SÃO PAULO STATE UNIVERSITY – UNESP

CAMPUS OF JABOTICABAL

ANTIOXIDANT METABOLISM OF *Panicum maximum* AND *Stylosanthes capitata* UNDER CLIMATE CHANGE

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AGRONOMIST

Jaboticabal - São Paulo

2019

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Dissertation submitted to the Faculty of Agricultural and Veterinary Sciences- UNESP Campus of Jaboticabal, as requirement for the degree of Doctor of Science in Agronomy (Crop Production).

Jaboticabal - São Paulo

2019

B734a

Borjas-Ventura, Ricardo Roberto
Antioxidant metabolism of Panicum maximum and Stylosanthes capitata under climate change / Ricardo Roberto Borjas-Ventura. -- , 2019
99 f. : il., tabs.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Farmacêuticas, Araraquara,
Orientadora: Priscila Lupino Gratão
Coorientadora: Carlos Martínez y Huamán

1. Estresse vegetal. 2. Gramíneas Forrageiras. 3. Leguminosas forrageiras. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Farmacêuticas, Araraquara. Dados fornecidos pelo autor(a).

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: ANTIOXIDANT METABOLISM OF *Panicum maximum* AND *Stylosanthes capitata* UNDER CLIMATE CHANGE

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Ricardo Roberto Borjas Ventura-nascido no dia 16 de janeiro de 1982, na cidade de Ica, Departamento de Ica-Perú, é filho de Ricardo Fernando Borjas Veja e de Melcy Ruth Ventura Maurtua. Cursou o ensino fundamental na escola "Colegio Parroquial Mixto San José de Cerro Alegre" na província de Cañete no departamento de Lima. Em 2001 ingressou à "Universidad Nacional Agraria La Molina" em Lima-Perú. Fez o trabalho de fim de curso no Departamento de Fitotecnia sob a orientação do Dr. Alberto Julca Otiniano. No ano de 2008 obteve o título de Engenheiro Agrônomo. Em outubro de 2010 iniciou o mestrado na "Universidad de Jaén" na "Comunidad autonoma de Andalucia" em España. Em 2011 obteve o título de mestre sob a orientação de Dra. Pilar Cordovilla Palomares, Dr. Víctor Aranda Sanjuan e Dr. Julio Calero Gonzalez. Em março 2016 começou o doutorado no programa de Produção Vegetal na Universidade Estadual Paulista campus Jaboticabal com bolsa da CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) sob a orientação da Dra. Priscila Lupino Gratão.

DEDICATÓRIA

Aos meus pais Ricardo e Melcy, às minhas irmãs Diana e Karina, aos meus sobrinhos Italo e Rodrigo.

AGRADECIMENTOS

Agradeço a Deus por todas as oportunidades que me dá todos os dias. Por dar-me a força para seguir lutando cada dia com mais força.

Às instituições UNESP, campus Jaboticabal por ter me aceito como estudante da pós-graduação. À UNALM (Universidad Nacional Agraria La Molina) por seu apoio institucional e a confiança durante meu tempo de estudo.

Aos meus pais (Ricardo e Melcy) e minhas irmãs (Diana e Karina) pela paciência e por todo o amor que sempre me têm.

À professora Priscila Lupino Gratão por ser minha orientadora e amiga. Agradeço por me dar a oportunidade de ser seu orientado, por tudo o que aprendi dela, contribuindo de forma importante na minha melhora pessoal e professional.

Ao professor Carlos Martínez y Huamán por ter confiado em mim e por ter me permitido ser parte de seu projeto de pesquisa. Por seu apoio durante todo este tempo no Brasil.

A Soninha Carregari por ser meu apoio em todo momento (dentro e fora da faculdade). Por seu tempo e paciência e todo o carinho que sempre mostrou para mim. Estarei eternamente agradecido. A todo o pessoal do laboratório, aprendi muito com eles. Obrigado pelo carinho e amizade.

Ao professor Arthur, Pedro Luiz e Ferraudo, pela amizade criada entre nós. Agradeço seus constantes conselhos e ensinos.

Ao professor Alberto Julca, pelos conselhos e os ânimos. São muitos anos apoiando-me e presenteando-me com sua amizade.

A minha família aqui no Brasil, o pessoal da república Kambuka (Jorge, Paulo, Gustavo, Yoandry, Bicudo, Juancito, Denis, Daniel “chefe”). Agradeço sua paciência, seu apoio e sua amizade, foram três anos nos quais me senti em casa. A meus amigos da faculdade, aprendi muito com eles e passe momentos inesquecíveis.

A todas as pessoas que contribuíram direta ou indiretamente com este estudo.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior Brasil (CAPES)-Código de financiamento 001.

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ANTIOXIDANT METABOLISM OF *Panicum maximum* AND *Stylosanthes capitata* UNDER CLIMATE CHANGE

ABSTRACT- Drought and heat stresses are considered the main climatic factors damaging the plant growth. In addition, according to Intergovernmental Panel on Climate Change (IPCC), the effect of combined drought and heat stresses will also be exacerbated in the next years, resulting in crop yield and economic losses. In spite of the consequences of the drought and heat combinations,, the investigations to reduce its detrimental effects are scarce, particularly under climate change conditions. In this sense, we carried out two experiments in field conditions to determine the plant antioxidant responses to drought (wS), elevated temperature (+2 °C above air temperature) (eT) and combined drought and elevated temperature stresses. We used *Panicum maximum* during the first experiment and *Stylosanthes capitata* during the second one. In order to increase the temperature, we used the Temperature Free-Air Controlled Enhancement **(**T-FACE) facility. Samplings of *P. maximum* were taken 13, 19 and 37 days after the treatments have begun in the first experiment; meanwhile, the samplings of *S. capitata* were taken 17, 24 and 46 days in the second experiment. In both experiment, the samplings were taken at 6:00 am and 12:00 pm. The following combined stresses displayed an increase of chlorophyll and carotenoid contents in *P. maximum* (particularly at 19 days after the treatments have begun). Furthermore, the occurrence of precipitation after the second sampling reflecting induced the decreased in MDA and carbonyl contents, such as observed in wS treatment at 6:00 am in the third sampling. In relation to antioxidant enzymatic responses, the combination of drought and heat stresses enhanced SOD and APX activities, whilst quantum efficiency (PSII) and quantum yield of non-regulated energy dissipation in PSII (Y(NO)) were not affected by stresses. By the other way, the combination of drought and elevated temperature decreased the chlorophyll content in *S. capitata* at 6:00 am, nevertheless, we noticed an increase in photosynthetic pigments at 12 pm. MDA and H_2O_2 contents were also increased by simultaneous stresses such as exhibited by third sampling, at 6:00 am. In

addition, combined stresses caused detrimental effect on antioxidant enzymatic responses (SOD, CAT and GR) at 46 days. SOD, GR and GSH have a crucial role to counteract the overproduction of MDA and H_2O_2 contents. In conclusion, *P. maximum* exhibited moderate response to combined stresses related to the good performance of its enzymatic antioxidant defence; whereas, *S. capitata* was severely affected by combined stresses.

Key words: Biochemistry response, combined stresses, T-FACE, pastures, field assessment.

Chapter 1.- General considerations

1. Introduction

Climate change, particularly the high temperatures may bring about great crop yield loss, especially in tropical and sub tropical regions (Rodríguez, 2007). In fact, in Brazil is expected an increase in the mean temperatures (26.3-38.9 ˚C) in the next 100 years. In addition, the variation of temperature will cause an increment of drought, resulting in crop yield significant reductions (Fagundes et al., 2010; Souza et al., 2012; Rio, 2014). The dramatic reduction of yields will cause great crise in the agricultural sector (Marengo, 2008), putting at risk the supply of food in the worldwide.

Likewise, some researchers also inform that the pastures will be affected by the elevated temperature and drought, for instance, a notable decline is expected in the production of *Panicum maximum* in the next years (Pezzopane et al., 2016). This scenario will threaten to the livestock farming (Nääs et al., 2010).

The pastures play a key role within the livestock farming and can occupy a large extension of land, for example, it is estimated 172 x 10^6 ha of pastures in Brazil (COOPEAVI, 2018), occupying 20% of agriculturable land.

In spite of the negative predictions, nowadays, there are few works studying how to diminish the climate change impact on the pastures, especially in *Panicum maximum* and *Stylosanthes capitata,* particularly in field conditions (Zhang et al., 2012; Approbato, 2015).

The yield reduction in crops subjected to drought and heat stresses is associated to overproduction of reactive oxygen species (ROS) (Superoxide ion: O_2^-; Synglet Oxygen: $^1O_2$; Hydrogen Peroxide: H_2O_2 and hydroxyl: •OH) (Mitller, 2017), attacking cellular membrane (lipid peroxidation), proteins and DNA (Das and Roychoudhury, 2014); increasing the membrane permeability, altering the protein function and structure, causing mutations and leading to plant death. We also have to mention that in normal conditions, ROS can occur as byproduct of physiological events, participating in the normal development of plants.

In order to reduce the detrimental effect of ROS, plants have developed a set of responses classified in enzymatic and non-enzymatic antioxidants. The enzymatic defence system is composed by Superoxide Dismutase (SOD, EC 1.15.1.1), Catalase (CAT, EC 1.11.1.6), Glutathione Peroxidase (GPX, EC 1.11.1.9), Glutathione Reductase (GR, EC 1.8.1.7), Monodehydroascorbate Reductase (MDHAR, EC 1.6.5.4), Ascorbate Peroxidase (APX, EC 1.11.1.11) and Dehydroascorbate Reductase (DHAR, EC 1.8.5.1) (Acosta et al., 2017).

In addition, non-enzymatic responses consist of Glutathione (GSH), Vitamin E (tocopherols and tocotrienols), Ascorbate (vitamin C) and Carotenoids (pro-vitamin A) (Asensi-Fabado and Munné-Bosch, 2010; Pintó-Marijuan and Munné-Bosch, 2013).

On the other hand, the combined stresses can not be considered like the sum of isolated stress since triggering different signalling pathways (Zhao et al., 2016; Zandalinas et al., 2018). The plant response also depends on the time and the magnitude of stress, and of specie/cultivar (Hu et al., 2010; Awasthi et al., 2017; Zandalinas et al., 2017).

Likewise, there are a plenty of works about combined stresses; however, most of them were carried out in greenhouse or climatic chamber (Zandalinas et al., 2017; Zhou et al., 2017; Correia et al., 2018). In addition, the researchers have used considerable temperature increase (15 °C-23 °C) for quantification of combined stresses (Suzuki et al., 2016; Silva et al., 2018). These types of works do not reflect realistic growth conditions (field conditions). In fact, experiments with a slight increment of temperature are scarce yet.

In light of lack of information about combined stress in pastures under field conditions (climate change mimic condition), this work was carried out in order to quantify the biochemical responses of *Panicum maximum* and *Stylosanthes capitata* to the increment of temperature (+2 °C above air temperature), drought and its combination.

2. Literature review

2.1. Global Warming

The Earth always showed equilibrium between the absorbed and emited energy. This balance allowed the life as we know it; however, the imbalance between energy absorbed and emited is causing the Earths warming (greenhouse effect) (EMBRAPA, 2016), leading to increment of temperature (Ring et al., 2012).

In order to study the increase of temperature and its effect on human activity, the nations created the Intergovernmental Panel on Climatic Change (IPCC) whose mission is “to evaluate the scientific, technical and socio-economic relevant information so as to understand the risks of climatic change on human population” (Orsini, 2007). IPCC also determined different future global scenerios (IPCC, 2007) (Table 1).

Each scenario will have different elevation of temperature depending on social and economic factors. For example, the scenario B1 corresponds to countries with rapid economic growth, and with few use of materials, and introduction of clean technologies. A1T is related to societies with rapid economic growth and use of non-fossil energy. In the case of B2 scenario, the world increases its population and seeks local solutions to economic ans social problems (IPCC, 2007).

The A1B scenario point out rapid economic growth and a balance in the use of all sources of the society. A2 scenario indicates a heterogeneous world with a rapid growth of population, but with slow and fragmented economic growth, and with few technological changes (IPCC, 2007).

Table 1. Climate change predicted by IPCC (2007).

Scenerios	T ˚C stimated	Range of T ˚C
B1	1.8	1.1-2.9
A1T	2.4	1.4-3.8
B2	2.4	1.4-3.8
A1B	2.8	1.7-4.4
A2	3.4	2.0-5.4
A1F1	4.0	2.4-6.4

Greenhouse gases (GHG) (Ring et al., 2012) provoke the increase of temperature. GSG embraces the dioxide (CO_2), nitrous oxide (N_2O), methane (NH_4), chlorofluorocarbon (CFC) and fluorinated gases (IPCC, 2007; Trenberth et al., 2014). In general, GHG absorb the infrared light, trapping the heat in the atmosphere causing an increment of temperature and consequently, the greenhouse effect (Power, 2009).

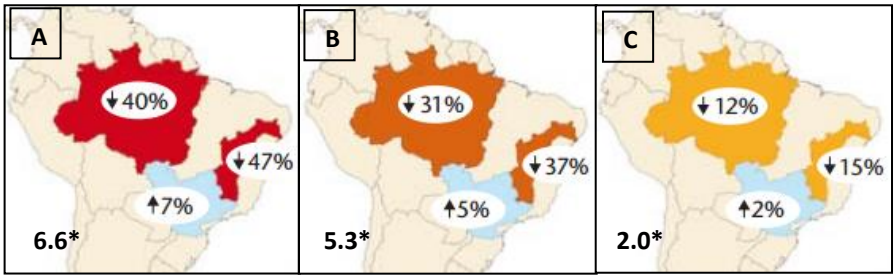
On the other hand, each gas composing GHG has different effects on global warming, in fact, CO_2, N_2O, NH_4 , CFC and fluorinated gases are responsibles for 64.3%, 6%, 17.0%, 11.9% and 0.8%, respectively (IPCC, 2007). In the same way, the GHG has different infrared radiation absorb capacity, for example, N_2O absorb 320 more times infrared radiation than CO_2 (Sandoval et al., 2003). Interestingly, if water vapor were considered as part of GHG, it would be responsible by 32-76% greenhouse effect and CO_2 by 9-26% (Braga and Pinto, 2009).

2.2. Increment of temperature in Brazil

Brazil had undergone an increase of minimum temperatures in 1.4 ˚C every 10 years from 1951 to 2002, meanwhile, the maximum temperatures increased in 0.4-0.6 ˚C (Orsini, 2007). In addition, São Paulo state presented a maximum temperature of 37.2 ˚C in 2014 (Marengo, 2014). Furthermore, for the

year 2100, under CO_2 high emission scenerio, Brazil will increase its mean temperature until 38.9 ˚C, whereas, under scenery of CO_2 low emission, this country will have a mean temperature of 26.3 ˚C.

The elevated temperature will bring variations in the precipitation of different regions and seasons (Trenberth et al., 2014), for instance, according to INPE (2016), Brazil will have a reduction of the precipitation, and an increase of temperature, causing a detrimental effect on the agricultural activity of tropical and subtropical countries (Rodríguez, 2007).



*Increment of mean temperature (˚C)
↑ Increment of precipitation ↓diminishing of precipitation

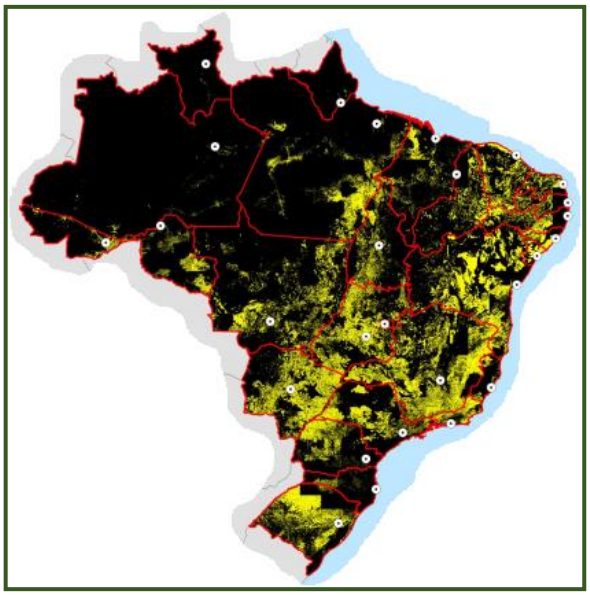
Figure 1. Relation between temperature and precipitation in Brazil (INPE, 2016). A) Scenerio 1: increment of mean temperature in 6.6 ˚C; B) Scenerio 2: increment of mean temperature in 5.3 ˚C; C) Scenerio 3: increment of mean temperature in 2.0 ˚C.

2.3. Pastures in the world and Brazil

The pastures are all those that can serve as grazing to animals, thus, it refers to grasses, legumes and other plants. Globally, the pastures cover an area of 3.5 x 10^9 ha (Tubiello et al., 2007). In Brazil, the pastures cover 172 x 10^6 ha (Figure 2), of which 90% are used to feed meet cattle. As well as, we have to mention that Brazil is the second meat worldwide producer (MAPA, 2016). These data display that the pastures play a crucial role into the production of meat.

In addition, in Brazil, the pastures are distributed in different states as Rio Grande do Sul, Paraná, Mato Grosso do Sul, São Paulo, Minas Gerais, Bahía,

Pernanbuco, Rio Grande do Norte and Ceará (Figure 2). On the other hand, one of the most used species as pasture is *Panicum* spp, which could be accompained by *Stylosanthes* spp. (Valle et al., 2009).



Yellow colour: Area with pastures in Brazil.

Figure 2. Distribution of pastures in Brazil (Ferreira et al., 2014).

2.4. Climate change and pasture productions

The agricultural activities are strongly linked to climatic factors such as precipitation and temperature (Araújo, 2012; Dias and Da Silva, 2015). On the other hand, in the next years, it is expected an increase of temperature and decrease in the precipitation, that could provoke great crises in agriculture (Marengo, 2008).

The global warming causes detrimental effects on pasture production (Al Faiz et al., 2010; Chaplin-Kramer and George, 2013); and consequently on the livestock production chain, especially on the meat cattle production. According to Marengo (2008), irregular precipitations bring about negative effects on livestock activity development, for example, drought causes a severe

diminishing on photosynthesis, dry mass yield, growth and forage quality, and consequently, on the performance of cattles (Durr and Rangel, 2003; Melo et al., 2009; Canesin, 2014; Brandão, 2016; ONU, 2019).

In spite of to have numerous informations about negative effects of climatic change on the crop production, there are few data of field experiments especially in pasture. Likewise, the field experiments are important because it supports the result of informatical simulations. In addition, the impact of climate change will be different in each place and for each crop, demonstrating the importance of carring out regional experiments (Barcellos et al., 2008).

2.5. *Panicum maximum*

P. maximum has special importance because it is use as feed of livestock. This specie has C4 metabolism. Additionally, it can grow in fertility medium and high soil; as well, it can grow in altitudes from 0 to 1500 m and produces 10-30 tons of dry mass year^{-1} ha^{-1} (Peters et al., 2010). It requires a mean temperature of 38 ˚C, with a minimum of 9 ˚C and a maximum of 50 ˚C (Del Pozo, 2002). As well as, the precipitation plays a crucial role in pasture quality indexes (Ramírez et al, 2011); in fact, it was estimated that this specie requires between 1000-3500 mm of precipitation per year.

Regarding to fertilization, *P. maximum* has a positive correlation with nitrogen application (Giacomini et al., 2014); however, this response depends on cultivar and nitrogen doses (Paciullo et al., 2016).

On the other hand, *P. maximum* cv Mombaça was introduced from Tanzania in 1984, and its yield is around of 15-20 tons per year. Futhermore, it also has high capability of extracting P from soil (Ruggieri, 2013).

2.6. *Stylosanthes capitata*

A specie that can accompany to *P. maximum* is *Stylosanthes capitata* (Pinheiro et al., 2014). The genus *Stylosanthes* comprises 41 species of which 37 are from America (in this continent, Brazil has high level of genetic diversity) (De Sousa and Schultze-kraft, 1993), *S. capitata* also occurs between 21 °S (Brazil) and 10 °N (Venezuela), displaying that this specie is adapted to high

temperatures (Tropical Forages, 2019). As well, it is able to grow from 0 to 1000 meters of altitude, and it requires 500-1500 mm of precipitation (FAO, 2016). On the other hand, *S. capitata* occur naturally on acid soils (pH<5) and has elevated yield in poor soils, and it can be used as a green manure (Karia et al., 2002; Rosa et al., 2011).

2.7. Temperature Free-Air Controlled Enhancement (T-FACE)

T-FACE facility elevates the air temperature, and hence the plant dossel temperature. T-FACE can also be used in field experiments to simulate global warming conditions (Figure 3).

On the other hand, T-FACE has heaters, which are placed above plant dossel. Likewise, so as to increase the temperature, T-FACE detects (with thermometers) the environmental air temperature, and, subsequently, the heaters emit infrared ligh increasing plant dossel temperature. In addition, T-FACE has Datalogger, which serve to control of temperature (Kimball, 2005).

Regarding to the use of T-FACE, there are many reports displaying that T-FACE is a useful tool for field experiments with different crops such as wheat (White et al., 2012), rice (Hasegawa et al., 2013) and soybean (Prior et al., 2006); as well as, it can be used in the ecology area (LeCain et al., 2015).

Figure 3. Temperature Free-Air Controlled Enhancement (T-FACE) system in *S. capitata* (A) and *P. maximum* (B). Photos by Carlos Martínez y Huamán.

2.8. Plant stress

According to Kranner et al., (2010), stress is defined as physiological alterations caused by factor (s) which induces an imbalance in the plant normal development. Regarding to type of stress, the scientific literature displays different classifications for this phenomeno; for example, we can find elastic (reversible stress) stress and plastic stress (non-reversible stress), and Eu-stress (positive effect) and Dis-stress (negative effect) (Kranner et al., 2010) (Figure 4). In the same way, the positive effect of stress depends on its intensity (Jansen and Potters, 2016).

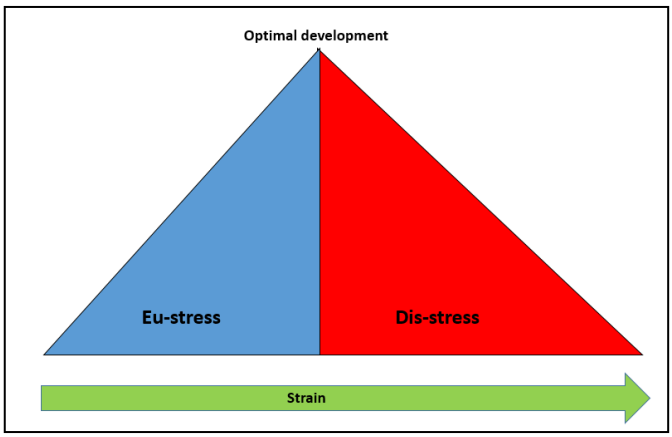


Figure 4. Two type of stress: Eu-stress and Dis-stress.

Furthermore, there are other classifications, for instance, biotic (for example: plant diseases) and abiotic stress. Abiotic stress is caused by non-optimal conditions of ligh, soil fertility, soil water, temperature and presence of heavy metals and other pollutants (Rhodes and Nadolska-Orczyk, 2001).

2.9. Heat stress

Currently, the Earth is undergoing an increment of temperature causing severe damage on crops (Hasanuzzaman et al., 2013; Pospisil, 2016). According to Zhu et al. (2010), heat stress provokes the increment of MDA and membrane permeability. In addition, numerous researches display detrimental

effects on growing, pollination, flowering and yield of wheat (Rosa, 2007; Oliveira et al., 2011).

Under heat stress, plants undergo of over production of ROS, reaching by 200%-1000%, killing to plants (Vacca et al., 2004) or decrease their performance.

In *Stylosanthes capitata*, an increment of 2 ˚C decreased electron transport, quantum yield, photosynthesis, leaf area and biomass (Martinez et al., 2014; Approbato, 2015). In *P. maximum*, the leave elongation was more predominant in plants under elevated temperature (2 ˚C) than in the Control (Prado et al., 2016).

On the other hand, plants have a set of mechanisms to face with elevated temperature conditions. Although, the plant responses depend on the time and intensity under heat stress, and the specie (Hasanuzzaman et al., 2013). Regarding to enzymatic response, in maize and wheat, Mengutay et al. (2013) reported that an increment of 10 ˚C elevated the activity of CAT, but decreased the SOD activity. Likewise, Li et al. (2014) informed that under prolonged heat stress, in *Zea mays*, the SOD activity was severely affected, nonetheless, under short time, SOD activity undergoes an increase. Furthermore, in *Stylosanthes capitata*, the increment of temperature (2 ˚C) did not enhance the SOD, CAT and APX activity (Martinez et al., 2014).

In relation to Ascorbic Acid (AA), it is increased by elevation of temperature (Zou et al., 2016; Ahmad et al., 2017). In addition, it plays a key role in the protection of cellular membrane under elevated temperature (Kamal et al., 2017). In some experiments, the tolerance to heat stress was associated to the AA synthesis (Khanna et al., 2016).

2.10. Drought stress

Other climatic factor causing severe damage on plants is the drought stress provoking biochemical and morphological changes, hence enormous decline of yield (20%-70%) in numerous crops (Figure 8).

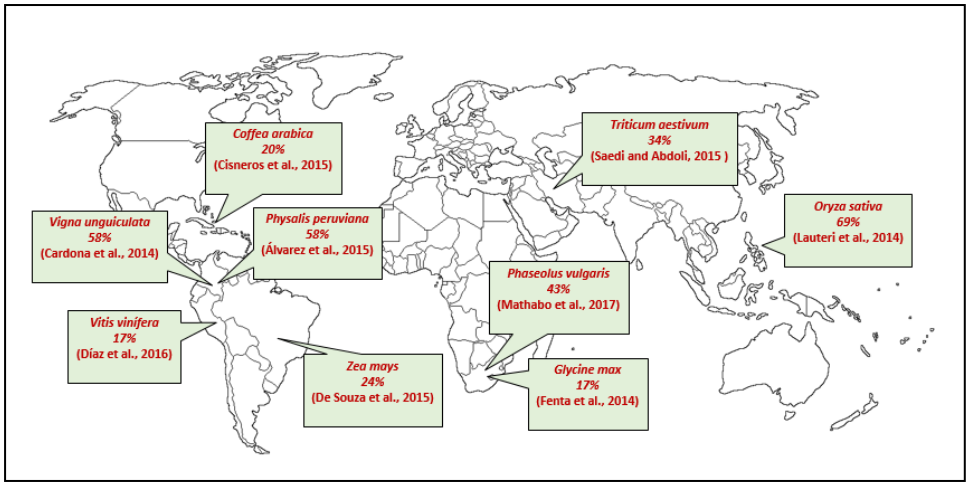


Figure 8. Yield loss in different crops caysed by drought stress (Forni et al., 2017).

The yield reduction is associated to over production of ROS, causing detrimental effects on the biochemical, physiological and morphological plant features, for instance, drought stress increases the content of MDA (Hameed et al., 2011), in fact, Vitkauskaite and Venskaityte (2011) determined that, under drought stress, *Panicum* sp. increased the MDA content by 24.7%. Additionally, the chlorophyll content underwent a diminished by this type of stress (Streit et al., 2005; Alemán et al., 2010; Silva, 2013). On the other hand, in *Panicum* sp., Meyer et al., (2014) reported a decrease in photosynthesis rate.

Morphological changes can be observed under drought situation, *Arachis hypogaea* underwent a decrease of root length (Sankar et al., 2014).

To cope with drought stress, plants have enzymatic and non-enzymatic defence systems. In relation to enzymatic response, in *Zea mays*, it was detected that, under moderate drought stress, SOD activity was increased by 42%-49%, however, under strong drought stress, the SOD activity increased by 53%-63% (respect to Control) (Lu et al., 2013). In *Zea mays*, Talaat et al. (2015) presented similar data.

CAT is crucial to limit the damage of ROS on plants (Yu et al., 2016; Farooq et al., 2016), in fact, in maize, Talaat et al. (2015) and Ghahfarokhi et al. (2015) reported an enhancement of CAT activity. As well as, the CAT activity depends on studied phenological stage (Ghahfarokhi et al., 2015).

Regarding to non-enzymatic respose, Ascorbic Acid (AA) is essencial to plant defence; nevertheless, its content vary depending on phenological stage in plant. For instance, in early growth of *Zea mays*, drought stress decresed the content of AA (Norman et al., 2015), however, in stages of vegetative growth and flowering the drought stress did not cause changes in the AA content (Darvisahn et al., 2013).

Additionally, in wheat and barley, Ghotbi-Ravandi et al., (2014) and Sharifi and Mohammadkhani (2015) informed that there is a positive relation between content of tocopherol and drought tolerance. Furthermore, Tammam et al. (2015), reported that shoot has more Tocopherol content than root.

In relation to GSH, in mung bean, Nahar et al. (2015) informed that the application of GSH reduced the formation of $^1O_2$ and MDA by 28% and 37% respectively. As well as, the exposure time is positively correlated to GSH content.

2.11. Combined stresses

Regarding to simultaneous stresses (drought and heat), it may not be considered like the sum of individual stress (Zandalinas et al., 2018). In this sense, the response to individual and combined abiotic stress involves different signalling pathways (Zhao et al., 2016). Likewise, due to the over production of ROS (for instance, hydrogen peroxide and superoxide) (Jin et al., 2016; Awasthi et al., 2017), the stress combination causes adverse effects on biochemical, physiology, and growth of plants (Fahad et al., 2017).

In relation to biochemical effects, in citrus, Zandalinas et al. (2017) reported that combined stresses (drought+elevated temperature) increased the MDA content, leading to pronounced electrolyte leakage (Awasthi et al., 2017). In *Portulaca oleracea*, Jin et al. (2016) documented that simultaneous stresses increased MDA content (almost two fold); as well as, electrolyte leakage increased by 25 percentage points (respect to control). On the contrary, Correia et al. (2018) did not establish significative shifts in the MDA content in *Eucalyptus globulus* subjected to the combination of heat and drought stress.

In the same way, the relative water content is negatively affected by simultaneous stresses, according to Zandalinas et al. (2017). Similar results were showed by Awasthi et al. (2017), in *Cicer arietinum*. The lower water content in plants provokes a damage on pigments and the photosyntethic apparatus followed by decline of photosynthetic rate (Zhou et al., 2017). In this sense, in *Cicer arietinum* was demonstred that simultaneous stresses reduced the content of pigment and photosynthetic efficiency by 25-60% and 68-83% respectively (Awasthi et al., 2017). In addition, in *Portulaca oleracea,* combined stresses decreased chlorophyll content by almost three times (Jin et al., 2016); nevertheless in other species as *Eucalyptus globulus*, the decline in chlorophyll content, caused by simultaneous stresses, can reach around 10 times (Correia et al., 2018).

The morphological features may undergo changes by the imposition of simultaneous stress, in fact, Zhou et al. (2017) reported that drought+heat stress considerably diminished shoot fresh weight, shoot dry weight, leaf area and internode length in tomato.

In relation to antioxidant response, Zandalinas et al. (2017) determined that SOD activity slightly increased under combined stresses. Neverthless, according to Awasthi et al., (2017), simultaneous stresses brought about the reduction of SOD activity, in *Cicer arietinum*. However, the imposition of combined stress on pepper plant provoked an increment of SOD activity (Hu et al., 2010).

In the case of CAT, when the cultivar is not tolerant or under strong combined stresses condition CAT activity can be drastically diminished, as it was observed for citrus (Zandalinas et al., 2017), *Cicer arietinum* (Awasthi et al., 2017) and *Jatropha curcas* (Silva et al., 2018).

For APX, in *Cicer arietinum*, Awasthi et al. (2017) reported that heat+drought stress decreased its activity. Conversely, in *Jatropha curcas* exposed to combined stresses, APX displayed high activity (almost 4 times compared to control) (Silva et al., 2018). On the other hand, simultaneous stresses decrased the GR activity in *Cicer arietinum* (Awasthi et al., 2017).

In the case of non-enzymatic antioxidant, in *Eucalyptus globulus*, Correia et al. (2018) displayed that combined stresses did not provoke changes in

carotenoid contents. Regarding to ascorbate, in citrus plant subjected to combined stresses, it was observed an increment of ascorbate (Zandalinas et al., 2017). On the contrary, Awasthi et al. (2017) determined that combined stress causes a detrimental effect to ascorbate and GSH.

2.12. Oxidative stress

Oxygen (O_2) is a crucial molecule in the Earth life; for instance, plants need it to for respiration. In fact, O_2 can serve as substract for formation of the reactive oxygen species (ROS) that in steady levels, play a key role in the plant development (Choudhury et al., 2016). These ROS is unstable and partially reduced forms of the atmospheric oxygen (O_2), formed during the aerobic cellular metabolism in all cell organelles that have the electron transport chain (the oxygen acts as the final electron acceptor in the chain) or a highly oxidized metabolic rate, such as mitochondria and peroxisomes (Gratão et al., 2005). On the other hand, the overproduction of ROS causes severe damages on the plant cell, leading, in extreme cases, to death cellular (Choudhury et al., 2016). The detrimental effect caused by the ROS is called oxidative stress.

2.12.1. Reactive oxygen species (ROS)

ROS results from the transfer of one, two or three electrons to the O_2 molecule, forming respectively the superoxide radical ($O_2^{-\bullet}$), the hydrogen peroxide (H_2O_2) or the hydroxyl radical ($OH^{\bullet}$) and also by the O_2 excitation processes generating the “Singlet” oxygen (Mitller, 2017). Each type of ROS has particular features and interacts with different cell parts. In the case of superoxide, it is resulted of the reduction of O_2 by one electron (Mattila et al., 2015), and has a life-span of 1-4 µs, and can move about 30 nm. As well as, it interacts with Fe-S protein (Mitller, 2017).

Singlet oxygen ($^1O_2$) has a lifetime of 1-4 µs, and can move around 30 nm. $^1O_2$ can also interact with proteins, lipids (causing the lipid peroxidation) and DNA (Mattila et al., 2015; Mitller, 2017). On the other hand, H_2O_2 has a life-

span of 1 ms and can move around 1µm, likewise, it poorly reacts with DNA (Das e Roychoudhury, 2014).

Regarding to hydroxyl radical (•OH), it has a timelife of 1 ns, it can move a distance of 1nm. This type of ROS has high reactivity and is extremely dangerous. It also reacts with protein, RNA, DNA and lipds (Mitller, 2017).

2.12.2. Targets of reactive oxygen species (ROS)

ROS is able to oxidize and to denature many other molecules and structures due to the unpaired number of electrons in their final outer shell, leading to the cell destruction. As mentioned above, ROS has mainly three targets: cellular membrane (lipid peroxidation), proteins and DNA (Figure 5) (Das and Roychoudhury, 2014).

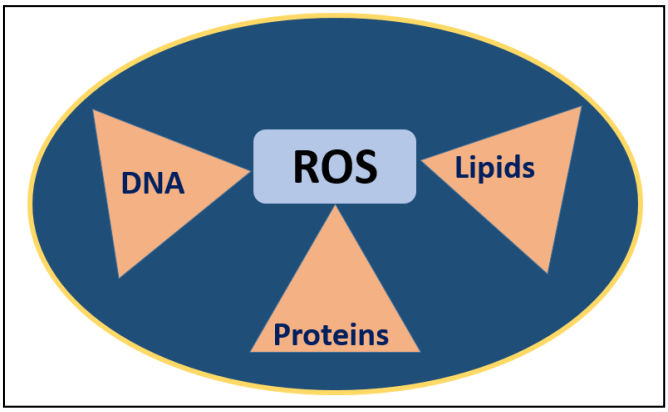


Figure 5. Targets of ROS in plant cell.

2.12.2.1. Lipid peroxidation

Under stress, ROS acts on fatty acid of the cellular membranes, causing a lipid peroxidation (LP). In the same way, the LP has three stages: a) Initiation, b) Propagation and c) ending (Repetto et al., 2012).

Regarding to Initiation, when ROS attacks the plant cell membranes, they cause that fatty acids lose one proton (and one electron), rendering a lipid radical (L•). L• can also react with O_2, resulting in the formation of peroxil radical

(LOO•). In relation to propagation stage, LOO• reacts with other fatty acids, leading to L• and hydroperoxide compounds formation interacting with Fe or Cu to form OH• and LOO•. In the ending stage, these radicals encounter with an electron donor and back to its basal state (Jablonska-Trypuc, 2017). Among products of lipid peroxidation, we can find Malondialdehyde (MDA) whose quantification is used to determine the degree of damage in plants, under drought and heat stress (Catola et al., 2015; Kong et al., 2016; Siddiqui et al., 2017).

2.12.2.2 Protein oxidation

Other target of ROS is the proteins that are severely damage in its biochemical constitution, hence in its functions (Davies, 2016). Currently, there are numerous studies showing the existence of four types of modifications in protein of stressed plant: Carbonylation (irreversible modification), S-Nitrosylation (irreversible modification), Sulfonylation and Glutathionylation (Choudhury et al., 2016).

Regarding to Carbonylation, it is foremost type of oxidation and it is used to quantify the damage caused by ROS (Trnková et al., 2015). During interaction between ROS and proteins is released a set of chemical groups among which is carbonyl. Once formed, it can be measured by formation of 2,4 dinitrophenylhydrazine (Cabiscol et al., 2014).

2.12.3. Sources of ROS

The overproduction of ROS can occur in different plant parts, for example in apoplast, chloroplast, mitochondria, peroxisomes, plasma membranes, cell wall and endoplasmatic reticulum (Das and Roychoudhury, 2014; Mattila et al., 2015), althought, chloroplast, mitochondria and peroxisomes are the foremost sources of ROS. In addition, in these three organelles, ROS mainly occurs in the electron transport chain (Paciolla et al., 2016).

2.12.3.1. Chloroplast

The sun's light is transformed in energy via the photosynthesis. Additionally, the photosynthetic process is composed by two stages: photochemistry and biochemical stage. The photochemistry stage is composed by the transport of electrons, where the formation of ROS (O_2^-, $^1O_2$, H_2O_2 and •OH) occurs (Mignolet-Spruyt et al., 2016; Dietz et al., 2016).

Regarding to O_2^-, it can be produced in three process: a) by partial reduction of water; b) mediated by semi plastoquinone (into Cytochrome B_6F) and c) during transference of electrons from Ferrodoxine to NADP (FSI) (Mattila et al., 2015).

In the case of $^1O_2$, it can occur in two places: antenna complex (FSII) and into FSII. In the antenna complex, the chlorophyll undergo over excitation and subsequently is formed the $^1O_2$. Into PSII, $^1O_2$ is directly produced by P680 (Telfer, 2014). H_2O_2 is also formed by spontaneous reactions, or by dismutation mediated by SOD (Niu and Liau, 2016). Other type of ROS formed in this organelle is •OH.

2.12.3.2. Mitochondria

The respiration is crucial reactions for plant survival; however, it can be a great source of ROS especially under abiotic stress (Huang et al., 2016). Normally, 2-5% the oxygen (in the mitochondria) is used in the O_2^- production. Furthermore, respiration comprises three stages: Glycolise, Krebs cycle and oxidative phosphorylation.

The foremost stage for ROS formation is the oxidative phosphorylation (electron transport chain) (Taylor et al., 2009), where, ROS are mainly produced in complexes I and III (Taylor et al., 2009; Zorov et al., 2014).

2.12.3.3. Peroxisome

In relation of this organelle, it is considered one of main sources of H_2O_2; for instance, the production of H_2O_2 in peroxisomes are 52 more times than

mitochondria and chloroplast, respectively. As well as, in general, peroxisomes account by around 35% of all production of H_2O_2 (Tripathi and Walker, 2016).

Into the peroxisomes, the production of H_2O_2 is triggered by β-oxidation of fatty acid and the transformation from glycolate to glyoxilate (Photorespiration) (Sandalio and Romero, 2015). In the same way, O_2^- production occurs in the membrane and the matrix. In addition, the presence of •OH was detected in this organelle (Del Río and López, 2016).

2.13. Scavenging ROS mechanism

In order to counteract the damages of ROS, the plants have a set of enzymes, and non-enzymatic compounds. The enzymatic detoxification is mainly composed by Superoxide Dismutase (SOD, EC 1.15.1.1), Catalase (CAT, EC 1.11.1.6), Glutathione Peroxidase (GPX, EC 1.11.1.9), Glutathione Reductase (GR, EC 1.8.1.7), Monodehydroascorbate Reductase (MDHAR, EC 1.6.5.4), Ascorbate Peroxidase (APX, EC 1.11.1.11) and Dehydroascorbate Reductase (DHAR, EC 1.8.5.1) (Acosta et al. 2017). Regarding to non-enzymatic scavenging, it includes to Ascorbate, Tocopherol, Carotenoids, Glutathione (GSH) and Proline.

2.13.1. Enzymatic antioxidant

Superoxide Dismutase (SOD, EC 1.15.1.1)

This enzyme is considered the first line defence in plants under abiotic stress (Ren et al., 2016). In addition, it is able to dismute O_2^- in H_2O_2 and O_2. SOD has three isoforms (FeSOD, MnSOD, Cu/ZnSOD), which are in different organelles, for instance, MnSOD is found in the mitochondria, FeSOD is found in the chloroplast and Cu/ZnSOD is found in cytosol, chloroplast and peroxisomes (Das and Roychoudhury, 2014) (Figure 6). Each isoform is composed by transition metals (TM), which are great electron donors (Singh et al., 2015), allowing the dismutation of O_2^-, mediated by gain or loss of electrons.

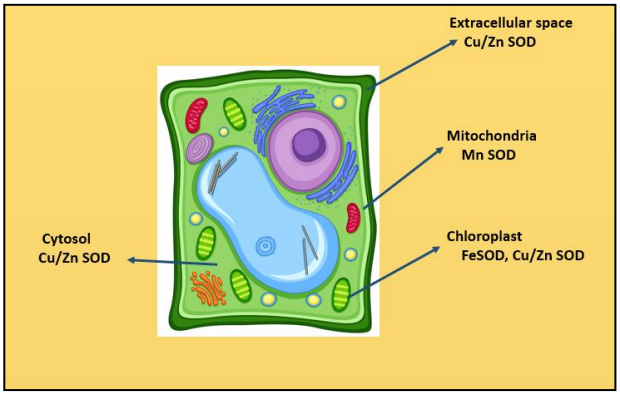


Figure 6. Distribution of SOD in different parts of plants.

On the other hand, the dismutation of O_2^- has two stages: 1) $TM_{oxidized}SOD + O_2^- = TM_{reduced}SOD + O_2$ and 2) $TM_{oxidized}SOD + O_2^- = H_2O_2$; where $TM_{oxidized}$: Cu^{2+}/Zn^{2+}, Fe^{3+}, Mn^{3+}; and, $TM_{reduced}$: Cu^{+}/Zn^{2+}, Fe^{2+} and Mn^{2+} (Miller, 2004).

Glutathiona Reductase (GR, EC 1.6.4.2)

Under stress conditions, the Glutathione (GSH) is crucial to maintain REDOX homeostasis, since it is capable of donating electron, and consequently scavenging of ROS. As result of oxidation of GSH, GSSG is formed. Thus, plants need to maintain high content of GSH (Glutathione disulfide). In order to, recycle GSSG and back to GSH, plants have the GR enzyme (Couto et al., 2016). In addition, to accomplish its function, GR needs a substrate (NAPDH), which donates an electron to GSSG (Figure 8). Likewise, the activity of GR is necessary to limit the damage of ROS, in plants under abiotic stress (Ding et al., 2016; Harshavardhan et al., 2017).

Catalase (CAT, EC 1.11.1.6)

To avoid the negative effect of H_2O_2 plants have a set of enzymes that transform H_2O_2 to water. Among these enzymes, Catalase (CAT) has a relevant role in the H_2O_2 detoxification. Due to heme group, CAT can dismute H_2O_2

(Mhamdi et al., 2010). In general, the dismutation of H_2O_2, mediated by the CAT, has two stages where water and O_2 are produced (Anjum et al., 2016).

Ascorbate peroxidase (APX, EC EC 1.11.1.11)

APX is other type of enzyme detoxifying H_2O_2. This enzyme may act under abiotic stress conditions (drought and heat). APX also has a high affinity for hydrogen peroxide, in other words, it needs few production of H_2O_2 to increase its activity. Thus, APX and CAT have a complementary role in the detoxification of H_2O_2 (Sofo et al., 2015).

Likewise, APX is part of Ascorbate-Glutahione cycle (Figure 7), where ascorbate is used as electron donor (Dietz, 2016). In addition, the oxidation of Ascorbate result in the formation of monohydroascorbate or dehydroascorbate, which may back to reduced state (Ascorbate).

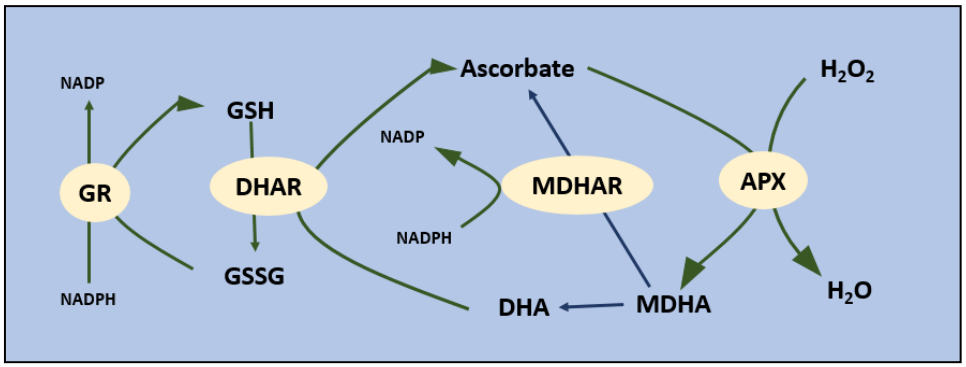


Figure 7. Ascorbate-Glutahione cycle in plants (Zechmann, 2014).

2.13.2. Non-enzymatic antioxidant

Ascorbate

Ascorbate plays key role in plant protection under stress conditions. It is considered a crucial water-soluble molecule to detoxify ROS. In the same way, it is part of the Ascorbate-Glutathione cycle (Akram et al., 2017), where it donates electron to APX. Furthermore, its quantification is used to meansure the degree of suceptibility to abiotic stress (Dwivedi et al., 2016).

Glutathione Reduced (GSH)

GSH is a molecule formed by Glutamate (Glu), Cysteine (Cys) and Glycine (Gly) (γ-Glu-Cys-Gly). This molecule limits the damage of ROS (O_2^-, H_2O_2, $^1O_2$ and •OH) (Das and Roychoudhury, 2014). It also participates in two defence systems: a) it can act in Glutathione Peroxidase cycle or b) it can be part of Ascorbate-Glutathione cycle (Figure 8) (Gill et al., 2013). Owing to the ability to done electrons, plants need to maintain high content of GSH, in other words, a high GSH/GSSG ratio.

Carotenoids

Carotenoids are part of complex antenna of photosystems. As well, it can be classified in carotens (formed by carbone and hydrogen, for instance: β-caroten) and xanthophyll (formed by carbone and oxygen, for instance: lutein) (Kiokias et al., 2016).

Likewise, this pigment has various functions for example it can absorb the high light energy (protecting the photosynthetic apparatus against formation of ROS) and moduling the PSII efficiency (Yamamoto and Bassi, 1996). It is the main way of $^1O_2$ quenching in chloroplast, as well as, it is capable to modulate response genes to oxygen singlet (Ramel et al., 2012). On the other hand, the carotenoid content may be affected by abiotic stress as drought and heat stress (Mibei et al., 2017; Bistgani et al., 2017; García-Plazaola et al., 2017).

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CHAPTER 2.-Impacts of warming and water deficit on antioxidant responses in *Panicum maximum* Jacq

ABSTRACT.- Agricultural activities are affected by many biotic and abiotic stresses associated with global climate change. Predicting the response of plants to abiotic stress under future climate scenarios requires an understanding of plant biochemical performance in simulated stress conditions. In this study, the antioxidant response of *Panicum maximum* Jacq. cv. Mombaça exposed to warming (+2 °C above ambient temperature) (eT), water deficit (wS) and the combination eT+wS was analysed under field conditions using a temperature free-air-controlled enhancement facility. Warming was applied during the entire growth period. Data were collected at 13, 19 and 37 days after the start of the water deficit treatment (DAT) and at two sampling times (6:00 and 12:00 h). A significant decrease in chlorophyll was observed under the wS treatment, but an increment in total chlorophyll was observed in eT+wS, particularly at 19 DAT. Significant increase in H_2O_2 content, malondialdehyde and protein oxidation was observed in the wS treatment at noon of the third sampling. In the combined wS+eT stress treatment, the activity of the enzymatic antioxidant system increased, particularly of superoxide dismutase (SOD; EC1.15.1.1) and ascorbate peroxidase (APX; EC1.11.1.11). The chlorophyll fluorescence images showed that the photochemical performance was not significantly affected by the treatments. In conclusion, under simulated future warming and water stress conditions, the photosystem II (PSII) activity of *P. maximum* acclimated to moderate warming and a water-stressed environment associated with a relatively favourable antioxidant response, particularly in the activity of APX and SOD.

Abbreviations

APX, ascorbate peroxidase (EC 1.11.1.11); DAT, water deficit treatment; DHAR, dehydroascorbate reductase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid; EDTA, ethylenediaminetetraacetic acid; C, control, the ambient canopy temperature + irrigated soil; Car, carotenoids; CAT, catalase; Chl, chlorophyll; DW, dry weight; eT, the elevated temperature at +2∘C above ambient canopy

This chapter was published in Physiologia Plantarum (2018) 165: 413-426

temperature + irrigated soil; FW, fresh weight; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; IR, infrared; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; PID, proportional integration device; ROS, reactive oxygen species; SOD, superoxide dismutase; SWC, soil water content; TBA, thiobarbituric acid; TCA, trichloroacetic acid; T-FACE, temperature free-air-controlled enhancement facility; wS, the ambient canopy temperature + water deficit with the suspension of irrigation; [Y(II)], effective PSII quantum yield; [Y(NO)], quantum yield of non-regulated energy dissipation.

1. Introduction

As a consequence of climate change and global warming, the mean of surface air temperature is expected to increase by more than 2.3 °C by 2046-2065 (Brown and Caldeira, 2017). Additionally, an increase in the intensity and frequency of extreme weather events such as heat waves, extreme precipitation and flooding and more intense droughts is also anticipated, producing severe effects on the production of economically important crops (IPCC 2014, Frank et al., 2015, Lesk et al., 2016).

Tropical countries that supply food will be severely affected by climate change. According to Marengo et al. (2017), in some regions of the Brazilian territory, climate change will cause an increase in temperature, a decrease in rainfall and lack of water in the coming years, affecting several economic sectors.

Because of the vulnerability of pastures to climate change, livestock farming will be one of the activities severely affected (Nääs et al., 2010). In fact, in tropical countries such as Brazil in which cattle-raising activity is essential, a considerable decline in production of pastures and yield of forages such as guinea grass (*Panicum maximum*) is expected for the next few years (Pezzopane et al., 2016).

Envisioning plant performance under future climate scenarios requires an understanding of how plants will cope with different stressors such as elevated

temperature and water deficit. Despite numerous studies examining the plant response to a water deficit or temperature increase (Ding et al., 2016, Shan and Ou, 2018), research focused on the combined effect of both stresses and its relationship to the antioxidant response remains scarce (Silva et al., 2010, Sekmen et al., 2014; Jin et al., 2016).

Plant responses to the combined effects of abiotic stresses such as water deficit and temperature increase are very complex. For example, the short-term response to drought such as closing stomata to reduce transpiration, developmental adjustments such as a decrease in leaf area to reduce water loss or the growth in depth of the root system to improve water uptake involve the modification of plant physiology, biochemistry and metabolism and alterations in the expression of many genes (Hu and Xiong, 2014).

Increases in both drought and temperature can cause adverse effects on plant development because of an overproduction of reactive oxygen species (ROS; superoxide, O_2^-; hydrogen peroxide, H_2O_2; hydroxyl radical, •OH; and singlet oxygen, $^1O_2$). ROS produced in excess reacts with cell membranes (lipid peroxidation), proteins, organic molecules and DNA and RNA (Mitller, 2017), causing mutations and loss of structure and function, which can lead to cell death (Sewelam et al., 2016). This phenomenon is called oxidative stress (Choudhury et al., 2017) and causes irreversible damage to plant growth and development, leading to a crop production decline (Neiff et al., 2016).

To control the excessive production of ROS and avoid damage caused by oxidative stress in plant metabolism, the synthesis of enzymatic and non-enzymatic compounds increases. Among the enzymatic compounds, we highlight superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione reductase (GR, EC 1.8.1.7), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), ascorbate peroxidase (APX, EC 1.11.1.11) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) (Acosta et al., 2017).

Other plant antioxidants are non-enzymatic types, such as glutathione (GSH), vitamin E (tocopherols and tocotrienols), ascorbate (vitamin C) and

carotenoids (pro-vitamin A) (Asensi-Fabado and Munné-Bosch, 2010; Pintó-Marijuan and Munné-Bosch, 2013). Moreover, some chemical priming agents such as hydrogen peroxide, sodium nitroprusside, sodium hydrosulfide, melatonin and polyamines can potentially improve plant tolerance to multiple abiotic stresses (Savvides et al., 2015).

Because of the intrinsic effect of climate change on plant metabolism, producing a severe threat to agricultural productivity, and because experimental data in the tropics and subtropics are scarce regarding the biochemical responses of tropical grass forages to future climatic changes, this study was conducted to analyse the enzymatic and non-enzymatic antioxidant responses of *P. maximum* Jacq. under a future 2 °C global warming scenario and water stress.

2. Material and Methods

2.1. Plant Material and growth conditions

Panicum maximum Jacq. cv. Mombaça seeds were sown and grown in 25m2 plots at the temperature free-air-controlled enhancement (T-FACE) facility located at the University of São Paulo (USP), Ribeirão Preto, SP campus (21∘17′83″ S, 47∘80′67″ W).

In this study, we followed field forage management procedures commonly utilised in tropical conditions. Before sowing, soil analysis was performed between 0 and 20 cm depth. Soil liming was conducted to adjust the soil pH to approximately 5.5. Then, the soil of each plot was differentially fertilised according to recommendations of Werner et al. (1997) to maintain a homogenous soil mineral composition in the experimental area.

2.2. Treatments

We established four treatments in a four-randomised blocks design with four replicates: (1) irrigation and ambient canopy temperature (Control, C); (2) soil water deficit and ambient canopy temperature (wS); (3) irrigation and elevated canopy temperature at +2 °C above ambient temperature (eT); and (4) the combination of soil water deficit and elevated canopy temperature (wS+eT). From sowing until the beginning of the soil water deficit, all plots were frequently

irrigated to maintain the soil water content (SWC) near 80% of field capacity. To induce the soil water deficit in treatments wS and wS+eT, we suspended the watering (on 6 November) 2 months after sowing. The SWC of all plots (Figure 1B) was recorded continuously using Theta Probe ML2X sensors located in the centre of each plot at a 10-cm depth and connected to a DL2e data logger (Delta-T Devices Ltd., Cambridge, UK). Precipitation data were obtained from a weather station located near the experimental field (Figure 1A).

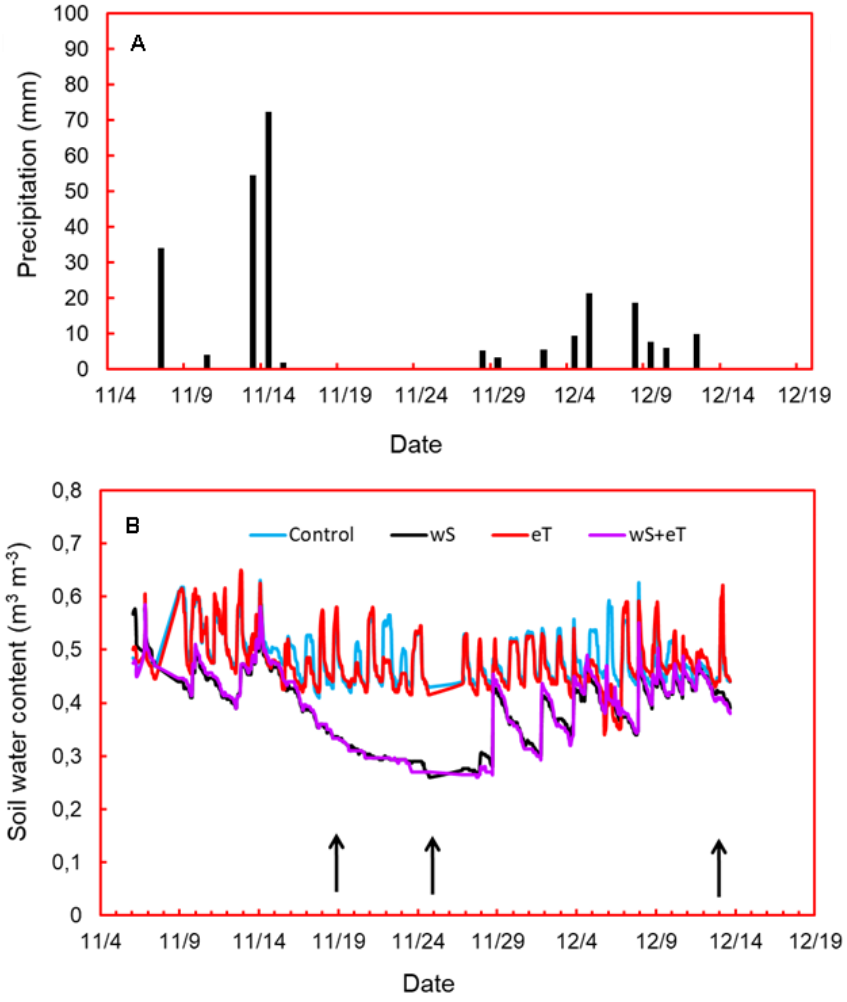

Figure 1. Precipitation at the experimental field of T-FACE (USP-Ribeirão Preto, SP) during the experimental period (A). SWC in treatments: irrigation and ambient canopy temperature (Control, C); soil water deficit and ambient canopy temperature (wS); irrigation and elevated canopy temperature at +2◦C above ambient temperature (eT) and the combination of soil water deficit and elevated canopy temperature (wS+eT) (B). The arrows indicate the first, second and third sampling of leaves for biochemical analysis conducted on 19, 25 November and 13 December 2016 respectively.

This chapter was published in Physiologia Plantarum (2018) 165: 413-426

2.2.1. Warming treatment

To increase the canopy temperature to +2 °C above the ambient temperature for the warming treatment, we used the T-FACE system developed by Kimball et al. (2008), as described in Martinez et al. (2014) and Prado et al. (2016). The T-FACE facility uses infrared (IR) heaters to apply a heating treatment to open-field plant canopies, simulating models of future global warming conditions. Each warming plot of the T-FACE contained six IR heaters (750W model FTE-750-240 Salamander ceramic IR heating element; Mor Electric Heating, Comstock Park, MI) placed at 0.80 m above the plant canopy in a hexagonal arrangement. Each IR heater was installed in an aluminium reflector model Salamander ALEX (Mor Electric Heating).To control the increase in temperature, the T-FACE uses a proportional-integrative-derivative control system installed in a data logger model CR1000 with AM2 5 T multiplexors (Campbell Scientific, Logan, UT). Apogee IR radiometers model SI-1H1-L20 (Apogee Instruments, Logan, UT) provided the data on control and heated plot temperatures (Kimball et al. 2008). The control of the T-FACE system was performed through the Logger net data logger support software (Campbell Scientific), which allowed communication with the data logger.

The maximum and minimum air temperatures were recorded using an automatic microclimate station WS-PH1 with sensors connected to a DL2e data logger (Delta-T Devices Ltd., Cambridge, UK) (Figure 2A). The plant canopy temperature of all plots (Figure 2B) was monitored using Apogee IR thermometers model SI-1H1 (Apogee Instruments).

2.3. Leaf sampling and quantification of pigments

The experiment began on 6 November, and the first sampling of leaves for biochemical analyses was on 19 November, the second on 25 November and the third on 13 December 2016 at 13, 19 and 37 days, respectively, after beginning of the water deficit treatment (DAT) (Figure 1B).At each sampling time, samples were collected at 6:00 and 12:00 h. After each sampling, leaf material was immediately placed in liquid nitrogen and stored in a freezer at −80

°C until biochemical analyses in the Laboratory of Plant Physiology, Department of Agriculture Applied Biology, UNESP, Jaboticabal Campus, Brazil.

Chlorophyll and carotenoids quantification was performed by maceration of tissue in 80% acetone in the dark. After extraction, the absorbance was read on a Perkin Elmer-Lambda spectrophotometer at wavelengths of 663 nm (chlorophyll a), 647 nm (chlorophyll b) and 470 nm [Carotenoids: carotene (c) + xanthophyll (x)]. To calculate the chlorophylls (Chl) and carotenoids (Car) concentration, we used the formulas proposed by Lichtenthaler (1987) as follows: Chl a = 12.25 A_{663}–2.79 A_{647}; Chl b = 21.50 A_{647}–5.10 A_{663}; Total Chl (a+b) = 7.15 A_{663}+18.71 A_{647}; Car (c+x) = (1000 A_{470}–1.82 Chl a – 85.02 Chl b)/198. The tissue content in chlorophylls and carotenoids is expressed in micrograms of the pigment per gram of dry weight (DW) (μg g^{-1}).

2.4. Lipid peroxidation

Lipid peroxidation, a widely used indicator of plant cell membrane stress, was estimated by malondialdehyde (MDA) concentration. The reaction between MDA and thiobarbituric acid (TBA) produces a reddish colour, which peaks at 532 nm. After maceration of leaf tissue, extraction was performed with 4 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifugation at 15000 g for 15 min at 4 °C. The supernatant was added to 1.5 ml of 0.5% TBA. The tubes were vigorously agitated and incubated at 95 °C for 30 min. The reaction was stopped, and the mixture was clarified by centrifugation at 10000 g for 5 min at 4 °C. Then, the sample absorbance was recorded at 532 nm and at a non-specific absorbance of 600 nm. The MDA molar extinction coefficient (155mM^{-1} cm^{-1}) was used for the calculations, and the results are expressed in nmol MDA g^{-1} of fresh weight (FW) (Shimizu et al., 2006).

2.5. Content of hydrogen peroxide in leaves

We determined the H_2O_2 content in leaves using the potassium iodide (KI) method (Alexieva et al., 2001). After maceration in TCA (0.1%), the samples were centrifuged at 12000 g for 15min at 4 °C. The supernatant was placed in 200 μl of 100 mM potassium phosphate buffer (pH 7.5) and 800 μl of a 1 M KI solution. The reaction tubes were then placed on ice and remained in

the dark for 1 h. The absorbance was read at the wavelength of 390 nm. The H_2O_2 amount is expressed in μmol g^{-1} FW.

2.6. Oxidation of proteins

The carbonyl content was quantified by the oxidation of proteins (Levineet al., 1990). We performed the extraction with phosphate buffer (25 mM) and centrifugation at 10000 g. The supernatant was reacted with 250 μmol 2,4-dinitrophenylhydrazina (DNPH 8 mM) in the dark for 1h with agitation every 10 min. Then, we added to the reaction mixture 500 μl of TCA (30%). Following protein precipitation, further centrifugation was performed for 5 min at 4 °C. Three washes were performed on the pellet glued to the Eppendorf, 10 min each, with an ethanol and ethyl acetate solution. After washing, we added 500 μl of guanidine and centrifuged for 5 min at 12096 g. The absorbance was read at the wavelength of 374 nm.

2.7. Reduced glutathione (GSH) content

To determine the GSH content, the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) was incubated in phosphate buffer at 143 mM and pH 7.5 for 10 min at 30 °C in a water bath. After the incubation period, the leaf extract was added. 2-nitro-5-thiobenzoicacid (TNB) production was determined with the absorbance read at the wavelength of 412 nm (Griffith, 1980).

2.8. Extraction and determination of protein

We performed total protein extraction quenching with 100 mM potassium phosphate buffer (pH 7.5) containing 1mM ethylenediaminetetraacetic acid (EDTA), 3 mM ditiotreitol (DTT) and 4% polyvinylpolypyrrolidone (PVPP). The mixture was centrifuged at 10000 g for 30 min at 4 °C. The supernatant was divided into aliquots, which were stored at −80 °C for use in the enzymatic assays (Azevedo et al., 1998), and protein quantification was performed using the spectrophotometric method of Bradford (1976).

2.9. Superoxido Dismutase (SOD) (EC 1.15.1.1) activity

The determination of SOD enzyme activity followed the protocol developed by Giannopolitis and Ries (1977).The buffer sodium chloride (50 mmol l^{-1}) pH7.8, methionine (13 mmol l^{-1}), nitro blue tetrazolium chloride (NTB; 75 mmol l^{-1}), EDTA (0.1 mmol l^{-1}) and riboflavin (µmol l^{-1}) were reacted under a fluorescent lamp at 25 °C for 5 min, which resulted in the formation of blue formazan compound. The absorbance was read at the wavelength of 560 nm.

2.10. Ascorbate Peroxidase (APX) (EC 1.11.1.11) activity

We quantified APX by reaction of a solution containing 650 µl of 80 mM potassium phosphate buffer pH 7.0 and 100 µl of 5 mM ascorbate with 100 µl of EDTA at 1 mM; the solution remained in a water bath at 30 °C. At reading, we added 100 µl of H_2O_2, 0.1 mM and 50 µl of extract. The absorbance was read at the wavelength of 280 nm for 1 min, considering the H_2O_2 decomposition (Gratão et al., 2008).

2.11. Glutathione Reductase (GR) (EC 1.8.1.7) activity

We determine GR activity from a mixture of 100 nM phosphate buffer pH 7.5 and 500 µl of DTNB in a water bath at 30 °C. Before reading, 1 mM oxidised GSH, 0.1 mM NADPH, and 50 µl of extract (Cakmak and Horst, 1991, Azevedo et al., 1998) were added. The absorbance was read at the wavelength of 412 nm.

2.12. Image fluorescence

Image fluorescence of the effective PSII quantum yield of the illuminated samples (Y[II]) and the quantum yield of non-regulated energy dissipation in PSII [Y(NO)]were obtained using an Imaging-PAM M-series chlorophyll fluorescence system (MINI-version model; Heinz Walz GmbH, Nuremberg, Germany). (Y[II]) reflects the capacity of PSII to obtain light energy for photosynthesis, whereas [Y(NO)] is related to non-regulated heat loss.

Leaves were detached from the plants and were adapted to the dark for 15min before measurements. All fluorescence measurements were conducted at room temperature. Images were calculated with Imaging Win software (Heinz Walz GmbH).

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2.13. Statistical analyses

The experiment consisted of a total of 16 plots that corresponded to a completely randomised block design with factorial arrangement. In this experiment, we tested two principal factors (Factor 1: water stress and Factor 2: elevated temperature). The Factor 1 is formed by two levels: (1) water stress-wS and (2) no water stress. Also, Factor 2 is formed by two levels: (1) normal temperature and (2) elevated temperature-eT. The combination of factor levels resulted in four treatments (C, wS, eT and wS+eT) with four replications. The results of each evaluated variable were the mean of triplicates. Data were submitted to analysis of variance (Test F), and comparison of means was carried out by the Tukey test at 5% of probability. For the statistical analyses, we used the AGROSTAT® software developed by the Department of Exact Sciences of UNESP (Jaboticabal, São Paulo, Brazil) (Barbosa and Junior, 2010).

3. Result

3.1. Microclimate

During the experimental period, the accumulated rainfall was 250 mm, with the highest level of precipitation recorded at the beginning of the experimental period (Figure 1A). After November 14, no precipitation occurred for the next 2 weeks. The decrease in precipitation caused decreases in SWC of the non-irrigated plots (Figure 1B). In control and eT plots, independent of precipitation, the SWC was maintained at approximately 0.50 m^3 m^{-3} during the experimental period using the irrigation system (Figure 1B). After the suspension of irrigation, as expected, the SWC in wS and wS+eT plots was reduced to the minimum value of 0.25 m^3 m^{-3} (Figure 1B). The period of the maximum difference in SWC between irrigated and non-irrigated treatments was from 10 to 26 days after the beginning of treatments, the period in which the first and second leaf sampling for biochemical studies took place (Figure 1B). During the third leaf sampling, a recovery of the SWC in water-stressed plots was observed, reaching values near those of the control plants (Figure 1B).

During the growing season, the average night-time relative air humidity was 91%; whereas the average daytime relative air humidity was approximately 75% (data not shown). The average maximum air temperature was 29.9 °C, with maximum and minimum values of 33.3 and 20.8 °C, respectively. The average minimum air temperature was 18.7 °C with maximum and minimum values of 20.9 and 10.8 °C, respectively (Figure 2A). Variation in air temperatures also had a significant effect on leaf canopy temperatures (Figure 2B). During the experimental period, the canopy temperature was 23.3, 23.5, 24.9, and 25.4 °C in the C, wS, eT and wS+eT treatments, respectively (Figure 2B).

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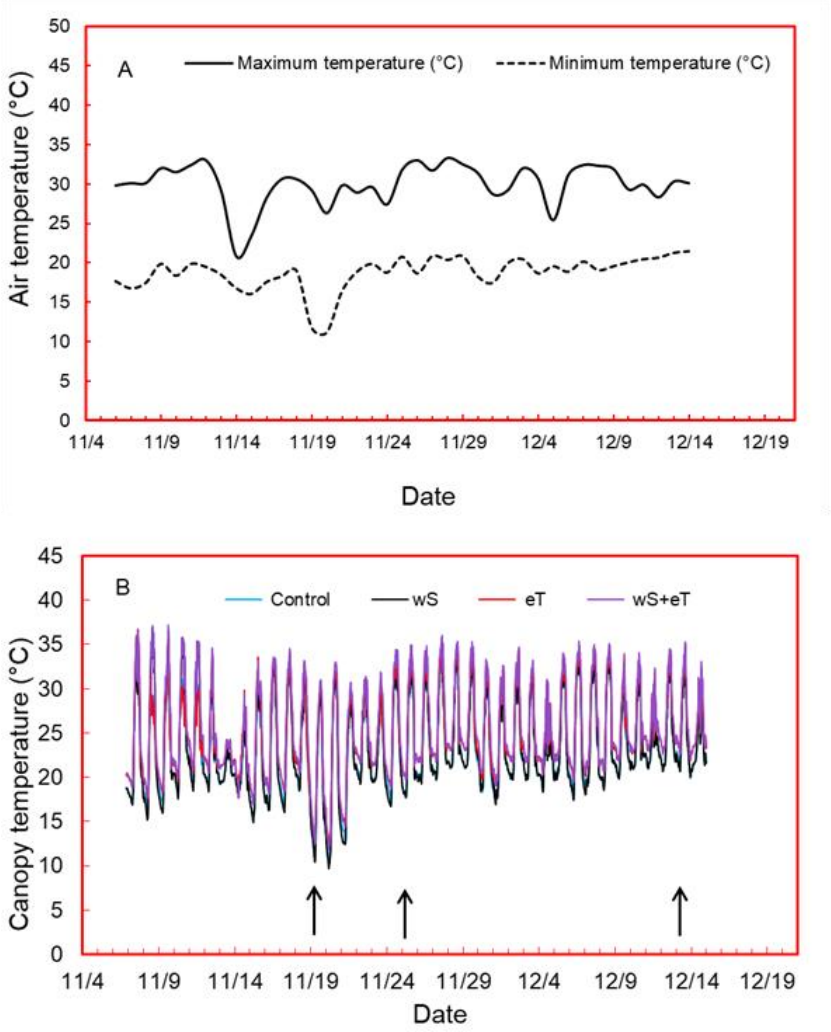

Figure 2. The maximum and minimum air temperatures during the experimental period (A). Plant canopy temperature in treatments: irrigation and ambient canopy temperature (Control, C); soil water deficit and ambient canopy temperature (wS); irrigation and elevated canopy temperature at +2◦C above ambient temperature (eT) and the combination of soil water deficit and elevated canopy temperature (wS+eT) (B). The arrows indicate the first, second and third sampling of leaves for biochemical analysis conducted on 19, 25 November and 13 December 2016 respectively.

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3.2. First sampling at 6:00 h

At the first sampling (6:00 h), the temperature of the canopy was 10.8, 10.6, 12.75 and 12.84 °C in the C, wS, eT and wS+eT treatments, respectively (Figure 3A). Also, all treatments presented the same content of pigments. However, in wS, the Chl/Carotenoids ratio increased by 47% (compared with that of the control; $P \leq 0.05$; Table 1).

Compared with the control, in wS+eT, the content of MDA, carbonyl and hydrogen peroxide increased by 113, 45 and 35%, respectively; whereas, in wS, the levels of carbonyl and hydrogen peroxide increased by 30 and 56%, respectively (compared with those in the control; $P \leq 0.05$; Table 2).

As shown in Table 3, no significant differences were detected in total leaf protein content. For the antioxidant enzymes, in eT, the SOD content increased by 18% (compared with that of the control). Additionally, APX activity increased in the wS treatment ($P \leq 0.05$). Moreover, the GSH content increased in all treatments.

3.3. First sampling at 12:00 h

At 12:00 h of the first sampling day, the temperature for the control and wS was 25.5 and 26 °C, respectively, whereas eT and wS+eT showed temperatures of 27.3 and 26.8 °C, respectively (Figure 3A). At this sampling, in eT and wS+eT, the content of Chl [a+b] increased by 11 and 21%, respectively (Table 1); whereas in wS, total pigments decreased by 26% ($P \leq 0.05$; Table 1).

However, in wS, MDA, carbonyl and H_2O_2 were not significantly affected. Additionally, in eT, MDA content decreased by 28.5%, whereas in wS+eT, MDA and H_2O_2 contents were reduced by 26 and 18%, respectively ($P \leq 0.05$; Table 2).

Table 3 shows that the protein content decreased significantly in all treatments compared with that of the control. However, in wS, SOD, APX, and GSH activities increased by 53, 90 and 65%, respectively, compared with those of the control. Similarly, in the wS+eT treatment, SOD content increased ($P \leq 0.05$) compared with that of the control (Table 3).

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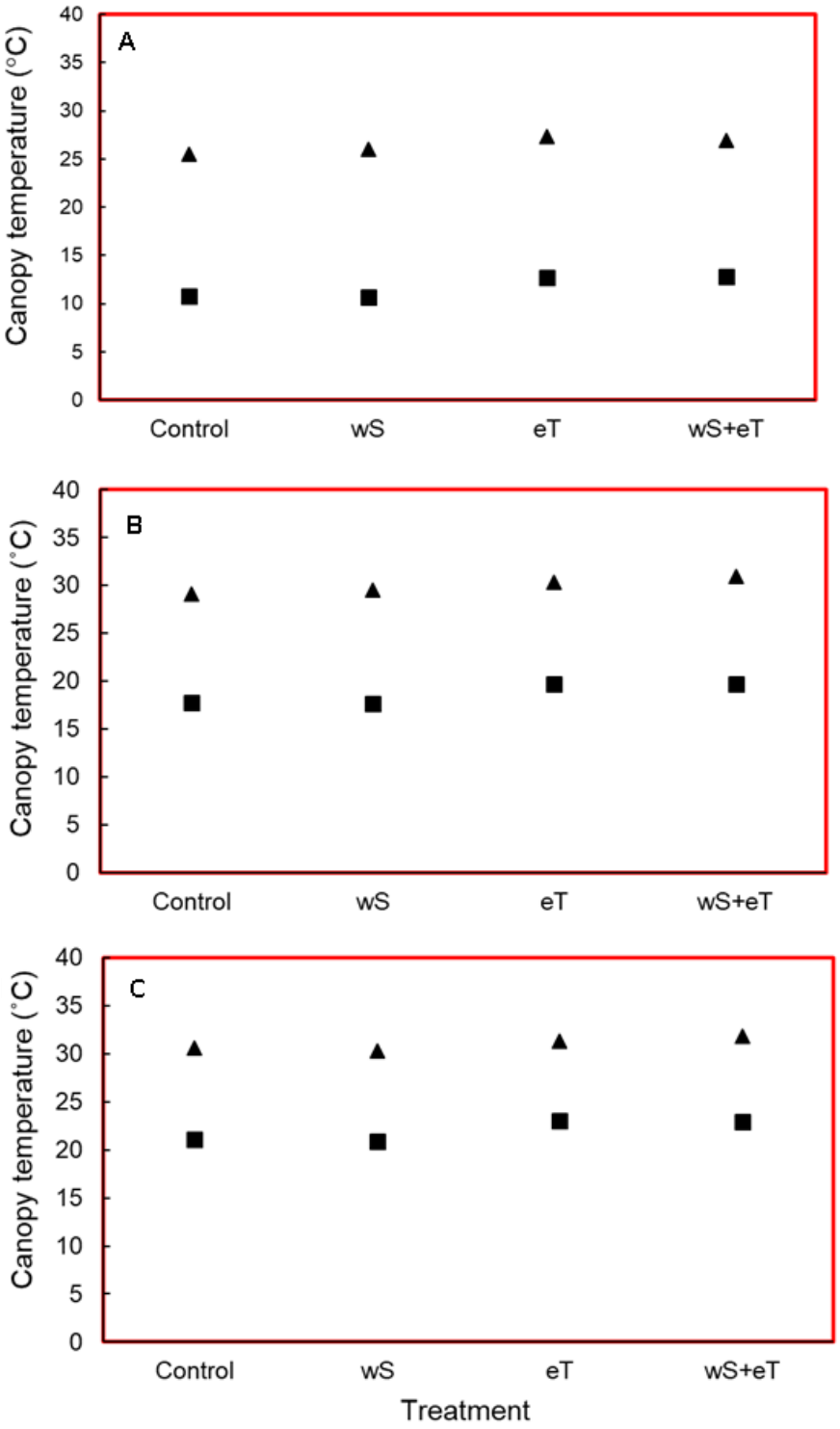

Figure 3. Plant canopy temperature at 6:00 h (■) and at 12:00 h (▲) on the three sampling dates: November 19(A), November 25(B) and December 13(C). Treatments: irrigation and ambient canopy temperature (Control, C); soil water deficit and ambient canopy temperature (wS); irrigation and elevated canopy temperature at +2 °C above ambient temperature (eT) and the combination of soil water deficit and elevated canopy temperature (wS+eT).

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3.4. Second sampling at 6:00 h

At 6:00 h of the second sampling day, the temperature of the canopy was 17.6, 17.7, 19.7 and 19.8 °C in the control, wS, eT and wS+eT treatments, respectively (Figure 3B). We observed that plants in wS+eT had higher Chl a, Chlb and Chl [a+b] contents (by 53, 37 and 48%, respectively) than those of the control plants ($P \leq 0.05$; Table 1).

The MDA content in wS was 32% higher than that of the control. However, in eT, carbonyl content increased and H_2O_2 content decreased by 40 and 26%, respectively, whereas in wS+eT, H_2O_2 content decreased by 33% ($P \leq 0.05$; Table 2).

Regarding antioxidant enzymes, we observed an increase of 30% in SOD and 43% in APX activities in the wS+eT treatment ($P \leq 0.05$; Table 3).

3.5. Second sampling at 12:00 h

At 12:00 h of the second evaluation, the canopy temperature in the control and wS treatment reached 29.1 and 29.5 °C, respectively, whereas in eT and wS+eT, the canopy temperatures were 30.3 and 30.9 °C, respectively (Figure 3B). In wS and eT, chlorophyll and carotenoids decreased, compared with those of the control. By contrast, wS+eT caused a significant increase in the pigments ($P \leq 0.05$; Table 1).

Additionally, in wS+eT, MDA content decreased by 21%, and carbonyl and H_2O_2 contents increased by 45 and 33%, respectively (compared with those of the control; $P \leq 0.05$; Table 2). Related to the antioxidant response of the plant, in wS, GSH and GSH/Oxidized glutathione (GSSG) decreased by 152 and 58%, respectively. Additionally, in eT, the content of SOD and GR increased but that of GSH and the GSH/GSSG ratio decreased ($P \leq 0.05$; Table 3).

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Table 1. Quantification of Chlorophyll a (Chl a), Chlorophyll b (Chl b), Chlorophyll a+b (Chl a+b), relation Chl a/Chl b, Carotenoids (Carot), and Chl/Carot ratio on each of the sampling of the *Panicum maximum* experiment under a climate change scenario.

	First Sampling											
	Chl a (µg Chl a g^{-1}DW)		**Chl b (µg Chl b g^{-1}DW)**		**Chl a+b (µg Chl a+b g^{-1}DW)**		**Chl a/b**		**Carot (µg Carot g^{-1}DW)**		**Chl/Carot**	
	6:00 h	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**
C	546.39 a	694.43 a	220.63 ab	115.90 b	767,02 a	810.35 b	2.53 b	6.18 a	261.69 a	257.33 ab	3.07 a	3.28 a
wS	667.90 a	464.06 b	179.44 ab	138.64 b	847.34 a	602.26 c	3.73 a	3.40 b	242.56 a	198.07 b	3.54 a	3.07 a
eT	551.07 a	729.66 a	169.42 b	171,05 ab	720.52 a	900,71 a	3.27 ab	4.33 ab	225.20 a	249.29 ab	3.22 a	3.75 a
wS+eT	656.68 a	753.16 a	231.88 a	224.15 a	888.56 a	977,31 a	2.85 ab	3.38 b	304.11 a	353.94 a	3.01 a	2.80 a
	Interactions											
wS	NS	**	NS	**	NS	**	NS	NS	NS	NS	NS	NS
eT	NS	**	NS	*	NS	NS	NS	*	NS	*	NS	NS
wSxeT	NS	**	**	NS	NS	**	*	NS	NS	*	NS	NS
	Second Sampling											
C	779.28 b	1031.97 b	235.95 b	281.89 a	1027.60 b	1313.41 b	3.31 a	3.67 a	355.02 ab	429.05 a	2.88 b	3.07 a
wS	645.23 b	509.36 c	173.30 c	135.77 b	841.28 b	645.14 c	3.90 a	3.78 a	302.70 bc	210.98 b	2.79 b	3.06 a
eT	620.34 b	586.52 c	163.33 c	169.26 b	783.66 b	755.79 c	3.88 a	3.49 a	218.32 c	230.99 b	3.64 a	3.31 a
wS+eT	1194.17 a	1278.60 a	324.13 a	306.89 a	1518.32 a	1585.49 a	3.67 a	4.21 a	421.63 a	482.05 a	3.63 a	3.32 a
	Interactions											
wS	**	*	**	NS	**	**	NS	NS	*	NS	**	NS
eT	**	**	**	*	**	NS	NS	NS	NS	NS	NS	NS
wSxeT	**	**	**	**	**	**	NS	NS	**	**	NS	NS
	Third Sampling											
C	526.54 ab	366.62 bc	203.83 a	138.71 ab	730.40 ab	505.23 bc	2.61 a	2.72 a	250.02 a	144.16 a	3.03 a	3.55 a
wS	450.50 b	523.27 a	130.46 b	207.39 a	580.95 b	730.66 a	3.44 a	2.66 a	182.18 a	229.14 a	3.19 a	3.32 a
eT	662.91 a	422.45 ab	199.05 a	118.98 b	920.45 a	541.43 b	3.39 a	3.56 a	251.88 a	186.67 a	3.65 a	2.92 b
wS+eT	601.58 ab	257.93 c	176.26 ab	97.15 b	773.84 ab	355.08 c	3.49 a	2.78 a	227.29 a	219.96 a	3.43 a	1.71 b
	Interactions											
wS	NS	NS	**	NS	*	**	NS	NS	*	NS	NS	**
eT	**	*	NS	**	NS	NS	NS	NS	NS	NS	NS	*
wSxeT	NS	**	NS	*	NS	**	NS	NS	NS	NS	NS	NS

Control (C): ambient canopy temperature+water availability; water stress (wS): ambient canopy temperature+water deficit with suspension of irrigation; elevated temperature (eT): +2 °C above ambient canopy temperature+water availability; water stress+elevated temperature (wS+eT): water deficit +2 °C above ambient canopy temperature. Different letters within columns indicate significant differences according to Tukey's test (P ≤0.05). NS, non-significant. *Significant at 0.05. **Significant at 0.01.

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Table 2. Quantification of MDA, protein oxidation, and peroxidation on each of the *Panicum maximum* samplings of the experiment under a climate change scenario.

	MDA (nmol MDA g^{-1}FW)		Protein Oxidation (nmol carbonil mg^{-1} protein)		Hydrogen Peroxide (µmol H_2O_2 g^{-1} FW)	
First Sampling						
	6:00 h	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**
C	1.48 b	4.00 a	56.35 c	91.11 ab	749.85 c	1173.98 a
wS	1.84 b	3.14 ab	73.07 ab	75.10 b	1172.47 a	1014.61 ab
eT	1.36 b	2.86 b	62.05 bc	70.47 b	883.29 bc	1037.63 ab
wS+eT	3.15 a	2.95 b	81.73 a	102.13 a	1014.62 ab	964.04 b
Interactions						
wS	**	NS	**	NS	**	*
eT	*	*	NS	**	NS	NS
wSxeT	**	NS	NS	NS	*	NS
Second Sampling						
C	1.70 b	3.33 a	57.68 b	48.78 b	1283.98 a	913.07 b
wS	2.25 a	3.25 a	66.78 ab	44.50 b	1244.32 a	1092.09 ab
eT	2.01 ab	2.58 b	80.86 a	64.73 ab	944.79 b	1012.75 ab
wS+eT	1.90 ab	2.64 b	60.97 b	70.89 a	876.02 b	1215.58 a
Interactions						
wS	NS	NS	NS	NS	NS	**
eT	*	**	NS	**	**	NS
wSxeT	NS	NS	**	NS	NS	NS
Third Sampling						
C	1.63 b	2.10 c	75.60 a	36.50 b	779.72 a	1285.98 b
wS	1.48 b	2.20 b	37.90 b	71.80 a	964.30 a	2294.90 a
eT	1.76 b	1.99 c	43.50 b	72.64 a	653.65 a	1106.30 b
wS+eT	2,61 a	3.00 a	47.20 b	56.98 ab	847.24 a	1121.99 b
Interactions						
wS	*	**	**	NS	NS	*
eT	**	NS	**	NS	NS	**
wSxeT	**	*	**	**	NS	*

Control (C): the ambient canopy temperature+water availability; water stress (wS): the ambient canopy temperature+water deficit by suspension of irrigation; elevated temperature (eT): +2 °C above ambient canopy temperature+water availability; water stress+elevated temperature (wS+eT): water deficit +2 °C above ambient canopy temperature. Different letters within columns indicate significant differences according to Tukey's test (P ≤0.05). NS, non-significant. *significant at 0.05. **significant at 0.01.

3.6. Third sampling at 6:00 h

At the morning of the third sampling day, the canopy temperature in the control and wS was 21 and 20.8 °C, respectively; whereas in the wS and wS+eT treatments, the temperature was 23 and 22.9 °C, respectively (Figure 3C). In this evaluation, all treatments had the same content of pigments, compared with that of the control (P ≤ 0.05; Table 1).

Table 3. Quantification of proteins, SOD, APX, GR, reducedGSH and GSH/GSSG ratio on each of the samplings of the *Panicum maximum* experiment under a scenario of climate change.

Treatment	Protein (mg)		SOD (U SOD mg^{-1} prot)		APX (µmol mg^{-1} prot)		GR (nmol mg^{-1} prot)		GSH (nmol g^{-1}FW)		GSH/GSSG	
First Sampling												
	6:00 h	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**
C	3.73 a	4.07 a	3.31 b	2.85 c	4.32 b	7.06 bc	48.80 a	51.81 b	643.68 c	1375.3 a	4.32 a	1.39 a
wS	3.50 a	2.51 c	3.68 ab	4.35 a	6.49 a	13.40 a	47.38 a	85.69 a	976.21 a	1260.7 ab	3.72 a	1.61 a
eT	3.36 a	3.17 b	3.91 a	3.57 b	3.21 b	8.31 b	54.89 a	56.89 b	1073.10 a	878.24 b	1.66 a	2.64 a
wS+eT	3.29 a	3.48 b	3.71 ab	3.61 b	5.11 ab	6.07 c	55.92 a	50.26 b	780.26 b	1126.07 ab	2.35 a	1.26 a
Interactions												
wS	NS	**	NS	**	**	**	NS	*	NS	*	NS	NS
eT	*	NS	*	NS	*	**	NS	**	**	NS	NS	NS
wSxeT	NS	**	*	**	NS	**	NS	**	**	NS	NS	NS
Second Sampling												
C	2.96 a	3.43 a	3.95 b	3.59 b	4.34 b	9.02 b	69.08 ab	46.48 b	872.10 a	1055.69 a	0.96 a	0.78 a
wS	2.79 a	2.87 ab	4.24 b	4.84 ab	4.91 b	12.01 ab	77.99 ab	72.69 ab	833.19 a	550.71 b	3.71 a	0.33 b
eT	2.84 a	2.29 b	4.12 b	5.26 a	4.76 b	12.06 ab	64.98 b	78.33 a	723.54 a	738.30 b	1.11 a	0.34 b
wS+eT	2.36 b	2.70 ab	5.16 a	5.00 ab	6.23 a	13.23 a	84.95 a	57.39 ab	871.78 a	735.54 b	5.14 a	0.68 a
Interactions												
wS	**	NS	**	NS	**	*	**	NS	NS	**	**	NS
eT	**	**	**	*	*	*	NS	NS	NS	NS	NS	NS
wSxeT	*	*	*	NS	NS	NS	NS	**	NS	**	NS	**
Third Sampling												
C	1.86 b	2.61 a	7.39 b	5.01 c	8.64 a	8.25 a	63.28 ab	68.55 a	956.1 a	1721.65 b	0.58 a	7,2 a
wS	2.24 a	2.37 ab	6.07c	5.94 bc	9.61 a	8.49 a	51.31 b	85.87 a	867.66 a	2181.13 a	0.61 a	5,08 ab
eT	1.56 c	2.18 b	8.01ab	8.97 a	11.7 a	9.07 a	60.15 ab	72.79 a	695.55 a	1146.90 c	0.72 a	0,77 b
wS+eT	1.92 b	2.33 ab	8.64 a	6.87 b	11.03 a	9.84 a	83.38 a	75.49 a	930.23 a	1264.11 c	0.56 a	0,87 b
Interactions												
wS	**	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS
eT	**	*	**	**	NS	NS	*	NS	NS	**	NS	**
wSxeT	NS	NS	**	**	NS	NS	*	NS	NS	NS	NS	NS

Control (C): the ambient canopy temperature+water availability; water stress (wS): the ambient canopy temperature+water deficit by suspension of irrigation; elevated temperature (eT): +2 ◦C above ambient canopy temperature+water availability; water stress+elevated temperature (wS+eT): water deficit +2◦C above ambient canopy temperature. Different letters within columns indicate significant differences according to Tukey's test (P ≤0.05). NS, non-significant. *significant at 0.05. **significant at 0.01.

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Regarding the stress characterisation, in wS, eT and wS+eT, the carbonyl content decreased significantly by 50, 42 and 50%, respectively ($P \leq 0.05$; Table 2). The wS treatment negatively affected SOD activity by 18%, but wS+eT induced an increment of 17% in SOD activity compared with that of the control ($P \leq 0.05$; Table 3).

3.7. Third sampling at 12:00 h

At 12:00 h of the third sampling day, the temperature of the canopy was 30.5, 30.3, 31.2 and 31.8 °C in the control, wS, eT and wS+eT treatments, respectively (Figure 3C).

At 12:00 h of the third evaluation, in wS, total pigments increased by 45% (Table 1). However, in wS, the content of MDA, carbonyl and H_2O_2 increased significantly compared with that of the control ($P \leq 0.05$; Table 2).

We observed that wS induced an increment in the GSH content of 26%, whereas in eT and wS+eT, SOD activity increased by 79 and 37%, respectively, compared with those of the control. Additionally, the GSH/GSSG ratio decreased in eT and wS+eT ($P \leq 0.05$; Table 3).

3.8. Chlorophyll fluorescence images

The chlorophyll fluorescence images showed that the effective PSII quantum yield [Y(II)] and the [Y(NO)] were not significantly affected by warming or soil water deficit (Figure 4).

4. Discussion

Climatic change could cause considerable increases in yield losses by an increment of temperature associated with drought (Wiebe et al., 2015). In this work, we determined the biochemical response of *P. maximum*, an important forage crop in tropical countries, subjected to water deficit (wS) and elevated temperature (eT) to understand the potential biochemical capacity of this C4 grass to face a future climate change scenario.

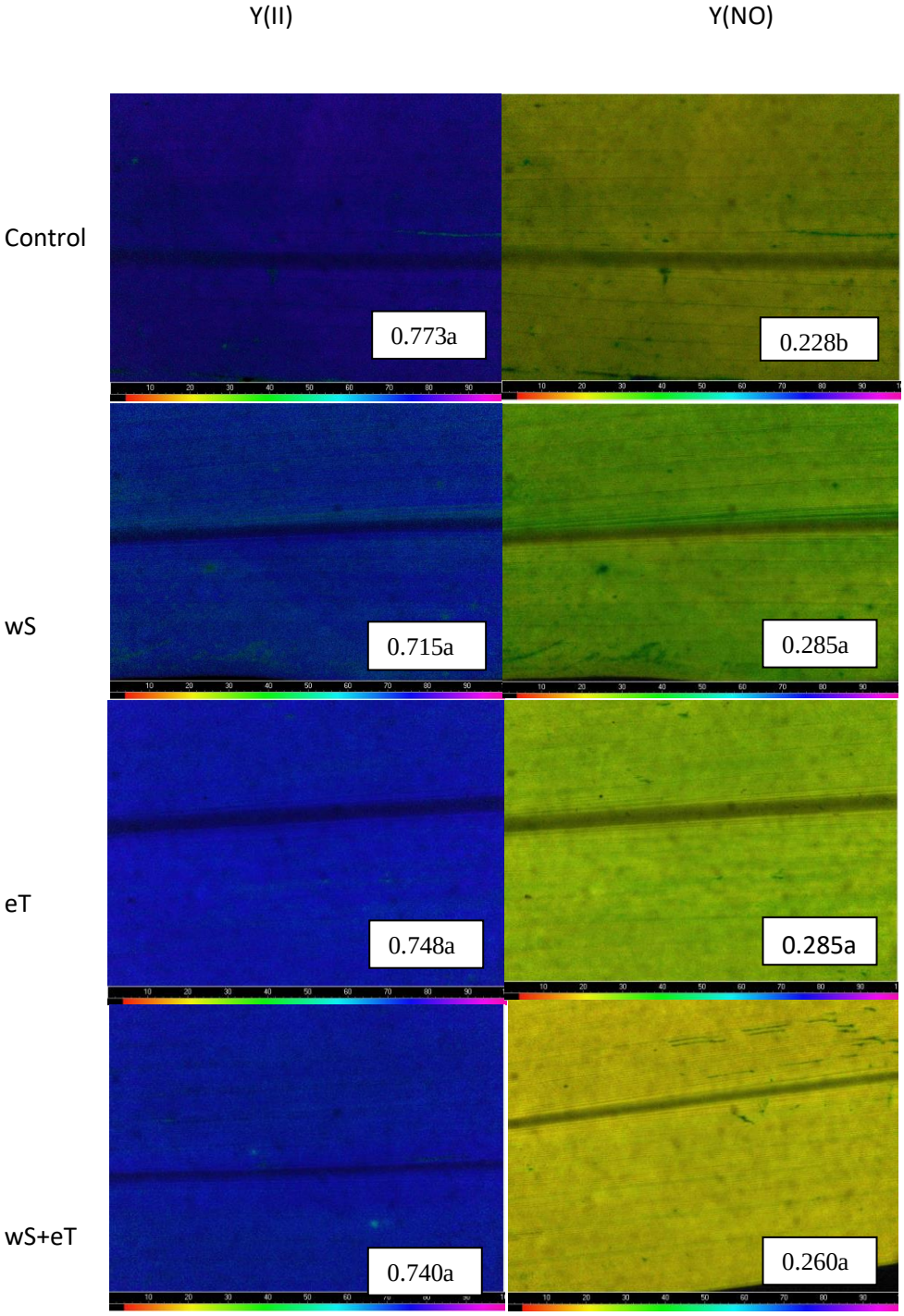

Figure 4. Chlorophyll fluorescence image of effective PSII quantum yield [Y(II)] and the [Y(NO)] in leaves of *Panicum maximum* Jacq. cv. Mombaça exposed to treatments: irrigation and ambient canopy temperature (Control, C); soil water deficit and ambient canopy temperature (wS); irrigation and elevated canopy temperatureat+2◦Caboveambient temperature (eT) and the combination of soil water deficit and elevated canopy temperature (wS+eT). The numbers inside the figures show the average values of Y(II) or Y(NO) (n=4). In each image of

fluorescence, means followed by the same letters in columns are not significantly different ($P < 0.05$).

A recent work (Zhou et al., 2017) showed that the sensitivity of plants to isolated stresses is different from that when exposed to the stresses combined, indicating that selection of plants for a single stress tolerance might not be correlated with a combined stress tolerance. In this work, we observed a detrimental effect of water stress on the chlorophyll content on two sampling dates; however, on the second sampling date, the photosynthetic pigment content in the combined wS+eT treatment increased. We also found that at the second sampling date, wS+eT treatment stimulated the content of carotenoids (non-enzymatic antioxidant system).

On the third sampling date, the content of chlorophyll and carotenoids in wS, eT, and combination wS+eT treatments reached normal levels, most likely associated with the increase in rainfall and resulting high SWC observed on this date. These results suggest that *P. maximum* can increase the pigment content as an adaptation to moderate water stress and warming conditions, as observed on the second sampling date. Nankishore and Farrell (2016) found that tomato plants more adapted to drought and heat stresses combined showed a high content of photosynthetic pigments, indicating a capacity to increase the efficiency of light utilisation (Fang and Xiong, 2014). Chlorophylls are essential to convert light energy into chemical energy, making photosynthesis possible, which in turn synthesises different compounds that are used by plants on the other hand, carotenoids act in the light antenna complex dissipating the excess of light energy, thereby protecting the photosynthetic apparatus against oxidative stress induced by ROS (Esteban et al., 2015). Thus, the capacity of a plant to adjust the photosynthetic pigment contents under water deficit and warming is a significant adjustment of the photosynthetic machinery to face abiotic stress (Gururani et al., 2015).

With the increment of carotenoids, *P. maximum* limited the oxidative damage in its plastids, maintaining the use of solar energy for photosynthesis (Smolikova and Medvedev, 2015). According to Czarnocka and Karpiński

(2018), plants subjected to abiotic stress form the triplet chlorophyll (^{3}Cl•), which transmits excess energy to O_2 thereby forming ROS; however, this excess energy can be captured by carotenoids to avoid ROS formation. This behaviour of carotenoids, as a protective agent against water deficit, is reported by Mibei et al. (2017), whereas Carvalho et al. (2016) observe an increase in carotenoid content in *Vitis vinifera* subjected to combined stresses (water stress + temperature increase).

However, the environmental factors evaluated (water stress and +2 °C in the canopy temperature) had a significant influence on the characterisation of stress. MDA is a by-product of lipid peroxidation and is used to quantify the damage caused by ROS to cell membranes (Correia et al., 2018). In this work, independent of treatment, the MDA content was low at 6:00 h and high at 12:00 h, as a result of the elevated level of solar radiation commonly observed at noon.

In our experiment, rainfall occurred after the second sampling, which reduced the possibility of oxidative damage as verified by the reduction in MDA and protein oxidation observed at 6:00 h in plants subjected to water stress. Thus, maintaining the proper frequency of irrigation during the growth of *P. maximum* can avoid the occurrence of oxidative stress.

The content of carbonyl is related to the damage to proteins. We observed that 19 days after the initiation of the water deficit, at 12:00 h, the carbonyl content increased in wS+eT, suggesting that under high temperature (31 °C), the antioxidant system of *P. maximum* lacked the ability to limit the damage to plant proteins caused by ROS (Das and Roychougury, 2014). Protein oxidation is considered as an irreversible damage, which is caused by an overproduction of ROS, leading to a decrease in the functionality of proteins, in addition to working as a quality control mechanism (Møller et al., 2011).

In this study, we evaluated the content of hydrogen peroxide to observe whether H_2O_2 was associated with oxidative stress damage. Because of its chemical characteristics, H_2O_2 has a critical role in signalling, particularly when

plants are under drought and heat stress; however, H_2O_2 is also involved in the formation of the hydroxyl radical (•OH). We observed that the content of H_2O_2 increased, particularly after 19 days, under the combination of water deficit and warming. This result might be related to the increase in protein oxidation observed in this experiment at the same time and sampling date. Regarding antioxidant activity, we quantified the enzymatic and non-enzymatic responses.

In this experiment, SOD, APX and GR represented the enzymatic response, and GSH and the GSH/GSSG ratio represented the non-enzymatic response against oxidative stress. We observed that P. maximum had the ability to increase the detoxification of superoxide radicals (O_2^-) by increasing the activity of SOD significantly under the combination of water deficit and warming (wS+eT). The disproportionation of O_2^- into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) catalysed by the SOD family of metalloenzymes is considered the first line of defence of the cell against oxidative stress (Del Río et al., 2018).

At the second sampling, we observed that in the wS+eT treatment, the activity of APX increased at 6:00 and 12:00 h, suggesting that *P. maximum,* under the combined stresses, increased the detoxification of H_2O_2, as related by Jin et al. (2016). APX has a high affinity for H_2O_2 and is a critical enzyme in the H_2O_2 detoxification as a part of the ascorbate-GSH cycle using ascorbate as an electron donor. According to Mitller (2002), APX limits the formation of the highly toxic hydroxyl radical (•OH).

We observed that at 12:00 h on the second and third samplings, the content of GSH decreased in wS+eT compared with that of the control, but the GSH/GSSG ratio was not affected by the treatments. In general, GSH is a versatile molecule that participates in ROS detoxification (Das and Roychougury, 2014), and equally, this molecule is related to drought and heat stress (Ren et al., 2016, Awasthi et al., 2017, Zandalinas et al., 2017). Moreover, GSH is a suitable electron donor and is part of the ascorbate-GSH cycle.

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The chlorophyll fluorescence images showed that the effective PSII quantum yield [Y(II)] and the [Y(NO)] were not significantly affected by warming or soil water deficit.

5. Conclusions

In summary, we concluded that the grass forage *P. maximum* was affected differently by water stress (wS), warming (eT) and the combined stresses (wS+eT),with effects influenced by the moment (13, 19, and 37 days after beginning DAT) and the time (6:00 and 12:00 h) of sampling. Our data indicated that *P. maximum* showed a relatively favourable response to the simulated future warming and water stress conditions responding well to the applied treatments. Plants showed in some cases an increase in photosynthetic pigment and carotenoids content and an increase in enzymatic activities, particularly those of SOD and APX. Here, we observed that PS II activity of *P. maximum* acclimated to warming and water stress maintaining membrane stability associated with efficient enzymatic free radical scavengers. Thus, the imposed conditions of warming and a short period of water deficit will not be detrimental to the survival of *P. maximum* under a future scenario of climate change.

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This chapter was published in Physiologia Plantarum (2018) 165: 413-426

CHAPTER 3.- Global warming: antioxidant responses to deal with drought and elevated temperature in *S. capitata*, a forage legume

ABSTRACT.- Drought is more frequent and severe along with elevated temperatures. To deal with the climate change impact on plants, we need to understand the plant biochemical performance. Nowadays, there is a lack of information on the plant antioxidant response, mainly in field and climate change conditions. Here, we quantified the antioxidant responses of the forage legume *Stylosanthes capitata* Vogel (in field condition), to deal with the elevated temperature (eT, +2ºC above ambient canopy temperature), and drought (wS) by water supply deprivation. T-FACE (Temperature free-air controlled enhancement) facility was used for warming, and samples were taken 17, 24 and 46 days after warming exposure at periods of 6:00 and 12:00 h. The eT+wS treatments decreased CAT, SOD and APX activities, especially at 46 days. Nevertheless, GSH content was not modified by eT+wS treatments. We also determined an important inverse relation between SOD-GR-GSH, and MDA-H_2O_2. In conclusion, combined stresses provoked detrimental effects of enzymatic antioxidant responses. Furthermore, we highlighted the importance of SOD GR and GSH to avoid damages of ROS in *S. capitata* grown under climatic change. In addition, under adequate irrigation, the antioxidant systems may face the elevated temperature, successfully.

Key words: Biochemistry responses, field assessment, combined stresses.

Abbreviations

APX, ascorbate peroxidase (EC 1.11.1.11); C, Control: The ambient canopy temperature + irrigated soil; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); DTT, ditiotreitol; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; eT, the elevated temperature in +2º above ambient canopy temperature + irrigated soil; GR, glutathione reductase (EC 1.8.1.7); GSH, glutathione; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NBT, Nitro blue tetrazolium chloride; O^{2-}, superoxide anion; $^{\bullet}OH$, hydroxyl radical; $^1O_2$, singlet

oxygen; PVPP, polivinilpolipirrolidona; ROS, reactive oxygen species; SOD, superoxide dismutase (EC 1.15.1.1); T-FACE, temperature free-air controlled enhancement facility; TNB, 2-nitro-5-thiobenzoic acid; wS, the ambient canopy temperature + water deficit by the suspension of irrigation.

1. Introduction

According to Brown and Caldeira (2017), the world temperature will increase 2.3 +/- 0.3°C threshold, during the next 50 years. With the elevated temperature is expected the increase of drought in different regions (Gateau-Rey et al, 2018; Liu et al., 2018), damaging crop yield (Fain et al., 2017; Neibergs et al., 2018).

On the other hand, the reactive oxygen species (ROS) are by products of plant development and growth; however, abiotic stress such as drought and the elevated temperature bring about the over production of ROS causing injuries in different parts of the plant, such as the cell membrane, DNA, RNA, and proteins leading to cell death, in extreme situations. The damage caused by ROS on the plant cell, is commonly named oxidative stress. (Choudhury et al., 2017).

Drought and heat stress are involved with the over production of reactive oxygen species (ROS) such as superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2), which have specific chemistry characteristics (Mitller, 2017).

To limit the effect of the ROS overproduction, plants have developed a set of antioxidant responses (enzymatic and non-enzymatic). The enzymatic antioxidant system is formed by Superoxide Dismutase (SOD, EC 1.15.1.1), Catalase (CAT, EC 1.11.1.6) Ascorbate Peroxidase (APX, EC 1.11.1.11), Guaiacol Peroxidase (GPX, EC 1.11.1.7), Glutathione Peroxidase (GPX, EC 1.11.1.9), Glutathione Reductase (GR, EC 1.8.1.7), Monodehydroascorbate Reductase (MDHR, EC 1.6.5.4), and Dehydroascorbate Reductase (DHR, EC 1.8.5.1) (Souza et al., 2017). In addition, non-enzymatic mechanisms include compounds such as tocopherols, carotenoids, ascorbate (AsA) and glutathione (GSH) may be also responsible for quenching excessively ROS (Gruszca et al., 2017).

On the other hand, there are so many investigations on drought, heat, and combined stresses but, most of them were carried out under greenhouse or climatic chamber (Zandalinas et al., 2017; Correia et al., 2018), where all other climatic variables were controlled. However, the plant biochemical responses to ambient stresses are better simulated in field experiment, where the result can be more realistic.

As well, most of the researches quantified the heat stress with a significant temperature increase, for example, 15 to 23°C (Suzuki et al., 2016; Silva et al., 2018); but the quantification of a slight increase of temperature, i.e., 2 to 4°C on plant biochemical response is scarce yet, especially when it is combined with water deficit, under field conditions.

Furthermore, we have to take into account that the response to abiotic stress is related to the specie and stress intensity and duration. Additionally, recent researches showed that isolated and combined stresses cause different plant biochemical responses (Zandalinas et al., 2018). In this sense, the determination of antioxidant response of *S. capitata* to a slight increase of temperature, water stress, and its combination is crucial because of it will allow understanding the biochemical mechanisms of defence in order to develop cultivars with high tolerance to climate change effects, primarily to heat and drought stress. The development of new tolerant cultivars can help us to mitigate the adverse effect of global warming on food production worldwide.

Taking into account the lack of information about of the antioxidant plant response to heat and drought stress under a global warming scenario, we carried out this work aiming to understand the biochemical responses of *Stylosanthes capitata* to elevated temperature (2°C above the environmental temperature) and different periods of water stress under field conditions.

2. Material and Methods

2.1. Plant material and Growth conditions

Seeds of *Stylosanthes capitata* cv Campo Grande were obtained from Wolf Seed Company (Ribeirão Preto, SP, Brazil), and they were sown on November 24th, 2017 in 12 experimental plots of 25m^2 each at the T-FACE (*Temperature free-air controlled enhancement*) facility located at the São Paulo

University, Ribeirão Preto campus (21˚17'83"S, 47˚80'67"W). The soil analysis was performed at 0–20 cm to determine the soil nutritional correction in each plot. The results of soil analysis indicated the necessity of supplemental fertilization in each plot.

The T-FACE system was used to increase the air temperature of +2ºC above the ambient on the canopy temperature according to Kimball et al. (2008). The T-FACE facility uses infrared (IR) heaters to apply a heating treatment to open-field plant canopies, simulating models of future global warming conditions. Each warming plot of the T-FACE contained six IR heaters (750 W model FTE-750-240 Salamander ceramic infrared heating element, Mor Electric Heating, MI, USA) placed at 0.80 m above the plant canopy in a hexagonal arrangement. Each IR heater was installed in an aluminum reflector model Salamander ALEX (Mor Electric Heating, MI, USA). For controlling the rise in temperature, the T-FACE uses a proportional-integrative-derivative (PID) control system installed in a datalogger model CR1000 with AM25T multiplexors (Campbell Scientific, UT, USA). Apogee infrared radiometers model SI-1H1-L20 (Apogee Instruments, UT, USA) provided the data on control and heated plot temperatures (Kimball et al., 2008). The controlling of the T-FACE system was done through the Loggernet datalogger support software (Campbell Scientific, UT, USA), which allowed the communication with the datalogger.

The experiment started on April 17th, 2018, when to induce the water deficit, we suspended watering continuously. In addition, we tested two conditions of soil water availability: irrigated plots (plants irrigated at 80% of field capacity, which is approximately 0.50 m^3 m^{-3}) and non-irrigated plots (plots without irrigation) and two levels of temperature: elevated (2° above ambient canopy temperature) and ambient. These two variables (with two levels each) were combined in a factorial arrange giving four treatments in a four-randomized blocks design: *Control* (irrigated plants and ambient canopy temperature); *wS* (soil water deficit and ambient canopy temperature); *eT* (irrigated and elevated canopy temperature) and *wS+eT* (soil water deficit and elevated canopy temperature). The air temperature (Figure 1A), the soil temperature (Figure 1 B C) and Soil water content (Figure 2) were monitored.

For the biochemical analysis, we carried out three samplings. The first sampling was made on May 04th, the second on May 11th and the third on June 2nd (17, 24 and 46 days after treatments exposure, respectively). At each sampling day, samples were collected at 6:00 and 12:00 h. After each sampling, the leaf material was immediately placed in liquid nitrogen and stored in a freezer at -80ºC for the biochemical analysis at the Laboratory of Plant Physiology, Department of Agriculture Applied Biology, UNESP, Jaboticabal Campus.

2.2. Quantification of pigments

We quantified the photosynthetic pigments according to Lichtenthaler (1987). The samples were macerated in 80% acetone for four days. After the maceration the reading was done by spectrophotometer at 663 nm (chlorophyll a), 647 nm (chlorophyll b), and 470 nm [Carotenoids – carotene (*c*) + xanthophyll (*x*)] wavelength. To calculate the chlorophylls (Chl) and carotenoids (Car) concentration we used the formulas as follow: Chl *a* = 12.25 A_{663} – 2.79 A_{647}; Chl *b* = 21.50 A_{647} – 5.10 A_{663}; Total Chl (*a*+*b*) = 7.15 A_{663} + 18.71 A_{647}; Car (*c*+*x*) = (1000 A_{470} – 1.82 Chl *a* – 85.02 Chl *b*)/198. The tissues chlorophyll and carotenoid content was expressed in µg of the pigment per fresh mass (µg g^{-1}).

2.3. Lipid peroxidation

To determine the effect of elevated temperature and water deficit on cellular membrane, we quantified the malondialdehyde (MDA) content according to Shimizu et al. (2006). After leaf tissue maceration in liquid nitrogen, the extraction was performed with four mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged for 15 minutes at 4°C. The supernatant was added in 1.5 mL of 0.5% TBA. The tubes were vigorously agitated and incubated at 95°C for 30 min. After that, the absorbance was recorded at 532 nm, and a non-specific absorbance of 600 nm. The MDA molar extinction coefficient (155 mM^{-1} cm^{-1}) was used for the calculations, and the results were expressed in nmol MDA g^{-1} of fresh mass (FM).

2.4. Hydrogen Peroxide

The H_2O_2 content was quantified according to Alexieva et al. (2001). The leaf tissue was macerated in TCA and centrifuged at 10000 rpm for 15 min under low temperature (5°C). Also, the supernatant was placed in 200 µl of 100 mM potassium phosphate buffer (pH 7.5) and 800 µl of 1M KI solution. After the reaction (in the dark place at low temperature), the reading was performed on a Perkin Elmer–Lambda spectrophotometer at the wavelength of 390 nm. The amount of H_2O_2 was expressed in µmol g^{-1} of fresh matter.

2.5. Reduced Glutathione (GSH)

The reduced glutathione (GSH) is an important non-enzymatic antioxidant. In this experiment to determine GSH content, we used DTNB and NADPH which was incubated in phosphate buffer 143 mM and pH 7.5 10 min at 30°C in a water bath. The TNB production was recorded on a Perkin Elmer – Lambda spectrophotometer at the wavelength of 412 nm (Griffith, 1980).

2.6. Extraction and determination of proteins

Samples were extracted using 100 mM potassium phosphate buffer (pH 7.5) containing 1mM EDTA, 3mM DTT, and 4% PVPP. Then, the mixture was centrifuged at 10 000 g for 30 min at 4°C. The supernatant was kept at -80°C. The reading of absorbance was performed on a Perkin Elmer–Lambda spectrophotometer at the wavelength of 595 nm. (Azevedo et al., 1998). Total protein was quantified according to Bradford (1976).

2.7. Superoxide Dismutase (SOD, EC 1.15.1.1) activity

The superoxide dismutase (SOD) is responsible for the dismutation of superoxide. To determine its activity we mixed the buffer sodium chloride (50 mmol l^{-1}) pH 7.8, methionine (13 mmol l^{-1}), NTB (75mmol l^{-1}), EDTA (0.1 mmol l^{-1}), and riboflavin (µmol l^{-1}). The mixture reacted under a fluorescent lamp at 25°C for 5 min, after which the blue formazan compound was formed. The reading was performed on a Perkin Elmer – Lambda spectrophotometer at the wavelength of 560 nm (Giannopolitis and Ries 1977).

2.8. Catalase (CAT, EC 1.11.1.6) activity

To determine the activity of Catalase, 1mL of phosphate buffer (100mM, pH 7.5) and 2.5µL H_2O_2 30% were mixed. We added 25µL of extract of protein in this misture. The enzymatic activity was determined on a Perkin Elmer – Lambda spectrophotometer at the wavelength of 240 nm, to 25◦C (Monteiro et al., 2012).

2.9. Ascorbate Peroxidase (APX, EC 1.11.1.11) activity

To calculate the APX activity, we used the protocol of Gratão et al. (2008). This determination was done by reaction of a solution containing 650 uL 80 mM potassium phosphate buffer pH 7.0 + 100uL 5mM ascorbate; 100 uL EDTA 1Mm, remaining in a water bath at 30°C. For reading, we added 100 uL of H_2O_2, 0.1 Mm, and 50 uL of extract. The reading was performed on a Perkin Elmer-Lambda spectrophotometer at the wavelength of 280 nm for 1 min, considering the H_2O_2 decomposition.

2.10. Glutathione Reductase (GR, EC 1.8.1.7) activity

The GR was determined according to Azevedo et al. (1998) and Cakmak and Horst (1991). We mixed 100nM phosphate buffer pH 7.5, 500 uL of 5'5-dithiobis (2-nitrobenzoic acid), DTNB in a water bath at 30°C. Before reading, we mixed 1 mM of oxidized glutathione, 0.1 mM NADPH, and 50 uL of extract. The reading was performed on a Perkin Elmer – Lambda spectrophotometer at the wavelength of 412 nm.

2.11. Statistical Analysis

The experiment consisted of 16 plots that correspond to a completely randomized block design with four treatments and four replications. Data were submitted to analysis of variance (Test F) and comparison of means by the Tukey test at 5% of probability. For the statistical analysis, we used the AGROSTAT® software which was developed by the Department of Exact Sciences of UNESP (Barbosa and Maldonado 2010).

We also determined the Factors that accounted for data variability. For this object, we submitted the data to Factor analysis. The Factor analysis studies the relationships among variables. To use this analysis, data were standardized (normal distribution, mean=0, variance=1). The coefficient obtained was used to interpret the meaning (it was considered the sign and size of each coefficient). The comparison of means of the main effects was made by Tukey test at 5% of probability. For this type of statistical analysis, we used the STATISTICA software.

3. Results

In this work, we quantified the plant response to elevated temperature (eT) (2°C above the environmental temperature), water deficit (wS) and the combined stresses (eT+wS). We also monitored the plant canopy temperature and soil water content for 48 days (Figure 1). We recorded temperatures from 12.5-14°C (first sampling), 12-14°C (second sampling), and 9-11.5°C (third sampling), at 6:00 h. Besides, the temperature of the first, second, and third samplings was from 31.8 to 33°C, 30 to 30.9°C and 31- 34°C respectively, at 12:00 h. Likewise, the soil water reached values ranging from 14%-17% and 48-58% in presence and absence of water deficit, respectively (Figure 2).

The effect of elevated temperature, water stress, and its combination on photosynthetic pigments, MDA, hydrogen peroxide, and antioxidant systems were evaluated. In each period (6:00 and 12:00 h) and each sampling date, the effect of treatments was compared by Tukey test (95%).

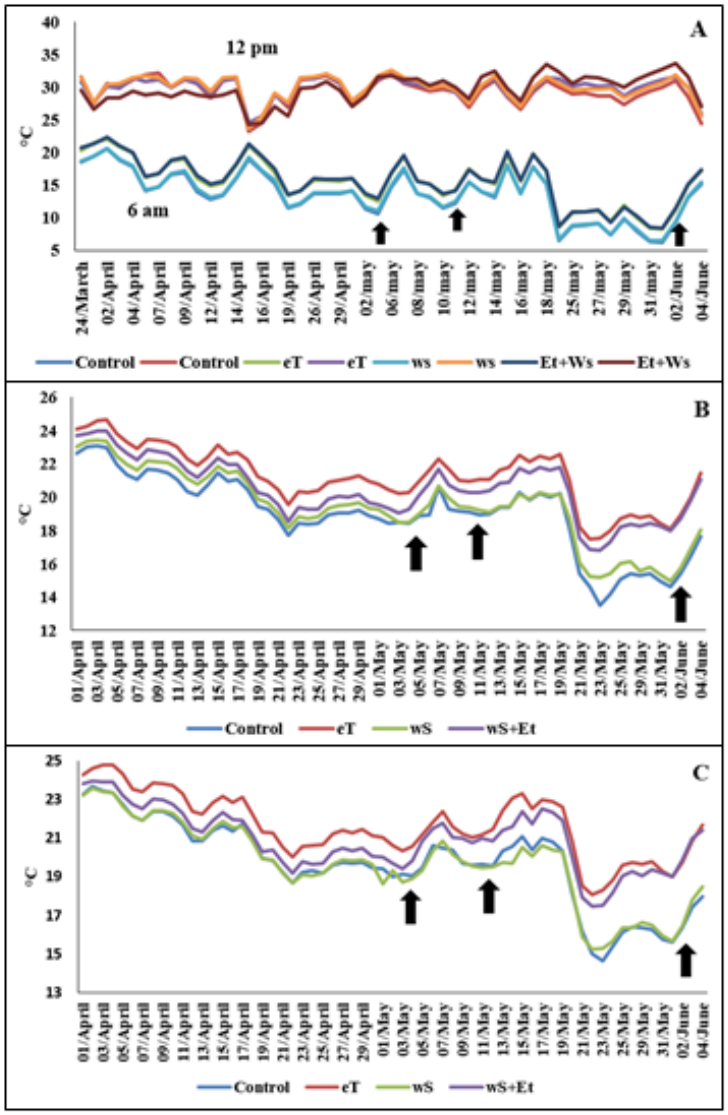

Figure 1. Air temperature (A), soil temperature at 6:00 h (B), and soil temperature at 12:00 h (C) recorded during the experiment with *Stylosanthes capitata* under field conditions. Arrows: Sampling days.

Besides, we found the interaction among the main factors (Temperature and irrigated soil), and detected the main Factors which accounted for data variability. Concerning the interaction of factors (Table 1), it was found that almost all treatments presented interaction between Temperature and Soil Water Content, as well the significance varied in each period and sampling.

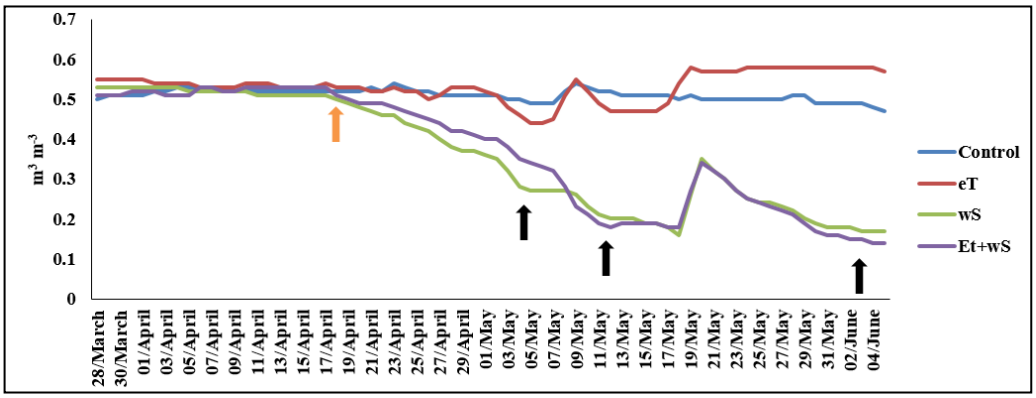

Figure 2. Soil water content recorded during the experiment with *Stylosanthes capitata* under field conditions. Orange arrow: starting of deprevation of water. Black arrows: Sampling days.

Table 1. Interaction among factors studied. Factor 1: Temperature (T) (Level 1: normal temperature; Level 2: +2°C above the environmental temperature). Factor 2: Soil Water Content (W) (Level 1: without water stress; Level 2: with water stress).

		First Sampling			Second Sampling			Third Sampling		
		T	W	TxW	T	W	TxW	T	W	TxW
Chl a	6:00 h	**	NS	*	**	**	*	**	**	NS
	12:00 h	**	**	*	*	**	**	**	**	**
Chl b	6:00 h	**	*	**	**	**	**	NS	**	*
	12:00 h	**	**	NS	**	*	**	NS	**	*
Chl a+b	6 00 h	**	NS	**	**	**	NS	*	**	*
	12:00 h	**	**	*	*	**	NS	NS	**	NS
Chl a/b	6:00 h	NS	NS	NS	**		**	NS	NS	NS
	12:00 h	**	**	*	**	**	**	NS	NS	NS
Carot	6:00 h	**	NS	**	*	**	NS	**	NS	*
	12:00 h	**	**	NS	*	**	**	*	NS	**
Chl/Carot	6:00 h	**	NS	**	**	*	NS	NS	**	NS
	12:00 h	**	NS	NS	NS	NS	**	**	**	**
MDA	6:00 h	NS	NS	*	**	NS	**	**	**	NS
	12:00 h	NS	**	NS	NS	NS	NS	*	**	**
H_2O_2	6:00 h	**	NS	NS	**	*	**	**	NS	**
	12:00 h	**	**	**	**	**	**	**	**	**
CAT	6:00 h	NS	**	NS	**	**	NS	**	**	**
	12:00 h	**	NS	**	**	*	**	**	NS	NS
SOD	6:00 h	**	NS	*	NS	**	**	*	**	**
	12:00 h	**	NS	**	**	**	**	**	**	**
APX	6:00 h	**	NS	*	NS	**	**	**	*	NS
	12:00 h	*	NS	*	**	**	**	**	**	NS
GR	6:00 h	**	**	NS	**	NS	*	**	**	**
	12:00 h	*	*	**	**	**	**	**	**	NS
GSH	6:00 h	NS	NS	*	NS	**	NS	NS	NS	NS
	12:00 h	NS	NS	NS	NS	**	NS	**	NS	**
GSH/GSSG	6:00 h	NS	NS	*	NS	**	NS	NS	NS	*
	12:00 h	NS	NS	**	*	**	NS	**	**	**

NS, non-significant. *significant at 0.05. **significant at 0.01 according to Tukey's test (P ≤0.05).

3.9. Photosynthetic Pigments

The pigment content was different in each sampling and period. In the Table 2 it is observed that temperature (eT) (2°C above the environmental temperature) had a positive effect on chlorophyll *a* and chlorophyll *b* content, at 6:00 h, leading to enhance of chlorophyll total, compared to others treatments ($P \leq 0.05$). eT also increased the levels of carotenoids significantly.

Likewise, wS decreased the content of chlorophyll [a+b] by 36% and 18%, at second and third sampling (Table 2), and, decreased the content of carotenoids by 19% at the third sampling, compared to Control.

Regarding to eT+wS, this treatment decreased the chlorophyll total content by 7% ($P \leq 0.05$) (compared to Control), at third sampling. Interesting, compared to wS, eT+wS increased the content of chlorophyll [a+b] by 35% and 58% at first and second sampling. In addition, compared to Control, eT+wS decreased the content of carotenoids by 20%, at third sampling.

It was noticed that *S. capitata* had favorable response when it was subjected to abiotic stress in the first evaluation at 12:00 h, in comparison to Control. Meanwhile, wS and eT+wS enhanced the content of Chl a+b ($P \leq 0.05$) and eT+wS increased de Chl/Carot ratio by 56%, at third sampling, compared to Control (Table 2).

3.1. Malondialdehyde (MDA) content

The cell damage was evaluated measuring the MDA content (Figure 3). Interestingly, at 6:00 h (Figure 3-A), we observed that all treatments incrased the MDA content (compared to Control) ($P \leq 0.05$) at second and third sampling, but only eT+wS increased remarkly the MDA content (compared to Control) by 44% and 53% ($P \leq 0.05$).

In the same way, at 12:00 h, eT decreased MDA levels by 13% ($P \leq 0.05$), however, eT+wS strongly stimulated the MDA content by 41% when compared to control, at third sampling (Figure 3-B).

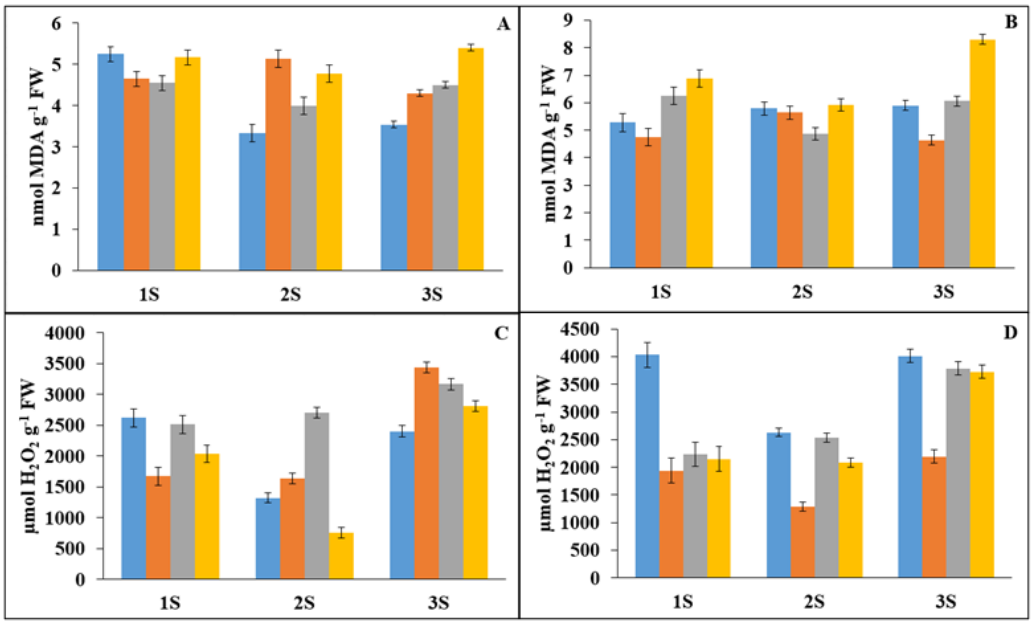

Figure 3. Content of MDA at 6:00 h (A), Content of MDA at 12:00 h (B), content of Hydrogen Peroxide at 6:00 h (C) and 12:00 h (D) during the experiment with *Stylosanthes capitata* under field conditions. 1S: First sampling; 2S: Second sampling; 3S: Third sampling. Light blue bar: Control; Orange bar: eT; Gray bar: wS; Yellow bar: eT+wS.

3.10. Hydrogen Peroxide (H_2O_2) content

In Figure 3-C, we noticed that the content of H_2O_2 in the third sampling was higher than in the second and first sampling at 6:00 h. As well, eT+wS presented lower H_2O_2 content than eT and wS ($P \leq 0.05$) at second and third evaluation.

In addition, eT showed lower content of H_2O_2, at 12:00 h in the three samplings (Figure 3-D). Likewise, eT, wS and eT+wS presented lower values of H_2O_2, in the first sampling, compared to Control, but, wS and eT+wS enhanced the hydrogen peroxide content, compared with eT ($P \leq 0.05$), in the third sampling.

Table 2. Quantification of Chlorophyll a (Chl a), Chlorophyll b (Chl b), Chlorophyll a + b (Chl a + b), relation Chl a/Chl b, Carotenoids (Carot), and Chl/Carot ratio in each of the sampling of the *S. capitata* experiment under a climate change scenario.

	Chl a (µg Chl a g^{-1}FW)		**Chl b (µg Chl b g^{-1}FW)**		**Chl a+b (µg Chl a+b g^{-1}FW)**		**Chl a/b**		**Carot (µg Carot g^{-1}FW)**		**Chl/Carot**	
	6:00 h	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**	**6:00 h**	**12:00 h**
First Sampling												
C	0.17 c	0.28 b	0.05 c	0.08 c	0.23 c	0.35 b	3.33 a	3.42 a	0.08 c	0.11 b	2.79 b	3.13 ab
eT	0.35 a	0.50 a	0.12 a	0.14 ab	0.47 a	0.65 a	3.00 a	3.75 a	0.15 a	0.22 a	3.04 ab	2.95 ab
wS	0.21 c	0.52 a	0.06 c	0.12 b	0.27 c	0.69 a	3.61 a	4.40 a	0.13 b	0.21 a	2.19 c	3.29 a
eT+wS	0.28 b	0.56 a	0.09 b	0.17 a	0.37 b	0.72 a	3.34 a	3.37 a	0.11 b	0.26 a	3.37 a	2.82 b
Second Sampling												
C	0.37 ab	0.39 b	0.08 b	0.11 b	0.43 b	0.525 ab	4.23 a	3.55 a	0.17 b	0.17 a	2.51 ab	2.95 b
eT	0.53 a	0.48 a	0.18 a	0.16 a	0.70 a	0.64 a	3.02 b	3.02 b	0.23 a	0.17 a	3.14 a	3.81 a
wS	0.37 ab	0.35 b	0.08 b	0.12 b	0.28 c	0.48 b	4.10 a	2.97 b	0.13 b	0.12 b	2.16 b	3.98 a
eT+wS	0.34 b	0.34 b	0.10 b	0.11 b	0.44 b	0.50 b	3.33 b	3.00 b	0.17 b	0.18 a	2.65 ab	2.89 b
Third Sampling												
C	0.41 ab	0.50 b	0.12 ab	0.13 b	0.51 b	0.65 b	3.16 a	4.09 a	0.19 b	0.27 b	2.66 a	2.42 b
eT	0.46 a	0.51 b	0.14 a	0.13 b	0.58 a	0.67 b	3.24 a	3.85 a	0.23 a	0.29 b	2.49 ab	2.30 b
wS	0.33 c	0.66 a	0.11 b	0.20 a	0.42 c	0.86 a	2.95 a	3.41 a	0.20 b	0.34 a	2.15 bc	2.44 b
eT+wS	0.37 bc	0.50 b	0.11 b	0.15 b	0.43 c	0.98 a	3.34 a	3.27 a	0.20 b	0.26 b	2.12 c	3.77 a

Control (C): the ambient canopy temperature + water availability; Elevated temperature (eT): +2 ˚C above ambient canopy temperature + water availability; Water Stress (wS): the ambient canopy temperature + water deficit by suspension of irrigation; Elevated Temperature + Water Stress (eT+wS): water deficit + +2 ˚C above ambient canopy temperature. Different letters within columns indicate significant differences according Tukey's test ($P \leq 0.05$).

3.11. Activity of Superoxide Dismutase (SOD, EC 1.15.1.1)

In general, we noticed that the SOD activity had an increment in the third sampling in all treatments (almost two fold). However, we also observed the adverse effect of eT+wS on SOD activity. In fact, in all samplings, the activity of this enzyme was decreased in response to combined stresses ($P \leq 0.05$), at 6:00 h (Figure 4-A).

As well, eT incrased the SOD activity significantly. Likewise, eT+wS decreased the SOD activity (compared to control) by 28% ($P \leq 0.05$) (Figure 4-B), at 12:00 h, in the third sampling,

3.12. Activity of Catalase (CAT, EC 1.11.1.6)

In this case, wS increased the activity of CAT by 32% and 15% ($P \leq 0.05$), respectively, when compared to Control in the second and third samplings at 6:00 h (Figure 4-C). Besides, eT+wS decreased the CAT activity significantly by 31% and 18%, respectively, at first and third samplings. Interesting, we noticed a gradual decrease of CAT ctivity from eT to eT+wT treatments, in the third sampling.

On the other hand, eT affected the CAT activity negatively in comparison to Control, especially at third sampling, where eT decreased the levels of CAT by 32%. Likewise, wS decreased the CAT content by 33% and 16% at second and third samplings respectively ($P \leq 0.05$) when compared to control (Figure 4-D). In addition, eT+wS increased and decreased the CAT activity stresses ($P \leq 0.05$), at second and third sampling at at 12:00 h.

3.13. Activity of Ascorbate Peroxidase (APX, EC 1.11.1.11)

In response to eT and eT+wS, APX showed a decline in its activity, at 6:00 h (Figure 4-E). For example at first, second and third samplings, compared to Control, eT dimished the APX activity by 21%, 36% and 45%, Meanwhile, eT+wS decreased the levels of APX by 45%, 33% and 45% ($P \leq 0.05$).

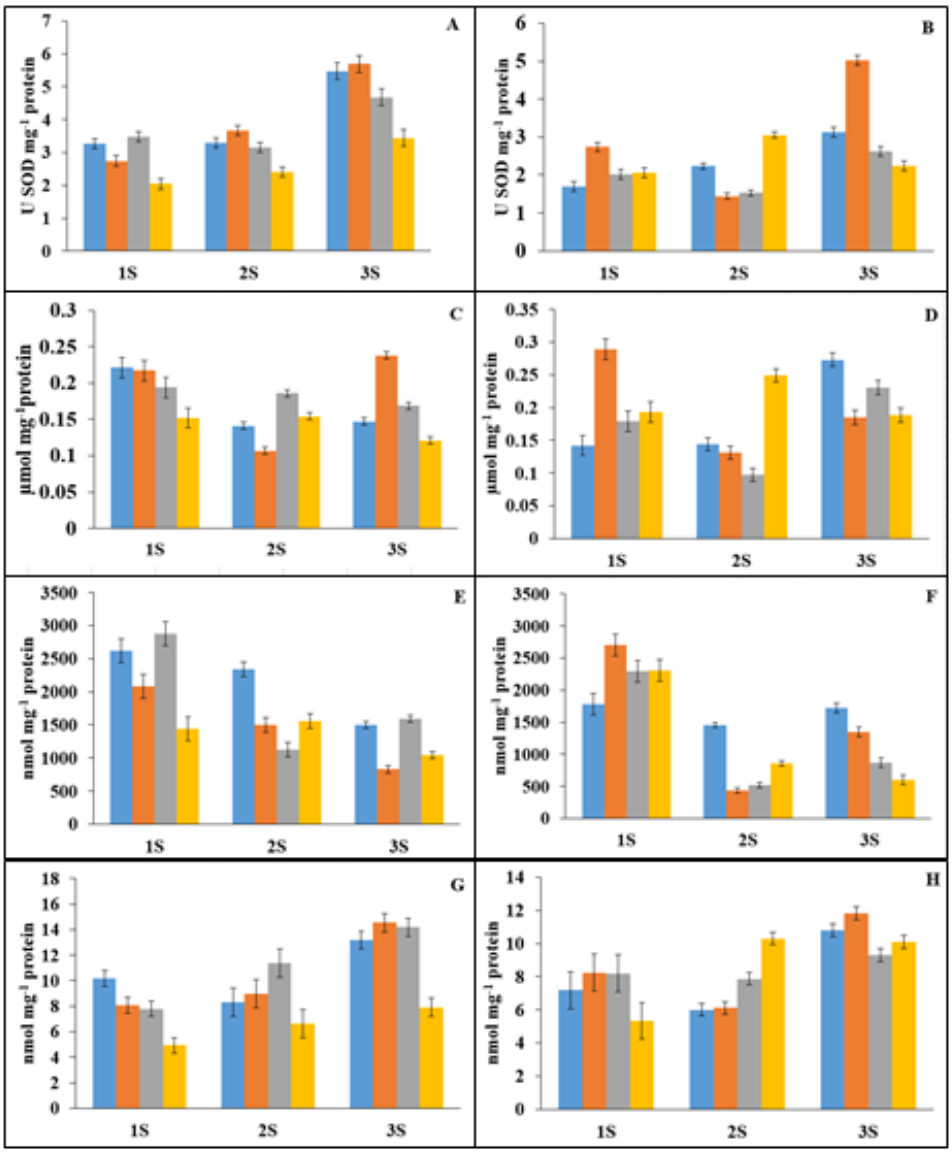

Figure 4. Activity of SOD at 6:00 h (A) and 12:00 h (B), Activity of CAT at 6:00 h (C) and 12:00 h (D), Activity of APX at 6:00 h (E) and 12:00 h (F), Activity of GR at 6:00 h (G) and 12:00 h (H). 1S: First sampling; 2S: Second sampling; 3S: Third sampling. Third sampling. Light blue bar: Control; Orange bar: eT; Gray bar: wS; Yellow bar: eT+wS.

In general, it was observed that eT, wS and eT+wS caused an increment of APX activity, in the first sampling compared to the second and third sampling (almost three fold), at 12:00 h. As well, all treatments declined the APX activity

significantly when compared to Control ($P \leq 0.05$), at second and third samplings. Likewise, APX decreased its activity by 49% and 65% in response to wS and eT+wS, respectively (Figure 4-F), in the third sampling and compared to Control.

3.14. Activity of Glutathione Reductase (GR, EC 1.6.4.2)

We also noticed that eT+wS declined the GR activity by by 51% and 40% ($P \leq 0.05$) at first and the third samplings at 6:00 h (Figure 4-G). It was also observed that GR activity was increased, almost two fold, in the plants subjected to eT+wS, in the second and third sampling compared to the first sampling, at 12:00 h. In addition, wS and eT+wS decreased the APX activity by 21% and 15%, respectively ($P \leq 0.05$) (Figure 4-H), when compared to eT.

3.15. Reduced Gluthatione (GSH) Content and GSH/GSSG ratio

Furthermore, high values of GSH content (approximately tree fold) was observed at the second sampling, compared to other samplings dates at 6:00 h and 12:00 h (Figure 5-A and 5-B respectively). Likewise, *S. capitata*, subjected to eT, wS, and eT+wS maintained the GSH levels similar to Control, in all evaluations. We found the same trends in the GSH/GSSG ratio (Figure 5-C and 5-D).

3.16. Factor analysis

The factor analysis showed that four Factors (F1, F2, F3 and F4) accounted for 95% of the overall variability (Table 3). The ANOVA model presented the interaction among treatments (T), Periods of sampling (P) (6:00 h and 12:00 h) and Sampling dates (S) (First, Second and Third Sampling dates).

The first Factor (F1) is related to use of light, in order to enhance the photosynthetic rate, and it is formed by chlorophyll a, chlorophyll b and chlorophyll [a+b], which have positive relationships. The F1 accounted for 31.70% of the data variability. In the same way, we noticed that treatments

caused the 16% of the variability. The eT stimulated strongly F1. Besides there was an increment of this process at 12:00 h and at third sampling.

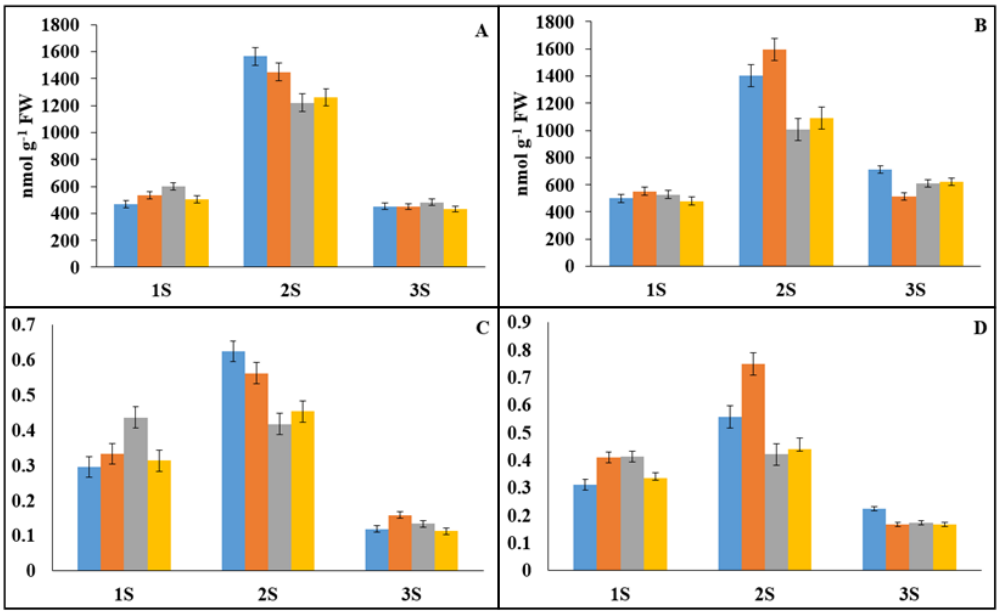

Figure 5. Content of GSH at 6:00 h (A) and 12:00 h (B), Content of GSH/GSSG at 6:00 h (C) and 12:00 h (D). 1S: First sampling; 2S: Second sampling; 3S: Third sampling. Third sampling. Light blue bar: Control; Orange bar: eT; Gray bar: wS; Yellow bar: eT+wS.

Second groups formed the second Factor (F2) reported in this work (Table 3). This group is composed by Chl/Carot ratio and MDA; likewise, they are negatively correlated with GR and SOD. The relation among these groups accounted for 26.2% of the data variability. As well, within this process, it was detected that treatments, periods, and samplings accounted for 14, 22, and 31% of result variability. The best performance of this process was observed in the response of eT, at 6:00 h and third sampling.

The third Factor (F3), is formed by GSH and GSH/GSSG, which are negatively correlated to H_2O_2 level. This process was more stimulated by eT at 12:00 h and at third sampling. F3 accounted for 24.26% of data variability. On the other hand, the Fourth biochemical process (F4) is formed by APX and Carotenoids, and both accounted for 12% of data variability.

Table 3. Factor analysis and Tukey mean multicomparison of variables evaluated in *Stylosanthes capitata.*

	F1	F2	F3	F4
Chl a	**0.9517**	0.0044	0.0077	-0.0452
Chl b	**0.9245**	0.0486	-0.0061	0.0187
Chl [a+b]	**0.9387**	-0.1315	0.0981	0.0594
Chl/Carot	0.1999	**-0.6720**	-0.1932	0.2413
MDA	0.4631	**-0.6330**	0.3371	0.1798
GR	0.0624	**0.8525**	0.2595	0.1763
SOD	0.0182	**0.9322**	0.1812	-0.0399
GSH	0.0324	-0.0927	**-0.8756**	0.2514
GSH/GSSG	-0.0938	-0.3357	**-0.8744**	0.0564
H_2O_2	0.0800	0.0313	**0.7897**	0.3356
APX	-0.2541	0.1239	0.0703	**-0.7097**
Carot	0.4394	-0.1271	0.0781	**-0.6386**
Variance Explained (%)	31.70	26.20	24.26	12.20
Interpretation	**Photosynthetic component**	**Membrane Protection**	**H_2O_2 Detoxification**	**APX and carotenoids**
ANOVA model Variance source				
Treatments (T)	16***	14***	9***	2^{NS}
Periods (P)	23***	22***	1***	1.5^{NS}
Sampling (S)	16***	31***	68***	16***
T x P	9***	5***	4***	5***
T x S	13***	11***	8*	13***
P x S	6***	3***	0.5***	6*
T x P x S	3***	4***	0.9*	14***
Mean multicomparison by Treatment				
C	C	B	B	A
eT	A	A	A	A
wS	B	B	B	A
eT+wS	B	C	B	A
Mean multicomparison by Periods				
6:00 AM	B	A	B	A
12:00 PM	A	B	A	A
Mean multicomparison by Sampling				
First Sampling	C	C	B	B
Second Sampling	B	B	C	A
Third Sampling	A	A	A	A

*significant at 0.05. **significant at 0.01. *** significant at 0.001. Different letters within columns indicate signicant differences according to Tukey's test (95%).

4. Discussion

The climate change causes crop yield loss, especially the high temperature and drought. Both provoke the over production of ROS, damaging different cell part (membrane, RNA, DNA and proteins) and leading to cell dead. In order to develop tolerant varieties to drought (wS) and heat stress (eT), is crucial determine the plant response to individuals or in combination stress (eT+wS).

Along with the increment of temperature, the climate change will increase the drought, resulting in a water stress in plants. According to Daryanto et al. (2016), the drought is considered the major abiotic factor that causes negative impact in the agriculture.

In this work, we determined the effect of wS, eT and eT+wS on photosynthetic pigments, damage on cellular membrane evaluated by the levels of MDA, production of hydrogen peroxide (H_2O_2), and the antioxidant responses (enzymatic and non-enzymatic). The chlorophyll is the pigment associated to plant photosynthesis, which can be used to quantify the effect of abiotic stress on plants (Alderfasi et al., 2017; Tyagi et al., 2017). On the other hand, carotenoids are pigments related to plant non-enzymatic antioxidant defence, especially under drought and elevated temperature (Carvalho et al., 2015; Durán et al., 2016).

The overall results showed an increment in the photosynthetic pigments content among sampling periods (Table 2), in fact, at midday we determined more chlorophyll than at 6:00 h. This increment could be related to that *S. capitata* need to improve the capture of light.

On the other hand, in general, eT+wS had not the same response as the single stresses. Interestingly, in the third sampling at 6:00 h, wS and simultaneous stresses decreased the content of chlorophyll. Our result can be related to the over production of ROS, which injured the chloroplast. In fact, we detected high values of MDA in this sampling (Figure 3), probably envolving the photosynthetic rate. Zhou, Yu, Ottosen, Rosenqvist et al. (2017) reported the additive and negative effects of elevated temperature and water stress in the pigments.

Nevertheless, eT+wS raised the content of pigments, at 12:00 h (third sampling); suggesting that *S. capitata* can adapt to combined stresses, in other species such as tomato, *Eucalyptus globulus* and *P. maximum* were observed this behaviour (Naskishore and Farrell, 2016; Correia et al., 2018; Borjas-Ventura et al., 2018). As well, the efficiency in the utilization of light is increased by increase in pigments (Fang and Xiong, 2014).

On the other hand, plants under abiotic stress (for example, under drought and heat stresses) showed an increase of ROS levels, which can attack the

cellular membrane (specifically the double bond of poly-unsaturated acid fatty), resulting a higher production of malondialdehyde (MDA) (Martínez et al., 2016; Nxele et al., 2017).

In general, it was detected that under single and combined stresses the MDA content was different, at 6:00 and 12:00 h. eT+wS also provoked an significant increment of MDA (Figure 3), in the third sampling. Our data indicate that *S. capitata* can not limit the production of ROS under combined stresses of 45 days. This behavior could be associated to low activity of antioxidant enzymes detected in this work. Our results are according to Zandalinas et al. (2017).

The hydrogen peroxide (H_2O_2) is a type of ROS that in steady levels it is related to plant growth and development. In addition, it plays a central role when the plant is facing stress condition (Sies, 2017). Conversely, high levels of H_2O_2 lead to oxidative stress, damaging DNA, proteins, and cellular membranes, and consequently affect the normal cellular function (Hossain et al., 2015; Martínez et al., 2018)

In this assessment, it was observed that wS and eT+wS raised the content of H_2O_2 in the third sampling, compared to first and second sampling (Figure 3), at 6:00 h and 12:00 h; however, it was noticed differences respect to control plants, at 6:00 h. Likewise, eT+wS could affect the signaling of antioxidant response (Niu and Liao, 2016), allowing the formation of other type of ROS (for example O_2^- or $^{\bullet}OH$). In fact, eT+wS presented high content of MDA (compared to wS), in spite of showing lower production of H_2O_2. Our data is according to Zandalinas et al. (2018).

One of the first ROS produced by abiotic stress is O_2^-, which in high levels cause damages to the proteins, DNA, and lipids, leading to cellular death (Gill et al., 2015). To prevent the negative effect of ROS on the cell, the plant requires an effective antioxidant system (Che et al., 2016).

SOD is the first plant defence line since it can catalyze O_2^- (first ROS produced) in H_2O_2 and O_2 (Wang et al., 2016). In current work, different responses were observed between single and combined stresses at 6:00 and 12:00 h (Fig 4). As well, in the third sampling, eT+wS showed lower activity of SOD when compared to single stresses, suggesting high presence of O_2^- like

oxidant agent. In coffee, SOD acitivy was affected negatively under 42°C (Martins et al., 2016).

Once formed, the H_2O_2, plants need an efficient enzymatic systems to reduce it, since that, high levels of H_2O_2 coud affect the REDOX homeostasis negatively. There are different plant enzymes that are in charge of H_2O_2 overproduction. Among these systems, we have CAT, APX and GR enzymes (Savvides et al., 2015).

Regarding CAT enzyme, many researchers reported a modification of its activity when the plant is under heat and drought stresses (Caverzan et al., 016; Silva et al., 2017). By the other way, APX enzyme, which is associated to H_2O_2 detoxification, using ascorbate as an electron donor (Caverzan et al, 2016). Along with APX, GR enzyme is related to Ascorbate-Glutathine cycle, as well as, it is responsible for the reduction of GSSG to GSH (Couto et al., 2016; Liu et al., 2017), and its activity is key under stress conditions (Harshavardhan et al., 2017; Soengas et al., 2018; Shan et al., 2018).

In this assessment, in the third sampling, eT+wS decreased the activity of CAT, APX and GR at 6:00 and 12:00 h. Therefore, *S. capitata* limits its ability to detoxify hydrogen peroxide under prolonged estress condition. Other researchers displayed detrimental effect of combined stresses on H_2O_2 detoxification (Awasthi et al., 2017). According to our results, combined stresses had an adverse impact on Ascorbate-Glutathione cycle (Kuźniak et al., 2017).

If is not counteracted, the over production of H_2O_2 is followed by the formation of other type of ROS such as $^{\bullet}OH$ (mediated Fenton reaction) (Pospíšil, 2016), which interact with the cell membrane, resulting in the over formation in MDA as observed in this assessment (Choudhury et al., 2017).

On the other hand, glutathione (GSH) is a low-molecule weight antioxidant type related to plant defense, for example against heat and drought stresses (Zandalinas et al., 2017; Bartoli et al., 2018; Xia et al., 2018). Under stress condition, GSH can donate electrons to the other molecules such as ROS. So, GSH can scavenge ROS (Couto et al., 2016). After GSH donates one electron, the oxidized glutathione is formed (GSSG) (Czarnocka and Karpiński, 2018). Besides, GSH participates in the ascorbate-glutathione cycle, which is

an important process related to H_2O_2 detoxification (Rahantaniaina et al., 2017). The determination of GSH levels is used to quantify the rate of oxidative stress (Hajdinák et al., 2018).

In our results, *S. capitata,* maintained acceptable GHS levels when it is subjected to elevated temperature, drought, and combined stresses at 6:00 and 12:00 h (Figure 5). The results observed at 6:00 and 12:00 h, pointed out that the treatments did not influence the REDOX state of the *S. capitata*. The maintenance of REDOX state is significant for the plant because it allows appropriate conditions to their proper development (Noctor et al., 2017).

Factor analysis have demonstrated the relation among variables studied. This work showed four factors (Table 3). We named them: Factor one (F1), Factor two (F2), Factor three (F3) and Factor four (F4). Likewise, the F1 is related to photosynthetic pigments. Meanwhile, F2, F3, and F4 are associated to plant defense systems.

In general, wS and eT+wS decreased the interaction of the variables in F1 (formed by chlorophyll a, chlorophyll b and chlorophyll [a+b]), probably affecting the photosynthesis rate. The correlation found in F2 (formed by group 1: Chl/Carot and MDA, which are negatively correlated with group 2: GR and SOD) shows the importance of antioxidant enzymes for limiting the damage on cell membrane.

We also noticed that the interaction of its variables was decreased by wS+eT, which could be explained by reduction in the activity of these enzymes caused by imposition of combined stresses.

In F3 we noticed negative correlation between GSH/GSSG and hydrogen peroxide suggesting that plants need high content of GSH to control the over production of ROS since GSH is a great donator of electrons.

On the other hand, the relation of variables determined in this work showed that SOD, GR and GSH enzymes are key components of *S. capitata* antioxidant system when it is growing under climatic change condition.

5. Conclusions

In summary, we observed that single and combined stress provoked differents responses in *S. capitata*. eT+wS also caused a detrimental impact on

the antoxidant enzymatic system, specially under strong condition (third sampling). Nevertheless, non-enzimatic response was not modified by the treatments. On other hand, the Factor analysis presented that GR and SOD enzymes and GSH content exhibited a key role to limit the damages of ROS in plant grown under climatic change conditions. Finally, we emphasize the proper management of irrigation since an increment of temperature by 2°C in the well-watered plant will promote biochemical processes to face the warming conditions**.**

Conflict of Interest

All authors declare that have no conflict of interest.

Author contributions

R. B. V. compiled and analysis data. A.S.F., C.A.M. and P.L.G. contributed in data analysis. C.A.M. designed and managed the T-FACE experiment. All the authors helped to conceived and commented the manuscript.

Acknowledgements

This work was supported by the CNPq/ANA/MCTI grant (Process 446357/2015-4) to C.A.M. and São Paulo Research Foundation, FAPESP (Thematic Project Grant 2008/58075-8 to C.A.M. which was included in the FAPESP Research Program on Global Climate Change). C.A.M. is CNPq fellowship (Process 306039/2016-8). Also, this study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. We thank Bruce Kimball and Franco Miglietta for assistance in installing the T-FACE and FACE infrastructure, respectively.

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