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PHYSIOLOGICAL RESPONSES OF *Bauhinia purpurea* L. TO HEAT STRESS IN THE CONTEXT OF CLIMATE CHANGE

RESPOSTAS FISIOLÓGICAS DE Bauhinia purpurea L. AO ESTRESSE TÉRMICO NO CONTEXTO DAS MUDANÇAS CLIMÁTICAS

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ABSTRACT

The response of trees to heat waves is uncertain, yet crucial for forest and urban ecosystems. *Bauhinia purpurea is a multipurpose woody species, but tolerance to heat stress remains unknown*. The aim of this study was to determine the effect of heat stress on photosynthesis, reactive oxygen species (ROS) production and antioxidant enzyme activity in *B. purpurea*. We hypothesize that heat stress inhibits photosynthesis in *B. purpurea*, through stomatal closure and alterations in the photosynthetic machinery, associated with the production of reactive oxygen species. One-year-old seedlings were placed in a growth chamber at 27°C/27°C, 38°C/27°C or 43°C/27°C, day and night, respectively, for seven days. At the end of the trial, gas exchange, chlorophyll a fluorescence emission, and biochemical indicators of oxidative stress were measured. A completely randomized experimental design with 10 replicates was used, and results were analyzed with ANOVA and Tukey's test. Heat stress inhibited net photosynthesis, but did not affect internal CO_2 concentration. It altered variables of chlorophyll a fluorescence emission, with a decrease in the rate of electron transport and photochemical quenching. Heat stress produced a low response in antioxidant enzymes, increasing malondialdehyde production and electrolyte loss. It is concluded that heat stress produces a non-stomatal inhibition of CO_2 fixation by inhibiting the photochemical stage of photosynthesis. Regimes of 43°C/27°C also produce photoinhibition and compromise light absorption. The species has an inefficient antioxidant enzyme response, resulting in oxidative stress and damage to cell membranes.

KEYWORDS: **Abiotic stress, Oxidative stress, Photosynthesis, Global warming**

RESUMO

A resposta das árvores às ondas de calor é incerta, mas crucial para os ecossistemas florestais e urbanos. *Bauhinia purpurea* é uma espécie lenhosa polivalente e sua tolerância ao estresse térmico é desconhecida. O objetivo deste estudo foi determinar o efeito do estresse térmico na fotossíntese, na produção de espécies reativas de oxigênio (ROS) e na atividade de enzimas antioxidantes em *B. purpurea*. Nós hipotetizamos que o estresse por altas temperaturas inibe a fotossíntese em *B. purpurea*, através do fechamento estomático e alterações na maquinaria fotossintética, associadas à produção de espécies reativas de oxigênio. Mudas com um ano de idade foram colocadas em câmara de crescimento a 27°C/27°C, 38°C/27°C ou 43°C/27°C, dia e noite, respectivamente, durante sete dias. Ao final do ensaio foram medidas trocas gasosas, emissão de fluorescência da clorofila a, e indicadores bioquímicos de estresse oxidativo. Foi utilizado delineamento experimental inteiramente casualizado com 10 repetições e os resultados foram analisados por ANOVA e teste de Tukey. O estresse térmico inibiu a fotossíntese líquida, mas não afetou a concentração interna de CO_2. Alterou variáveis de emissão de fluorescência da clorofila a, com diminuição da taxa de transporte de elétrons e extinção fotoquímica. O estresse térmico produziu uma baixa resposta nas enzimas antioxidantes, aumentando a produção de malondialdeído e a perda de eletrólitos. Conclui-se que o estresse térmico produz uma inibição não estomática da fixação de CO_2 ao inibir a etapa fotoquímica da fotossíntese. Regimes de 43°C/27°C também produzem fotoinibição e comprometem a absorção de luz. A espécie apresenta resposta enzimática antioxidante ineficiente, resultando em estresse oxidativo e danos às membranas celulares.

PALAVRAS-CHAVE: **Estresse abiótico, Estresse oxidativo, Fotossíntese, Aquecimento global**

INTRODUCTION

Since the industrial revolution, the emission of greenhouse gases such as carbon dioxide, nitrous oxide and methane has increased. Consequently, global warming and alterations in the hydrological cycles have occurred. By 2017, there was an increase in the average annual global temperature of approximately 0,8ºC (DUSENGE et al., 2019). The Intergovernmental Panel on Climate Change (IPCC) predicts that the concentration of carbon dioxide will reach values from 730 ppm to 1000 ppm by the year 2100, with increases in global average temperature in the order of 0,3°C to 4,8°C (PACHAURI et al., 2014). An increase in the frequency, intensity and duration of heat waves is also expected, reducing the growth, development and survival of plants (ORTIZ-BOBEA et al., 2019).

The response of trees to extreme heat waves is uncertain, yet crucial in both forest and urban ecosystems (TESKEY et al., 2015). High temperatures recorded during heat waves can exceed the thermal threshold of plants. This results in severe physiological alterations and mortality, unless they can quickly adjust to these conditions (O'SULLIVAN et al., 2017). A key factor is this process of carbon dioxide and water exchange during these events. Consequently, net photosynthesis is usually decreased by stomatal closure and/or by increased mitochondrial respiration and photorespiration (CHAUDHARY et al., 2020). However, some studies have reported that heat stress increases stomatal aperture, allowing leaf cooling and thus preventing damage to cell metabolism (ROGERS et al., 2017; URBAN et al., 2017).Extreme temperatures lead to the production of reactive oxygen species (ROS), such as superoxide radical, hydroxyl radical, singlet oxygen and hydrogen peroxide. ROS exert a dual effect on plants, depending on their concentration, subcellular localization and period of action (CHAUDHRY & SIDHU, 2022). At low concentrations, they act in signaling processes, mediating stress response. However, at high concentrations they can produce photosynthetic pigment degradation, protein degradation, lipid peroxidation, altered gene expression, and programmed cell death (PETROV et al. 2015, SINGH et al. 2019). ROS are detoxified by antioxidants of enzymatic and non-enzymatic nature (ZHOU et al., 2019).

Bauhinia purpurea L. (Fabaceae) is an intermediate-sized tree native to Southeast Asia. Its wood is used in the construction of beams, and the leaves can be used as fodder. Roots, flowers and leaves have medicinal properties, being used in the treatment of digestive disorders. Worldwide, it is frequently used in urban areas, due to its size and the beauty of its conspicuous flowers (SINGH, 2020). Because of its use as an ornamental species in large cities, it is of interest to know its response to high temperatures.

The aim of this work was to determine the effect of heat stress on photosynthesis, ROS production and antioxidant enzyme activity in *B. purpurea*. We hypothesize that heat stress inhibits photosynthesis in *B. purpurea*, through stomatal closure and alterations in the photosynthetic machinery, associated with the production of reactive oxygen species.

MATERIAL AND METHODS

Plant material and experimental conditions

The tests were carried out in November 2023 at the Faculty of Agronomy and Agroindustries of the National University of Santiago del Estero, located in El Zanjón, Santiago del Estero, Argentina (27°51’59”S 64°14’49”W). One-year-old seedlings (about 40 cm in height and 0,3 cm in diameter) were grown in 3 L plastic pots containing equal proportions of loam and vermiculite. Seedlings were placed in a growth chamber at 27°C/27°C (control), 38°C/27°C or 43°C/27°C, day and night, respectively. The photoperiod was adjusted to 12 h, with 1000 µmol m^{-2} s^{-1} photosynthetic photon flux density. Seedlings were watered daily with distilled water to maintain the substrate at field capacity. After seven days of trial, gas exchange and modulated chlorophyll a fluorescence emission were measured, and oxidative stress variables were quantified. The experiment was replicated twice.

Measurement of gas exchange and modulated chlorophyll a fluorescence

Gas exchange measurements were performed on the first three fully developed leaves from the apex (three leaves per plant). For this purpose, an infrared gas analyzer was used in an open system (IRGA-LCpro+ System ADC, BioScientific Ltd.), under conditions of saturating artificial light (1000 µmol m^{-2} s^{-1}) and CO_2 concentration of 40 Pa. The following variables were measured: net photosynthesis (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration (J). Water use efficiency (WUE) was calculated as the A/J ratio. Three measurements were performed on each leaf, in a total of 10 plants.

Modulated chlorophyll a fluorescence emission was measured with a PAM-2500 Walz portable chlorophyll fluorometer, on the same leaves on which the gas

exchange variables were measured. Measurements were performed according to the methodology described by Martins et al. (2020). The following variables were measured: electron transport rate (ETR), photochemical quenching (qP), non-photochemical quenching (NPQ) and variable fluorescence/maximal fluorescence ratio (F_v/F_M).

Total chlorophyll quantification

Total chlorophyll concentration was performed on the same leaves on which gas exchange and chlorophyll a fluorescence emission were measured. An extract was made with 80% acetone, and pigment concentration was determined spectrophotometrically, according to the technique described by Martins et al. (2019). Results were expressed in µg g^{-1} FW.

Enzymatic determinations

For enzyme extraction, leaves were homogenized in a buffer solution 100 mM Tris-HCL (pH 7,5) in presence of 5 mM Dithiothreitol, 1 mM EDTA, 10 mM $MgCl_2$, Polyvinylpyrrolidone (1,5%), 5 mM magnesium acetate, and aprotinin (1 µg/ml). The extract was centrifuged at 10.000×g for 15 min, and the supernatant was used for enzymatic assays (RAJA et al., 2020). The concentration of total soluble proteins was quantified as described by Bradford (1976), using bovine serum albumin as standard. Superoxide dismutase activity (SOD, E.C. 1.15.1.1) was determined according to the technique described by Giannopolitis & Ries (1977): one unit of SOD corresponded to the amount of enzyme necessary to inhibit by 50% the photoreduction of nitro blue tetrazolium chloride; results were expressed in U mg $protein^{-1}$. Catalase activity (CAT, E.C. 1.11.16) was determined using the method developed by Havir & Mchale (1987), following the oxidation of H_2O_2, at 240 nm and 30ºC and monitoring the absorbance over 300 s. The activity was calculated using the molar extinction coefficient of H_2O_2 (36 $M^{-1}.cm^{-1}$); results were expressed in µmol H_2O_2 mg^{-1} protein min^{-1}. Ascorbate peroxidase activity (APX, E.C. 1.11.1.11) was determined according to the method described by Nakano & Asada (1981) through the oxidation of ascorbate by H_2O_2 at 290 nm using a 2,8 mM^{-1} cm^{-1} extinction coefficient; results were expressed as µmol ascorbate mg^{-1} protein min^{-1}.

Determination of $O_2^{\cdot-}$ concentration, lipid peroxidation and membrane damage

$O_2^{\cdot-}$ concentration was quantified by nitrite formation from hydroxylamine in the presence of superoxide (ELSTNER & HEUPEL, 1976); results were expressed in nmol min^{-1} g^{-1} FW.

Malondialdehyde (MDA) concentration was quantified spectrophotometrically, according to the method described by Cakmak & Horst (1991), using an extinction coefficient of 155 mM^{-1}. cm^{-1}; results were expressed in µmol.g^{-1} FW.

Membrane damage was estimated through the measurement of electrolyte loss, according to the method described by Silva et al. (2019), and was expressed as percentage.

Experimental design and statistical analysis

A completely randomized experimental design with 10 replications was used; results were analyzed with ANOVA and Tukey's test. The experimental unit consisted of one plant growing in a pot.

RESULTS AND DISCUSSION

Temperature regimes of 38ºC/27ºC and 43ºC/27ºC reduced net photosynthesis of *B. purpurea* by 17% and 58%, respectively, with respect to the control (Figure 1).

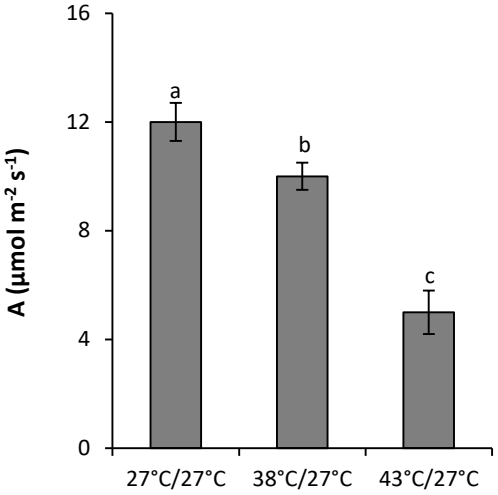

Figure 1. Net photosynthesis in leaves *of B. purpurea* grown at different temperatures. For each variable, different letters indicate significant differences by Tukey's test at 5%.

Inhibition of photosynthesis in *B. purpurea* under heat stress may compromise its growth in these conditions. Photosynthetic rate has been used as a variable in the selection of heat-stress tolerant cultivars in agronomically important species such as *Solanum lycopersicum* (ZHOU et al., 2018), Glycine max (DJANAGUIRAMAN et al., 2019) and *Cicer arietinum* (MAKONYA et al., 2019).

On the other hand, stomatal behavior depended on the severity of heat stress. Thus, whereas 38ºC/27ºC

reduced stomatal conductance by 39%, 43ºC/27ºC increased it by 146% with respect to the control (Figure 2A). However, CO_2 concentration was not limiting for the photosynthetic process, since C_i remained constant in all treatments (Figure 2B). Therefore, the inhibition of photosynthesis was of the non-stomatal type.

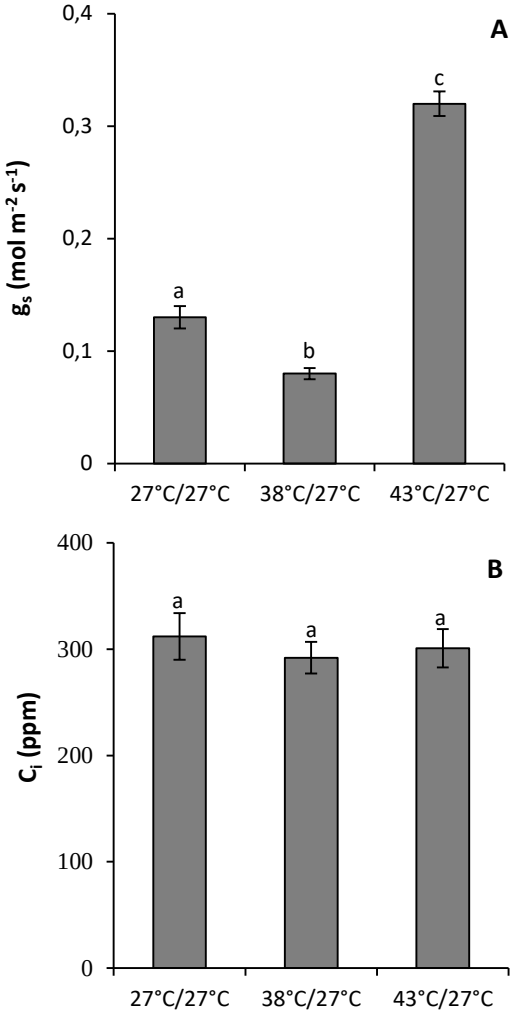

Figure 2. Stomatal conductance (A) and Intercellular CO_2 concentration (B) in leaves *of B. purpurea* grown at different temperatures. For each variable, different letters indicate significant differences by Tukey's test at 5%.

The response of stomatal conductance to increasing temperature varies depending on the species and genotype involved (MOORE et al., 2021). In general, g_s increases with increasing temperature until a tipping point is reached, after which it declines. At extremely high temperatures, it may increase again (TRICKER et al., 2018). The temperature at which each of these events occurs depends on the species, and is probably given by water viscosity, hydraulic conductivity and photosynthetic demand (MOORE et al., 2021). Stomatal conductance has also been used as a selection variable for heat-stress tolerant genotypes. Traub et al. (2018) selected high-temperature tolerant *Phaseolus vulgaris* cultivars using physiological variables, including stomatal conductance. They tested three temperature conditions: 35ºC/30ºC, 40ºC/35ºC and 45ºC/40ºC in greenhouse. In the high-temperature tolerant genotype, stomatal conductance increased with increasing temperature, up to 40ºC/35ºC.

In *Vigna radiata*, g_s increased up to 40ºC/30ºC, contributing to leaf cooling, but decreased at 43ºC/30ºC and 45ºC/32ºC. (KAUR et al, 2015). Sita et al. (2017) used g_s to select high-temperature tolerant cultivars of *Vigna radiata* and *Lens culinaris*; in tolerant genotypes, that variable increased with increasing temperature. Similar results were reported in the selection of high-temperature and water stress tolerant cultivars of *Solanum lycopersicum*. Thus, tolerant cultivars had higher g_s and lower leaf temperature under stress conditions (NANKISHORE & FARRELL, 2016).

Transpiration followed the pattern of stomatal conductance, decreasing under the 38oC/27oC treatment and increasing under the 43oC/27oC treatment (Figure 3).

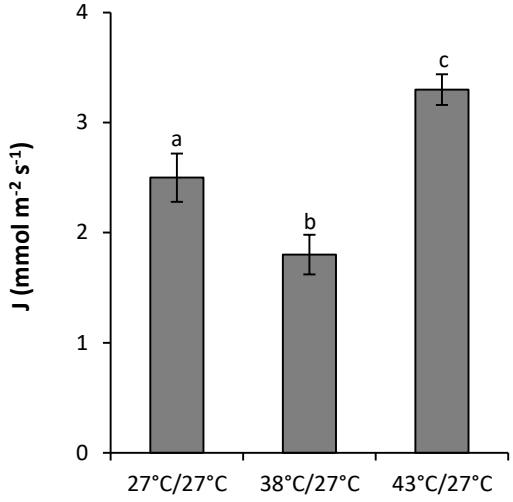

Figure 3. Transpiration in leaves *of B. purpurea* grown at different temperatures. For each variable, different letters indicate significant differences by Tukey's test at 5%.

As a consequence of the changes in net photosynthesis and transpiratory rate, the 38ºC/27ºC regime increased water use efficiency by 26%, whereas the 43ºC/27ºC regime reduced it by 49%, compared to the control (Figure 4).

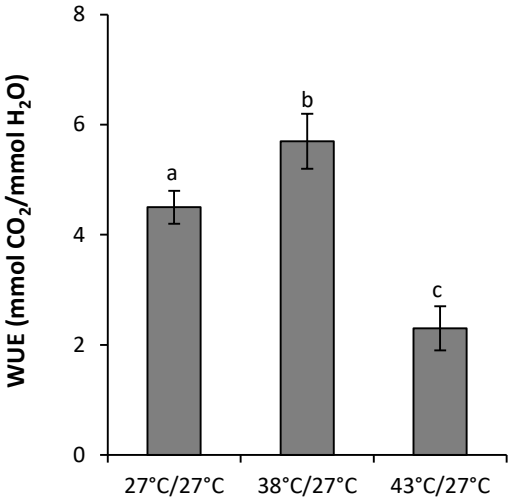

Figure 4. Water use efficiency in leaves *of B. purpurea* grown at different temperatures. For each variable, different letters indicate significant differences by Tukey's test at 5%.

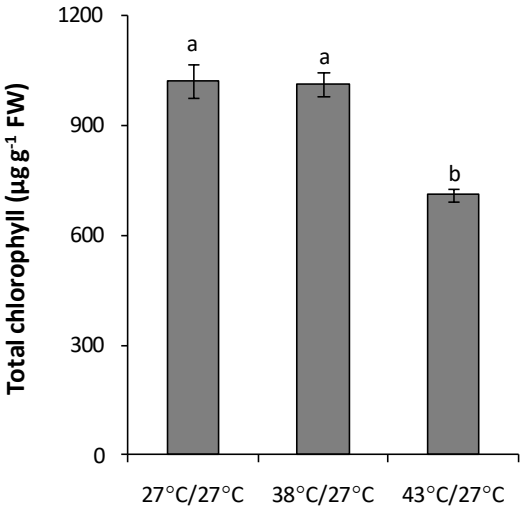

Figure 5. Total chlorophyll concentration in leaves *of B. purpurea* grown at different temperatures. For each variable, different letters indicate significant differences by Tukey's test at 5%.

The increase in g_s at high temperatures observed in *B. purpurea* seedlings subjected to 43ºC/25ºC regimes increases transpiratory flux and its cooling effect on leaves. However, increased water loss through transpiration may compromise plant water status (MATTHEWS & LAWSON, 2019). Because high temperatures generally coincide with water deficit, stomatal closure allows maintaining the water status of tissues at the expense of CO_2 demand by photosynthesis (MOORE et al., 2021).

In experiments with *Pinus taeda* and *Populus deltoides* seedlings, when water was not a limiting factor, a decoupling between photosynthesis and transpiration was observed at high temperatures. Thus, high transpiration rates were measured, with net photosynthesis equal to zero or with negative values. This decoupling allows leaf cooling, avoiding damage to cell metabolism (ROGERS et al., 2017). This response was also observed in a field trial with *Eucalyptus parramattensis* (DRAKE et al., 2018). This phenomenon also has an impact at the environmental level, since transpiration influences soil temperature, and in models predicting heat wave intensities (KALA et al., 2016).

In agreement with the observation of a non-stomatal inhibition of photosynthesis, the 43ºC/27ºC treatment reduced the concentration of total chlorophyll in leaves by 30% (Figure 5).

It has been reported that high temperatures can decrease total chlorophyll concentration, with a concomitant reduction in the size of the antenna complex and thus a reduced ability to absorb light energy (CHAUDHARY et al., 2020). Therefore, senescence delay has been used in selection programs for high-temperature tolerant *Zea mays* (SINGH et al., 2020), *Lens culinaris* (SITA et al., 2017) and *Solanum lycopersicum* (ZHOU et al., 2017) cultivars. High temperatures for short periods can inhibit chlorophyll synthesis; in longer periods, in addition to inhibiting its synthesis, its degradation is activated (ANTONIOU et al., 2017). In *Apium graveolens*, the inhibition of photosynthetic pigment synthesis occurs at the genetic level (HUANG et al., 2017). In *Hordeum vulgare*, the inhibition in chlorophyll synthesis by high temperatures is attributed to the inhibition of the activity of enzymes involved in the synthesis of pyrrole rings or protochlorophyllide (MATHUR et al., 2014).

Fluorescence emission of chlorophyll a is sensitive to abiotic stresses, including high temperatures. It provides information on photosynthetic capacity and acclimation of plants under stress (KALAJI et al., 2018). In agreement with the observation of non-stomatal inhibition of photosynthesis, all chlorophyll a modulated fluorescence variables were altered by heat stress (Figures 6 and 7). Temperatures of 38°C/27°C produced 20% and 17% reduction in electron transport rate and photochemical quenching, respectively, with respect to the control (Figure 6 A, B). In the 43°C/27°C treatment, the reduction of both variables was 40% and 44%, respectively.

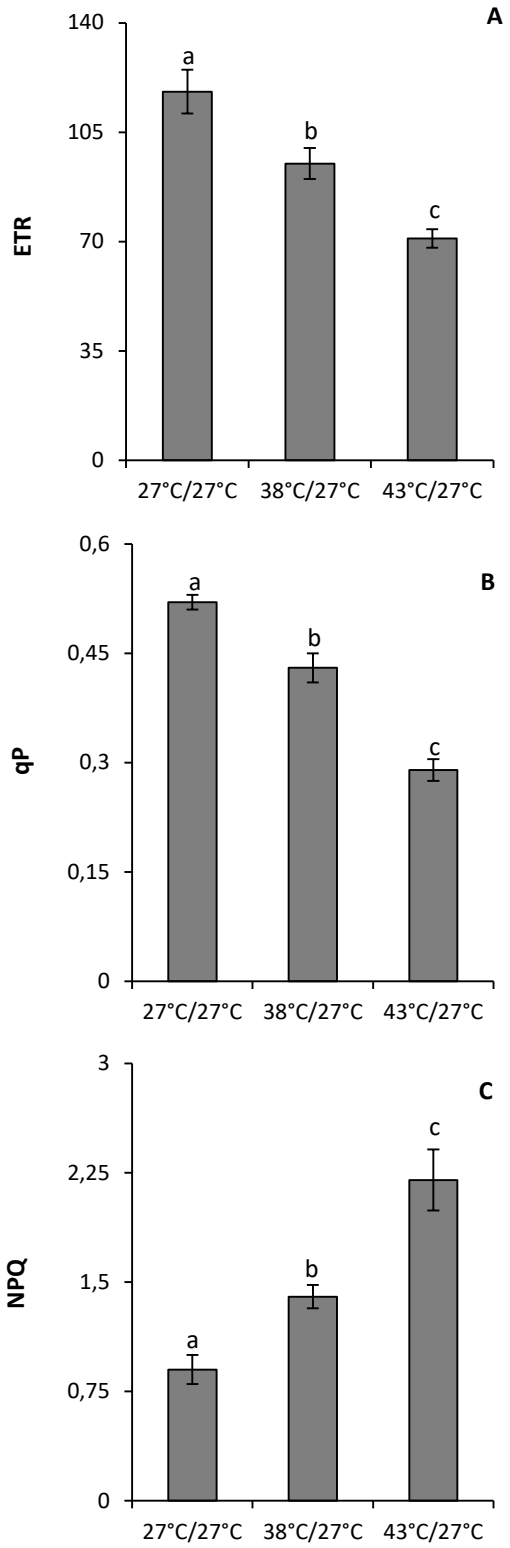

Figure 6. Electron transport rate (A) Photochemical quenching (B) and Non-photochemical quenching (C) in leaves of *B. purpurea* grown at different temperatures. For each variable, different letters indicate significant differences by Tukey's test at 5%.

In contrast, non-photochemical quenching increased by 55% and 144% in the 38°C/27°C and 43°C/27°C treatments relative to the control, respectively (Figure 6C). These results demonstrate that heat stress reduced photochemical conversion and electron transfer capacity in leaves of *B. purpurea* (WANG et al., 2018).

Temperatures of 38°C/27°C did not affect the F_v/F_M ratio, which remained around 0,72. In contrast, temperatures of 43°C/27°C significantly reduced the F_v/F_M ratio to values of approximately 0,49 (Figure 7).

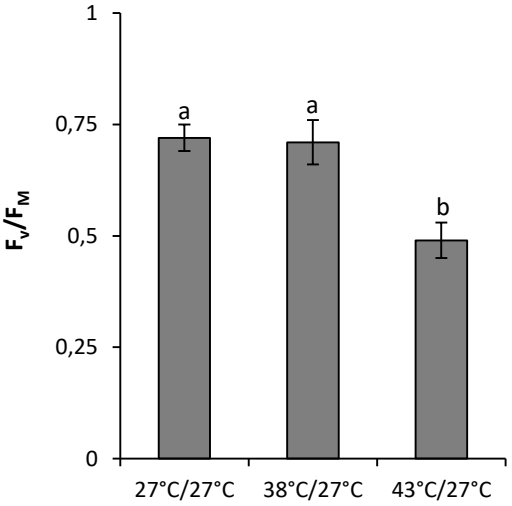

Figure 7. F_v/F_M ratio in leaves of *B. purpurea* grown at different temperatures. For each variable, different letters indicate significant differences by Tukey's test at 5%.

The F_v/F_M ratio represents the maximum quantum efficiency of photosystem II (PSII). In healthy plants, its value is approximately 0,8; a decrease in the F_v/F_M ratio indicates photoinhibition (WANG et al., 2018). Therefore, the non-stomatic inhibition of photosynthesis in the 43°C/27°C treatment could be partly due to photoinhibition. Although there are discrepancies about the molecular basis of photoinhibition, considerable evidence indicates that it involves the degradation of D_1 protein in PSII (RAJA et al., 2020). In agreement with these results, Sharma et al. (2014) exposed *Triticum aestivum* cultivars to 40°C for three days; the most tolerant cultivars had higher F_v/F_M values than the sensitive ones. A similar response was observed in *Oryza sativa* (SAILAJA et al., 2015), *Hordeum vulgare* (OUKARROUM, et al., 2016) and *Vigna radiata* (SHARMA et al., 2016)

Heat stress did not affect the activity of the enzymes superoxide dismutase (SOD) and catalase (CAT) (Table 1).

Table 1. Activities of superoxide dismutase (SOD, U mg^{-1} protein min^{-1}), catalase (CAT, µmol H_2O_2 mg^{-1} $protein^{-1}$ min^{-1}) and ascorbate peroxidase (APX, µmol ascorbate mg^{-1} protein min^{-1}), in leaves of *B. purpurea* grown at different temperatures.

Temperatures	SOD	CAT	APX
27ºC/27ºC	2,1±0,03 a	79,4±3,5 a	0,49±0,02 a
38ºC/27ºC	1,9±0,01 a	73,1±2,9 a	0,51±0,01 a
43ºC/27ºC	2,3±0,07 a	75,7±1,4 a	0,65±0,04 b

Means followed by the same letter in the column do not differ statistically at p<0.05 (Tukey's test).

Ascorbate peroxidase (APX) activity was not affected by the 38°C/27°C treatment, and increased by 33% in the 43°C/27°C regime (Table 1).

In agreement with the inefficient response of the antioxidant enzymes, heat stress significantly increased the concentrations of superoxide radical (Table 2). Oxidative stress generated by high temperatures produced an increase in malondialdehyde concentrations, as a result of lipid peroxidation (Table 2). In agreement with this observation, damage to cell membranes was also observed, as measured by the loss of solutes (Table 2).

Table 2. Superoxide radical concentration ($O_2^{\cdot-}$, nmol min^{-1} g^{-1} FW), malondialdehyde concentration (MDA, µmol g^{-1} FW) and membrane damage (MD, %), in leaves of *B. purpurea* grown at different temperatures.

Temperatures	$O_2^{\cdot-}$	MDA	MD
27ºC/27ºC	8,2±0,3 a	19,3±0,81 a	9,1±0,36 a
38ºC/27ºC	15,9±0,51 b	38,7±1,44 b	12,9±0,52 b
43ºC/27ºC	45,6±1,97 c	86,1±2,69 c	45,6±1,99 c

Means followed by the same letter in the column do not differ statistically at p<0.05 (Tukey's test).

The enzymes SOD, CAT and APX are involved in ROS detoxification. Their activity is usually higher in heat stress tolerant genotypes compared to sensitive ones (WANG et al., 2020). Thus, Zhou et al. (2019) reported that in *Solanum lycopersicum*, the cultivar Sufen 14 was more tolerant to heat stress (38°C /30°C) than the cultivar Jinlingmeiyu, presenting higher activity of the enzymes SOD, POD, APX and lower malondialdehyde concentrations. This approach also allowed the selection of *Brassica juncea* cultivars tolerant to 45°C. These cultivars had higher activity of the enzymes POD, CAT and GR and lower lipid peroxidation than moderately tolerant and sensitive ones, respectively (WILSON et al., 2014). In *C. sativus*, high temperatures increased superoxide radical concentration by 79,9%, producing oxidative stress (DING et al., 2016). These authors also observed that heat stress produced an abrupt drop in qP values, measured as the proportion of PSII reaction centers capable of photochemistry, and a decrease in CO_2 assimilation.

CONCLUSIONS

Heat stress produces a non-stomatic inhibition of CO_2 fixation by inhibiting the photochemical stage of photosynthesis. Regimes of 43°C /27°C also produce photoinhibition and compromise light absorption through a decrease in total chlorophyll concentration. The species has an inefficient antioxidant enzyme response, resulting in oxidative stress and damage to cell membranes. In future research, heat waves of different duration and intensity will be simulated.

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