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RESEARCH ARTICLE

Rising CO_2 in the field does not offset warming or drought constraints on leaf growth of a C_3 forage

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Abstract

The regrowth of a C_3 forage *Stylosanthes capitata* in a rain-fed field provided the weekly data collection in the southeast of Brazil during a heat event in autumn. A system named Trop-T-FACE simulated the free-air carbon dioxide enrichment and the enhanced temperature in four climatic regimes: current atmospheric conditions (Control), CO_2 enrichment (600 ppm, eC), warming (2 °C above ambient canopy temperature, eT), and a combination of eC + eT. The area and biomass of foliage per shoot decreased, and the number of flowers per shoot and flowered ramification increased under single eC treatment besides the increment in palisade parenchyma of leaves. Increased investment in flowering in eC occurred notably when the soil water content was higher than 0.30 m^3 m^{-3}. Single eT treatment also impaired the area or biomass of foliage production per shoot, raised the shoot mortality, and promoted the increment of the spongy leaf parenchyma. There was some mitigation of the adverse effects of foliage production of eT or eC in eC + eT, but under this combined treatment, the shoot mortality also increased. Changes in leaf tissues under eC or eT or some mitigation of adverse effects in eC + eT did not offset the constraints on leaf growth per shoot. The harmful impact on foliage production by eC, eT, or eC + eT under rain-fed conditions indicated no advantages for feeding the livestock with the C_3 forage *S. capitata* in expected climate change under field conditions.

Keywords: Flowering; Leaf area; Leaf biomass; Shoot mortality; Soil water content

Introduction

The atmospheric concentration of CO_2 ($[CO_2]$) has grown exponentially since the Industrial Revolution and now exceeds 410 ppm, about 30% greater than 50 years ago (Mauna Loa Observatory, 2016). Burning fossil fuels and changing land use are the major causes of increased greenhouse gas emissions (IPCC, 2014) followed by an increase in the atmospheric temperature (Lacis et al., 2010). Along with increases in the global, seasonal, and daily average atmospheric temperature (Bloom, 2010), there will be increment in frequency and intensity of extreme climatic events, such as heat waves and changes in rainfall patterns causing drought or flooding in many regions of the Earth (Boers et al., 2019).

The current atmospheric CO_2 available is lower than that for saturating C_3 photosynthesis (Bloom, 2010; Bowes, 1993). Concentrations of 600 ppm of CO_2 could increase C_3 photosynthesis by 40% (Long et al., 2004). *Stylosanthes hamata*, a C_3 legume, produced 85% more fresh and dry biomass under 650 ppm of CO_2 growing in consortium with *Panicum maximum*, a C_4 grass, in open-top chambers (Bhatt et al., 2010). Raised $[CO_2]$ increased 30% the dry biomass production in a pasture containing C_3 forage species *Lolium perenne* and *Trifolium repens* grown in experimental enclosures (Newton, 1991; Newton et al., 1994). These increments involved not only physiological adaptations but also leaf anatomical alterations, such as the increased number of chloroplasts per cell and mesophyll size (Pritchard et al., 1999).

Field experiments conducted with free-air carbon dioxide enrichment (FACE) facility showed lesser stimulation of photosynthesis in C_3 species than in enclosure ambient (Ainsworth and Long, 2005; Leakey et al., 2009). FACE results are near to those in natural settings because the concentration of CO_2 is usually lower than in open-top chambers (Long et al., 2004), and there is a direct influence of external factors (Ainsworth and Long, 2005; Mauri, 2010). Regardless the approach for measuring the effect of $[CO_2]$ on plants, elevated CO_2 concentration usually alleviates the harmful effects of warming on C_3 plants by increasing carbon uptake via photosynthesis and better water use (Albert et al., 2011). Nevertheless, for each 1.0 °C increment in seasonal temperature, a reduction of between 3 and 16% in crop yield is expected (Battisti and Naylor, 2009) due to increased respiration and photorespiration in C_3 species. Also, growth temperature above a finely tuned threshold can trigger flowering, bypassing the need for other inductive stimuli (Balasubramanian et al., 2006).

Increased temperature may decline the impact of CO_2 by augmenting the water demand (Easterling et al., 2007). The combination of 450 ppm CO_2 and 0.8 °C temperature increment enlarged yield by approximately 5.3% in rain-fed spring wheat, but the combination of 450 ppm and 1.8 °C above ambient reduced yield by roughly 5.7% (Xiao et al., 2005). Knowing how C_3 forage species will respond in the field to a warmer, carbon-enriched atmosphere is necessary for predicting the foliage availability for livestock. According to the Ministry of Agriculture, Livestock, and Supply, Brazil has the second-largest livestock population in the world, with about 200 million head (MAPA, 2015) being a leading exporter responsible for 20% of all internationally traded meat involving over 180 countries since 2004. Despite the great importance of pastures on the food industry, there is no sufficient information to estimate the impact of the future climate change on tropical grasslands. As most Brazilian pastures are rain-fed, their production 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 (Carter et al., 2007).

The C_3 legume forage *S. capitata* is similar to that of other species in the genus, such as *Stylosanthes macrocephala*, *Stylosanthes bracteata*, and *Stylosanthes pilosa* being highly resistant to the anthracnose and free of compounds interfering in cattle health (EMBRAPA, 2007). Also, *S. capitata* is perennial and responded positively to 2 °C temperature increase when grown in monoculture free of water shortage and nutritional impairments during vegetative stage (Martinez et al., 2014). Our experiment was conducted during the early autumn at the beginning of the dry season in a rain-fed field in the southeast of Brazil when *S. capitata* was about to flower (Fortuna-Perez et al., 2011) simulating a heat event based on the RCP6 climate scenario outlined by the Intergovernmental Panel on Climate Change (IPCC) for the year 2050 (rain-fed, 600 ppm CO_2, and +2 °C). We employed a free-air temperature-controlled enhancement combined with a FACE system named Trop-T-FACE to simulate future climate change conditions of warming and elevated $[CO_2]$, respectively, in *S. capitata* pasture.

It was hypothesized that plants of *S. capitata* grown under 600 ppm of $[CO_2]$ would increase biomass production (Ainsworth and Rogers, 2007; Albert et al., 2011; Bowes, 1993), enlarge leaf thickness (Miyazawa et al., 2011; Xu et al., 2012), and intensify flowering by short-term changes in plant development (Ahuja et al., 2010). Under the increase of 2 °C, severe impairment of the vegetative and reproductive growth of *S. capitata* was expected because of the synergism between drought and elevated temperature causing low stomatal conductance to CO_2 and a reduction in photosynthetic capacity by decreasing CO_2/O_2 specificity of Rubisco (Jordan and Ogren, 1984). Although *S. capitata* is adapted to an elevated temperature (Martinez et al., 2014), the combination of +2 °C increase and drought in the rain-fed field probably requires leaf modification to mitigate this stressful situation (Tomás et al., 2014). Under treatment with both 600 ppm

CO_2 and warming, the increase in air temperature may cancel the benefits of elevated CO_2 for photosynthesis (Battisti and Naylor, 2009). However, because high atmospheric CO_2 concentration is related to stomatal closure and further reduces transpiration (Bowes, 1993), enriched atmosphere effects can mitigate water stress by increasing water use efficiency. Thus, under 600 ppm and +2 °C, some aspects of initial regrowth in *S. capitata* probably will 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 leaf anatomy, early vegetative regrowth, and flowering of *S. capitata* could be affected in short term by a heat event and atmospheric CO_2 enrichment in monoculture without irrigation. Studies of climate impacts on forage productivity are pivotal to support pasture management, particularly in areas where livestock production makes a significant contribution to the economy, as it does in Brazil.

Materials and Methods

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, São Paulo State, Brazil (21°10′08.0″S and 47°51′49.5″W, 546 m.a.s.l.) showing an Aw climate, tropical with rainy summers (Köppen and Geiger, 1928). Historical data from 1982 to 2012 showed 21.9 °C as the average annual temperature, with minimum and maximum between 13.8 and 26.8 °C (Climate-Data.org, 2016). The soil in the experimental area is an Oxisol, dystrophic Red Latosol (Soil Survey Staff, 1999). After soil analysis, we performed land liming to correct the pH from the initial average values of 4.0–4.5 to 5.0–5.5 and chemical fertilization with NPK 4-14-8 fertilizer at a dose of 1 t ha^{-1}.

On the 14th and 15th of January 2015, the C_3 subshrub *S. capitata* was sown by rows surrounding and in the center of the plots of every treatment. Seeds of *S. capitata* were placed into holes 30 cm apart in 12 m × 12 m plots. We maintained only three plants per hole after germination. The genus *Stylosanthes* Sw. (Leguminosae) comprises 48 species with a pantropical distribution (Fortuna-Perez et al., 2011). *S. capitata* Vogel is a perennial, 12–25 cm tall, with trifoliate leaves. Inflorescences are terminal or axillary, simple or composed of two to four stalks, with 11–20 flowers each (Fortuna-Perez et al., 2011).

On 17 March 2015, 62 days after planting, we fertilized the area 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 CO_2 fumigation and warming treatments. Irrigation occurred only on 2 days during seedling growth, on 24 and 29 April 2015, to stimulate early growth. Subsequently, the plantation was rain-fed, with the most significant rains occurring on 4, 7, 10, and 19 May 2015 with 41, 14, 21, and 16 mm, respectively.

Treatments in the Trop-T-FACE facility

The Trop-T-FACE facility is a combined free-air temperature-controlled enhancement (T-FACE) and mini-free-air carbon dioxide enrichment (miniFACE) system used to evaluate the performance of tropical pastures under future climate-change scenarios of elevated [CO_2] and warming (Prado et al., 2016). The treatments were the current atmospheric conditions (Control), CO_2 enrichment (600 ppm, eC), warming (2 °C above ambient canopy temperature, eT), and a combination of eC + eT, with four replications. We established 16 plots, each taking the form of a 2-m diameter ring.

The miniFACE component for simulating high CO_2 climate scenarios under field conditions was a modification of the POPFACE sonic injection system of pure CO_2 designed by Miglietta et al. (2001). The miniFACE provided the treatment of elevated CO_2 on eight of these plots. The eight miniFACE plots were placed randomly in the experimental area and located 10 m away from each other to minimize cross-contamination (Prado et al., 2016). An air pump sampled the CO_2 concentration at canopy height in the center of each miniFACE plot. Each plot contained a CO_2 transmitter sensor model GMT222 (Vaisala, Helsinki, Finland) to measure [CO_2] for manipulating the supply of pure CO_2 gas in each plot. Individual valves using an ITV series automatic pressure regulator (SMC Corporation, Japan) controlled the amount of CO_2 released in the plant canopy. A Proportional Integration Device (PID algorithm) controlled the CO_2 supply to maintain automatically the ~600 ppm set point of elevated [CO_2] in each fumigated plot.

An anemometer located 2 m above the ground in the center of the Trop-T-FACE facility determined the wind speed to assist in calculating the amount of CO_2 to be injected into the plots. The CO_2 supplementation began on 24 April 2015 at sunrise and ended at sunset on each day, until 17 June 2015. A protected computer on the site recorded all the variables (CO_2 concentration, valve voltage, and wind speed) every 5 s during the experiment. Four of the eight miniFACE plots were also placed under high canopy temperature treatment (2 °C above the ambient) by using the free-air temperature-controlled enhancement (T-FACE) as a component of the Trop-T-FACE facility. Ceramic infrared heaters (model FTE-750-240 Salamander, Mor Eletric Heating, Alpine Ave NW, Comstock Park, MI 49321, USA) suspended above the ground of the warmed plots increased canopy temperature as described by Kimball et al. (2008). Salamander ALEX-F reflectors (Mor Electric) were mounted with the heaters on a triangular aluminum pole system. There were six heater units per plot, with a heater at each point of the hexagon. To produce a consistent amount of shade between the warmed and reference plots, we installed a similar array of dummy heaters in the reference plots consisting of aluminum reflectors without a heating element.

We established eight warmed plots keeping the heaters at 0.8 m above the canopy and eight reference plots (Controls). Infrared thermometers (model SI-1H1-L20, Apogee Instruments, Logan, UT, USA) measured the canopy temperature. A PID system installed in a Model CR1000 data logger with AM25T multiplexors (Campbell Scientific, Logan, UT, USA) controlled the heater voltage as recommended by Kimball et al. (2008). The LoggerNet software (Campbell Scientific) collected the data. For communication, an NL201 network link interface (Campbell, Scientific) and a wired Ethernet network connection with the data logger were used. The Trop-T-FACE system provided, continuously and automatically, an elevated temperature up to 2 °C above the ambient canopy temperature (Control) in the warmed plots.

Theta Probe soil moisture (ML2x) and temperature (ST2) sensors connected to a DL2e data logger (Delta-T Devices, Cambridge, UK) monitored, respectively, the soil water content and the soil temperature in each plot. An automatic microclimatic station (WS-HP1) monitored and stored continuously climatic data (air temperature, relative humidity, total irradiance) using specific sensors. A rain sensor located 5 km from the Trop-T-FACE area provided the precipitation data.

In summary, we designed the experiment to evaluate *S. capitata* leaf anatomy, initial regrowth, and biomass yield under an atmosphere enriched with CO_2 (600 ppm, eC), a warming level 2 °C above the ambient temperature (eT), and under the combination of both CO_2 enriched atmosphere and warming (eC + eT), for a total of 16 plots.

Measurements of the shoots

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

The number of flowering ramifications and the number of flowers on each ramification on the marked shoots were recorded to analyze the reproductive growth. The total number of flowers divided by the total number of marked shoots resulted in the number of flowers per shoot, indicating the flowering intensity in each atmospheric regime. The total number of flowers divided by the total number of ramifications with flowers resulted in the number of flowers per flowered ramification in each treatment indicating the intensity of flowering in the flower-bearing ramifications. We also recorded the shoot length, the number of leaves, and the number of ramifications per shoot. The number of leaves and the number of ramifications were also expressed per centimeter of the shoot, representing their distribution along with the shoot.

On 17 June 2015, the individuals with marked shoots were collected by cutting at the ground level identifying 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. The foliage and stalk of each shoot were separated in paper bags and dried in a forced-air circulation oven at 60 °C. The data of leaf and stalk dry biomass per shoot resulted in total shoot biomass. Thus, shoot biomass did not include the reproductive portion, that is, the inflorescences. Leaf and stalk biomass were also expressed per centimeter of the shoot, representing their distribution along with the shoot. The numbers of dead and living marked shoots were counted 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.

Leaf anatomy measurements

It was possible to determine the thickness of the entire leaf and tissue layers in cross-sections from three central leaflets of sun leaves in each plot. The first leaf below the inflorescence was sampled between 13:00 h and 14:00 h on 17 June 2016, fixed in FAA for 24 h (Johansen, 1940), washed with 50% ethanol for 2 h, and stored in 70% ethanol. Samples 5 mm in diameter from the middle of each leaf were kept in terpineol oil for 2 days and embedded in glycol methacrylate (Leica Instruments, Heidelberg, Germany) (Kraus and Arduin, 1997). Cross-sections (6 μm) were prepared with a microtome (Leica RM2245), stained with 1% toluidine blue for 5 min (O'Brien et al., 1964), mounted on glass slides, and then digitally photographed (Leica DFC 500) with a light microscope (Leica DM4000 B) at 400× magnification. It was possible to determine the distance between the secondary vascular bundles as well as the thickness of different tissues always at the same points in the cross-sections. From each sample, we evaluated five sections and measured five cross-section points in each of them. The images analyzed with ImageJ software made possible to determine the thickness of the following leaf tissues: adaxial and abaxial cuticle and mucilage layer, adaxial and abaxial epidermis, palisade parenchyma, spongy parenchyma, and total leaf thickness. We also calculated the proportion of each tissue proportionally to the total leaf thickness.

Statistics

The XLStat software Version 2014.5.03 (Addinsoft® 1995–2014) performed the data analysis. We used at $p < 0.1$ a Chi-square test to compare the percentage of survivorship of shoots and the Mann–Whitney test to compare the average values for the growth parameters between atmospheric regimes. The discriminant analysis (multivariate) was applied to demonstrate the overall growth differences at $p = 0.05$ among atmospheric regimes. Each measured parameter related to vegetative and reproductive growth was graphically represented and considered together in the discriminant analysis. The leaf anatomical data were analyzed according to a randomized blocks design with an analysis of variance (ANOVA) to compare the effects of CO_2 enrichment (eC), warming (eT), and the combined treatments (eC + eT) at $p < 0.10$. ANOVA analyses were performed using R software (R Core Team, 2013). In situations where there were outliers (detected by a residue analysis), we estimated a new value with the ANOVA statistical model used.

Results

Climate, canopy temperature, soil water content, and CO_2 concentration

The experimental period was usually free of clouds with solar irradiance between 0.70 and 0.90 kW m^{-2} at midday, whereas cloudy or rainy days showed 0.50–0.20 kW m^{-2} at noon on 7 May and 2 June (Figure 1a). The highest (0.89 kW m^{-2}) and lowest (0.20 kW m^{-2}) irradiances at noon happened on 5 May and 2 June, respectively. The reduced relative humidity typically occurred with peaks of soil temperature and solar irradiance around midday, mainly after 2 June when the rainy period ended (Figure 1a, Figure 1b and Figure 2). The minimum relative humidity at noon was 33.0% on 13 June (Figure 1b), and the average of the experimental period was 85.1%. Wind speed rarely exceeded 1.5 m s^{-1} and only once reached 2.0 m s^{-1} on 14 June (Figure 1c). The maximum canopy temperature on 17 June in the warmed plot (33.5 °C, Figure 2) was not stressful for *S. capitata* because its optimum temperature for growth is between 30 and 40 °C (Date, 1989). Soil temperature varied between 16.5 and 24.0 °C under Control and warmed regimes (Figure 1d). The maximum soil temperature (24 °C) on 3 May was below the 30/40 °C night/day root temperature for optimal growth and nitrogen fixation in *Stylosanthes* (Date, 1989). Under the heated regimes, the soil temperature was 1.5 °C higher, especially during the night (Figure 1d). The T-FACE system successfully maintained the warmed plots near the set point of +2 °C most of the time (Figure 2). The canopy temperature varied between 8.4 and 33.5 °C with the lowest and the highest values recorded at night and day, respectively (Figure 2).

Peaks of soil water content were recorded in rainy events not exceeding 0.43 m^3 m^{-3} (Figure 1e). Accumulated rainfall from 30 April to 17 July was 113 mm. High values of soil water content were obtained under the eC treatment most of the time, especially from 22 May to the end of the experiment (Figure 1e). On 28 May, the soil water content in the Control, eT, and eC + eT treatments reached 0.30 m^3 m^{-3}, while under eC it was close to 0.40 m^3 m^{-3} (Figure 1e). Immediately after 1 June 2015, under the control and warming treatments, soil water content markedly declined and by the end of the experimental period reached close to 0.20 m^3 m^{-3}. Nevertheless, under eC, soil water content values never fell below 0.30 m^3 m^{-3} (Figure 1e).

The average ± standard deviation of [CO_2] in the enriched plots was 595 ± 20 ppm during the daytime. The daily average [CO_2] in Control plots was 395 ± 15 and 584 ± 15 ppm during the day and night, respectively. The higher [CO_2] during the night was associated with nocturnal heterotrophic soil respiration.

Vegetative and reproductive growth

There were no differences in stalk biomass per shoot or total shoot biomass across atmospheric regimes (Table 1). However, leaf biomass per shoot was 39 and 32% lower than Control in eC and eT, respectively. Leaf area per shoot and leaf area by a centimeter of the shoot was 20–37% inferior under all treatments except for eC + eT that did not change the leaf area per shoot significantly compared to Control (Table 1). Single eT was more unfavorable to leaf biomass per shoot and leaf area per shoot than the combined eC + eT treatment. Also, the survivorship of shoots decreased only in heated plots (eT and eC + eT). The flowering was more intense under eC than Control expressed as the number of flowers per shoot (+23%) or by the number of flowers per flowered ramification (+33%, Table 1). Contrastingly, the number of flowers per flowered ramification decreased under eT (−32%, Table 1). The flowering stimulation of rising [CO_2] and the flowering impairment by warming was evident with the similar and low average values regarding the number of flowers per flowered ramification in eC + eT and eT, respectively.

The discriminant analysis confirms those results of vegetative and reproductive growth by outputting differences among atmospheric regimes at $p = 0.05$ (Figure 3). There are four sets of symbols representing the three treatments and the Control with arrows pointing to the centroids of each set. The symbols representing eT stand out to the right of the others, indicating

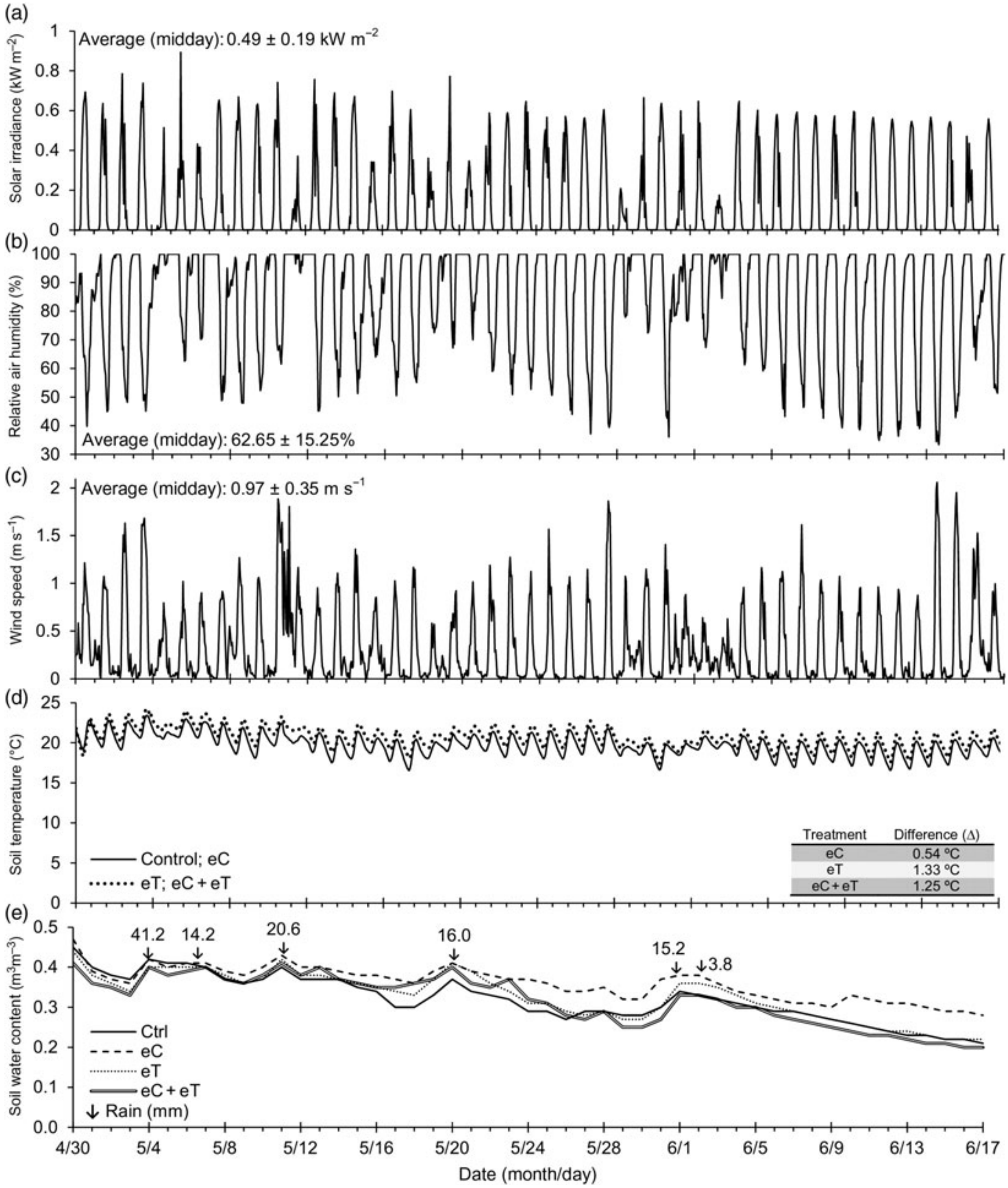
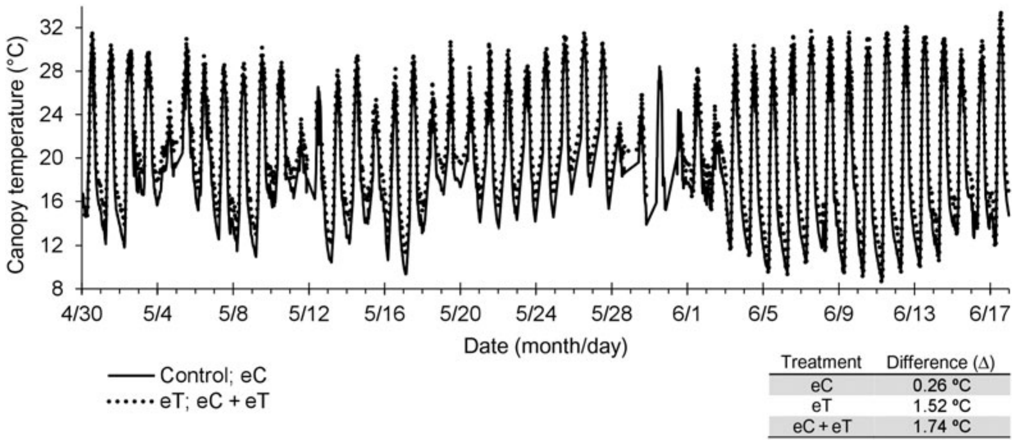


Figure 1. The daily and midday average values (±standard deviation) of total solar irradiance (a), relative air humidity (b), and wind speed (c) from 30 April to 17 June 2015 at the center of the experimental area. The soil temperature in heated and non-heated treatments (d), with the average of the differences (Δ) between those treatments and the Control. The continuous line represents the averages in plots with natural conditions of CO_2 and temperature (Control) and enriched CO_2 at 600 ppm (eC). The dotted line represents the averages of enhanced temperature +2 °C above ambient (eT), and the combined treatments (eC + eT). The soil water content (e) was obtained in Control (Ctrl), eC, eT, and eC + eT. The arrows with numeric values in (e) indicate rainfalls (mm) during the experimental period.

a more differentiated plant response. It corroborates with results described earlier in Table 1 being warming harmful to some vegetative and reproductive growth. The set of symbols representing eC + eT appeared between those referring to eC and eT and overlapping some Control symbols. This intermediate position of eC + eT symbols is consistent with the mitigation by eC of some

Table 1. Percentage of shoot survivorship and average ± standard error of vegetative and reproductive growth parameters of *Stylosanthes capitata* grown under different atmospheric regimes: ambient CO_2 and temperature (Control), 600 ppm of CO_2 (eC), canopy temperature 2 °C above (eT), and eC + eT. Different letters after percentage of survivorship or ± standard error indicate significant differences at $p < 0.1$ by Chi-square test for shoot survivorship and Mann–Whitney test for the other parameters. Values in brackets indicate the percentage of increasing (+) or decreasing (−) about Control

Growth parameters		Control	eC	eT	eC + eT
Vegetative	Shoot survivorship (%)	75 A	70 AB	53 B	40 B
	Leaf biomass per shoot (mg)	223 ± 31A	136 ± 14 B (−39)	151 ± 26 B (−32)	170 ± 34 AB (−24)
	Stalk biomass per shoot (mg)	253 ± 28A	215 ± 23 A (−15)	305 ± 57 A (+21)	290 ± 40 A (+15)
	Total shoot biomass (mg)	476 ± 63A	351 ± 34 A (−26)	455 ± 74 A (−4)	460 ± 74 A (−3)
	Leaf area per shoot (cm^2)	42.2 ± 5.6 A	27.9 ± 2.5 B (−34)	30.5 ± 5.6 B (−30)	35.7 ± 5.6 AB (−15)
	Leaf area per centimeter of shoot (cm^2 cm^{-1})	1.32 ± 0.06 A	1.05 ± 0.09 B (−20)	0.83 ± 0.07 B (−37)	0.99 ± 0.06 B (−25)
Reproductive	Number of flowers per shoot	18.3 ± 3.4 B	22.5 ± 2.7 A (+23)	15.8 ± 2.7 B (−14)	17.7 ± 4.0 B (−3)
	Number of flowers per flowered ramification	6.4 ± 0.77 1.2 B	8.5 ± 0.84 A (+33)	4.3 ± 0.55 C (−32)	5.4 ± 1.0 BC (−16)



Treatment	Difference (Δ)
eC	0.26 °C
eT	1.52 °C
eC + eT	1.74 °C

Figure 2. Daily canopy temperatures recorded from 30 April to 17 June 2015. The averages of the plots with ambient CO_2 and temperature (Control), and with 600 ppm of CO_2 (eC), are represented by a continuous line. The averages of the plots with the temperature at 2 °C above ambient (eT), and with the combined treatments (eC + eT), are represented by a dotted line. The average of the differences between the treatments and the Control are shown as the Δ values.

adverse eT on vegetative (leaf biomass and area per shoot) and reproductive (number of flowers per flowered ramification) growth shown in Table 1. The distinct effects imposed by eC and eT on vegetative and reproductive growths are represented by the great distance of the centroids and the opposition of the areas occupied by the corresponding symbols of both treatments (Figure 3).

In Figure 4 it is possible to identify four periods of flowering (P1–P4). After irrigation at the beginning of the experiment from 30 April to 8 May, the average number of flowers per shoot was similar in all climatic regimes with soil water content (SWC) higher than 0.30 m^3 m^{-3} (P1). The differences of soil temperature (ΔT) and soil water content (ΔSWC) between non-warmed and

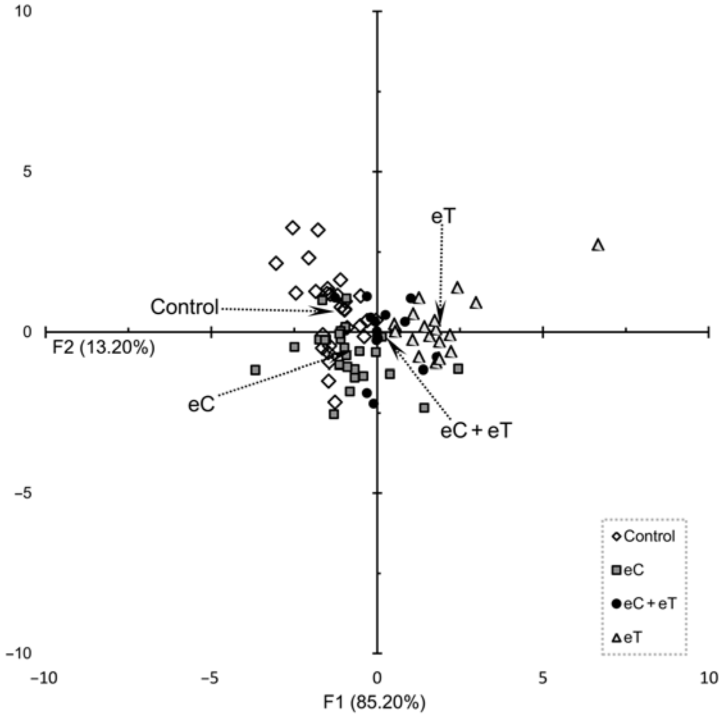


Figure 3. Discriminant analysis considering all vegetative and reproductive growth parameters of *Stylosanthes capitata* grown under ambient CO$_2$ and temperature (Control), under 600 ppm of CO$_2$ (eC), under foliar temperature 2 °C above ambient (eT), and under eC + eT. Symbols represent a synthesis of all parameters measured in each shoot. Different symbols indicate the treatments applied: control (◇), eC (▣), eT (△), and eC + eT (●). Arrows point to the centroid of the distribution of observations in each treatment. The proximity of symbols or centroids indicates how close the atmospheric regimes are associated.

warmed regimes varied broadly with high soil water moisture due to initial irrigation and rains along P1 (Figure 4c). The average number of flowers per shoot was higher in warming than in non-warming regimes in P2 (Figure 4a). Heated regimes probably favored flowering along P2, when the rains of 20.6 and 16 mm kept SWC above 0.30 m^3 m^{-3}. ΔT and ΔSWC showed a broader range of daily variation in high soil moisture between P1 and P2 than in the last P4 period, which was free of rain (Figure 4c). At the beginning of P3, SWC reached 0.30 m^3 m^{-3}, but it was 0.35 m^3 m^{-3} in eC, with a new flowering behavior without particular divergence between warming and non-warming regimes (Figure 4a, Figure 4b). From P1 to P4, the SWC was higher than 0.30 m^3 m^{-3} only in eC with flowering increasing or showing slight decreasing resulting at the end of the experiment in the highest average values regarding the number of flowers per shoot (Table 1). In P3 there was a reduced oscillation of ΔT, but ΔSWC increased before the rains displaying that the transient water availability affected more ΔSWC than the soil ΔT. Without rains at P4, the SWC dropped to values lower than 0.30 m^3 m^{-3} in all regimes with an exception in eC. The flowering promotion was evident in eC, probably because of the favorable availability of carbon from the atmosphere and water from the soil.

Leaf anatomy measurements

The anatomy of the adaxial cuticle and mucilage layer, and the adaxial epidermis in *S. capitata*, along with total leaf thickness, did not show any alterations under elevated CO$_2$ and warming (Figure 5). However, the leaves grown under elevated CO$_2$ showed a palisade parenchyma 9%

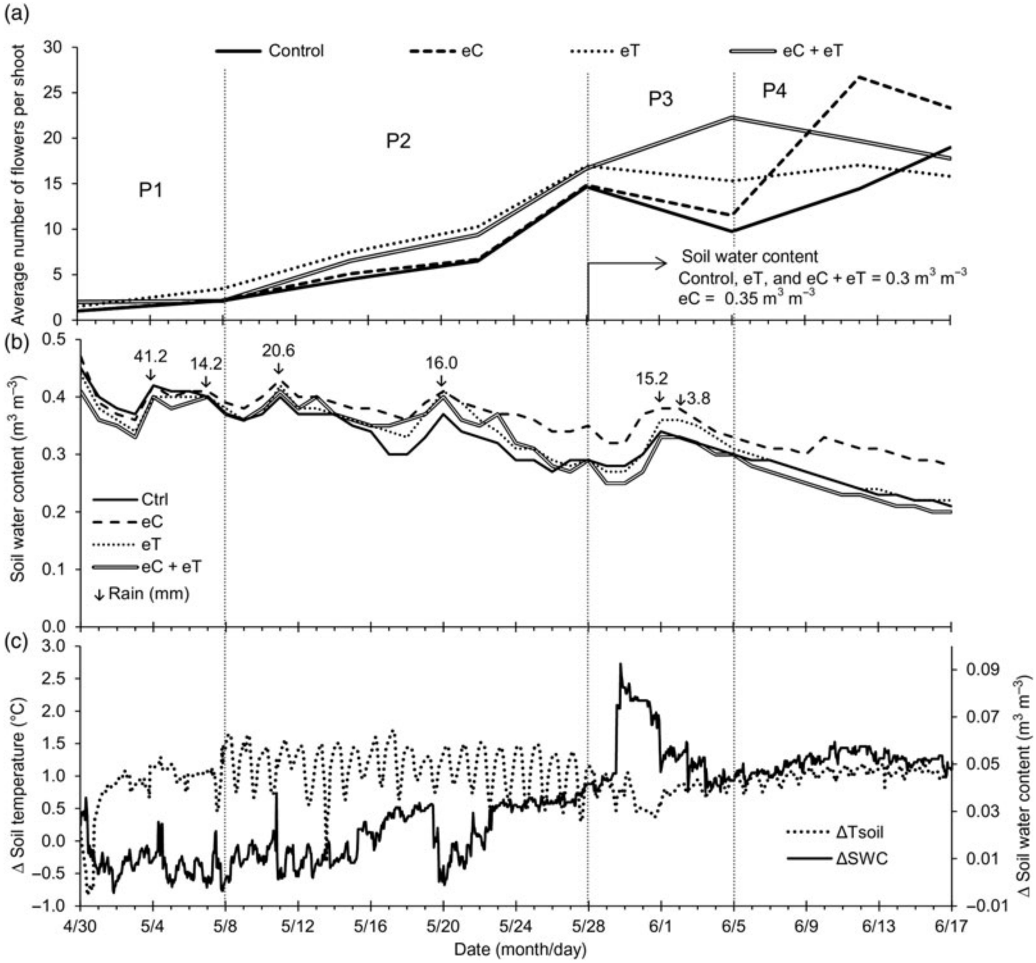


Figure 4. Flowering, daily soil water content, and differences of soil temperature and soil water content during the experimental period from 30 April 2015 to 17 June 2015. Four periods (P1–P4) were depicted according to flowering behavior. (a) Average number of flowers per shoot in *Stylosanthes capitata* under ambient CO_2 and temperature (Control, solid line), under 600 ppm of CO_2 (eC, dashed line), under foliar temperature 2 °C above ambient (eT, dotted line), and under both treatments (eC + eT, open line). (b) Soil water content in plots under Control, eC, eT, and under eC + eT. Arrows with numeric values represent the rainfall intensity (mm). (c) Average soil temperature differences (ΔTsoil) between non-warmed (control and eC) and warmed (eT and eC + eT) regimes and the average of the differences in soil water content between eC and the other atmospheric regimes.

thicker than the Control leaves. The eT treatment reduces the spongy mesophyll by 15% and the abaxial cuticle and mucilage layer by 5%, compared with leaves grown under Control. No interaction between CO_2 and temperature was detected for any of these leaf anatomical traits.

Discussion

The vast majority of studies of growth and biomass accumulation in C_3 plants conducted in an atmosphere enriched with CO_2 showed enhanced C_3 plant growth and yield (Bhatt et al., 2010; Leakey et al., 2009; Marabesi, 2007; Wand et al., 1999). Commonly, these studies used a different type of growth chambers or open-top chambers to maintain elevated [CO_2] besides ample water availability to roots overestimating the C_3 plants' gains (Ainsworth and Long, 2005). In such studies, the reported increases in total biomass are approximately 40% (Poorter, 1993; Wand et al., 1999). Here, despite the investment in palisade parenchyma leading to an increase in leaf thickness, the leaf production was not benefited on mass or area per shoot in eC. Leaf parenchyma is one of the most massive sinks of carbon in a leaf (Bolhar-Nordenkampf, 1985). The larger cells and thicker palisade layer in plants grown under elevated CO_2 often exhibit an increase in the number and size of chloroplasts, and this provides more gas exchange area since the number of chloroplasts facing the cell wall also increases (Xu et al., 2012). Beyond these changes, a thicker palisade layer may also be related to an enhanced ability to spread light to internal regions of the mesophyll (Terashima and Hikosaka, 1995) increasing the photosynthetic rate, decreasing the likelihood of photoinhibition (Nishio and Whitmarsh, 1993) and consequently enhancing biomass accumulation. However, stalk and shoot biomass remained similar to the Control besides the foliage production per shoot decreasing in eC. As shoot biomass only included stalk and leaf and did not add the flowers, the more magnificent flowering indicated more photoassimilates allocation in the reproductive organs under eC.

The lack of irrigation probably was not the primary factor in the reduction of leaf production per shoot since there was more favorable soil water availability in eC until the end of the experiment (usually greater than 0.30 m^3 m^{-3}). *S. capitata* reduced the leaf production per shoot despite the potentially increased CO_2 uptake capacity by thicker palisade parenchyma and by higher water and CO_2 availability, respectively, to roots and leaves, indicating a down-regulation about foliage production per shoot in eC. Also, if eC contributed to higher water use efficiency (Bowes, 1993; Long and Ort, 2010), this contribution *per se* did not result in a gain of autotrophic tissues per shoot. Since the leaf biomass provides the food supply for the cattle, elevated CO_2 in the atmosphere would not be favorable for supplying livestock by rain-fed *S. capitata* even under favorable soil moisture due to the down-regulation of leaf production per shoot.

In general, elevated temperature harms C_3 plants (Fagundes et al., 2010). However, in a previous study with *S. capitata* in the same experimental area using irrigation, Martinez et al. (2014) found increased biomass production by 16% under moderate warming (+2 °C) during the vegetative stage. Warming without water and nutritional restrictions were beneficial for the physiological and biophysical processes involved in *S. capitata* leaves (Habermann et al., 2019; Martinez et al., 2014). Our study was conducted under similar temperature and soil nutritional conditions as carried out by Martinez et al. (2014), but rain-fed instead of being irrigated. The leaf water potential at noon and leaf water content at pre-dawn is typically 9% lower in eT than in Control (Habermann et al., 2019). The values of soil water content decreased from 0.45 to 0.25 m^3 m^{-3} at the end of P4 in eT. By comparing our results of foliage production with those of Martinez et al. (2014), it is evident that the lower soil water content under eT was hampering the foliage regrowth of *S. capitata* since shoot survivorship and the biomass and area of leaves per shoot diminished.

Therefore, when the temperature was moderately elevated, *S. capitata* invested less in vegetative growth and even in reproduction (lower number of flowers per flowered ramification) in rain-fed conditions. It was the absence of irrigation aggravated by the warming that was responsible for the impairment of the vegetative regrowth and flowering under eT rather than the single warming (LeCain et al., 2015; Wall et al., 2011). The increased foliage temperature and water stress probably nullified the benefits of elevated CO_2 concentration for photosynthesis in the rain-fed plantation of *S. capitata* by increasing respiration, photorespiration, mesophyll resistance to carboxylation and stomatal resistance to CO_2 access (Battisti and Naylor, 2009; Monteiro and Prado, 2006). These stressful conditions resulted in less number of flowers per flowered ramification and inferior area and mass of leaves per shoot in eT. In short, the absence of irrigation nullified the potentially favorable conditions under moderated warming +2 °C, being the effect of eT detrimental to vegetative and reproductive growth in rain-fed circumstances. It is an alarming result for using *S. capitata* as forage in the future, since its initial regrowth may be impaired by higher temperatures under the heat waves expected to accompany the global climate change in south-eastern Brazil (Andrade, 2013; IPCC, 2014).

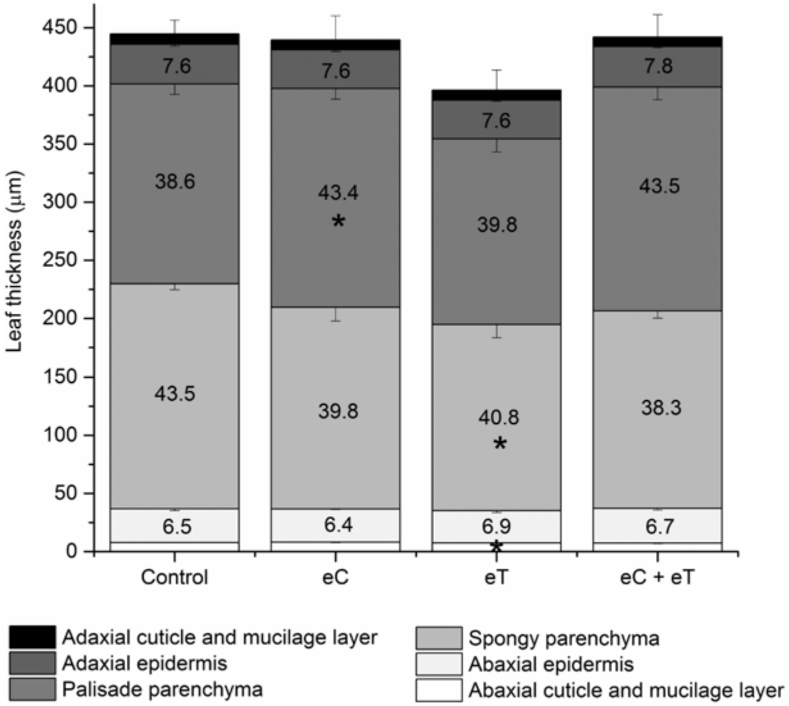


Figure 5. Isolated and combined effects of elevated CO_2 and temperature on leaf anatomical characteristics of *Stylosanthes capitata*. Each leaf tissue is represented by a different color, and the measurements given (µm) are averages of four replications ($n = 4$). The percentage (%) of each tissue concerning total leaf thickness is shown for the adaxial and abaxial epidermis and palisade and spongy parenchyma in the columns. The negative bars (−SE) represent the standard error for leaf layer thickness (µm) and the positive bars (+SE) for total leaf thickness (µm). Treatments: Control (ambient CO_2 and temperature), eC (600 ppm CO_2 concentration and ambient temperature), eT (ambient CO_2 concentration and 2 °C above ambient canopy temperature), and eC + eT (600 ppm CO_2 concentration and 2 °C above ambient canopy temperature). Significant differences ($p < 0.1$) between the treatments and the control are indicated by an asterisk (*).

The thinner spongy parenchyma under moderate warming was related to spongy cell number and volume diminishing (Chabot and Chabot, 1977; Tomás et al., 2014). Due to the relationship between cell wall elasticity and cell size, small cells can support a higher negative turgor pressure than large cells (Chartzoulakis et al., 2002), reducing the probability of cell collapse under drought conditions, a regular adaptation of xeromorphic species (Oertli et al., 1990). Total leaf thickness under moderate warming decreased by approximately 10% as expected (Figure 5), with thinner spongy parenchyma contributing to this. This phenomenon is essential in eT because thinner leaves lose more energy by conduction processes due to their higher capacity for thermal conductivity (Chandra, 2004). Moreover, thinner spongy parenchyma provides less resistance to carboxylation because it shortens the path of the CO_2 from the atmosphere to the palisade parenchyma. However, these alterations in spongy parenchyma did not avoid the declining of survivorship and the production of foliage in mass or area per shoot in eT.

Therefore, in grazing areas without irrigation and subject to elevated CO_2 or warming, *S. capitata* will experience significant unfavorable conditions for growing as in the future climate changes. Nonetheless, irrigation of an extensive area may cause the loss of water courses, soil salinization, and contamination (Khan and Hanjra, 2008). Our results specify a disturbing situation since the future climate will change rainfall patterns in south-eastern Brazil (Marengo, 2006), the region with the second-largest Brazilian cattle herd (MAPA, 2015).

The adverse effects of eC associated with the down-regulation of foliage production and the adverse effect of eT regarding the access and assimilation of CO_2 were partially compensated in the combined treatment given that the leaf area and leaf biomass per shoot were similar to the Control in eC + eT. Other compensatory effects in eC + eT are evident by percentage values less negative in combined than in eT treatment. However, foliage production per shoot in eC + eT was also similar to eC and eT. Moreover, the shoot survivorship was the lowest in eC+eT. In the combined treatment, the down-regulation of leaf production and the constraints to CO_2 assimilation probably are associated with the decreasing shoot survivorship. Therefore, each atmospheric regime acts differently ($p = 0.05$) on vegetative and reproductive growth of *S. capitata*, but neither none of these (eC, eT, and eC + eT) promoted the leaf area or leaf biomass production per shoot.

In conclusion, the elevated [CO_2] and leaf temperature associated with decreasing of soil water content in rain-fed plantations of *S. capitata* will impair foliage production and increase shoot mortality during Brazilian autumn. The effects of high atmospheric CO_2 concentrations on the C_3 photosynthesis pathway will not be enough to offset the adverse effects of warming and water shortages in autumn in expected future climate change. Despite significant differences in leaf tissue related to drought and elevated temperature, they will not also be sufficient to compensate for the stressful conditions in the field under eC or eT. Single CO_2 enrichment (600 ppm) will down-regulate foliage production and promote flowering, especially during the period of favorable soil water content. The eT foliage enhancement (+2 °C) will also decrease leaf production and increase shoot mortality. The combined treatment eC + eT will increase shoot mortality without promoting foliage production in remaining shoots.

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