location. For the months of April and May, historical data (1982-2012) reveals an average temperature of 21.15 °C, with minimum and maximum temperatures of 14.95 °C and 27.35 °C, respectively (68).

The soil in the area is a dystrophic Red Latosol (Oxisol) (69). The area is fenced, and soil analysis, contouring, railing, and soil pH correction by liming had been performed previously. The value of the average initial pH was 4.0 to 4.5 and remained at 5.0 to 5.5 after liming. Chemical soil fertilization was realized after pH correction according to the initial nutrient availability. Therefore, the soil was nutritionally appropriate and homogeneous at the time of planting.

In the area of 2500 m², the C₄ grass *Panicum maximum* Jacq. cv. Mombaça (Poaceae) was intercropped with C₃ legume *Stylosanthes capitata* Vogel (Fabaceae). *S. capitata* was planted on 15th December 2013, and the consortium with *P. maximum* was established on 13rd February 2014. Both were sowing by rows. Seeds of Guinea grass, *Panicum maximum* Jacq. (Poaceae), were planted into holes 30 cm apart in 12 m × 12 m plots. NPK fertilizer at 40-140-80 kg ha⁻¹ was applied into the holes during planting. Only three plants per hole were maintained after germination. Two standardization cuts were realized on 10th and 21st April 2014. The plants of *P. maximum* and *S. capitata* were cut at a height of 30 cm from the ground. This trimming established a full, similar canopy among treatments as the

standard practice for managing the consortium in natural conditions. The beginning of the experiment with CO₂ fumigation and heating plots was on 22nd April 2014. On 29th April 2014, plants were fertilized with urea (120 kg ha⁻¹) on the soil surface. Fertilization and the irrigation were done to study *P. maximum* growth in the consortium without water and nutritional constraints.

2.2.2 THE TROP-T-FACE SYSTEM, THE WATERING FACILITIES, AND THE TREATMENTS

The Trop-T-FACE system was previously described (Section ‘The Trop-T-FACE system’, pages 22 to 29). The experiment occurred from 23rd April to 24th May 2014, during autumn, in irrigated consortium with *S. capitata*. The data collection occurred at initial phase of the consortium.

The design of this experiment to evaluate the *S. capitata* growth and biomass gain included four treatments with four replicates each: ambient conditions (Control), elevated atmospheric CO₂ concentration to 600 ppm (eC), elevated canopy temperature +2°C above the ambient temperature (eT), and elevated CO₂ concentration and temperature (eC+eT), totaling 16 experimental units.

2.2.3 FOLIAGE MEASUREMENTS AND DATA ANALYSIS

Foliage growth and stages of leaf development were set in the context of the tiller, the basic unit of growth in grasses. Each plot was divided into quadrants because the experimental area has been used by different research teams. Each team was responsible for one quadrant. Ten randomly equidistant tillers of *P. maximum* were selected inside our quadrant in each plot. Since the experiment was performed with four replicates per treatment, 40 tillers were measured per treatment. Data collection took place in the months of April (days 24th, 30th) and May (days 07th, 14th, and 21st) 2014, during the early autumn in Brazil.

Foliage traits recorded on each date included the number of expanded and expanding leaves (with and without visible ligule, respectively), the length of the expanding and expanded leaves, the width of the intact expanding and expanded leaves, and the length of the stem. From these measurements, we obtained average values per tiller in each treatment, and we calculated the following parameters.

The number of leaves that emerged in each period between data collection days was named the leaf appearance rate (LAR, day⁻¹). We calculated the number of emerged leaves as

the number of leaves from the current data collection day minus the number of leaves from the previous data collection day, with only positive values considered. We obtained the final LAR in a similar treatment by summing all positive values from each measurement time divided by the number of days in the entire experiment (28 days). This parameter indicated the rate of the appearance of leaves per tiller. Among the original 40 tillers marked per treatment, 27, 33, 39, and 39 in the Control, eC, eT, and eC+eT treatments, respectively, were usable for obtaining the average value of LAR.

The leaf elongation rate (LER, cm day⁻¹) per tiller was calculated as the length of expanding leaves on the current data collection day minus the length of these leaves from the previous data collection day, divided by the number of days between the two data collection times. All the original 40 marked tillers per treatment were usable for obtaining the average value of LER through the end of the experiment. An average value was calculated for the entire experimental period to show how fast the expanding leaves grew in length per tiller per day.

The combined number of expanded and expanding leaves (leaves with and without a ligule, respectively) resulted in the number of green leaves per tiller (NGL), disregarding senescent leaves. Senescent leaves were identified as those with more than 50% of their area without green color. The NGL was calculated as the total number of expanded and expanding leaves on each data collection day. An average NGL value was obtained for the entire experimental period to show how many functional leaves were maintained in each treatment. From the five data collection days, it was possible to calculate the average number of green, expanded, and expanding leaves per tiller in each treatment.

We used LAR to calculate the phyllocron (PHY, LAR⁻¹). PHY and NGL to estimate the leaf lifespan (LLS, day) according to the equation:

$$LLS = NGL \cdot PHY$$

proposed by Lemaire & Chapman (80). LLS represents the period between the appearance of a leaf to its senescence, when the leaf exhibit yellowing or loss of initial green or to natural leaf abscission.

As the foliage went through a standardization pruning at 30 cm from the ground at the beginning of the experiment, we observed leaves with cut apex and non-cut apex (named intact leaves) during the experimental period. We calculated the final leaf length (FLL, cm) from the average length of all intact expanded leaves present in the tiller. We measured the

length of the intact expanded leaves from the leaf ligule to its apex. FLL revealed the average final length of *P. maximum* leaf in each treatment.

We used the intact expanding leaves to take the measures of length and leaf width to get the results of leaf ontogeny. We considered the length of expanding leaves from the point where the leaf emerges from the sheath to the apex of the leaf. We measured the width of intact expanding leaves in leaf spot of the maximum leaf width (MLW, cm).

We also measured the stem length (SL, cm) from the ground to the last expanded leaf ligule.

We measured the leaf area of *P. maximum* using the WinDIAS Leaf Image Analyses System (Delta-T, UK). We collected the marked tillers of *P. maximum* in the field intact by cutting their stem base at the ground level at the end of the experiment (23rd May). We detached the leaves to measure its area per tiller. The foliage and the stems of each identified tiller were dried separately in a stove with forced air circulation at 60 °C to obtain the leaf and the stem biomass.

We performed statistical analyses with the open software BioEstat, version 5.3 (Instituto Mamirauá, Brazil). We used D'Agostino-Pearson test to verify whether data were normally distributed. Since the datasets did not show a normal distribution, we used the non-parametric Mann-Whitney test to comparing the datasets among treatments. We determined significance at $p < 0.1$ for the tiller measurements to compare the datasets among treatments. We created trend lines in Microsoft Exceltm to perform the correlation analysis between leaf area and leaf biomass and between leaf length and width in each treatment for each leaf category, considering the best fit line for each correlation. We selected the function that best fits, that is, the one with the highest R-value.

2.3 RESULTS

2.3.1 METEOROLOGICAL DATA

The course of the daytime was usually free of clouds with solar irradiance between 0.7-0.9 kW m⁻² around midday during the period of the experiment (Fig 2.1A). Nonetheless, cloudy days with solar irradiance around 0.5 kW m⁻² occurred at 18th and 23rd May 2014. The highest solar irradiance value (around 0.87 KW.m⁻²) was registered at 24th April 2014 at 12:45 PM (Fig 2.1A). At this day, canopy temperature reaches approximately 32.3 °C at warmed plots, around 12 °C above average (Fig 2.1E), when the relative air humidity reached 62.2% (Fig 2.1B). Air relative humidity values at midday were never less than 29.5% (Fig 2.1B). Average air relative humidity during the experiment was around 75.9%. Reduced air relative humidity occurred together with peaks of temperature and solar irradiance, usually around midday, as can be seen in Figure 2.1 (A, B, and F).

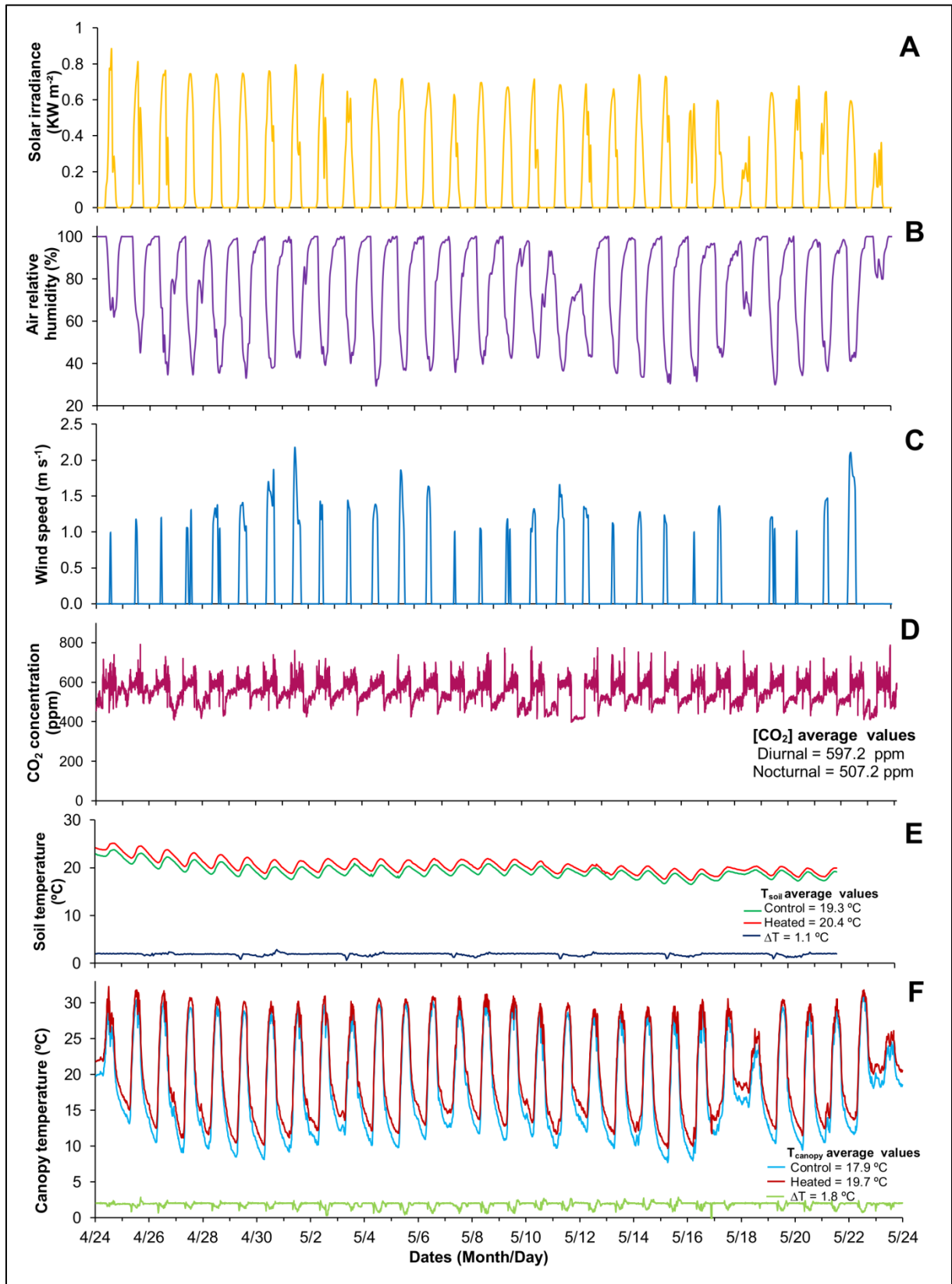


Fig 2.1 The daily courses of total solar irradiance (A), relative air humidity (B), and wind speed (C) recorded from April 24th 2014 to May 24th 2014 at the center of the experimental area. It is also showed the CO_2 concentration in the enriched atmosphere (D) with average values of nocturnal and diurnal CO_2 concentration, and the soil (E) and the canopy (F) temperatures in heated and in control regimes, respectively, with the averages and the temperature difference between them (Δ).

Soil temperature varied between 16.3 °C to 25.3 °C in Control and warmed regimes (Fig 2.1E). The average soil temperature was 19.3 °C in Control regime and 20.4 °C in warmed plots (Fig 2.1E). The target soil temperature reached +2°C in heated regime almost all the time (Fig 2.1E). The maximum value of canopy temperature (32.7 °C at 22th May 2014, Fig 2.1F) occurred during the experimental period was not stressful for *P. maximum* even in warmed plots since the C₄ photosynthetic metabolism is advantageous in warmer temperatures (81,82). The optimum temperature for *P. maximum* growth is estimated to be 19.1 °C to 22.9 °C (83). More precisely, the optimum temperature is estimated to be around 21 and 21.9 °C for Mombaça grass (84). Average temperatures values obtained (17.9 °C at control and 19.7 °C at warmed plots, Fig 2.1F) was below this range of optimum temperatures and it was lower than historical data (1982-2012) for the months of April and May (average temperature of 21.15 °C with minimum of 14.95 °C and maximum of 27.35 °C, (24)). The canopy temperature varied between 32.7 °C and 7.6 °C during the daily courses (Fig 2.1F) with the target canopy temperature reaching +2°C in the heated regime, especially during the night (Fig 2.1F). Since *P. maximum* is a tropical grass that occupies regions with temperatures ranging between 25 °C and 30 °C, lower temperatures tend to be more harmful to *P. maximum* than elevated temperatures (83). At temperatures lower than the base temperature, the growth of the crop is paralyzed. The lowest values of canopy temperature occurred at night, and they were below the base temperature for Mombaça grass, estimated to be around 14.2 to 15.6 °C (84,85). Canopy and soil temperatures were more closely correlated to solar irradiance than with wind speed (Table 2.1). Indeed, soil temperature had no correlation with wind speed (Table 2.1). The wind speed rarely exceeds 2 m s⁻¹ and only on 1st May 2014 it was higher than this value (2.2 m s⁻¹, Fig 2.1C).

Table 2.1. Pearson correlation coefficient (R) between the maximum temperature values in soil (T_{soil}) and canopy (T_{canopy}) at control and heated plots and the maximum values of solar irradiance and wind speed.

Pearson correlation coefficient (R)	T _{soil}	T _{soil}	T _{canopy}	T _{canopy}
	Control	Heated	Control	Heated
Solar irradiance	0.61**	0.65**	0.50**	0.56**
Wind speed	-0.04 ∅	0.03 ∅	0.48*	0.44*

The greater the R-value (positive or negative), the greater the correlation between the parameters. The R-values from 0.5 to 0.7 (positive or negative) indicate moderate correlation and are marked by a double asterisk (**). The R-values from 0.3 to 0.5 (positive or negative) indicate weak correlation and are marked by a single asterisk (*). R-values from 0 to 0.3 (positive or negative) indicate an insignificant correlation between the parameters and are marked by ∅ symbol.

The average values of CO₂ concentration at enriched plots (Fig 2.1D) was 507.6 ppm during daytime and 597.2 ppm at night. Nocturnal CO₂ concentration was due to plant respiration as the FACE system was off at night. CO₂ concentration fluctuations observed at Fig 2.1D was a result of FACE system mainly by the photosynthetic CO₂ assimilation in plants and wind CO₂ dispersing.

2.3.2 GROWTH RESULTS

The elevated atmospheric CO₂ concentration was the main responsible for stimulating leaf appearance. Compared to the control, under eC and eC+eT there was an increase in LAR (Fig 2.2A) with no statistical difference from Control under eT. In fact, LAR was slightly higher than control under eT, but the statistical difference was not significant (Fig 2.2A).

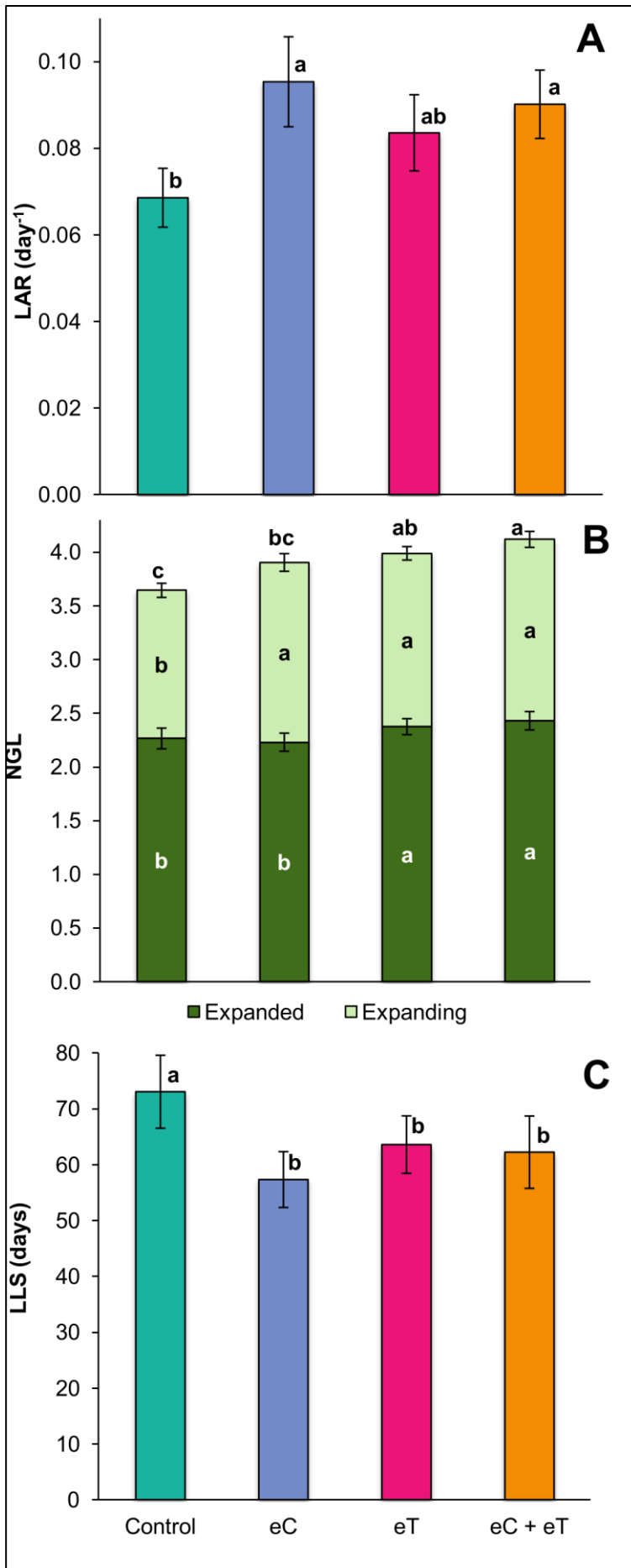


Fig 2.2 Leaf appearance rate (LAR, A), number of green leaves (NGL, B), and leaf lifespan (LLS, C) per tiller of *Panicum maximum* under ambient CO₂ and temperature (Control), under 600 ppm of CO₂ (eC), under elevated temperature at 2°C above ambient (eT), and under both treatments (eC+eT). Bars show average values and lines at the top of bars the standard error. Different letters above and inside the bars indicate significant differences among data sets after the Mann-Whitney test at $p < 0.1$. The two different colored bar of (C) shows two categories of leaves (expanded and expanding leaves). The size of these two bars considered together represents the NGL.

The carbon enrichment on eC treatment provoked an increase in the number of expanding leaves, not in expanded leaves (Fig 2.2B). There was a slight increase in NGL (Fig 2.2B) under eC, caused by the greater presence of younger leaves. Therefore, the higher NGL under eC was a consequence of the elevated LAR (Fig 2.2A), a greater number of expanding leaves (Fig 2b), and maintaining the number of expanded leaves (Fig 2.2B).

Warming increased the number of expanded and expanding leaves, as can be seen in eT and eC+eT treatments (Fig 2.2B). As a result, under these treatments, NGL was higher than control (Fig 2.2B). We also obtained the largest NGL under eC+eT (Fig 2.2B) which showed a synergistic effect of eC and eT on the leaves number in this treatment.

Under eC, the elongation of leaves (Fig 2.3A) was faster than in control, but leaves had a reduced estimated LLS (Fig 2.2C) and, consequently, shorter FLL (Fig 2.3C). In other words, the leaf phenology was accelerated under eC at the expense of FLL and estimated LLS. The higher carbon concentration under eC also provoked reduction on MLW (Fig 2.3C) and SL (Fig 2.3D).

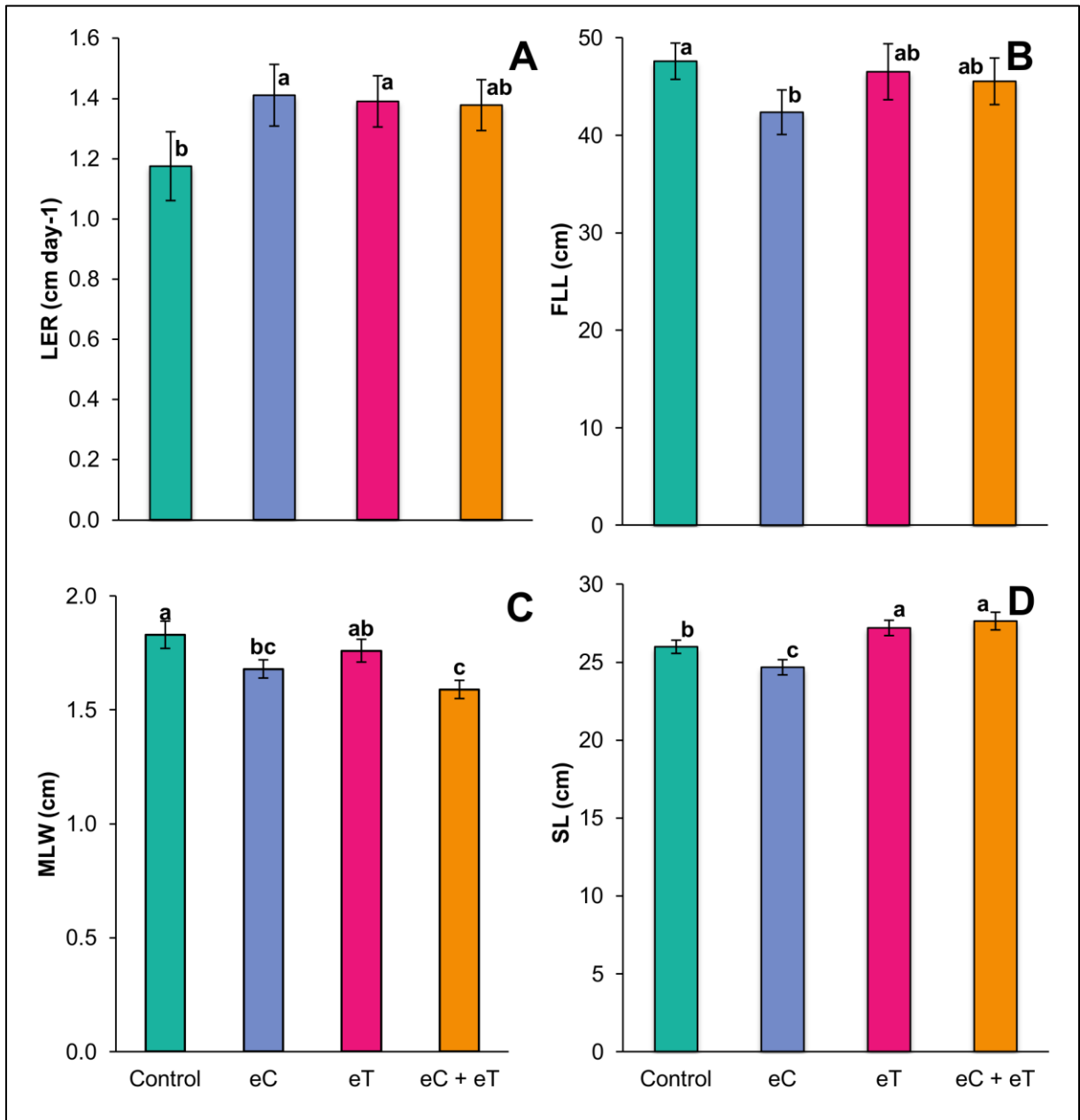


Fig 2.3 Leaf elongation rate (LER, A), final leaf length (FLL, B), average maximum expanding leaf width (MLW, C), and stem length (SL, D) per tiller of *Panicum maximum* under ambient CO₂ and temperature (Control), under 600 ppm of CO₂ (eC), under elevated temperature at 2 °C above ambient (eT), and under both treatments (eC+eT). Bars show average values and lines at the top of bars the standard error. Different letters above bars indicate significant differences among datasets after the Mann-Whitney test at $p < 0.1$.

Likewise, LLS (Fig 2.2C) was reduced, and LER (Fig 2.3A) was higher under eT. Nonetheless, isolated warming did not alter FLL (Fig 2.3B) nor MLW (Fig 2.3C) despite this parameters had shown a slight decrease regarding control.

Under the combined treatment (eC+eT), there were no alterations in LER (Fig 2.3A) nor in FLL (Fig 2.3B) compared to control. The LLS (Fig 2.2C) was as reduced as the other treatments. As in eC treatment, the expanding leaves were narrower than control (Fig 2.3C). It

is noteworthy that the width of expanded leaves was not influenced by the treatments (data not shown). Also, the FLL did not suffer a significant impact of the temperature increase. Differently, the SL (Fig 2.3D) was strongly affected by warming showing bigger size under eT and eC+eT than under control and eC.

As SL (Fig 2.3D) was shorter than the control, and NGL was slightly higher (Fig 2.2B), leaf biomass under eC was larger than stem biomass under this treatment, as demonstrated by the relationship between leaf:stem biomass ratio more than 1.0 (1.2, Fig 2.4B). Under eT, leaf and stem biomass behave just as control, with a slight increase in leaf biomass (Fig 2.4B). Therefore, leaf:stem biomass ratio (Fig 2.4B) is little higher than control, with no statistical differences.

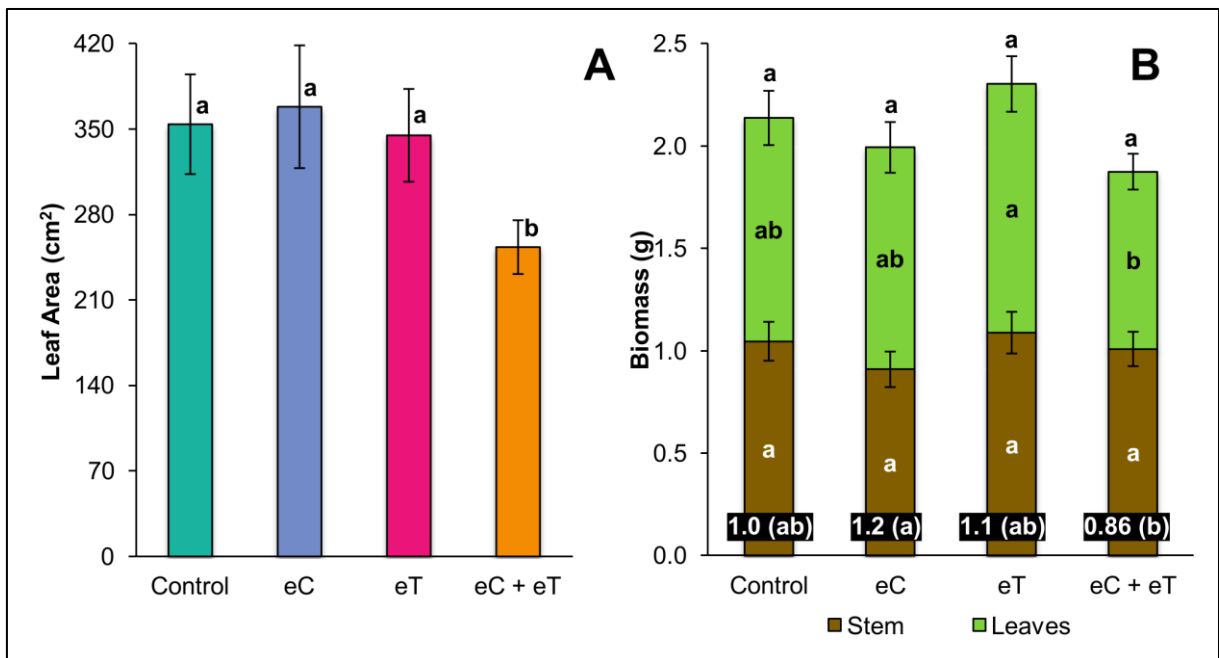


Fig 2.4 Leaf area (a) and biomass (b) per tiller of *Panicum maximum* under ambient CO₂ and temperature (Control), under 600 ppm of CO₂ (eC), under elevated temperature at 2 °C above ambient (eT), and under both treatments (eC+eT). Bars show average values and lines at the top of bars the standard error. Different letters above and inside bars indicate significant differences among data sets after the Mann-Whitney test at p<0.1. The two different colored bar of (b) indicate leaves or stem biomass and lines at the top of bars the standard error. The size of these two bars considered together represents the tiller biomass. The number and letters inside the black boxes below bars represent leaf:stem biomass ratio with statistical comparisons.

Under eC+eT, even with a greater NGL (Fig 2.2B), the leaf biomass (Fig 2.4B) was the lowest observed. It happened due to individual responses of *P. maximum* under eC+eT. The LER (Fig 2.3A) was similar to control and less than under eT and eC in absolute terms. It contributed to a reduced FLL (Fig 2.3B) but not so reduced as under eC. Also, the estimated LLS (Fig 2.2C) was also lower under eC+eT. However, the primary cause of reduced leaf

biomass (Fig 2.4B) was a translocation of biomass to the stem of the plant. As can be seen in Fig 2.3D, the stem length is greater in the treatments at elevated temperature (eT and eC+eT). Together with a reduced leaf area (Fig 2.4A) under eC+eT, the biomass translocation to the stem under this treatment resulted in a leaf:stem biomass ratio lower than 1.0 (0.86, Fig 2.4B), which indicates that the stem biomass was greater than the leaf biomass under eC+eT. Reduced leaf area (Fig 2.4A) was probably a result of narrower expanding leaves since we did not observe a significant reduction of FLL (Fig 2.3B).

Both leaf area and biomass of leaves, stem, and tiller (Fig 2.4) were similar to the control under eC. However, in the control, the increase in biomass due to the increase in leaf area exhibited linear correlation (Fig 2.5, Control). The same correlation under eC appeared as a polynomial of 2nd degree (Fig 2.5, eC). It indicates that, under eC, when the leaf area per tiller reaches approximately 550 cm², leaf biomass gain per tiller tends to stabilize with increasing leaf area. When the leaf area per tiller is about 800 cm², leaf biomass per tiller increase is practically stable. It indicates that there was inhibition of investments on leaves under eC as the leaf area increases.

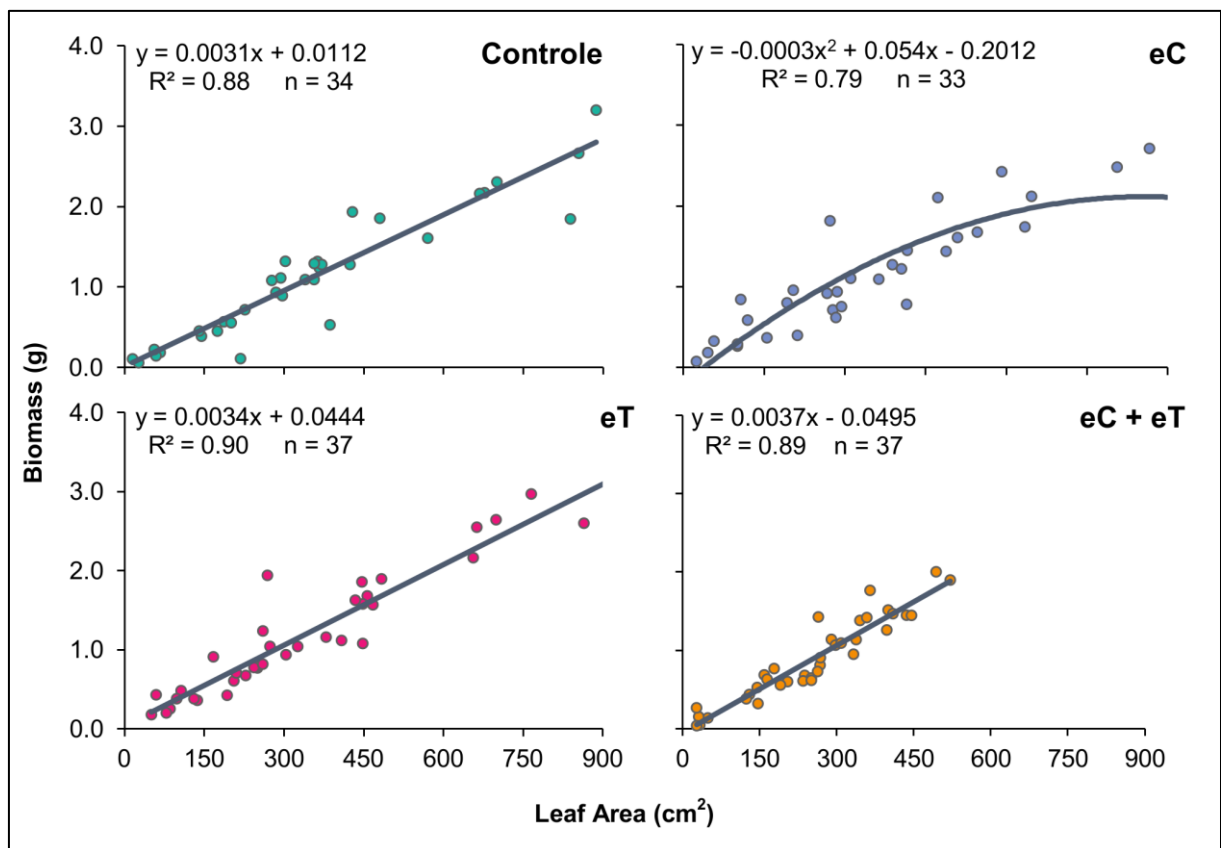


Fig 2.5 Biomass as a function of leaf area per tiller of *Panicum maximum* under ambient CO₂ and temperature (Control), under 600 ppm of CO₂ (eC), under elevated temperature at 2 °C above ambient (eT), and under both treatments (eC+eT). In the graphic are demonstrated the equation of the function, the R-square value, and the n value as the number of leaves evaluated.

The biomass of stem and leaves (Fig 2.4B) remains similar to the Control and eC as well as leaf area (Fig 2.4B) despite the NGL (Fig 2.2B) was higher, and the stem was longer than the Control and eC (Fig 2.3D). Similarly as in Control, biomass continues to increase linearly as a function of the leaf area (Fig 2.5, eT). It explains the highest leaf biomass values found in this treatment compared to others.

Under eC+eT, it is observed that the values of biomass (Fig 2.4B) and leaf area (Fig 2.4A) are lower than in other treatments. The points on the graph are located closer to the origin of the axes, and the leaf area was not higher than 550 cm². The correlation between these points displays linear function as the Control and under eT. It means that with the increase in leaf area results in increased leaf biomass, but the leaf area was limited in 550 cm².

2.3.3 LEAF ONTOGENY

Intact expanding leaves showed different responses in the correlation between width and length of the leaves in each treatment (Fig 2.6). However, in all treatments, the function that describes the correlation between length and width of the expanded leaves was outlined by a root function in the Control and all the treatments (data not shown). The occurrence of the same type of function means that changes in environmental conditions caused modest modifications in the ontogeny of leaves that have completed their expansion and will slightly affect the meristems operation in these leaves.

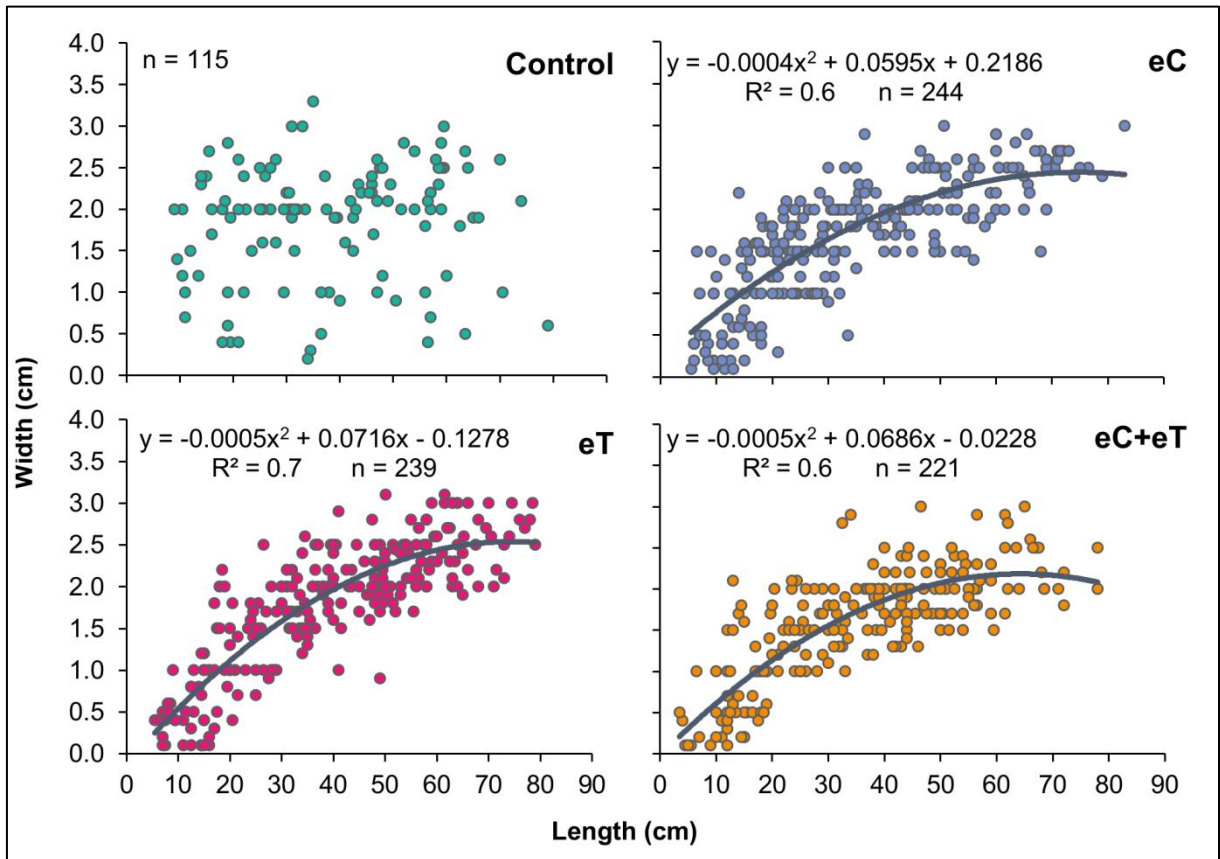


Fig 2.6 Width as a function of length of intact expanding leaves per tiller of *Panicum maximum* under ambient CO₂ and temperature (Control), under 600 ppm of CO₂ (eC), under elevated temperature at 2 °C above ambient (eT), and under both treatments (eC+eT). In the graphic are demonstrated the equation of the function, the R-square value, and the n value as the number of leaves evaluated.

However, the meristematic activity of intact expanding leaves showed to be strongly influenced by environmental conditions. In the Control, there was a weak correlation between growth in width and length (Fig 2.6). When the treatments were applied, meristematic activity became more coordinated and responded in different ways depending on the treatment.

Under eC, there was a curve flattening of the function that describes the correlation between length and width of expanding leaves. It means that the leaves show signs of narrowing reaching almost 2.5 cm of width (Fig 2.6). Under eT there were no signs of temperature limiting meristematic activity and the leaves easily reached 2.5 cm (Fig 2.6). However, under eC+eT, we observed substantial limitation of width increase, represented by a drop at the end of the curve and a curve flattening (Fig 2.6). It resulted in narrower leaves, as showed in Fig 2.3C.

These results demonstrated that the elevated CO₂ concentration was limiting the increase in leaf width throughout its growth in length. Furthermore, the presence of elevated CO₂ concentration together with elevated temperature (eC+eT) has aggravated this situation

and kept the smallest average width growth of the leaf (Fig 2.3C). Narrower leaves may be the main responsible for the reduction in leaf area under eC+eT treatment (Fig 2.4A).

2.4 DISCUSSION

There are two main meristems responsible for the formation of a grass leaf: the shoot apical meristem and the leaf meristem (48). The shoot apical meristem is located at the stem apex and initiates leaf primordia. Its activity is characterized by the leaf appearance rate (48). In the developmental system of a leaf grass, the width of the leaf is first determined along the circumference of the shoot apical meristem, followed by elongation along the longitudinal axis drive by leaf meristem (48). The leaf meristem is located at the leaf base and drives the growth in length and development of the leaf by producing new cells (48).

There are three ways to environmental conditions control the leaf meristem. First, there is a regulation on the number of leaf meristems. This regulation can be detected by the number of leaves and leaf appearance rate. Secondly, there is a modulation of meristematic activity, given by leaf elongation rate and final leaf length. Finally, there is the control of the size and shape of the leaf meristem, indicated by the leaf width. The leaf area and biomass are resultants of combinations of these three conditions.

The simulated future scenario provided the highest leaf appearance rate and a greater number of green leaves, thus increasing the number of meristems. Elevated CO₂ concentration was the main factor acting on increasing leaf appearance rate and on the number of expanding leaves. However, this accelerated phenology shortened the leaf lifespan of *S. capitata*. The presence of more resources (CO₂, temperature, and in the case of the experiment, water, and nutrients) should shorten the time required for the leaf pays back the investment made on it in the future climate scenario. This payback should happen via positive carbon balance, provided by favorable environmental conditions. Therefore, the payback will be faster, and the leaf will die sooner when there are more resources.

The changes observed in leaf ontogeny demonstrated that environmental variables in this experiment also performed modifying the meristematic activity of *P. maximum* leaves. In expanded leaves, future climate will little affect the meristems operation probably because leaf expansion is complete. We observed discreet differences between the width x length functions in Control and under eC+eT of expanded leaves (data not shown). However, in the leaves at the beginning of their development (rapid expansion), the atmospheric variables provoked several alterations on leaf ontogeny. Leaf meristem activity was stimulated by the high CO₂ concentration and warming as isolated treatments accelerating the elongation of the leaf. In the simulated climate scenario, the joint action of CO₂ and warming will not affect the

functioning of leaf meristem after its formation, and the leaves will have similar final length as currently climate.

Nonetheless, in the future will be modifications in the size and shape of leaf meristem. Elevated CO₂ and concomitant warming will be responsible for a smaller leaf area caused by narrower leaves, provoked mainly by the high CO₂ concentration. Under eC and eC+eT, the shoot apical meristem produced a shorter leaf meristem. In other words, the leaf meristem is formed by a smaller half-circle around the shoot apical meristem under conditions of high CO₂ concentration. High CO₂ concentration in warmed leaves aggravated this situation by a substantial limitation of leaf meristem formation around shoot apical meristem. The drop at the end of the curve that describes the relationship between length and width (Fig 2.6, eC+eT) indicates the production of narrower leaves and with a markedly reduced leaf area.

Those relations showed the presence of two meristems triggered differently along the leaf growth, and they are suffering the effects of abiotic factors. Their action was combined and dependent on the stage of leaf development and environmental conditions. The influence of future climate scenario is clear about the number of leaf meristems and their formation around shoot apical meristem. The future climate will lead to the production of a small leaf meristem in the expanding leaves mainly generating narrower leaves, hardly with a width larger than 2 cm. It will reflect on reduced leaf area and can affect the diet of cattle. Forage quality is based on the composition of digestible or fermentable compounds and forage consumption by ruminants (46). Forage nutritive value is highly associated with leaf blade anatomy and its tissues, which may be highly or poorly digestible or even indigestible (46). Thus, the relative quantity of these tissues determines forage quality and is related to leaf morphology (46). Therefore, as the anatomy of the grasses is related to morphology and chemical composition of leaf blades, alterations in leaf width and length may influence consumption and digestibility, thus interfering with the forage quality (46).

Nevertheless, in the simulated climate scenario with widely available resources, although *P. maximum* leaf area was reduced, leaf biomass is practically the same, probably compensated by the higher number of leaves. These responses suggest downregulation since it is not necessary to invest in leaf area to capture more carbon when atmospheric CO₂ concentration is elevated. Because it is a plant with C₄ metabolism, *P. maximum* has a carbon concentration mechanism, being less responsive to changes in atmospheric CO₂ concentration. Recent evidence from free-air CO₂ enrichment (FACE) experiments suggests

that elevated CO₂ concentration does not directly stimulate C₄ photosynthesis (72). The downregulation probably occurred at leaf meristem level during its formation.

The response of aboveground biomass to climate change is critical to livestock because it reflects the availability of food for the cattle. Aboveground biomass of *P. maximum* suffered few modifications under climate change, demonstrating plasticity in an environment with abundant resources. It is possible to observe the inhibitory effect of high concentration of CO₂ in the leaf biomass gain, only when comparing eT and eC+eT treatments. This inhibitory effect was evident when it was analyzed leaf biomass as a function of leaf area in the eC treatment. In this case, the carbon undeniably limited the biomass accumulation when the leaf area per tiller reached about 550 cm². On the other hand, warming does not affect the biomass gain and the leaf area enhancement, because C₄ plants are well adapted to warmer climates (71).

C₄ plants, such as *P. maximum*, generally tend to show little or no growth response to elevated CO₂ concentration under well-watered conditions (25,77). However, global warming and changes in precipitation patterns are likely to expose many ecosystems, including C₄-dominated ones, to increasing soil and atmospheric water stresses (1,3,86). Evidence indicates that C₄ photosynthesis is highly sensitive to water stress (86). A reduced leaf area may favor the water balance by decreasing the transpiration surface of the leaf. In fact, when C₄ plants experience drought in their natural environment, elevated CO₂ concentration alleviates the effect of water stress on plant productivity indirectly via improved soil moisture and plant water status as a result of decreased stomatal conductance and reduced leaf transpiration (86). Thus, C₄ plants photosynthesis and productivity could be stimulated by elevated CO₂ concentration in times and places of drought stress (72) caused by global climate changes. However, it has been argued that a mere doubling or tripling of ambient CO₂ concentration is not enough to overcome the stomatal limitation caused by water stress, and that very high CO₂ concentration is needed to force CO₂ to diffuse across the whole leaf surface (86).

However, even in a condition free of water stress, changes in *P. maximum* canopy caused by heating and CO₂ enrichment are significant and important to evaluate the use of this plant as forage for livestock. The changes were detected at various levels of the canopy and are spatial and temporal. Changes in time were revealed by increased leaf appearance rate. Changes in space were evidenced by a reduced leaf area and a greater number of green leaves. As a final result, *P. maximum* displays a new canopy, with changes in the level of individual leaves and leaf population. The new leaf may have an adverse influence on the cattle feed

(46), but it may be mitigated by the largest population of leaves, which prevents a reduction in aboveground biomass. These changes indicate an adaptation of *P. maximum* to the simulated climate scenario and require more detailed studies with this plant to establish the best management strategies for their future use in livestock.

2.5 CONCLUSIONS

Global climate change will cause significant space-time variations in the canopy of *P. maximum*. The treatments modified the way the leaf grows. This study revealed the CO₂ and warming effects in the two meristems responsible for leaf growth. It gave an overview of two-dimensional leaf growth under future climate. The leaf biomass allowed complement this picture with a sense of three-dimensional leaf growth.

The changes observed in *P. maximum* canopy to climate change also took place at the level of leaf population. Larger population leaves allow maintaining leaf biomass. However, changes in individual leaf level, such as reducing the leaf area may hamper the use of *P. maximum* as pasture in the future by interfering in forage quality.

- 3 -

GROWTH OF FORAGE C₃ LEGUME INTERCROPPED WITH C₄ GRASS UNDER SIMULATED FUTURE CLIMATE

ABSTRACT

We studied growth and biomass production of a C₃ forage, *Stylosanthes capitata* Vogel intercropped with a C₄ grass, *Panicum maximum* Jacq. under subtropical field conditions in a simulated climate scenario predicted for 2050. Plant growth measurements were carried out weekly in autumn between April-May 2014 during 27 days. Plants grew with permanent irrigation under two combined systems, the free-air carbon dioxide enrichment and the free-air temperature controlled enhancement, where plants were subjected to four climatic regimes: current temperature and CO₂ concentration (Control), high CO₂ concentration (600 ppm, eC), high canopy temperature (2 °C above canopy ambient temperature, eT) and the combination eC+eT. Under eC, there was a stimulation of foliage growth but without a consistent increase in the biomass of leaves. There were fewer leaves and ramifications per shoot caused probably by vigorous growth and shade of the C₄ foliage under warming (eT). On the other hand, under eC+eT, there was a higher density of leaves per shoot. As the number of leaves per shoot did not change among treatments, the highest leaf density was a consequence of shorter shoots under eC+eT. Besides, the apical flowering of *S. capitata* was more intense in eC+eT with side gems unfolded in additional ramifications. Even without water and nutritional shortage, eC+eT treatment did not result in any increase to *S. capitata* leaf biomass gain. The consortium of *P. maximum* with *S. capitata* already is rarely used, and did not appear to be an alternative pasture at Southwest of Brazil since the future climate predicted by 2050 with 2 °C increment in temperature and atmospheric CO₂ concentration elevated until 600 ppm will hardly bring benefits to *S. capitata*.

Keywords – CO₂ concentration, FACE, flowering, global warming, leaf biomass, pasture

3.1 INTRODUCTION

Several activities have contributed to rising global atmospheric concentration of carbon dioxide. These activities are, primarily, the increasing use of fossil fuels, industrial processes as the concrete production and land-use change such as deforestation, forest burns, and agriculture. The cement industry is responsible for approximately 3% of global emissions of greenhouse gases (GHG) and for almost 5% of total CO₂ emissions (6). According to Brazilian Ministry of Technology, Science, and Innovation (MTCI), the contribution of energy production for emissions of GHG has increased from 16% in 2005 to 32% in 2010 in Brazil (7). The Brazilian Institute of Geography and Statistics (8), stated that forest burns contributed for more than 75% of the CO₂ emission in Brazil. Deforestation resultant from the woods burns to deploy pastures and agriculture further aggravates the situation by reducing the uptake of carbon from the atmosphere by trees and the amount of water transported daily to the atmosphere by plant transpiration (10). GHGs emissions from agriculture correspond to 35% of all Brazilian GHG emission mostly due to the use of nitrogen fertilizers and the methane resulting from the digestive processes of cattle (7).

In March 2016, it was recorded the monthly average value of 404 ppm of CO₂ in the atmosphere (2). The increase in CO₂ atmospheric concentration and other GHG lead to unprecedented changes in the Brazilian climate according National Institute for Space Research (INPE) from Brazil and the UK Met Office-Hadley (13). The A2 emission scenario designs a future CO₂ atmospheric concentration of 600 ppm for the year 2050 (3). Along with increase CO₂ atmospheric concentration there are the rise of global average atmospheric temperature (1) and the changes in rainfall patterns in many regions of the Earth (11). In less pessimistic scenario (B1) outlined by IPCC (1) the temperature of Earth surface will increase by 2 °C by 2050. These climate changes will have a significant economic and ecological impact on grassland and forests (15).

Changes in the availability of CO₂ concentration, water, and temperature will affect the whole trophic chain of the planet (26). CO₂ is the single carbon source for photosynthesis, and the current atmospheric concentration of CO₂ is lower than ideal especially for C₃ plants. Thus, C₃ species respond positively to increased atmospheric CO₂ concentrations (29). Stimulation of photosynthesis by increasing CO₂ concentration has been widely described (4,27,30–33). On the other hand, as a consequence of CO₂ increase, plant photorespiration may reduce. Recently, has been suggested that the reduction of

photorespiration/photosynthesis ratio in response to the ~100 ppm CO₂ increase over the 20th century was 25% (34).

Mathematical models predict that the increase in the CO₂ concentration from 400 to 600 ppm could increase C₃ photosynthesis to around 40% (4). *Panicum maximum* (C₄) and *Stylosanthes hamata* (C₃) produced 67 and 85% more fresh and dry biomass, respectively, under 600 ± 50 ppm CO₂ in open top chambers (OTCs, (35)). Duplication in the atmospheric concentration of CO₂ provokes a rise of 30% in the dry biomass production of a C₃ forage growing in experimental rooms (36,37). Indeed, doubling the CO₂ concentration caused 25% increase in yield of C₃ legume *Arachis glabrata* growing intercropped with C₄ grass *Paspalum notatum* in a temperature-gradient greenhouse (38).

However, in field experiments conducted on free-air carbon dioxide enrichment systems (FACE) the stimulation of photosynthesis in C₃ plants under 500-600 ppm CO₂ was significantly lower, *ca.* 14% (19,31,40). The FACE systems provide a more realistic scenario of a future atmosphere with high CO₂ concentration (31). Studies in chambers have used a mean CO₂ concentration of 700 ppm while studies with FACE use 550 to 600 ppm (4). Certainly, plants growing on FACE are under the influence of external factors setting the results in FACE closer to the reality (41).

Beneficial effects of high CO₂ atmospheric concentration may compensate in part the climate change damaging on C₃ crops. However, an increase in air temperature of 1°C may reverse the benefits of increased CO₂ concentration on yield (16). For each 1°C increment in seasonal temperature may result in a reduction of 3 to 16% in crop yield (43) due to increased respiration and photorespiration in C₃ species. Thus, high temperatures could be harmful to C₃ plants growth because it causes carbon loss. Moreover, the growth temperature above a finely tuned threshold can rapidly trigger flowering, bypassing the need for other inductive stimuli such as day length (49). As the increase in CO₂ atmospheric concentration also leads to a rise in temperature, forecast how C₃ plants would respond in the future under field conditions is still uncertain.

The consortium between *Stylosanthes guianensis* cv. Mineirão (C₃ legume) and *Panicum maximum* cv. Mombaça (C₄ grass) may be an alternative pasture for fertile soils in a vast area of Brazilian Cerrado vegetation (54). *P. maximum* cv. Mombaça produced approximately 3200 kg ha⁻¹year⁻¹ of dry matter in monoculture. In a consortium with *S. guianensis* cv. Mineirão, this production has increased to approximately 4500 kg ha⁻¹year⁻¹ (54). Here, we tested three hypotheses of C₃ forage in field conditions under future climate

change in consortium with *Panicum maximum* cv. Mombaça. 1 - In high CO₂ concentration treatment (600 ppm), we expected a significant growth increase and higher biomass production due to the greater supply of carbon, particularly for C₃ photosynthesis. However, the competition for resources with *P. maximum* (C₄) in the consortium could offset the advantages promoted by the atmospheric CO₂ increased due to faster growth of C₄ grass. 2 - The C₄*P. maximum* under high temperature will present faster growth than in Control regime extinguishing resources in soil and shadowing *S. capitata*. However, the slight increase in temperature by 2 °C with additional water and nutritional input may benefit *S. capitata* as reported by Martinez *et al.* (87) resulting in gain of biomass despite the competition with the C₄ grass. 3 - Under combined atmospheric regime (600 ppm of atmospheric CO₂ and temperature +2 °C above ambient temperature), we expected a higher biomass production of *S. capitata* than in Control atmospheric regime. The increase in the supply of carbon for photosynthesis will mitigate possible direct constraints by respiration and photorespiration on growth and indirect impairments by the competition with *P. maximum*. Besides, the increment in temperature by 2 °C with additional water and nutritional input may benefit *S. capitata* in field conditions (87).

The objective of this research was to determine the effects of B1 climate scenario (1) on initial growth, biomass production and the flowering of *Stylosanthes capitata* (C₃ legume) growing intercropped with *Panicum maximum* (C₄ grass) under field conditions. Studies on climate impacts on forage productivity are crucial for supporting the pasture management under adverse atmospheric conditions, particularly in areas where livestock have a significant contribution to the economy. To achieve this goal, the Trop-T-FACE (Free-air Temperature and CO₂ Controlled Enhancement) system provided the B1 scenario climatic conditions, that means 600 ppm of CO₂ in atmosphere and +2 °C increase in air temperature (1). The experiment was conducted during the early autumn in Brazil when *S. capitata* was near to flowering (88).

3.2 MATERIALS AND METHODS

3.2.1 EXPERIMENTAL AREA, SPECIES, PLANTING, AND STANDARDIZATION

The experimental area was located in the campus of the University of São Paulo (USP) in Ribeirão Preto city, state of São Paulo, Brazil (21°10'S and 47°48'W, 500 to 800 m altitude). According to the Köppen-Geiger classification Ribeirão Preto shows the Aw climate, tropical climate with rainy summer (63). Historical data from 1982 to 2012 show the average annual temperature of 21.9 °C, with minimum and maximum annual temperatures of 18.4 °C and 23.9 °C, respectively, and a average annual rainfall of 1508 mm at this location. For the months of April and May, historical data (1982-2012) reveals an average temperature of 21.15 °C, with minimum and maximum temperatures of 14.95 °C and 27.35 °C, respectively (68).

The soil in the area is a dystrophic Red Latosol (Oxisol) (69). The area is fenced, and soil analysis, contouring, railing, and soil pH correction by liming had been performed previously. The value of the average initial pH was 4.0 to 4.5 and remained at 5.0 to 5.5 after liming. Chemical soil fertilization was realized after pH correction according to the initial nutrient availability. Therefore, the soil was nutritionally appropriate and homogeneous at the time of planting.

In the area of 2500 m², the C₃ legume *Stylosanthes capitata* Vogel (Fabaceae, C₃) was intercropped with *Panicum maximum* Jacq. cv. Mombaça (Poaceae, C₄). *Stylosanthes* Sw. comprises 48 species with a pantropical distribution (88). *S. capitata* (Leguminosae-Dalbergiae) species is perennial, with 12 to 25 cm tall and trifoliolate leaves. Inflorescences are terminal or axillary, simple or composed of 2 to 4 stalks, with 11 to 20 flowers each (88). *S. capitata* was planted on 15th December 2013 and the consortium with *P. maximum* was established on 13rd February 2014. Both were sowing by rows. Seeds of *S. capitata*, were planted into holes 30 cm apart in 12 m × 12 m plots. NPK fertilizer at 40-140-80 kg ha⁻¹ was applied into the holes during planting. Only three plants per hole were maintained after germination. Two standardization cuts were realized on 10th and 21st April 2014. The plants of *P. maximum* and *S. capitata* were cut at a height of 30 cm from the ground. This trimming established a full, similar canopy among treatments as the standard practice for managing the consortium in natural conditions. However, *P. maximum* grew faster than *S. capitata* and remained higher than *S. capitata* from the standardization pruning. The beginning of the experiment with CO₂ fumigation and heating plots was on 22nd April 2014. On 29th April 2014

plant were fertilized with urea (120 kg ha^{-1}) on soil surface. Fertilization and the irrigation were done to study *S. capitata* growth in the consortium without water and nutritional constraints.

3.2.2 THE TROP-T-FACE SYSTEM, THE WATERING FACILITIES, AND THE TREATMENTS

The Trop-T-FACE system was previously described (Section ‘The Trop-T-FACE system’, pages 22 to 29). The experiment occurred from 23rd April to 24th May 2014, during autumn, in irrigated consortium with *Panicum maximum*. This experiment occurred at the same time and place of the one in Chapter 2, but evaluating *S. capitata* growth. The data collection occurred at initial phase of the consortium.

The design of this experiment to evaluate the *S. capitata* growth and biomass gain included four treatments with four replicates each: ambient conditions (Control), elevated atmospheric CO_2 concentration to 600 ppm (eC), elevated canopy temperature $+2 \text{ }^\circ\text{C}$ above the ambient temperature (eT), and elevated CO_2 concentration and temperature (eC+eT), totaling 16 experimental units.

3.2.3 MEASUREMENTS ON CROWN AND DATA ANALYSIS

Data collection occurred at day 1 after start of treatments (April, 24th) and at 16 (May, 09th), 23 (May, 16th) and 30 (May, 23rd) days after start of treatments, during the early autumn in Brazil. Each ring of treatment was divided into quadrants. We considered the shoot as the unit to determine the vegetative and reproductive growth. The selected buds of shoots unfolded on five bushes of *S. capitata* randomly chosen in one quadrant of each ring in every treatment. The sample unit (shoot) was composed of the main branch and its ramifications and components (leaves and inflorescences). Three buds with maximum 2 cm long were chosen in each of five bushes, resulting in 15 selected shoots per ring, totaling 60 shoots for treatment.

The vegetative growth traits recorded on in each period of measurements on marked shoots were the number of leaves per shoot, number of ramifications per shoot and shoot length. To achieve the number of leaves per shoot we considered the sum of the number of leaves on the main branch and its subsequent ramifications. This parameter allowed evaluating the number of leaves per unit of shoot length in each treatment. The number of ramifications emerged in each shoot indicated the branching intensity advanced by lateral buds in each treatment. The shoot length was considered as the sum of the length of the main

branch plus the length of its ramifications. This parameter indicated the linear shoot growth promoted by the intensity of apical meristem activity in each treatment.

We recorded the number of flowered ramifications and the number of flowers in each ramification apiece of initially marked shoot for analyzing the reproductive growth. We considered the presence or absence of flower in each ramification to obtain the number of flowered ramifications. From these measurements, the percentage of flowering per initially marked shoot in every treatment was calculated as the number of ramifications on the shoot that flowered divided by the total number of ramifications on the shoot in each treatment. This parameter indicated the flowering capacity of ramifications in each treatment. The number of flowers per shoot was calculated as the total number of flowers present in each treatment divided by the total number of shoots. It indicated the flowering intensity on shoots in each treatment. The number of flowers per flowered ramification was calculated as the total number of flowers found in each treatment divided by the total number of ramifications with flowers. This parameter indicated the intensity of flowering in ramifications carrying flower in every treatment.

On 30th May 2014 (37 days after starting treatments), we collected the selected shoots for growth analyzes in each ring cutting them at its point of origin (from the place where it was located the gem labeled the beginning of the experiment) and they remained intact. Foliage and stalk of each marked shoot have been identified separately and we took them to dry out in a forced air circulation stove at 60°C for obtaining the dry weight of leaves and stalks of each shoot. We calculated the data of leaf and stalk biomass per shoot and the average daily gain in leaf biomass after the drying process. We obtained the average daily gain by dividing the leaf biomass values per number of days of the experiment.

We used Pearson correlation coefficient to identify which environmental factor is correlated to the increase in soil and canopy temperature. For this, we utilized the maximum temperature values in soil and canopy at Control and heated plots and analyzed their correlation with the maximum values of wind speed and solar irradiance. The p-value considered for these correlation was 0.05, where $p < 0.05$ indicates significant correlation. We performed this analysis with BioEstat software, version 5.3 (Instituto Mamirauá, Brazil).

We also performed the vegetative growth data analysis with BioEstat 5.3 software (Instituto Mamirauá, Brazil). We used D'Agostino-Pearson test to verify the distribution of datasets of vegetative growth. Since the datasets were not normally distributed, we used the nonparametric Mann-Whitney test with p-value at 0.1 to compare average values of

vegetative growth among treatments. We performed the reproductive growth data analysis using the GraphPad InStat software version 3.01 (GraphPad Software Inc., USA). We utilized Fisher's exact test with P-value at 0.1 to compare the flowering among treatments. When P-values are lower than 0.1 the differences of mean values among treatments were considered significant.

3.3 RESULTS

3.3.1 METEOROLOGICAL CONDITIONS

The experiment happened at the same time and place that one of Chapter 2. So, the meteorological conditions are identical to those of Chapter 2 as we describe below.

The course of the daytime was usually free of clouds with solar irradiance between 0.7-0.9 kW m⁻² around midday during the period of the experiment (Fig 3.1A). Nonetheless, cloudy days with solar irradiance around 0.5 kW m⁻² occurred at 23 and 30 days after start of treatments (DAT) (on 18th and 23rd May 2014, respectively). The highest solar irradiance value (around 0.87 KW.m⁻²) was registered on 24th April 2014 at 12:45 PM (Fig 3.1A). At this day, canopy temperature reaches approximately 32.3 °C in warmed plots, around 12 °C above environment average temperature (Fig 3.1E), when the air relative humidity reached 62.2% (Fig 3.1B). Air relative humidity values at midday were never less than 29.5% (Fig 3.1B). Average air relative humidity during the experiment was 75.9%. Reduced air relative humidity occurred together with peaks of temperature and solar irradiance, usually around midday (Fig 3.1A, B, and F).

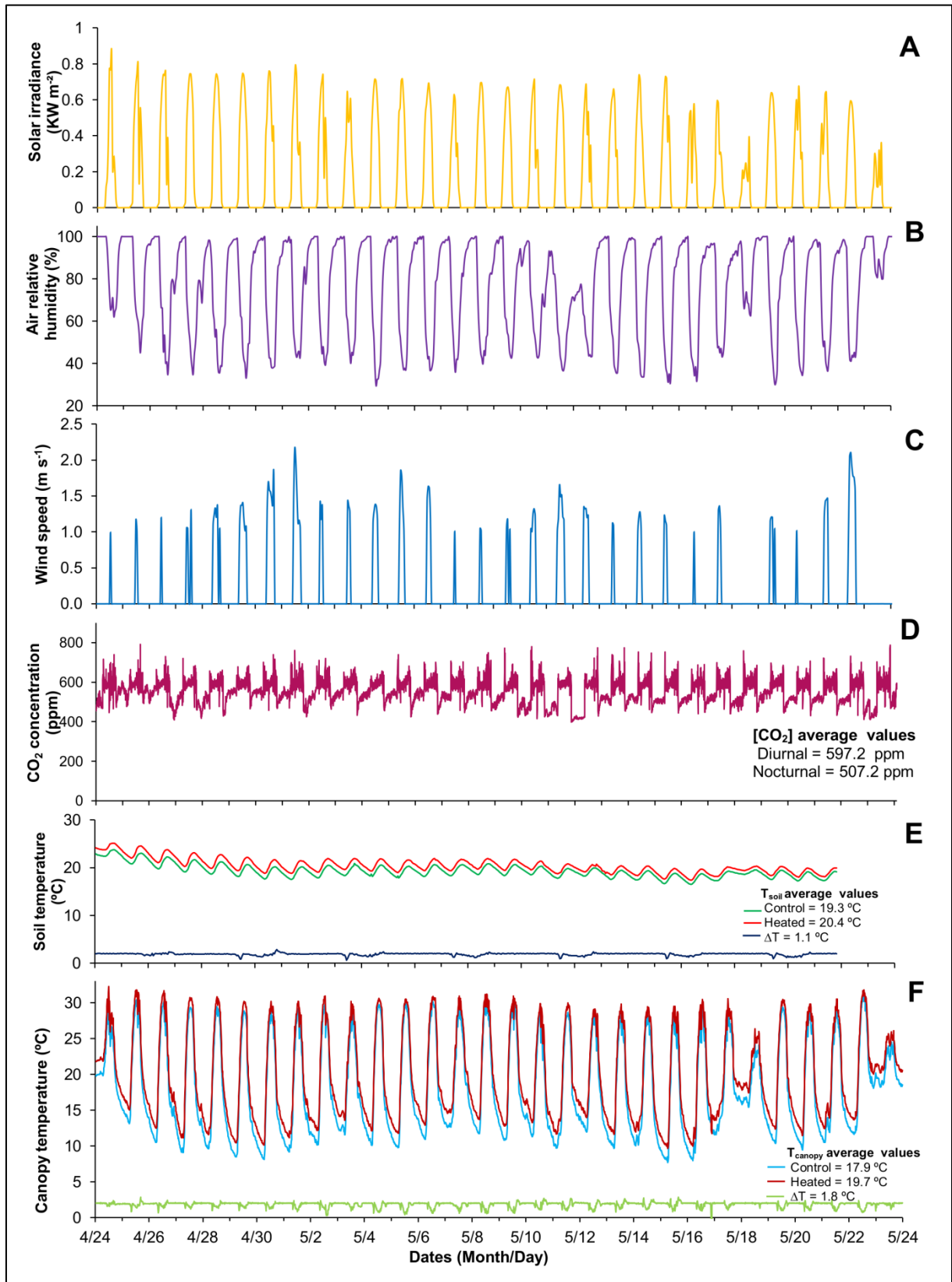


Fig 3.1 The daily courses of total solar irradiance (A), air relative humidity (B), and wind speed (C) recorded from April 24th 2014 to May 24th 2014 at the center of the experimental area. It is also showed the CO_2 concentration in the enriched atmosphere (D) with average values of nocturnal and diurnal CO_2 concentration, and the soil (E) and the canopy (F) temperatures in heated and in control regimes, respectively, with the averages and the temperature difference between them (Δ).

The maximum value of canopy temperature (32.7 °C) during the experimental period occurred at 29 DAT (on 22th May 2014, Fig 3.1F) and was not stressful for *Stylosanthes* even in warmed plots since it is tolerant to high temperatures (growth temperatures of 30-40 °C, Martinez *et al.*, 2014). Soil temperature varied between 16.3 °C to 25.3 °C in Control and warmed regimes (Fig 3.1E). The average soil temperature was 19.3 °C in Control plots and 20.4 °C in warmed plots (Fig 3.1E). The maximum value of soil temperature (25.3 °C, Fig 3.1E) was observed in the beginning of experiment on 24th April 2014. During experimental period the maximum soil temperature was under the better temperature ranges for growth and nitrogen fixation of *Stylosanthes* even in warmed plots (40/30 °C, day/night root temperature, respectively (89)).

Average air temperatures values obtained at experimental period was lower than historical data (1982-2012) for the months of April and May. Historical data brings average temperature of 21.15 °C and minimum and maximum temperature of 14.95 °C and 27.35 °C respectively (68). The canopy temperature varied between 32.7 °C and 7.6 °C during the daily courses among all treatments (Fig 3.1F) with the target canopy temperature reaching +2 °C in heated regime, especially during the night (Fig 3.1F). The soil temperature varied between 19.3 °C and 20.4 °C during the daily courses (Fig 3.1E) with the difference between heated and control soil temperature reaching +1.1°C over the period of experiment (Fig 3.1E). Canopy and soil temperatures were more closely correlated to solar irradiance than with wind speed (Table 3.1). Indeed, soil temperature had no correlation with wind speed (Table 3.1). The wind speed rarely exceeds 2 m s⁻¹ and only at 8 DAT (1st May 2014) it was 2.2 m s⁻¹ (Fig 3.1C).

Table 3.1. Pearson correlation coefficient (R) between the maximum temperature values in soil (T_{soil}) and canopy (T_{canopy}) at control and heated plots and the maximum values of solar irradiance and wind speed.

Pearson correlation coefficient (R)	T _{soil}	T _{soil}	T _{canopy}	T _{canopy}
	Control	Heated	Control	Heated
Solar irradiance	0.61**	0.65**	0.50**	0.56**
Wind speed	-0.04 ∅	0.03 ∅	0.48*	0.44*

The greater the R-value (positive or negative), the greater the correlation between the parameters. The R-values from 0.5 to 0.7 (positive or negative) indicate moderate correlation and are marked by a double asterisk (**). The R-values from 0.3 to 0.5 (positive or negative) indicate weak correlation and are marked by a single asterisk (*). R-values from 0 to 0.3 (positive or negative) indicate an insignificant correlation between the parameters and are marked by ∅ symbol.

The average values of CO₂ concentration at enriched plots (Fig 3.1D) was 597.2 ppm during daytime and 507.2 ppm at night. Nocturnal CO₂ concentration was higher than average value of 404 ppm of CO₂ in the Earth atmosphere (2) due to plant respiration as the miniFACE system was off at night. In Control plots, the average diurnal CO₂ concentration was 393±10 ppm and the average nocturnal CO₂ concentration was 438±20 ppm (data not shown).

3.3.2 VEGETATIVE GROWTH

The changes on vegetative growth of *S. capitata* in each treatment were subtle. There were no significant differences among atmospheric regimes regarding the biomass of shoot, leaf, or stalk (Fig 3.2A). Indeed, we found no significant differences in daily gains of shoot nor stalk (data not shown) biomass among atmospheric regimes.

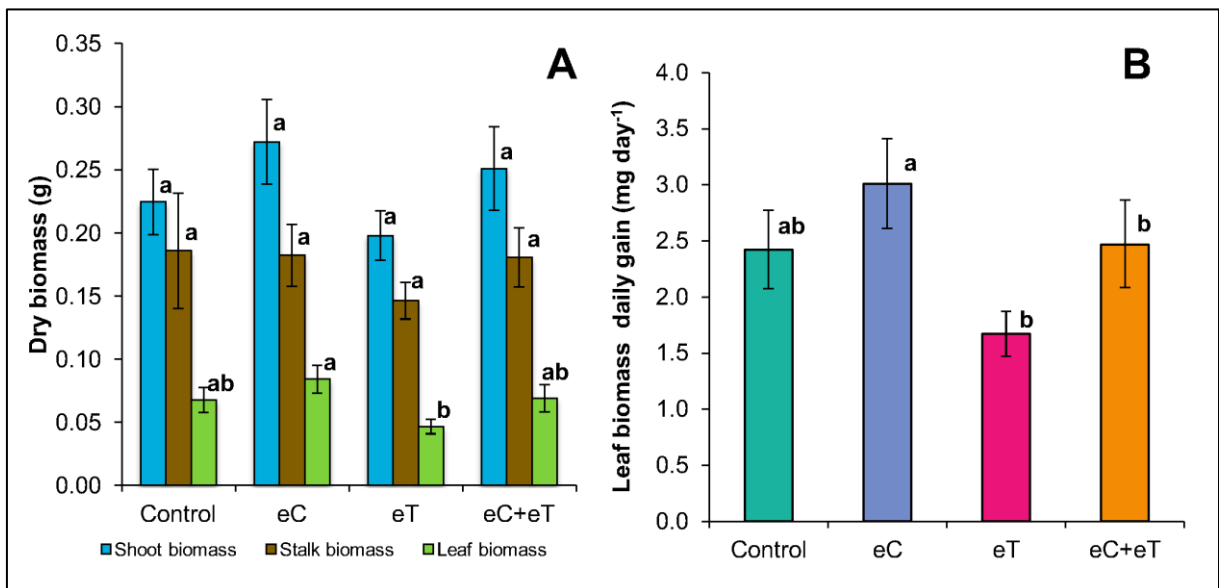


Fig 3.2 Total dry biomass per shoot (A) and daily dry biomass gain of leaf per shoot (B) of *Stylosanthes capitata* growing in consortium with *Panicum maximum* under ambient CO₂ and temperature (Control), 600 ppm of CO₂ (eC), elevated temperature at 2 °C above ambient (eT), and the combined treatments (eC+eT). Bars show the averages and lines at the top of the bars the standard error values. Different letters above bars indicate significant differences among datasets after the Mann-Whitney test at p<0.1.

Under eC, there were no significant increments in the leaf biomass (Fig 3.2A), number of leaves (Fig 3.3B), or even in number of leaves per length of shoot (Fig 3.3D). Despite some larger values under eC, there was no difference between eC and Control regimes in any trait considered on shoot bases (Figs. 3.2 and 3.3).

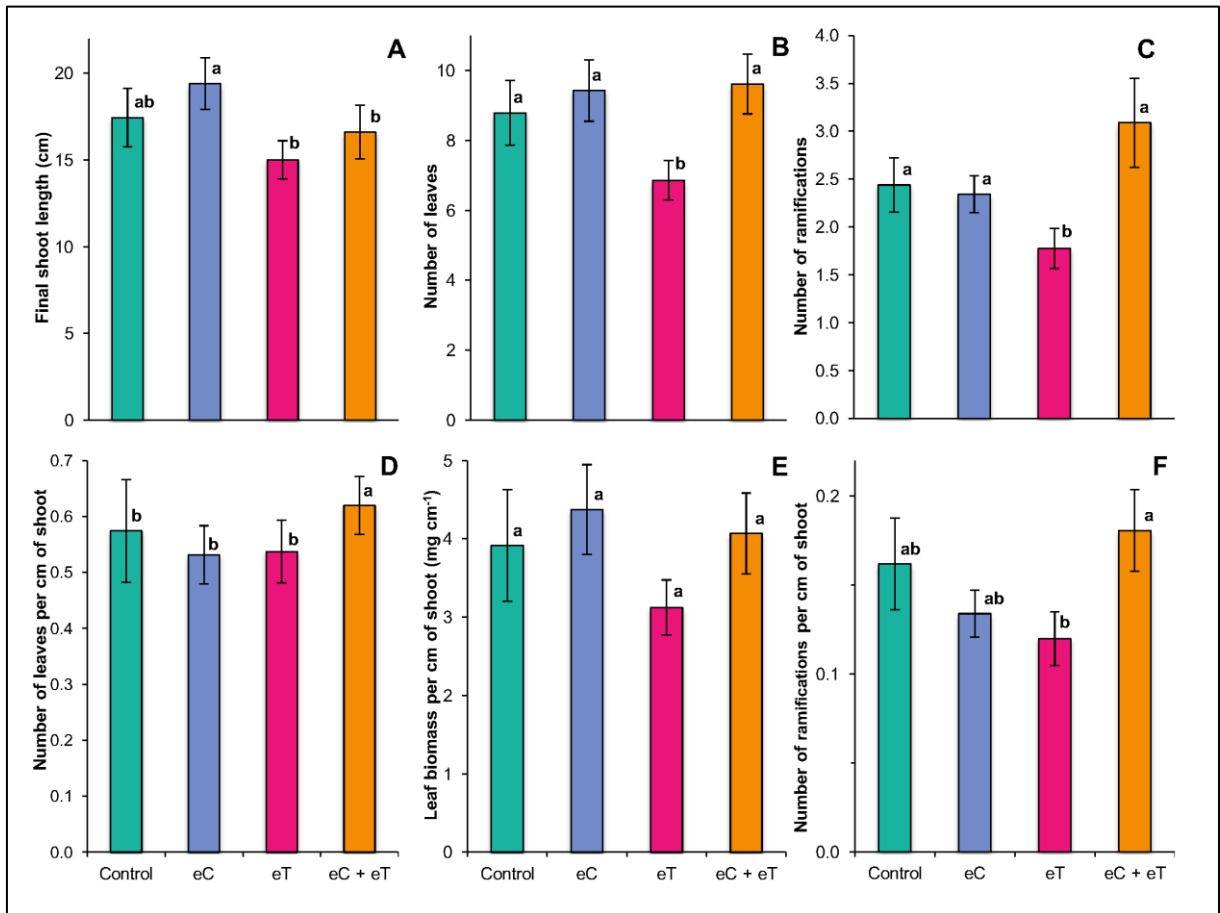


Fig 3.3 Final shoot length (A), and the number of leaves (B) and ramifications (C) per shoot of *Stylosanthes capitata* growing in consortium with *Panicum maximum* under ambient CO₂ and temperature (Control), 600 ppm of CO₂ (eC), elevated temperature at 2 °C above ambient (eT), and under combined treatments (eC+eT). It is also showed the number of leaves (D), the leaf biomass (E) and the number of ramifications (F) per centimeter of shoot. Bars show average values and lines at the top of bars the standard error. Different letters above bars indicate significant differences among datasets after the Mann-Whitney test at $p < 0.1$.

On the other hand, there were some significant differences between Control and eT in some measurements. Under eT, there were fewer leaves per shoot (Fig 3.3B) and fewer ramifications (Fig 3.3C) than Control. Also, the daily gains of leaf biomass (Fig 3.2B) and the shoot length (Fig 3.3A) were smaller in eT than eC. Indubitably, the increased temperature acted negatively on foliage and inhibited the *S. capitata* shoot growth. Thus, there was a contrasting behavior under eC and eT about the features measured on shoot bases. The increase in CO₂ concentration acted positively on the characteristics determined per shoot, and the rise in temperature showed the opposite effect (Fig 3.2 and 3.3).

The vegetative growth stimulation by eC and the inhibition by eT become even more evident when both regimes were compared with eC+eT. In treatments in which eC was applied (eC and eC+eT), the number of leaves (Fig 3.3B) and the number of ramifications (Fig 3.3C) were larger than eT. Therefore, the adverse effects of eT on *S. capitata* vegetative

growth appeared to be mitigated when eC was combined with warming. Again, leaf biomass (Fig 3.2A) was larger in eC than eT, but under eC+eT it remained similar to the Control. Indeed, in general, under eC+eT, there were no significant statistical differences in vegetative growth (Fig 3.2 and 3.3) comparing to the Control. On the other hand, the combination eC+eT modified the distribution of leaves on shoots of *S. capitata* (Fig 3.3D). Therefore, the contrasting effects between eC and eT, promoting and impairing biomass production, respectively, on shoots of *S. capitata* resulted in a set of data equivalent to the Control under combined treatments eC+eT (Fig 3.2 and 3.3). Notwithstanding, the combination eC+eT changed the display of foliage on shoots (Fig 3.3D).

In summary, the increase in temperature had the antagonistic effect of increased CO₂ concentration on growth of *S. capitata* during autumn in the consortium. The rise in temperature somehow impaired the vegetative shoot growth while the increase of CO₂ concentration promoted it. Positive effects of elevated CO₂ concentration appeared clearly mitigating the adverse impacts of elevated temperature under combined treatment. On the other hand, eC+eT affected shoot development changing the display of leaves on shoots. Despite the promotion of biomass production, single elevated concentration of CO₂ could not increase significantly the biomass of foliage or stalk on shoots under field conditions.

3.3.3 REPRODUCTIVE GROWTH AND PHENOLOGY

Fig 3.4 shows the percentage of flowered ramifications in relation to the total number of ramifications on the shoots in each treatment (see ‘shoot’ definition on ‘measurements on crown and data analysis’ at ‘Materials and Methods’ section). Besides, in Fig 3.4 there are the number of flowers per shoot and the number of flowers per flowered ramification on the shoot. Under eC+eT, it was observed higher values of flowering per shoot (Fig 3.4). Hence, the increase of temperature combined with elevated CO₂ concentration promoted the amount of ramifications carrying flowers and the number of flowers in flowered ramification per shoot. On the other hand, the flowering percentage under eC+eT (40.5%, Fig 3.4) did not show statistical difference in relation to control and eT (Fig 3.4), but it was larger than eC (16.2%, Fig 3.4) indicating that the temperature is promoting flowering in this case.

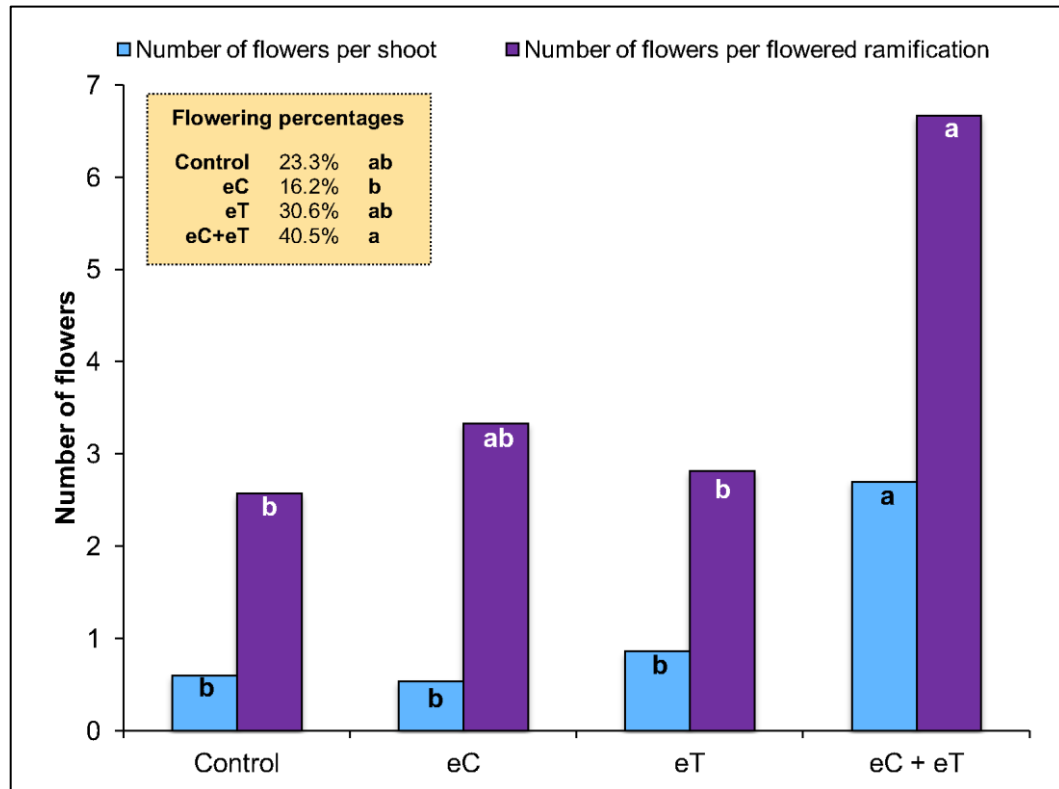


Fig 3.4 Number of flowers per shoot and number of flowers per flowered ramification of *Stylosanthes capitata* growing intercropped with *Panicum maximum* under ambient CO₂ and temperature (Control), 600 ppm of CO₂ (eC), elevated temperature at 2 °C above ambient (eT), and in combined treatments (eC+eT). Bars show average values and different letters at the top of bars indicate significant differences. The numbers in the panel at the left corner of the graph indicate the flowering percentage and different letters in front of the number indicate significant differences. Differences were tested by Fisher exact test at $p < 0.1$.

The eC and eT treatments featured the percentages of flowering equal to control (Fig 3.4). The analysis of the number of flowers and the number of ramifications showed better the differences found among atmospheric regime. Under eC+eT there were more flowers per flowered ramification (6.7, Fig 3.4) than under control (2.6, Fig 3.4) and eT (3.3, Fig 3.4). When we considered the relation between the number of flowers on the total evaluated ramifications (Fig 3.4) it is clear that there were differences between eC+eT and the others regimes. Considering the appearance of flowers per flowered ramification, there were 4.5 times more flowers per shoot in treatment that reproduces the predicted climatic conditions (eC+eT, Fig 3.4) than in the current climatic conditions (Control, Fig 3.4). Therefore, under future condition in 2050, *S. capitata* will have more flowered shoots, more flowers per flowered ramification and more flowers per shoot than now (Control) growing free of water and mineral shortage. On the other hand, the increase in CO₂ concentration (eC) did not influence the appearance of flowers per flowered ramification, being this value statistically equal to all other treatments (Fig 3.4).

The flowering increased and was most intense from 16 DAT (May 9th) in all treatments (Fig 3.5B). Similarly, the branching process was intensified after that time in eC and eC+eT (Fig 3.5). Under eT, the branching process was somehow impaired since the average number of ramifications increases only from 9 to 16 DAT (02nd to 09th May, respectively) (Fig 3.5A).

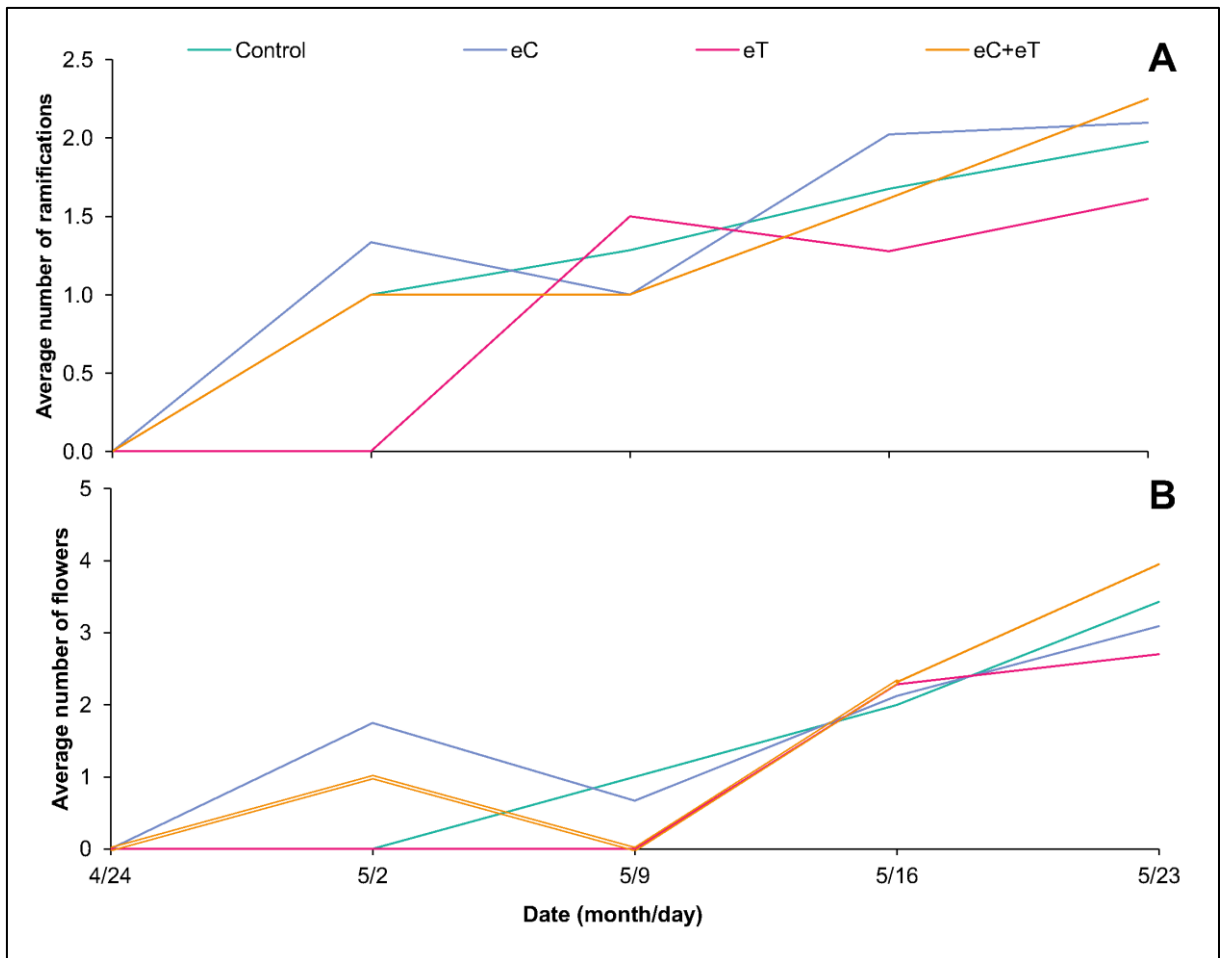


Fig 3.5 Average values of number of ramifications (A) and flowers (B) per initial marked shoots of *Stylosanthes capitata* growing intercropped with *Panicum maximum* under ambient CO₂ and temperature (Control), 600 ppm of CO₂ (eC), elevated temperature at 2 °C above ambient (eT), and in combined treatments (eC+eT) throughout the experimental period.

3.4 DISCUSSION

3.4.1 VEGETATIVE GROWTH AND ENVIRONMENTAL CONDITIONS

The environmental conditions directly influence plant growth. T-FACE systems allow study of the effects of elevated temperature and CO₂ concentration on plants and ecosystems grown under natural conditions without enclosure (31,59). The above ground biomass gain obtained with FACE system are below to those achieved in CO₂ chambers (31,33) mainly because phytotrons and open top chambers impose limited soil volume to root growth and mitigate natural factors such as temperature variation and wind (31). In addition, phytotrons and chambers have been working with a mean CO₂ concentration of 700 ppm while FACE systems use 550 to 600 ppm (4). Using T-FACE system, we were able to control CO₂ concentration enrichment at predicted levels and canopy temperature without wind attenuation or over increase temperature. Besides, plants growth in field, not in pots, such as in a pasture, approaching our experiment closer to natural conditions as we proposed.

To perform this experiment, we provide three conditions that promoted plant growth: 1. CO₂, present in the atmosphere in the Control (around 400 ppm) and at a concentration of 600 ppm maintained by the FACE system in the treatments with CO₂ enrichment; 2. Water, supplied by irrigation system; and 3. Nutrients in the soil, deposited during fertilization at planting. In addition, the environmental conditions during the experimental period were not enough to cause nutritional or water stress to the plants (87,89). Moreover, meteorological conditions were consistent with the historical average weather (1982-2002) for the months of April and May at the experimental location (68). However, in this experiment, the plants of *S. capitata* were cultivated in consortium with *P. maximum*. The nitrogen fertilization during the implementation of the experimental area is much more favorable to *P. maximum* than to *S. capitata*, and the grass grows even faster. So, the competition by light between both crops would have effects on the responses of both *S. capitata* and *P. maximum* to elevated CO₂ and warming treatments.

The relative humidity and the temperatures of canopy and air are abiotic factors that influence gas exchange between the plant and the atmosphere and the plant growth (90). In our experiment, the solar irradiance appeared as the environmental factor that was determining the canopy temperature in the plots and, less strongly, the relative humidity. The maximum solar irradiance values were correlated with the canopy temperature in all treatments (Table 3.1). The wind, in turn, was weakly correlated with the canopy temperature,

and did not influence the canopy temperature in this experiment (Table 3.1). However, the wind is responsible for remove the gaseous volume of the boundary layer saturated with water vapor near leaf epidermis renewing this air with a non-saturated amount of water vapor over the transpiring surface (90). With increasing wind speed, the boundary layer resistance decreases (90,91). A wind speed of less than 2.0 m s^{-1} , as in our experimental condition most of the time, is unable to remove the boundary layer of water vapor between the leaf and the atmosphere (90). Therefore, gas exchange tends to be reduced in light winds conditions because resistance to gas exchange between the leaf and the atmosphere are elevated in that conditions (91). Only wind speed of 2.0 m s^{-1} or more are able to cause boundary layer resistance less than 0.5 s cm^{-1} , insignificant compared to the stomatal resistance (90), thus improving transpiration and another gas exchange.

Despite several conditions being favorable to plant growth, we did not observe a significant positive growth response. However, the increase in CO_2 concentration under eC in autumn directly influenced *S. capitata* leaf development in the consortium. The impact in leaf biomass is positive and evident only when eC is compared with eT. Under eC, there was greater leaf biomass at the end of the experiment (Fig 3.2A) and higher daily gains in leaf biomass (Fig 3.2B) than eT. Plant height and biomass production of a consortium of *Stylosanthes hamata* with *P. maximum* were influenced significantly under elevated CO_2 ($600 \pm 50 \text{ ppm}$) in open top chambers (35). *S. hamata* height increased by 49.0% and the consortium biomass production increased 85% over the control under elevated CO_2 (35). Growth increase is expected for C_3 plants under CO_2 atmospheric enrichment (53,92,93). Nonetheless, the increments in foliage under eC that we obtained were not sufficient to cause significant differences in regarding to Control.

On the other hand, the adverse effects of elevated temperature in the consortium were mitigated by the elevated carbon supply for photosynthesis under eC+eT. Heat increment combined with high CO_2 concentration promoted the number of leaves per shoot length (Fig 3.3D). However, the number of leaves (Fig 3.3B) and the gains in shoot and leaf biomass (Figs. 3.2B and 3.2D) are similar to the control. Elevated CO_2 concentration in the FACE system stimulates net photosynthesis by increasing the intracellular concentration of carbon (42). Night-time warming combined with elevated CO_2 concentration synergistically increased plant carbon uptake in early season as seen in the C_3 grass *Deschampsia flexuosa* (42). Here, enhancing CO_2 concentration was only sufficient to mitigate negative effects of increasing temperature on *S. capitata* growth.

S. capitata is adapted to tropical climate and elevated temperatures as the achieved on our experiment were in tolerance range for the species (87,89,94). However, *S. capitata* was intercropped with *P. maximum* and because it was a consortium there was competition between species. Interspecific competition may be responsible by the responses that we obtained. The difference between *S. capitata* growing individually and in consortium can be established by comparing the results of the present experiment with those obtained at same experimental area with same procedures of irrigation and fertilization in the autumn 2013 by Martinez *et al.* (87). These authors obtained a biomass gain of 16% in *S. capitata* cultivated in monoculture under eT during the autumn 2013. In autumn season, *S. capitata* received light and heat without attenuating by *P. maximum* and showed a vigorous growth under eT in monoculture. However, in consortium the C₄ *P. maximum* grows faster than *S. capitata* in the beginning, especially under eT. This condition generated shading between C₃ and C₄ species. Besides, as C₄ grew faster under eT, *P. maximum* captured the most part of heat increment of +2 °C. In fact, the soil temperature measurements (Fig 3.1E) indicates that the heat is attenuated by the leaves of *P. maximum* above *S. capitata* leaves ($\Delta T_{\text{soil}} = 1.1$ °C and $\Delta T_{\text{canopy}} = 1.8$ °C, Figs. 3.1E and 3.1F, respectively). The effects of competition in heated plots probably were intensified favoring *P. maximum*. In a previous work, Prado *et al.* (14) found significant effects of warming on leaf growth and leaf development in *Panicum maximum* grown in consortium. Therefore, the impairment to C₃ growth under eT was probably about the competition with C₄ species, not because the elevated temperature *per se* (87). Bazzaz *et al.* (92) studied a C₃ and a C₄ plant growing in a consortium and individually under atmospheric CO₂ enrichment. When C₃ and C₄ grow separately in a CO₂-enriched atmosphere, the C₃ accumulates more biomass than C₄. When planted together the differences between C₃ and C₄ under increased CO₂ tended to disappear (92). Besides, as C₄ have a faster growth the C₃ species loss competition with C₄ above and below ground (92).

The differences observed between eC and eT treatments here is not due the gains provided by the increased CO₂ nor the direct negative effect of heat under eT treatment during autumn in the consortium. The light wind condition, the competition with C₄ in consortium and the mitigation effect of high concentration of CO₂ in increased temperature resulted in non significant differences in *S. capitata* growth under eC+eT in relation to Control in autumn.

3.4.2 REPRODUCTIVE GROWTH AND PHENOLOGY

S. capitata flowering is apical or axillary (88). As apical dominance stops after apical flowering, the lateral buds unfold into new ramifications or flowers. Higher temperatures contributed to hastened bud formation (95,96). Under eC+eT treatment, there was a greater flowering percentage (Fig 3.4) probably due the lowest leaf area of *P. maximum* under eC+eT (Chapter 2) and better access of *S. capitata* to light and heat in the consortium. Also, we observed more ramifications of *S. capitata* (Fig 3.3C) under eC+eT, probably stimulated by the cessation of apical dominance by unfolding the apical meristem into flower. The analysis of the production of flowers and ramifications under eC+eT throughout the experimental period allowed to confirm that the branching process intensifies when flowering becomes more vigorous at 16 DAT (May 9th, Fig 3.5A and B). The formation of new branches was made possible by the presence of more carbon to build the buds and then the flowers and branches. In the eC+eT treatment, the presence of more carbon enabled the plant to branch more intensely.

Under eT, although the number of ramifications was the lowest observed (Fig 3.3C), there was a differentiated flowering percentage (30.6%, Fig 3.4) in relation to Control. The elevated temperature favored the flowering of *S. capitata* and the process of ramification is clearly hampered under eT throughout time (Fig 3.5A). Under eT, *S. capitata* were investing on reproductive growth instead vegetative growth. High temperatures can induce changes in the phenology of plants and change the networks of interaction between plants and pollinators (97–99). For example, elevated temperatures are responsible for the decrease in the number of flowers of some species (100), but warming may increase flowering in others (101). These opposite effects caused by the elevated temperature in the production of flowers suggest that some particular species are more susceptible to high temperatures while others are more tolerant (102). Typically, plants that depend on temperature signals to regulate flowering and those that are more limited by the availability of nitrogen than water may be better adapted to respond to warming (103,104).

Even with stimulated ramifications formation (Fig 3.3C) under eC+eT, the leaf biomass remained similar to current climatic conditions (Fig 3.2A). In drought seasons, cattle prefers *S. capitata* leaves before consuming *P. maximum* due to the better nutritional value (105). With more flowers and no increase in leaf biomass under eC+eT, *S. capitata* will be not an suitable forage in consortium with *P. maximum*.

3.4.3 FINAL CONSIDERATIONS

With increased concentration of CO₂ and subsequent increase in global temperatures, an increase in canopy temperature regardless of the amount of water available to the consortium is expected even with irrigation. In other words, the canopy temperature will increase even with irrigation, because of the increase of the Earth surface temperature. Moreover, our experimental area have wind speed below 2 m s⁻¹ most of the time keeping elevated the boundary layer resistance to gas exchange such as transpiration, the main process of heat dissipation on leaves. Thus, the wind speed in this case did not condition canopy temperature. If solar irradiance condition canopy temperature at this time of year and wind speed did not, canopy temperature were a response to solar irradiance, not wind speed, at the irrigated consortium.

The experiment was accomplished in an area with fertilized soil and with constant irrigation, ensuring the supply of nutrients and water to plants. By deploying a grassland area, the soil is corrected and fertilized, but irrigation of an extensive pasture area is economically unrealistic and put the ecosystem on risk. Salinization of soil, contamination, and loss of water courses are some consequences of water over use (106). Considering a situation in which grazing areas are not irrigated and are subject to elevated CO₂ concentration and temperature, *S. capitata* will find a much less favorable condition for growth with *P. maximum* in the near future climate in autumn. Irrigation is required for the consortium, but is innocuous for the purpose of lowering the temperature. Without irrigation, the consortium will probably be unfeasible. With irrigation, it will be probably economically unfeasible. It is predicted that the future climate will change rainfall pattern in the Southeast of Brazil (107), which has the second largest Brazilian cattle herd (18). Under water shortage the growth of *S. capitata* might be further reduced, which might compromise the use of this C₃ as forage in consortium with a C₄ *P. maximum*. However in recent years, in an attempt to increase the production of forage during the dry season and mitigate the effects of water stress, irrigation of pastures has been deployed in numerous properties in Central Brazil. However, little is known about the productivity of grasses, especially in winter. In *P. maximum* (108) and *Brachiaria brizantha* (109) there was a positive effect of irrigation on the fresh leaf production from the end of dry season and winter, when the photoperiod was probably not a limiting factor. Pizarro *et al.* (110) found that under irrigation was produced up to three times more pure seed of *Stylosanthes guianensis* in the Brazilian Cerrados in the dry season than when irrigation was not applied.

In short, competition with *P. maximum* in consortium under increased temperatures was the main factor influencing the growth of *S. capitata* during autumn. Under treatment that simulates future climate change scenario in autumn and free of water and nutritional stresses, the vegetative growth of *S. capitata* showed no significant increments of leaf biomass in consortium with C₄. On the other hand, under elevated temperature conditions there was flowering increment. Promotion of flowering may be advantageous to increase the population of plants through the formation of a denser seed bank ensuring the survival of the species. However, the promotion of flowering occurred at the expenses of leaf biomass, with no advantage for using *S. capitata* as forage.

3.5 CONCLUSION

We conclude that climate change will not bring benefits to *S. capitata* use as pasture in consortium with *Panicum maximum* during autumn under future climate change. Even without water and nutritional shortage, the predicted climate to 2050 will not result in any increase to *S. capitata* leaf biomass gain as a consequence of elevated temperature and CO₂ concentration in atmosphere. On the contrary, elevated temperature and CO₂ concentration in 2050 will promote an increment of flowering at the expense of biomass accumulation in leaves in *S. capitata* even growing without water and nutritional stresses. The consortium of *Panicum maximum* with *S. capitata* already is rarely used, and did not appear to be an alternative pasture at Southwest of Brazil at near future.

- 4 -

RAIN-FED GROWTH AND FLOWERING OF FORAGE C₃ LEGUME IN FUTURE CLIMATE

ABSTRACT

Increasing emissions of greenhouse gasses are resulting in climate change with significant impacts on agriculture. Our objective was to evaluate the growth and biomass production of a C₃ shrub, *Stylosanthes capitata* under field conditions in a simulation of the climate predicted for 2050. Plant growth measurements were carried out weekly in autumn from April–June 2015. Plants grew without irrigation under a Trop-T-FACE system, which provides free-air carbon dioxide enrichment and enhanced temperature control under field conditions. We implemented four climatic regimes: control with current atmospheric conditions, high CO₂ concentration (600 ppm, eC), elevated canopy temperature (2 °C above ambient canopy temperature, eT), and a combination of eC+eT. Atmospheric CO₂ enrichment did not increase foliage area or biomass. Under eC, there was an increased investment in flowering, notably when soil water content was higher than 0.3m³m⁻³. Warming impaired vegetative growth and raised shoot mortality. Under eC+eT, there was no mitigation of the adverse effects of warming, and growth was even more impaired. The climate predicted for 2050 will be harmful to *S. capitata* growing without irrigation during autumn. The elevated temperature and the lower soil water content will impair vegetative growth and increase shoot mortality. Elevated atmospheric CO₂ concentration acting on the C₃ photosynthesis pathway will be not enough to mitigate the adverse effects of warming and autumn water shortages. CO₂ enrichment will only promote flowering during the period with favourable soil water content, and an increase in the number of flowers per shoot instead of an increase in leaf biomass will not be beneficial to livestock.

Keywords – climate changes, foliage biomass, leaf area, leaf tissues, *Panicum maximum*, shoot compartments, soil water content, *Stylosanthes capitata*

4.1 INTRODUCTION

The atmospheric concentration of CO₂ has grown exponentially since the Industrial Revolution and now exceeds 400 ppm, 30% greater than 50 years ago (2). Atmospheric CO₂ could exceed 600 ppm in 2050 and double by the end of the century (4). The fourth and fifth reports of Intergovernmental Panel on Climate Changes IPCC (1,3) indicated a high correlation between the increasing of greenhouse gas (GHG) concentrations and the average increase in the Earth's surface temperature. Anthropogenic activities, such as burning fossil fuels and land use, are the major causes for increased GHG emissions (3). Along with increases in the global, seasonal and daily average atmospheric temperature (1,28) there will be alterations in rainfall patterns in many regions of the Earth (11).

Plants are dependent on various environmental factors such as solar irradiance, temperature, nutrient supply, and water availability. Any changes in these factors will affect the whole trophic chain of the planet and will have a significant economic and ecological impacts on grassland and forests (27). Carbon dioxide plays a major role in plant metabolism because it is the only carbon source for the photosynthetic production of carbohydrates. The current atmospheric concentration of CO₂ is lower than the saturating amount for C₃ photosynthesis (28), and so C₃ species tend to respond positively to increased atmospheric CO₂ levels (29). Mathematical models predict that CO₂ concentration of 600 ppm could increase C₃ photosynthesis up to 40% (4). Indeed, *Stylosanthes hamata* (L.) Taub, a C₃ legume, produced 85% more fresh and dry biomass under 650 ppm CO₂ when grown in concert with *Panicum maximum* Jacq., a C₄ grass, in open top chambers (OTCs, (35)). An increase in atmospheric CO₂ concentration caused an increase of 30% in the dry biomass production in a pasture containing two C₃ forages species (*Lolium perenne* L. and *Trifolium repens* L., a grass and a legume, respectively) grow in experimental enclosures (36,37). Elevated CO₂ concentrations also caused a 25% increase in yield in the C₃ legume *Arachis glabrata* Benth. when intercropped with the C₄ grass *Paspalum notatum* Flügge in a temperature-gradient greenhouse (38). These increments involve not only physiological adaptations but also leaf anatomical alterations, such as increased mesophyll size and increased numbers of chloroplasts per cell (39).

On the other hand, the stimulation of photosynthesis in C₃ plants under CO₂ concentrations from 500–600 ppm was significantly lower (14%) in field experiments

conducted with free-air carbon dioxide enrichment using the FACE system (Ainsworth and Long, 2005; Leakey *et al.*, 2009; Long *et al.*, 2006). This occurs because the concentration of CO₂ is lower in FACE than in OTCs (4), and because in FACE systems there is a direct influence of external factors on the plants (41), bringing the results of FACE closer to those in natural settings (31,41). Elevated CO₂ concentration may partially offset the harmful effects of warming on C₃ plants by synergistically increasing carbon uptake via photosynthetic capacity up-regulation and by better access to water (42). Nonetheless, for each 1.0 °C increment in seasonal temperature, a reduction of between 3–16% in crop yield is expected (Streck, 2005; Battisti and Naylor, 2009) due to increased respiration and photorespiration in C₃ species. Also, a growth temperature above a finely tuned threshold can trigger flowering, bypassing the need for other inductive stimuli (49). High temperature during flowering may lower the effect of CO₂ by reducing grain number, size, and quality (50). Increased temperatures may also reduce the effects of CO₂ indirectly by increasing water demand (50). The combination of 450 ppm CO₂ and a 0.8 °C temperature increment increased yield by approximately 5.3% in rain-fed spring wheat. Nonetheless, the combination of 450 ppm CO₂ and a 1.8 °C increment reduced wheat yield by roughly 5.7% (51). Forecasts of how C₃ forage plants in the field will respond to a warmer, carbon-enriched atmosphere are necessary for predicting foliage production.

According to the Ministry of Agriculture, Livestock, and Supply (MAPA), Brazil has the second largest livestock population in the world, with about 200 million head (18). Since 2004, Brazil has been a leading exporter, responsible for 20% of all internationally traded meat, with sales in over 180 countries (18). The tropical climate and the size of the country contribute to this, as they allow cattle breeding in pastures over a significant part of the national territory. However, despite the great importance of pasturage to the food industry, there is not sufficient information to estimate the impact of climate change on tropical grasslands. As most Brazilian pastures are rain-fed, they probably will be impaired by the predicted changes in rainfall patterns in the tropics, such as poorly and unevenly distributed rainfall with long periods of drought (11). Physiological responses to drought include stomatal closure, decreased photosynthetic activity, altered cell wall elasticity, and even the generation of toxic metabolites that cause plant death (21). In addition, insect herbivory increases and plant biomass declines in water-stressed plants (22). Studies that investigate the

effects of water deficit from predicted future climates in tropical pastures are therefore relevant.

Stylosanthes capitata Vogel (C₃) is a forage legume of high quality, and when grown together with *P. maximum* Jacq. (C₄) its biomass production increases as does the nutritional quality of the forage (55). The *S. capitata* genome is similar to that of other species in the genus, such as *S. macrocephala* M.B. Ferreira & Souza Costa, *S. bracteata* Vogel, and *S. pilosa* M.B. Ferreira & Souza Costa (56). *S. capitata* has agronomic potential in Brazil because it is highly resistant to the anthracnose found in related species such as *S. macrocephala* (56). According to the Brazilian Agricultural Research Corporation (EMBRAPA), this forage is well accepted by livestock, has no compounds that interfere with livestock health, and is widely used in pastures (57). *S. capitata* is a perennial and responds positively to temperature increases when grown in monoculture free from water and nutritional impairments (87). However, when grown in concert with *P. maximum* under the same conditions, *S. capitata* had its growth impaired by temperature increase (LHG Camargo-Bortolin *et al.*, USP, Ribeirão Preto, Brazil, unpubl. res.).

Our experiment was conducted during the early autumn at the beginning of the dry season in southeastern Brazil when *S. capitata* was about to flower (88). We simulated the RCP6 climate scenario, a medium stabilization scenario for the year 2050 outlined by the IPCC (rain-fed, 600 ppm CO₂, and +2 °C). We employed a Free-air Temperature Controlled Enhancement system and a Free-air Carbon Dioxide Enrichment system for the tropics named Trop-T-FACE in a rain-fed *S. capitata* pasture to test the following three hypotheses.

First, at an atmospheric CO₂ concentration of 600 ppm, we expect *S. capitata* will have a significant growth increase and greater biomass production under rain-fed conditions due to a higher supply of carbon for C₃ photosynthesis (42). Elevated CO₂ concentrations are related to increases in net photosynthesis (32,42) and to strategies that withstand drought stress (29,32). The elevated CO₂ concentration will also affect flowering and may be as influential as the temperature increase in determining future changes in plant developmental timing (21).

The second hypothesis pertains to atmospheric warming in the absence of irrigation. Under these conditions, we expect severe impairment of the vegetative and reproductive growth of *S. capitata* and higher mortality of shoots. Drought may reduce net photosynthesis via low stomatal conductance and a reduction in photosynthetic capacity (42), while elevated

temperature may decrease the CO₂/O₂ specificity of Rubisco (111), thereby increasing photorespiration and carbon loss and so offsetting biomass gain. Although *S. capitata* is adapted to an elevated temperature (87), warming contributes to increasing water loss from the soil by evaporation (112,113).

Finally, under the treatment with both 600 ppm CO₂ and warming, the increase in air temperature may cancel the benefits of elevated CO₂ for photosynthesis (43). However, because high atmospheric CO₂ concentration is related to stomatal closure and further reduces transpiration (29), enriched atmosphere effects can mitigate water stress by increasing water use efficiency. Thus, under this treatment, we expect some aspects of *S. capitata* growth to be similar to the control, while still being hampered by the reduced water in the soil and by heating.

By testing these hypotheses, we aimed to demonstrate how the vegetative growth and flowering of *S. capitata* could be affected by increased temperature and CO₂ concentration in monoculture without irrigation. Studies of climate impacts on forage productivity are key to supporting pasture management, particularly in areas where livestock production makes a significant contribution to the economy, as it does in Brazil.

4.2 MATERIAL AND METHODS

4.2.1 EXPERIMENTAL AREA, SPECIES, PLANTING, AND STANDARDIZATION

The experiment was carried out at the Trop-T-FACE facility located on the campus of the University of São Paulo (USP) in Ribeirão Preto city, São Paulo state, Brazil (21°10'08.0"S and 47°51'49.5"W, 546 m a.s.l.). According to the Köppen-Geiger classification (64), Ribeirão Preto has an Aw climate, tropical with rainy summers (63). Historical data from 1982 to 2012 show 21.9 °C as the average annual temperature, with minimum and maximum annual temperatures of 18.4 °C and 23.9 °C during July and January, respectively (68). From April to June, historical data show an average temperature of 20.3 °C, with minimum and maximum temperatures of 13.8 °C and 26.8 °C, respectively, and a total annual rainfall of 1508 mm (68).

According to the U.S. Department of Agriculture (69), the soil in the experimental area is a dystrophic Red Latosol (Oxisol). After soil analysis, we performed soil liming to correct the pH from initial average values of 4.0–4.5 to 5.0–5.5. Chemical fertilization was applied in keeping with initial nutrient availability after pH correction, making the soil appropriate to *S. capitata* at the time of planting. On 14 and 15 Jan. 2015, the C₃ subshrub *S. capitata* was sown by rows in the centre of and surrounding the plots of every treatment. Seeds of *S. capitata* were put into holes 30 cm apart in 12 m × 12 m plots together with NPK 4-14-8 fertilizer at a dose of 1 t ha⁻¹ applied in the holes. We maintained only three plants per hole after germination. The genus *Stylosanthes* Sw. (Leguminosae) comprises 48 species with a pantropical distribution (88). *Stylosanthes capitata* Vogel is a perennial, 12 to 25 cm tall, with trifoliolate leaves. Inflorescences are terminal or axillary, simple or composed of two to four stalks, with 11 to 20 flowers each (88).

On 17th March 2015, 62 days after planting, the area was fertilized with urea in doses of 150 kg ha⁻¹ on the soil surface. On 10 April 2015, we cut the plants 35 cm above the ground and began the CO₂ supplementation and warming treatments. Irrigation was provided only on two days during seedling growth, on 24th and 29th April 2015, to stimulate early growth. Subsequently the plantation was rain-fed, with the most significant rains occurring on 4 (42 mm), 7 (14 mm), 10 (21 mm), and 19 May 2015 (16 mm).

4.2.2 TREATMENTS IN THE TROP-T-FACE FACILITY

The Trop-T-FACE system was previously described (Section ‘The Trop-T-FACE system’, pages 22 to 29). The experiment occurred from 30th April to 17th June 2015, during autumn, in monoculture of *S. capitata* without irrigation.

We designed the experiment to evaluate *S. capitata* growth and biomass yield under an elevated atmospheric CO₂ concentration of 600 ppm (eC), or a warming level 2 °C above the ambient temperature (eT), or under the combination of both high atmospheric CO₂ concentration and warming (eC+eT), for a total of 16 investigational units.

4.2.3 MEASUREMENTS OF THE SHOOTS

We measured vegetative and reproductive structures weekly during the Brazilian autumn, from 30th April 2015 to 17th June 2015. The sample unit, a shoot, was defined as the central stalk and its ramifications, leaves, and inflorescences originating from the same marked initial lateral bud. We selected buds of unfolded shoots on five randomly chosen individuals of *S. capitata* in one quadrant of each plot per treatment. We chose three buds with a maximum length of 2 cm on each of five individuals, resulting in 15 selected shoots per plot, totalling 60 shoots per treatment.

We recorded the number of flowering ramifications and the number of flowers on each ramification on the marked shoots to analyse reproductive growth. We calculated the mean number of flowers per shoot as the total number of flowers present in each treatment divided by the total number of marked shoots; this indicated the flowering intensity on the shoots in every treatment. We calculated the mean number of flowers per flowered ramification as the total number of flowers found in each treatment divided by the total number of ramifications with flowers. This parameter indicated the intensity of flowering in the flower-bearing ramifications in every treatment.

On 17th June 2015, we collected the individuals with marked shoots by cutting at ground level. In every individual we identified each marked shoot and its corresponding ramifications with stalk, foliage and, eventually, inflorescences. We scanned the leaves to obtain the leaf area with ImageJ open-source software. We separated the foliage and stalk of each shoot in paper bags and dried them in a forced-air circulation stove at 60 °C. We calculated the data of leaf and stalk dry biomass per shoot and summed them to calculate total

shoot biomass. Thus, shoot biomass did not include the reproductive portion, *i.e.* the inflorescences.

We also counted the number of dead and living marked shoots throughout the experimental period to determine their survivorship, calculated as the number of living shoots divided by the total number of marked shoots in each treatment.

4.2.4 STATISTICS

We performed our data analysis with BioEstat 5.3 software (Instituto Mamirauá, Brazil). We used the D'Agostino-Pearson test to verify data normality, and since the data were not normally distributed, we used the nonparametric Mann-Whitney test with $p < 0.1$ to compare the data sets from each treatment. We used a Chi-square test ($p < 0.1$) to compare survivorship on a percentage basis in each treatment.

4.3 RESULTS

4.3.1 CLIMATE AND SOIL WATER CONTENT

During the period of the experiment, the days were usually free of clouds and solar irradiance was approximately $0.7\text{--}0.9 \text{ kW m}^{-2}$ at midday (Fig. 4.1A). Nonetheless, cloudy or rainy days with solar irradiance between $0.5\text{--}0.2 \text{ kW m}^{-2}$ occurred on 4th, 6th, 11th, 15th, 18th, 20th, and 28th May and 2nd and 15th June 2015. The lowest solar irradiance value around midday (near 0.17 kW m^{-2}) occurred on the rainy day 2nd June 2015. The highest solar irradiance value (around 0.89 kW.m^{-2}) was registered on 5th May 2015 at 12h15 (Fig. 4.1A). On this day, canopy temperature reached approximately $31.5 \text{ }^{\circ}\text{C}$ in the warmed plots (about $11.5 \text{ }^{\circ}\text{C}$ above the average value; Fig. 4.2), relative air humidity reached 60% (Fig. 4.1B), and soil water content was $0.4 \text{ m}^3 \text{ m}^{-3}$ (Fig. 4.1E). Reduced relative humidity usually occurred together with the peaks in temperature and solar irradiance around midday (Fig. 4.1A, B, and Fig. 4.2), and mainly after 2nd June 2015 with the cessation of the rains. The minimum relative humidity value at noon was 33% on 13th June 2015 (Fig. 4.1B). The average value of relative humidity during the whole experiment was 85.1%.

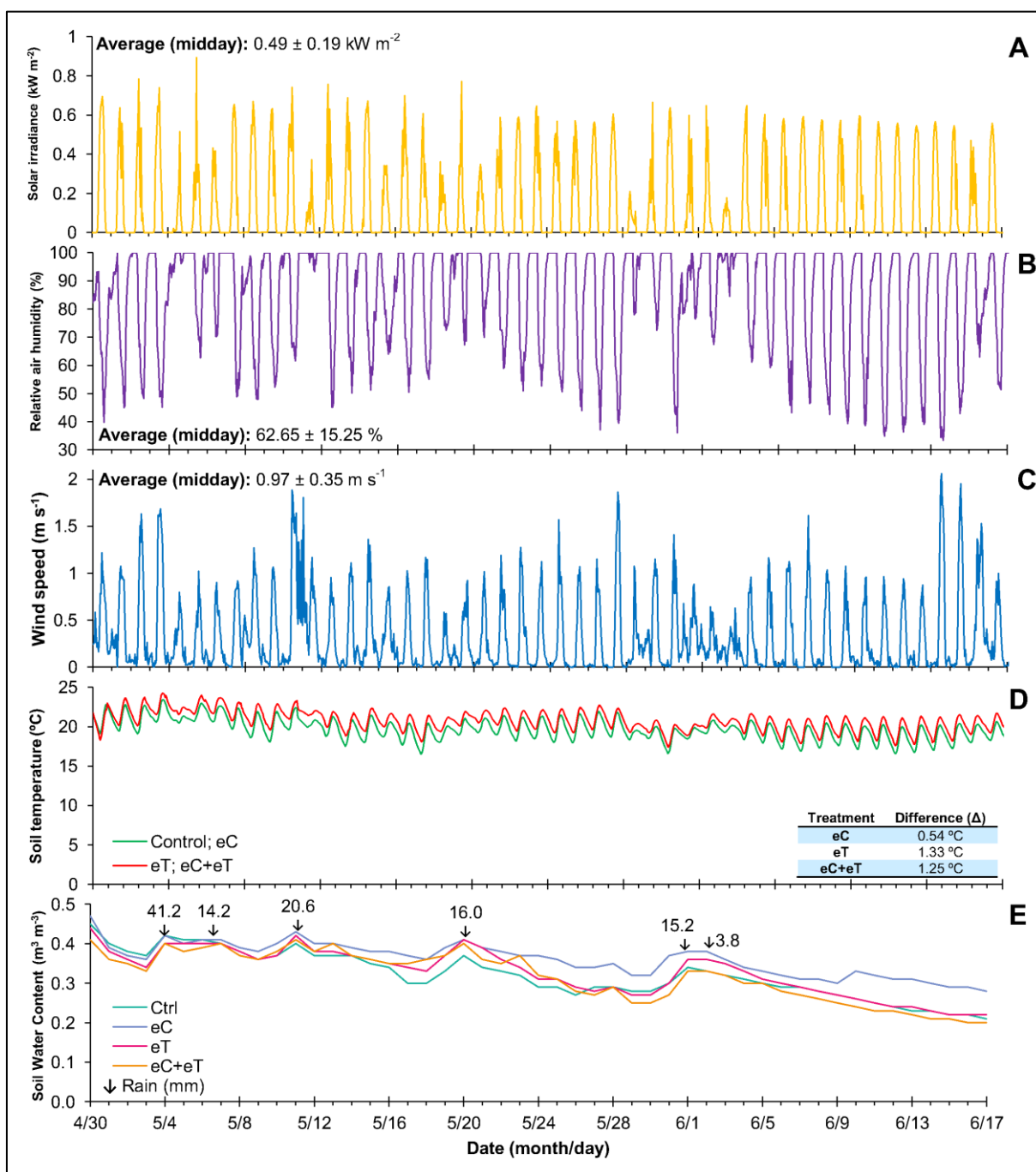


Fig 4.1 The daily courses and the average values \pm standard deviation at midday of total solar irradiance (A), relative air humidity (B), and wind speed (C) recorded from April 30th to June 17th 2015 at the center of the experimental area. It is also showed the soil temperature (D) in each treatment with the average differences between the treatments and Control (Δ). The average between the plots with ambient CO₂ and temperature (Control) and with 600 ppm of CO₂ (eC) is represented by a continuous line. The average between the plots with elevated temperature at 2°C above ambient (eT) and with the combined treatments (eC+eT) is represented by a dotted line. The soil water content (E) was obtained in plots with ambient CO₂ and temperature (Control), 600 ppm of CO₂ (eC), elevated temperature at 2°C above ambient (eT), and in the combined treatments (eC+eT). The arrows with numeric values on (E) represent the most significant rainfall (mm) along the experimental period.

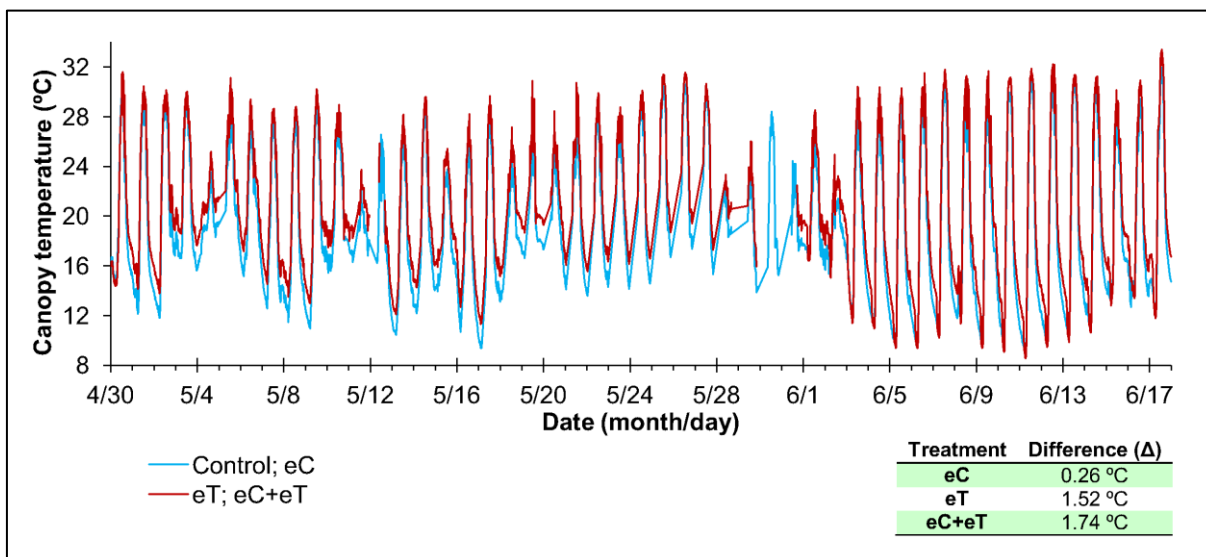


Fig 4.2 Daily courses of canopy temperature recorded from April 30th to June 17th 2015. The average between the plots with ambient CO₂ and temperature (Control) and with 600 ppm of CO₂ (eC) is represented by a dotted line. The average between the plots with elevated temperature at 2°C above ambient (eT) and with the combined treatments (eC+eT) is represented by a continuous line. The average difference between the treatments and Control are also showed as the Δ value.

The maximum value for canopy temperature (33.5 °C on 17th June 2015; Fig. 4.2) was not stressful for *S. capitata* even in the warmed plots. The temperature range for optimum growth of *S. capitata* is 30–40 °C (Martinez et al., 2014). Soil temperature varied from 16.5 °C to 24 °C under control and warmed treatments (Fig. 4.1D). The average soil temperature was 19.7 °C in the control plots and 20.7 °C in the warmed plots (Fig. 4.1D). The maximum soil temperature during the experimental period (24 °C on 3rd May 2015; Fig. 4.1D), even in the warmed plots, was below the 40/30 °C day/night root temperature for optimal growth and nitrogen fixation in *Stylosanthes* (Date, 1989).

The canopy temperature varied from 8.4–33.5 °C (Fig. 4.2) during the course of the day, with the canopy temperature reaching 2 °C higher under the heated treatment, especially during the night (Fig. 4.2). In fact, the T-FACE system successfully maintained the set point of 2 °C most of the time. Soil temperature varied between 19.3 °C and 20.4 °C during the course of the day (Fig. 4.1D), and was 1.5 °C higher under the heated regime, especially during the night (Fig. 4.1D). Wind speed rarely exceeded 1.5 m s⁻¹ and only once reached 2.0 m s⁻¹ (14th June; Fig. 4.1C).

Peaks of soil water content were recorded on rainy days, and they did not exceed 0.55 m³ m⁻³ (11th May 2015; Fig. 4.1E). Accumulated rainfall from 30th April to 17th July 2015 was 113 mm, below the historical average (1982–2012), which was 170 mm of

accumulated rainfall (68). Higher values of soil water content were obtained under the eC treatment most of the time, especially from 24th May 2015 to the end of the experiment (Fig. 4.1E). On 28th May 2015, soil water content in the control, eT, and eC+eT treatments reached a value of $0.3 \text{ m}^3 \text{ m}^{-3}$, while under eC it remained close to $0.4 \text{ m}^3 \text{ m}^{-3}$ (Fig. 4.1E). Immediately after 1st June 2015, under the control and warming treatments, soil water content markedly declined and by the end of the experimental period reached values close to $0.2 \text{ m}^3 \text{ m}^{-3}$ (Fig. 4.1E). Under eC, soil water content values never fell below $0.28 \text{ m}^3 \text{ m}^{-3}$ (Fig. 4.1E).

The average CO₂ concentration in the enriched plots was 583 ppm during the daytime and 584 ppm during the night. The higher nocturnal CO₂ concentration was due to plant and soil respiration as the FACE system was off at night.

4.3.2 VEGETATIVE AND REPRODUCTIVE GROWTH

The survival of shoots of *S. capitata* growing under eC tended to be similar to the control, and was lower under warming treatments (Fig. 4.3). In fact, warming treatments markedly decreased the percentage of shoot survivorship of *S. capitata* (eT and eC+eT; Fig. 4.3). There was a negative synergistic effect when eC was combined with eT, and this combination displayed the lowest shoot survivorship percentage (Fig. 4.3).

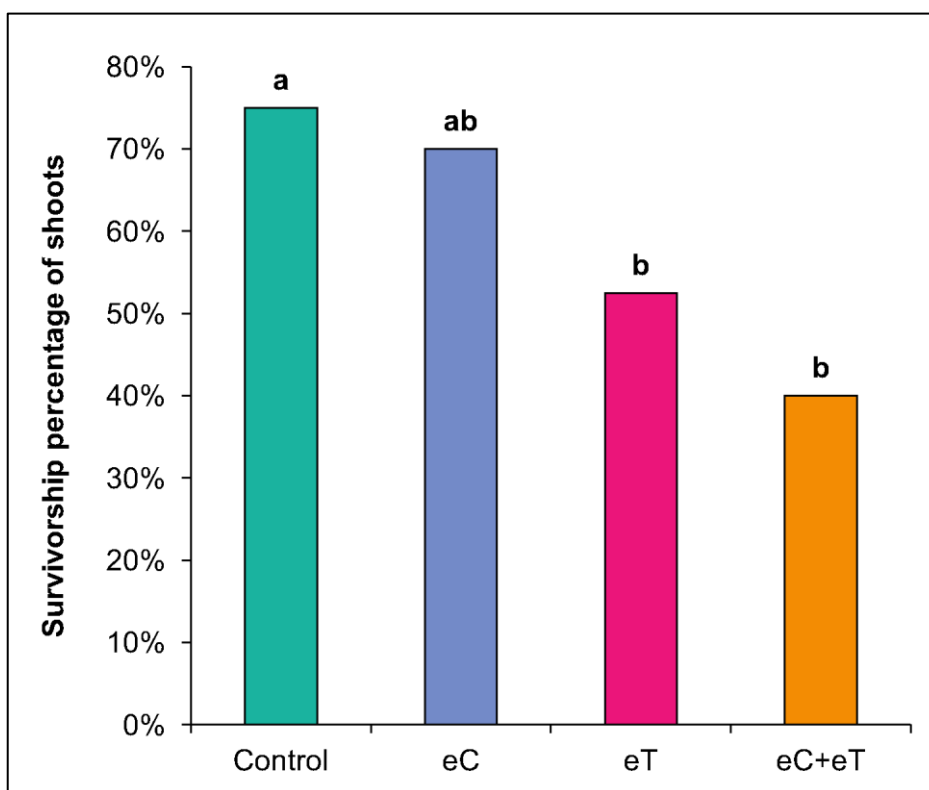


Fig 4.3 Survivorship percentage of shoots of *Stylosanthes capitata* growing under ambient CO₂ and temperature (Control), 600 ppm of CO₂ (eC), elevated temperature at 2°C above ambient (eT), and the combined treatments (eC+eT). Bars show the percentages and different letters at the top of the bars indicate significant differences among them after Chi-square test ($p < 0.1$).

On the other hand, warming had an evident negative effect on leaf biomass and leaf area (Figs 4.4 and 4.5, respectively). The lowest relative leaf biomass value was observed under eT (33% of shoot biomass; Fig. 4.4). Under eC, leaf biomass was lower than under the control and accounted for 39% of shoot biomass (Fig. 4.4). In addition, under eC, leaf area and leaf area per centimetre of shoot (Fig. 4.5) were lower than in the control plots. However, it was possible to notice some compensatory effects of eC that mitigated the adverse effects of eT on leaf area and biomass. Under eC+eT, leaf biomass (Fig. 4.4) and leaf area per shoot (Fig. 4.5A) tended to be similar to those of the control. Therefore, the absence of negative synergism in the eC+eT treatment indicated that the major losses would be in the number of shoots (shoot survivorship), not in leaf area or leaf biomass under the predicted future climate scenario.

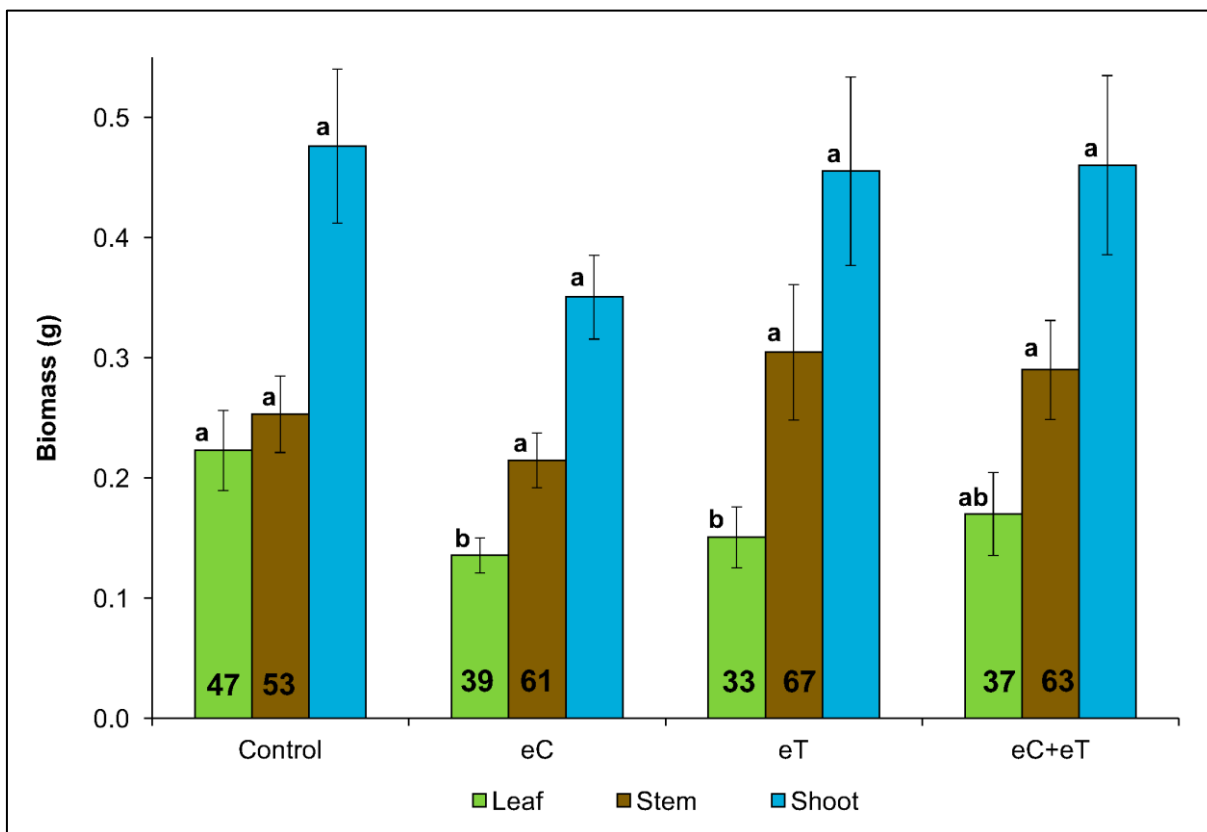


Fig 4.4 Leaf, stem and total shoot biomass of *Stylosanthes capitata* growing under ambient CO₂ and temperature (Control), 600 ppm of CO₂ (eC), elevated temperature at 2°C above ambient (eT), and the combined treatments (eC+eT). Bars show the averages and lines at the top the standard error values. Different letters above bars indicate significant differences among corresponding datasets after the Mann-Whitney test ($p < 0.1$). Numbers at the base of the bars representing leaf and stem are the percentages in relation to the total shoot biomass.

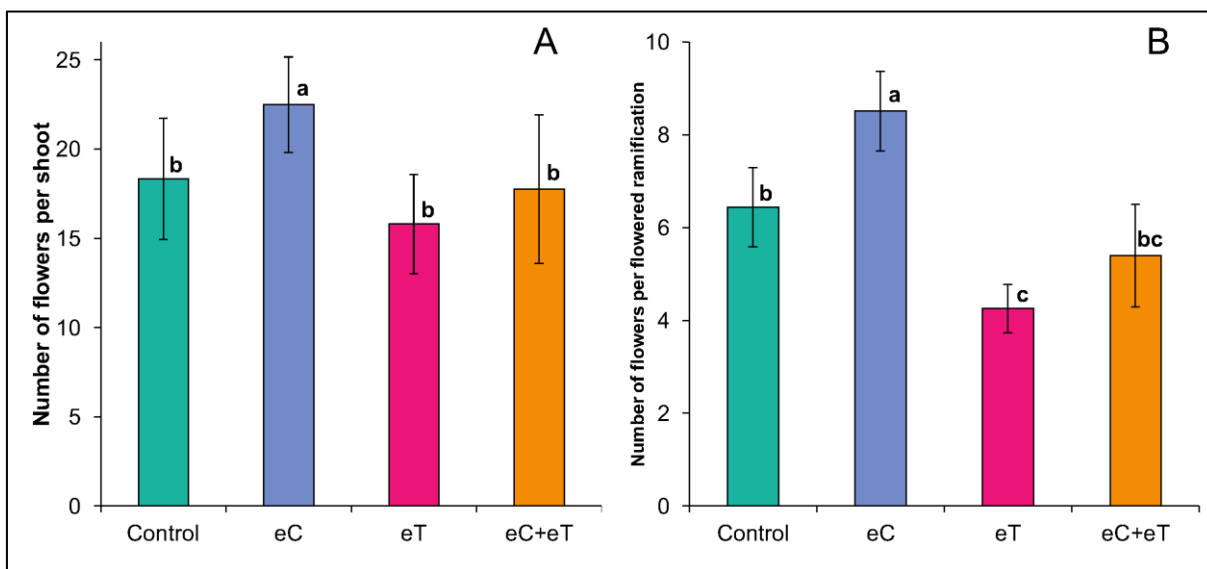


Fig 4.5 Leaf area per shoot (A) and leaf area per cm of shoot (B) of *Stylosanthes capitata* growing under ambient CO₂ and temperature (Control), 600 ppm of CO₂ (eC), elevated temperature at 2°C above ambient (eT), and the combined treatments (eC+eT). Bars show the averages and lines at the top the standard error values. Different letters above bars indicate significant differences among datasets after the Mann-Whitney test ($p < 0.1$).

Under eT and eC+eT, the number of flowers per shoot (Fig. 4.6A) did not change, but there were fewer flowers per flowered ramification (Fig. 4.6B). In fact, the lowest number of flowers per flowered ramification was observed under eT (Fig. 4.6B). By contrast, flowering was most intense under eC, with the highest values for number of flowers per shoot (Fig. 4.6A) and flowers per flowered ramification (Fig. 4.6B). The flowering stimulation and impairment under eC and eT, respectively, became evident when we analysed flowering under eC+eT. Under eC+eT, eC mitigated the impairment effect on number of flowers per flowered ramification seen under eT (Fig. 4.6B). The positive and negative effects of eC and eT, respectively, on the flowering process of *S. capitata* in the absence of irrigation were thus evident.

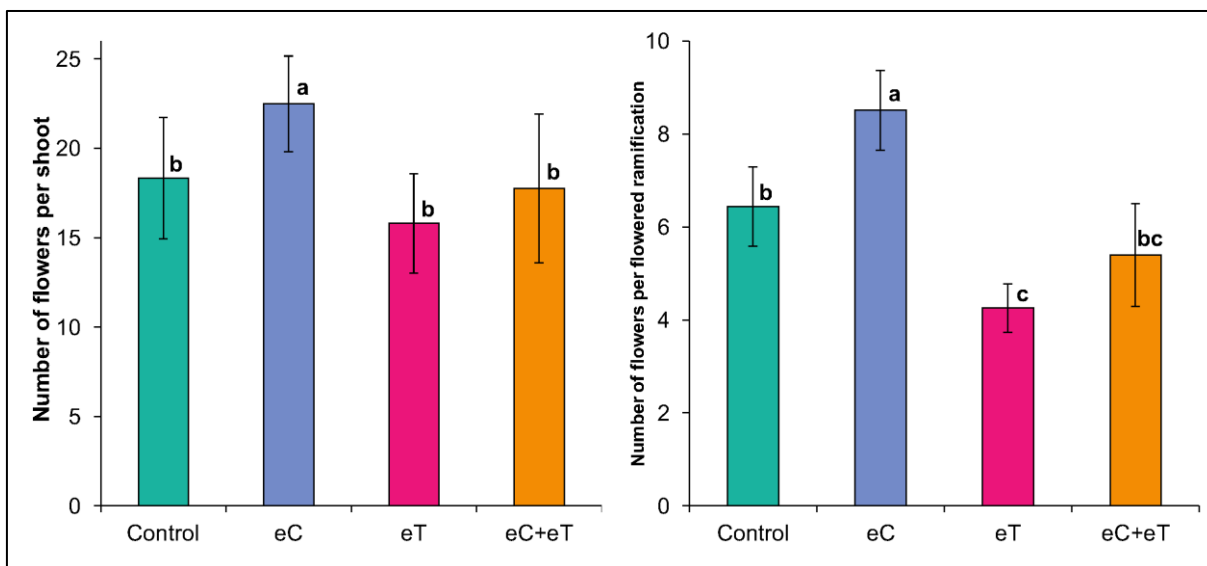


Fig 4.6 Number of flowers per shoot (A) and number of flowers per flowered ramification (B) of *Stylosanthes capitata* growing under ambient CO₂ and temperature (Control), 600 ppm of CO₂ (eC), elevated temperature at 2°C above ambient (eT), and the combined treatments (eC+eT). Bars show the averages and lines at the top the standard error values. Different letters above bars indicate significant differences among datasets after the Mann-Whitney test ($p < 0.1$).

Under eC, the soil retained more water for a longer time than under the other atmospheric regimes, especially from the cessation of rains on 2nd June 2015 until the end of the experiment (Fig. 4.7B). It is evident that when the values of soil water content reached 0.3 m³ m⁻³ (28th May 2015; Fig. 4.7B), the average number of flowers per shoot began to diverge among the treatments (Fig. 4.7A).

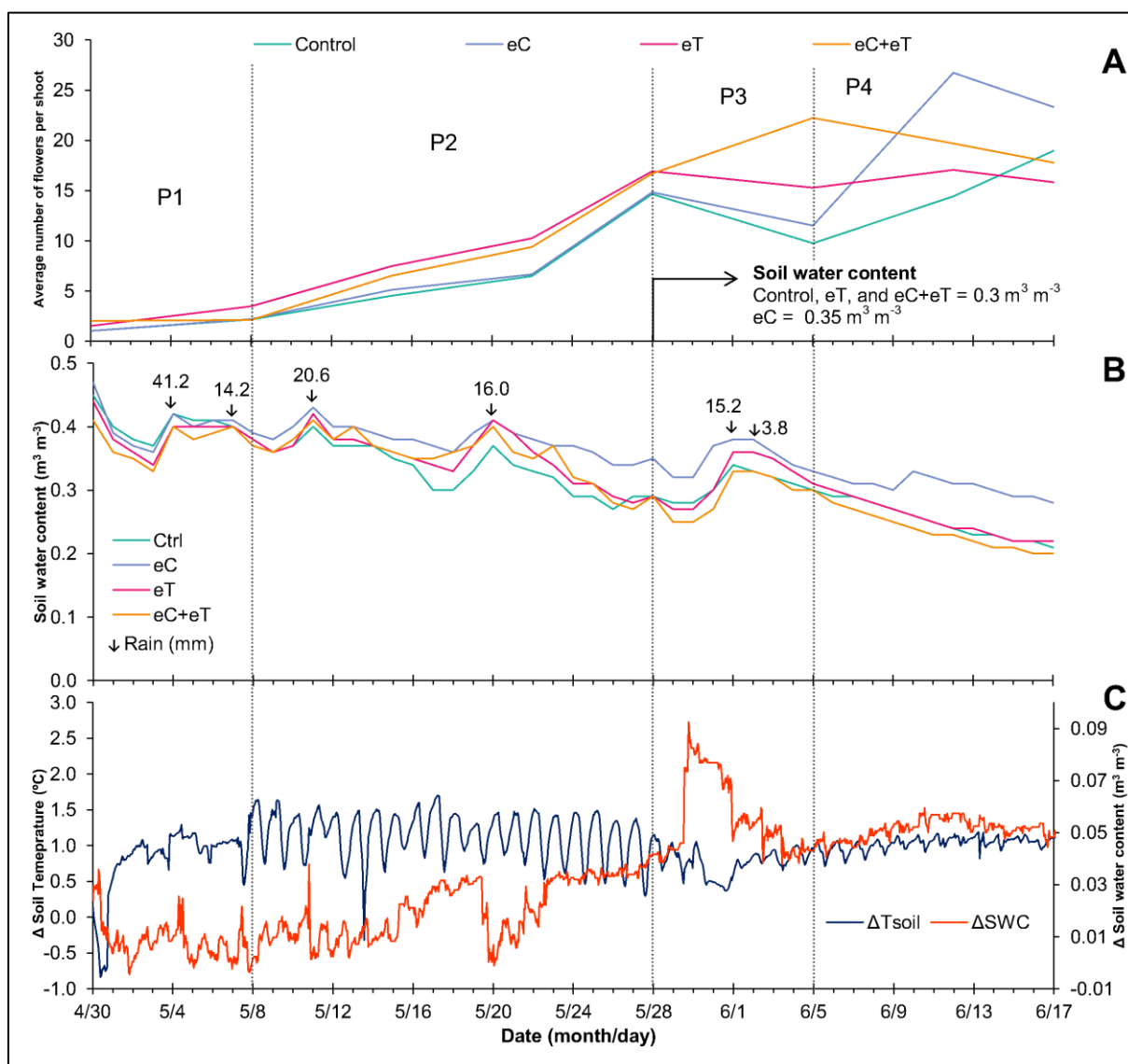


Fig 4.7 Flowering and daily courses of soil water content along the experiment, from April 30th 2015 to June 17th 2015. The four highlighted periods (P1, P2, P3 and P4) represents significant changes in soil water content and flowering. (A) Average number of flowers per shoot of *Stylosanthes capitata*. Ambient CO₂ and temperature (Control, solid line), 600 ppm of CO₂ (eC, dashed line), elevated temperature at 2°C above ambient (eT, dotted line), and the combined treatments (eC+eT, open line). (b) Soil water content obtained in plots with ambient CO₂ and temperature (Control, solid line), 600 ppm of CO₂ (eC, dashed line), elevated temperature at 2°C above ambient (eT, dotted line), and in the combined treatments (eC+eT, open line). The arrows with numeric values on (b) represent the most significant rainfall (mm) in the experimental area. (c) Average values of soil temperatures differences (ΔTsoil) between non-warmed (Control; eC) warmed treatments (eT; eC+eT). Average values of the differences of soil water content between eC and the other treatments.

We identified four significant periods following flowering (Fig. 4.7). After the irrigation at the beginning of the experiment, from 30th April to 8th May, the average number of flowers per shoot was similar across treatments, although the number of flowers in the warming treatments began to diverge at the end of this period (P1 period; Fig. 4.7A). In this

period, the soil water content was higher than $0.3 \text{ m}^3 \text{ m}^{-3}$ in all climatic regimes (Fig. 4.7B). The soil temperature varied slightly due to the presence of more water in the soil (Fig. 4.7C).

From 8th to 28th May, the flowering data under the warming treatments (eT and eC+eT) stood out, with higher values than those observed under the non-warming treatments (control and eC; Fig. 4.7A). This means that warming favoured flowering during the P2 period when the rains kept the soil water content values above $0.3 \text{ m}^3 \text{ m}^{-3}$ (Fig. 4.7B). After the last rainfall during P2, the soil water content decreased significantly in the warming treatments and the control, but not in the eC treatment (Fig. 4.7B). The combination of heating and soil water content values higher than $0.3 \text{ m}^3 \text{ m}^{-3}$ during the whole P2 period (Fig. 4.7B) probably favoured flowering in the warming treatments (eT and eC+eT; Fig. 4.7A). Soil temperature varied more broadly with soil water content oscillation (Fig. 4.7C).

During the P2 and P3 periods, soil water content was clearly favourable under the eC treatment. From 28th May to 5th June, the differences in soil water content among the climatic regimes were significantly higher. Soil water content reached $0.3 \text{ m}^3 \text{ m}^{-3}$ under all the atmospheric regimes except eC. Flowering was strongly divergent among the atmospheric regimes, but there was no particular divergence between warming and non-warming regimes. Thus, during the P3 period, soil water content below $0.3 \text{ m}^3 \text{ m}^{-3}$ in the control, eT, and eC+eT treatments, not simply the warming, established a new flowering behaviour. The results thus indicate that heating and soil water content are involved in the determination of the intensity of flowering in *S. capitata* shoots.

In the P4 period, which ran from 5th June to the end of the experiment, flowering remained stable under eT, but there was a reversal in flower production under eC, eC+eT, and the control. In other words, under eC and the control there was increased flowering, and under eC+eT flowering decreased (Fig. 4.7A). More carbon and water was available under the eC treatment, and so it exceeded all atmospheric regimes with respect to flowering. The soil water content of the soil dropped below $0.3 \text{ m}^3 \text{ m}^{-3}$ for all treatments except eC. The rapid increase in the production of flowers under eC and the significant reduction in the number of flowers under eC+eT resulted in the eC treatment exhibiting the highest number of flowers at the end of the experiment (Fig. 4.6). The decrease in flowering under the eC treatment in the final days of the experiment (Fig. 4.7A) did not alter this picture. Soil temperature varied slightly due to the lack of water in the soil (Fig. 4.7C).

In summary, the increase in temperature impaired vegetative growth and promoted shoot mortality even in the presence of elevated atmospheric CO₂ concentrations. The elevated temperature regime also reduced soil water content, probably by increasing evaporation and leaf transpiration. On the other hand, elevated CO₂ concentrations contributed to retaining more water in the soil, most likely because of stomatal closure and a consequent reduction in leaf transpiration. The CO₂ enriched atmosphere also promoted intense flowering but did not stimulate leaf biomass gain. Heating was able to promote flowering only when soil water content was above 0.3 m³ m⁻³. CO₂ atmospheric enrichment counteracts the adverse effects of increased temperature on growth and especially on leaf biomass gain.

4.4 DISCUSSION

The vast majority of studies of growth and biomass accumulation in C_3 plants conducted in atmospheres enriched with CO_2 show that C_3 plant development is benefitted under these conditions (Bazzaz, 1990; Bowes, 1993; Coleman *et al.*, 1993; Poorter, 1993; Ross *et al.*, 1996; Wand *et al.*, 1999; Streck, 2005; Marabesi, 2007; Leakey *et al.*, 2009; Bhatt *et al.*, 2010). These studies usually use chambers to concentrate atmospheric CO_2 , and they provide plants with ample water. Studies in CO_2 concentration chambers often overestimate the C_3 plants' gains (31). In such studies, the reported gains in total biomass are approximately 40% (Poorter, 1993; Wand *et al.*, 1999). In contrast to what we hypothesized, leaf development of rain-fed *S. capitata* did not benefit from the increasing atmospheric CO_2 concentration. Instead, leaf biomass accumulation was lower, as was leaf area. Moreover, the percentage of leaf biomass under eC was 39% while under the control it was about 50% of shoot biomass (Fig. 4.4). Thus, the assimilated carbon was not invested in an increment of the biomass of leaves, stalk, or shoot under eC. As shoot biomass only included stalk and leaf biomass and did not include the flowers, the greater flowering under this treatment shows that the biomass that had been produced in the leaves probably was invested in the reproductive organs. Furthermore, the lack of irrigation should not have been the primary factor in this leaf growth reduction under eC. The soil water content was higher than $0.3 \text{ m}^3 \text{ m}^{-3}$ during most of the experimental period under CO_2 enrichment (Fig. 4.1), and only values below $0.3 \text{ m}^3 \text{ m}^{-3}$ were detrimental to *S. capitata* growth (mainly flower production). In fact, CO_2 enrichment enables plants to withstand drought stress better and delays its onset (29).

S. capitata exhibited reduced leaf area under eC in addition to potentially increased CO_2 supply. This is an indication of down-regulation in leaf development. In other words, leaf area was smaller under eC while the leaf having access to more abundant supply of CO_2 provided by an enriched atmosphere. Acclimation to elevated CO_2 is the rule rather than the exception in C_3 plants (29). In our case, since *S. capitata* did not invest in leaf formation, there was spare biomass available to invest in flowering.

We partially confirmed our hypotheses for the eC treatment. The isolated increment in CO_2 concentration, instead of an increase leaf biomass, caused a down-regulation in leaf biomass gain. As a consequence, *S. capitata* translocated the saved assimilated carbon to flowers. Even if eC contributed to greater water use efficiency (29,117,118), this contribution

per se did not promote leaf biomass gain under eC. Since the leaf biomass provides the food supply for the cattle (54), elevated CO₂ in the atmosphere would be not favourable for supplying livestock with rain-fed *S. capitata*.

As other studies have shown, elevated temperature harms C₃ plants (16,119). However, in a previous study with *S. capitata* in the same experimental area, Martinez *et al.* (87) found that biomass production increased by 16% under moderate warming (2 °C). Warming without water and nutritional restrictions was beneficial for the physiological and biophysical processes involved in *S. capitata* plant growth (87). Our study was conducted under similar temperature and soil nutritional conditions as Martinez *et al.* (87), except that the soil was not irrigated. We cannot state that the conditions we provided for growing *S. capitata* under eT were entirely non-stressing. Indeed, the soil water content was low under eT and leaf water potential at noon and leaf water content at pre-dawn was 9% lower than in the control (E. Habermann, USP, Ribeirão Preto, Brazil, unpubl. res.).

The values of soil water content decreased primarily at the end of the experimental period, to less than 0.25 m³ m⁻³ (Fig. 4.1). By comparing our results for leaf biomass with those of Martinez *et al.* (87), it is evident that the lower soil water content under eT (Fig. 4.1) was hampering the growth of *S. capitata* since shoot mortality was elevated (Fig. 4.3), leaf biomass (Fig. 4.4) and leaf area (Fig. 4.5) were reduced, and flowering was impaired (Fig. 4.6). This is an alarming result, since longer periods of drought, higher temperatures, and heat waves are expected to accompany global climate change in southeastern Brazil (3,120).

In a previous experiment with *S. capitata* grown in concert with *P. maximum* (C₄) under eT, the legume produced fewer leaves and ramifications per shoot in relation to the control (Chapter 3). The shade caused by the C₄ foliage under eT could be involved in this impaired vegetative development (Chapter 3). On the other hand, under moderate warming, *S. capitata* increased the allocation resources devoted to flowering, just as the control plants growing in irrigated conditions in concert with *P. maximum* did (Chapter 3). Thus, moderate warming did not impair *S. capitata* flowering (Chapter 3) and did promote foliage biomass gain in irrigated conditions (87). However, when soil water was scarce and the temperature was moderately elevated, *S. capitata* invested less in vegetative growth and even in reproduction. Therefore, rather than the increased temperature, it was the absence of irrigation aggravated by the warming (112,113) that was responsible for the impairment of flowering and vegetative growth of *S. capitata* under eT.

Despite the observed positive effects of eC, under the rain-fed eC+eT environment that simulated the RCP6 climate scenario (1) *S. capitata* was not able to offset the adverse effects of warming by making use of the greater atmospheric CO₂ concentration. Elevated CO₂ levels in the FACE system stimulate net photosynthesis by increasing the intracellular concentration of carbon (42) and promoting biomass accumulation. However, *S. capitata* remains growth impaired under eC+eT, even though the elevated atmospheric CO₂ concentration represents an attempt to mitigate the adverse effects of warming. As increased CO₂ concentration is related to stomatal closure and reduced transpiration (29), the effects of an enriched atmosphere can mitigate the impairments caused by elevated temperature, especially in drought conditions. Nonetheless (Fig. 4.3), the greater shoot mortality observed under eC+eT indicates that heating combined with elevated CO₂ concentration was even more harmful to *S. capitata* than eT alone was. Even though the higher carbon supply under eC+eT provided conditions to mitigate the adverse effects of warming, it was not sufficient under water stress. In this context, we could assert that the main reason for impaired growth under warming was the lack of water in the soil since *S. capitata* is adapted to warm climates, provided it is growing under suitable water and nutritional soil conditions (87). In this case, not only may the increase in air temperature have nullified the benefits of elevated CO₂ concentration for photosynthesis (43), but the lack of water in the soil may also have exacerbated the harmful effect of warming.

In a previous experiment with *S. capitata* irrigated and grown in concert with *P. maximum*, during the same season and in the same experimental area, eC+eT treatment provoked the most abundant flowering (Chapter 3). Moreover, investment in reproduction occurred at the expense of vegetative growth under eC+eT (Chapter 3). In analysing Fig. 4.7, we can see that from 28th May 2015 onward, the number of flowers began to diverge among the treatments (Fig. 4.7A). At the same time, the soil water content decreased in both the control and the heated treatments (Fig. 4.7B). Under eC, the soil water content remained at comparatively high levels during this period and flowering abruptly increased, thereby draining biomass. Under the warming regimes, flowering was clearly hampered, as was vegetative growth. The benefits of eC attenuated the flowering impairment caused by warming in eC+eT, and flowering remained as it was in the control.

From these results, it is possible to infer that soil water content is fundamental to the flowering process and is limiting at approximately 0.3 m³ m⁻³.

As warming increases water demand, the benefits of CO₂ will be indirectly reduced (50) under the RCP6 scenario. The absence of irrigation and the possible low soil water content should aggravate this situation for both the vegetative and reproductive development of *S. capitata*. When working in a grassland area, the soil can be corrected and fertilized, but irrigation of an extensive pasture area puts the ecosystem at risk for consequences such as soil salinization and contamination, and loss of water courses (106), and irrigation of an extensive pasture area is economically unfeasible. In grazing areas that are not irrigated and are subject to elevated CO₂ concentrations and warming, *S. capitata* will experience much less favourable conditions for growth under future climate changes. This study thus corroborates the assumption made in Chapter 3.

Since it is predicted that the future climate will change rainfall patterns in southeastern Brazil (107), the region with the second largest Brazilian cattle herd (18), this is a disturbing situation. New pasture management technologies are needed to mitigate this expected instability, as well as reductions in the global emission of greenhouse gasses.

4.5 CONCLUSION

The climate predicted for 2050 will be harmful to *S. capitata* grown without irrigation in southeastern Brazil during autumn. The elevated temperature and the lower soil water content will impair vegetative growth and increase shoot mortality. The effects of elevated atmospheric CO₂ concentrations on the C₃ photosynthesis pathway will not be enough to mitigate the adverse effects of warming and autumn water shortages. CO₂ enrichment will only promote flowering during the period of favourable soil water content, and an increase in the number of flowers per shoot instead of an increase in leaf biomass will be not be beneficial to livestock.

- CONCLUSÕES -

- As mudanças climáticas provocarão alterações significativas no crescimento, desenvolvimento e fenologia reprodutiva (C₃) e vegetativa (C₃ e C₄) das forrageiras estudadas.
- Ocorreram mudanças significativas nas duas espécies estudadas em todos os níveis de organização avaliados, de população de folhas (C₃ e C₄) e de ramos (C₃) até ao nível molecular (concentração de nitrogênio na folha, C₄).
- O consórcio com o arbusto C₃ e a gramínea C₄ não se apresentou como uma alternativa de pastagem em clima futuro, pois não apresentou vantagens relevantes ao crescimento, desenvolvimento e incremento de biomassa da forragem em nenhum regime ou época do ano estudada.
- A irrigação se mostrou extremamente necessária para o mínimo desenvolvimento das forrageiras nas condições estudadas. Como a pecuária no Brasil é frequentemente realizada em extensas áreas de pastagens, a irrigação nesse porte se torna inviável economicamente e de elevado risco ambiental.
- Como são previstas alterações desfavoráveis nos regimes de chuvas com as mudanças climáticas no Sudeste brasileiro, torna-se urgente o desenvolvimento de estratégias de manejo e, em último caso, de irrigação de pasto com menor impacto ambiental possível.
- Novas alternativas de manejo de pastagem devem ser prontamente estudadas e experimentadas antes de 2050.

- CONCLUSIONS -

- Climate changes will cause significant modifications in growth, development and reproductive (C_3) and vegetative (C_3 and C_4) phenology of the studied forage.
- Significant changes occurred in both species at all evaluated levels of organization, from leaf (C_3 and C_4) and branches (C_3) population to the molecular level (nitrogen concentration in the leaf, C_4).
- The consortium with the C_3 legume and the C_4 grass did not appear as a grazing alternative in future climate, because it showed no significant advantages to the growth, development and forage biomass increment in any studied regime or time of year.
- Irrigation proved to be extremely necessary for the minimal development of forage in the studied conditions. As the livestock farming in Brazil is often held in large areas of pastures, irrigation in this scale becomes economically unfeasible and of high environmental risk.
- Since there are predicted unfavorable changes in rainfall patterns with climate change in the Brazilian Southeast, it is urgent to develop management strategies and, ultimately, pasture irrigation with minimal environmental impact.
- New pasture management alternatives should be promptly investigated and experimented before 2050.

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