UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BOTÂNICA

NICOLÁS DE PALMA

Metabolismo de braquicerina em *Psychotria brachyceras* Müll Arg. (Rubiaceae) frente a múltiplos estresses e prospecção de alcaloides correlatos em espécies congêneres mexicanas

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Tese apresentada como requisito parcial para obtenção do título de Doutor em Botânica na Universidade Federal do Rio Grande do Sul.

Orientador: Prof. Dr. Arthur Germano Fett-Neto.

Porto Alegre

2023

CIP - Catalogação na Publicação

De Palma, Nicolás
Metabolismo de braquicerina em Psychotria brachyceras Müll Arg. (Rubiaceae) frente a múltiplos estresses e prospecção de alcaloides correlatos em espécies congêneres mexicanas / Nicolás De Palma. -- 2023.
92 f.
Orientador: Arthur Germano Fett-Neto.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Programa de Pós-Graduação em Botânica, Porto Alegre, BR-RS, 2023.

1. alcaloides. 2. braquicerina. 3. estresse osmótico. 4. estresse térmico. 5. Psychotria. I. Fett-Neto, Arthur Germano, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
Programa de Pós-Graduação em Botânica - PPGBOT
Av. Bento Gonçalves, 9500 - Bairro Agronomia - CEP 91501970 - Porto Alegre - RS - www.ufrgs.br
Prédio 43433 s 214

ATA DE DEFESA DE TESE N°10/2023

Aluno: **Nicolás De Palma**, com ingresso em 01/04/2019
Título: "Metabolismo de braquicerina em *Psychotria brachyceras* Müll Arg. (Rubiaceae) frente a múltiplos estresses e prospecção de alcaloides correlatos em espécies congêneres mexicanas".
Orientador: Dr. Arthur Germano Fett-Neto

Data: 5 de outubro de 2023
Horário: 9h
Local: Anfiteatro da Botânica

Banca Examinadora:
Dr. Felipe Klein Ricachenevsky (UFRGS)
Dr. Giancarlo Pasquali (UFRGS)
Dra. Tatiana Schäffer Gregianini (SES/RS)

Avaliação Geral da Banca: (X) Aprovado ( ) Reprovado
Correções solicitadas: ( ) Sim (X) Não
Indicação de Voto de Louvor: ( ) Sim (X) Não

Observação: Esta Ata não pode ser considerada como instrumento final do processo de concessão de título ao aluno.

Porto Alegre, 5 de outubro de 2023.

Arthur Germano Fett-Neto

Felipe Klein Ricachenevsky

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Tatiana Schäffer Gregianini

“Mucha gente pequeña, en lugares pequeños, haciendo cosas pequeñas, puede cambiar el mundo”

~Eduardo Galeano~

AGRADECIMENTOS

A Deus, pela vida, por me guiar e me abençoar a cada dia.

Aos meus pais, Claudia e Oscar, e à minha irmã, Carolina, que nunca deixaram de me alentar para que alcance meus objetivos, e por estarem sempre presentes apesar da distância.

Aos meus avós que me guiam desde o céu, obrigado por cuidar de mim sempre.

À minha namorada, Aneliz, pelo amor e pelo apoio constante, e à sua família pelo carinho que recebo.

Ao Professor Dr. Arthur Germano Fett-Neto, pela orientação, paciência, profissionalidade e, acima de tudo, pela qualidade humana.

Aos Professores Drs. Janette Palma-Fett, Felipe dos Santos Maraschin, Tatiana Schäffer Gregianini, Felipe Klein Ricachenevsky e Giancarlo Pasquali, membros da banca de exame de qualificação e exame de defesa de Tese, pelas suas sugestões e contribuição ao meu trabalho.

Aos meus colegas do Laboratório de Fisiologia Vegetal da UFRGS pela convivência cotidiana, parceria, pelos momentos bons, os ruins, os de catarse coletiva, e pelas palavras de apoio constante diante das dificuldades.

Ao Professor Dr. Robert Winkler, responsável pelo Laboratório de Análise Bioquímica e Instrumental (LABI) do CINVESTAV, que foi meu coorientador no México, por me receber no seu laboratório e por seu profissionalismo. A seus auxiliares de pesquisa, M.Sc Hilda Ramos e Enrique Ramírez, pela disponibilidade para me ajudar a todo momento e pelo acolhimento.

Aos meus colegas do Laboratório de Análise Bioquímica e Instrumental (LABI) (México), pela parceria, pelas alegrias e os momentos compartilhados.

À Capes, pelo apoio financeiro.

À Organização dos Estados Americanos (OEA), pela bolsa concedida para a realização do Doutorado.

À Agência Mexicana de Cooperação Internacional para o Desenvolvimento (AMEXCID), pela bolsa concedida para realizar o Doutorado Sanduíche no Exterior.

Muito obrigado a todos vocês por serem parte deste sonho!

Esta Tese está dedicada aos meus pais, à minha irmã e aos meus avós.

SUMÁRIO

Capítulo 2

Artigo publicado na revista Journal of Plant Research.

Objetivo específico deste capítulo: avaliar o metabolismo de braquicerina e as mudanças bioquímicas em discos foliares de *Psychotria brachyceras* Müll Arg. sob condições de estresse individual, sequencial e combinado.

Journal of Plant Research
https://doi.org/10.1007/s10265-023-01441-z

REGULAR PAPER – PHYSIOLOGY/BIOCHEMISTRY/MOLECULAR AND CELLULAR BIOLOGY

Biochemical responses in leaf tissues of alkaloid producing *Psychotria brachyceras* under multiple stresses

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Received: 23 November 2022 / Accepted: 8 February 2023

Abstract

Under natural conditions plants are generally subjected to complex scenarios of combined or sequential environmental stresses. Among the various components of plant biochemistry modulated by abiotic variables, a pivotal role is played by antioxidant systems, including specialized metabolites and their interaction with central pathways. To help address this knowledge gap, a comparative analysis of metabolic changes in leaf tissues of the alkaloid accumulating plant *Psychotria brachyceras* Müll Arg. under individual, sequential, and combined stress conditions was carried out. Osmotic and heat stresses were evaluated. Protective systems (accumulation of the major antioxidant alkaloid brachycerine, proline, carotenoids, total soluble protein, and activity of the enzymes ascorbate peroxidase and superoxide dismutase) were measured in conjunction with stress indicators (total chlorophyll, ChA/ChB ratio, lipid peroxidation, H_2O_2 content and electrolyte leakage). Metabolic responses had a complex profile in sequential and combined stresses compared to single ones, being also modified over time. Different stress application schemes affected alkaloid accumulation in distinct ways, exhibiting similar profile to proline and carotenoids, constituting a complementary triad of antioxidants. These complementary non-enzymatic antioxidant systems appeared to be essential for mitigating stress damage and re-establishing cellular homeostasis. The data herein provides clues that may aid the development of a key component framework of stress responses and their appropriate balance to modulate tolerance and yield of target specialized metabolites.

Keywords Abiotic stress · Combined stress · Heat stress · Osmotic stress · *Psychotria* · Sequential stress

Abbreviations

MIA	Monoterpene indole alkaloids
TIA	Terpene indole alkaloids
ROS	Reactive oxygen species
MS	Culture medium (Murashige and Skoog culture medium)
PEG	Polyethylene glycol 6000
HPLC	High performance liquid chromatography
UV–Vis	Ultraviolet–visible
DW	Dry weight
ChA	Chlorophyll A
ChB	Chlorophyll B
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
FW	Fresh weight
TSP	Total soluble proteins
CPE	Crude protein extract
APX	Ascorbate peroxidase
SOD	Superoxide dismutase
NBT	Nitro blue tetrazolium
EL%	Relative electrolyte leakage

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Published online: 21 February 2023

Introduction

Psychotria L. is the largest genus of the Rubiaceae family showing wide pantropical distribution and including approximately 1,600 species (Berger et al. 2022), 142 of them endemic of Brazil (Flora do Brasil 2020 2022). Ethnopharmacological studies have shown that species of this genus are used in traditional medicine for their properties in the treatment of gastrointestinal and bronchial diseases and reproductive problems (Formagio et al. 2014). Probably the most popularly known species of this genus is *Psychotria viridis* Ruiz & Pav., one of the main components of the hallucinogenic drink Ayahuasca, consumed in rituals by indigenous Amazonian tribes.

Psychotria spp. are characterized by the production of different specialized metabolites, mainly monoterpene indole alkaloids (MIA) (Carvalho et al. 2016). From a physiological point of view, alkaloids play an important role in protecting against ultraviolet radiation, oxidative stress and in chemical defense against predators and pathogens (Matsuura and Fett-Neto 2015).

Brachycerine is the main MIA produced by *Psychotria brachyceras* Müll Arg. (Nascimento et al. 2007), an endemic species from southern Brazil (Flora do Brasil 2020 2022). Although not toxic to several herbivores and yeast (Magedans et al. 2019), this MIA has UV protection capacity (Gregianini et al. 2003), as well as antioxidant and antimutagenic activities (Nascimento et al. 2007). Previous studies have shown that the production of this alkaloid increases after exposure of leaves and leaf discs to different isolated biotic and abiotic stresses, a fact that highlights its protective function against oxidative damage caused by several factors, such as UV radiation, heat, osmotic and mechanical damage (Gregianini et al. 2003, 2004; Magedans et al. 2017; Nascimento et al. 2007, 2013; Porto et al. 2009). Brachycerine has significant antioxidant capacity, being able to quench reactive oxygen species (ROS) such as peroxide and superoxide. This is likely because of the presence of a glucose residue, OH group, double bonds, and a secondary amine (Gregianini et al. 2003). The potential of MIAs as non enzymatic antioxidants has also been reported in other species (Matsuura et al. 2014). Indeed, *P. brachyceras* has been shown to be highly tolerant to several stresses (Magedans et al. 2019).

Under natural conditions, plants often face adverse abiotic factors such as extreme temperatures, high irradiance, ultraviolet radiation, water deficiency or excess, nutrient deprivation or toxicity, and salinity among others (He et al. 2018). Although these stresses are widely studied in isolation, there is a great need to better understand the physiological mechanisms triggered when these unfavorable conditions occur in a sequential and combined manner. Knowledge about these stress responses may contribute both to the genetic improvement of crops of agronomic interest, and to elicit improved yields of bioactive metabolites.

Heat and drought are the two stresses that most affect crop growth and yield (Fahad et al. 2017). Drought stress causes reduced growth, slow cellular metabolism, stomatal closure, inhibition of photosynthesis, and destabilization of membranes and proteins (Fahad et al. 2017). Heat stress is known for increasing membrane fluidity and protein degradation, as well as photosynthetic and respiratory inhibition (Hemantaranjan et al. 2014). Although these stresses may trigger different morphological, physiological, and biochemical responses, the production of ROS is a shared feature (Fahad et al. 2017). Combined and sequential stresses add another level of complexity to metabolic adjustments needed to mitigate stress caused damage, which are manifested at multiple levels of regulation, such as transcriptome, proteome, and metabolome (Anwar et al. 2021).

Herein, physiological changes in *P. brachyceras* leaves under individual, sequential, and combined osmotic and heat stress conditions were investigated. A previously validated system using leaf disks was employed providing uniformity and reproducibility (Nascimento et al. 2013). Several stress-mitigating systems, including non enzymatic (particularly brachycerine) and enzymatic antioxidants were quantified along with stress damage parameters. Metabolic responses had a complex profile in sequential and combined stresses, compared to single ones. The complementary action of the antioxidant systems appeared to be essential for mitigating stress damage and reestablishing cellular homeostasis.

Materials and methods

Plant material and foliar disks preparation

Cuttings with healthy and fully expanded leaves from three different populations of *Psychotria brachyceras* Müll. Arg. were collected in the summer at Morro Santana (30°03′56.5″S 51°07′19.5″W), Porto Alegre (RS), Brazil. They were acclimated for one week in 10% (v/v) Murashige and Skoog culture medium (MS) (pH 5.8) (Murashige and Skoog 1962) in the presence of fluorescent light, at 25 °C ± 3 °C, under irradiance of 100 µmol photons m^{-2} s^{-1} and photoperiod of 16 h/8 h (day/night). A voucher specimen of the collected plant material was deposited in the ICN Herbarium of the Biosciences Institute of the Federal University of Rio Grande do Sul (UFRGS) (ICN 203679).

After acclimatization, the leaves were immersed in 1.5% (v/v) sodium hypochlorite for 15 min and washed three times with distilled water. From this material, leaf disks of approximately 1 cm in diameter were made. These leaf disks

were placed in Petri dishes (30 disks/dish of 9 cm diameter) containing filter paper soaked in the same autoclaved culture medium (20 mL per plate). Then, the plates were kept in a growth chamber for 5 days (Magedans et al. 2017).

Acclimation of leaf disks and exposure to abiotic stress conditions

Leaf disks were maintained in a grown chamber for 24 h under control conditions (25 °C ± 3 °C) under irradiance of 70 µmol of photons m^{-2} s^{-1} and photoperiod of 16 h/8 h (day/night) to acclimate. Lower irradiance was used for disk assays relative to the one applied to cuttings during the acclimation week to avoid possible photooxidative stress. During the following 4 days, isolated, sequential, and combined abiotic stresses were applied. Heat stress (40 °C) and osmotic stress (0.025 M Polyethylene glycol 6,000 (PEG), circa $\Psi_w = -0.36$ MPa) were imposed as follows (Table 1):

*Individual stresses: T° (4 days, 40 °C) (T). PEG (4 days, 25 °C) (PEG).

*Sequential stresses: T° (2 days, 40 °C), followed by PEG (2 days, 25 °C) (T-PEG). PEG (2 days, 25 °C), followed by T° (2 days, 40 °C) (PEG-T).

*Combined (simultaneous) stresses: T° and PEG (4 days, 40 °C) (T + PEG).

The control treatment was devoid of PEG and kept for 4 days at 25 °C (control).

For those conditions initially shared by different treatments (T and T-PEG, PEG, and PEG-T) the same batch of samples were used for the various analyses.

Biochemical parameters for measurement were selected based on expected relative importance and representativeness among the several possible indicators of the two main classes investigated in the assays: stress damage and stress protective systems. In addition, after preliminary experiments, parameters that showed adequate reproducibility and measurement reliability were selected also considering the availability of leaf disk biomass for each treatment. All physiological parameters detailed below were evaluated on the second and fourth day after exposure to stress conditions.

Table 1 Osmotic stress (PEG 0.025 M, $\Psi = -0.38$ MPa) and heat stress (T) (40 °C) applied during the experiment

Treatment	0–2 days	2–4 days	Type of stress
Control	25 °C	25 °C	None
T	40 °C	40 °C	Individual stress
PEG	PEG (25 °C)	PEG (25 °C)	Individual stress
T-PEG	40 °C	PEG (25 °C)	Sequential stress
PEG-T	PEG (25 °C)	40 °C	Sequential stress
T + PEG	PEG (40 °C)	PEG (40 °C)	Combined stress

In sequential stresses, the shifts of treatments were done on the second day

Brachycerine analysis

The concentration of the alkaloid brachycerine was analyzed by high performance liquid chromatography (HPLC) according to Magedans et al. (2017) with minor modifications. Leaf disks samples (150 mg) were ground in liquid nitrogen, mixed with 1 mL of methanol (HPLC grade), and then ultrasonicated for 30 min at 25 °C. After centrifuging the extract at 16,000×*g* at 4 °C for 20 min, the supernatant was recovered for further analysis in a Shimadzu HPLC chromatograph with UV–Vis photodiode array detector. Stationary phase was a C8 reversed-phase HPLC column Shimpack CLC-C8 (M) (150 × 4.6 mm) and corresponding guard column. Linear gradient separation used a mobile phase of methanol/water/trifluoroacetic acid in 35 min, at a flow rate of 1 mL min^{-1}, starting with water: methanol (81:19), and ending with 100% methanol. Both eluents contained trifluoroacetic acid (Sigma) at 0.05% (v/v). Monitoring wavelength was 280 nm. Brachycerine content (dry weight basis, DW) was calculated based on an external calibration curve prepared with authentic brachycerine.

Pigments analysis

Chlorophyll A (ChA), Chlorophyll B (ChB) and carotenoids concentrations were analysed according to Schlindwein et al. (2006), with minor modifications. Leaf disks (50 mg) were ground in liquid nitrogen and homogenized with 1 mL of cold acetone/0.1 M tris buffer (80/20, v/v) (pH 7.8). These extracts were ultrasonicated in ice water for 15 min and then placed in the dark at 4 °C for 24 h. After that they were removed from the refrigerator and again ultrasonicated for 15 min. The extracts were centrifuged for 5 min at 10,000×*g* and the supernatant was recovered and diluted with the same extraction solution (1/3 ratio). Absorbances readings were performed in a SpectraMax M2 UV–Vis spectrophotometer at 470, 537, 647 and 663 nm. Total chlorophyll content, ChA/ChB ratio and carotenoid content were estimated according to the equations proposed by Sims and Gamon (2022).

Proline quantification

Proline content was determined according to Lee et al. (2018) with minor modifications. Leaf disks (120 mg) were ground in liquid nitrogen and placed in 400 µL of 3% (w/v) sulfosalicylic acid. The extract was then vortexed and centrifuged at 16,000×*g* for 10 min. Sixty-six µL of the supernatant were mixed with 132 µL of 1.25% (w/v) ninhydrin in 80% (v/v) glacial acetic acid, and incubated in the oven

at 100 °C for 60 min. The reaction was stopped by transferring the mixture to ice for 10 min. Absorbance was read in spectrophotometer at 510 nm. Proline content was calculated from a standard curve and expressed on a DW basis.

Lipid peroxidation analysis

Lipid peroxidation was estimated as previously described by Velikova et al. (2000). Leaf disks (100 mg) were ground in liquid nitrogen and homogenized with 667 µL of 0.1% (v/v) trichloroacetic acid (TCA). The extract was then vortexed and centrifuged at 16,000×*g* at 4 °C for 20 min. Next, 200 µL of the supernatant were recovered and mixed with 400 µL of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (v/v) TCA solution. The reaction was incubated in a hot water bath at 100 °C for 30 min and then stopped by transfer to ice bath. Absorbances readings were performed at 532 and 600 nm. Lipid peroxidation was estimated from the MDA content, which was calculated and expressed on a fresh weight (FW) basis.

Hydrogen peroxide (H_2O_2) analysis

Hydrogen peroxide content was estimated in the extract prepared for lipid peroxidation analysis. For this, 100 µL of 0.1% (v/v) TCA extract were mixed with 100 µL of 10 mM potassium phosphate buffer and 400 µL 1 M KI. The mixture was incubated in darkness for 1 h. Absorbance was read at 390 nm, and H_2O_2 content was calculated and expressed on a FW basis (Alexieva et al. 2001).

Quantification of total soluble proteins (TSP)

For the quantification of total soluble proteins, a protein extract was obtained as follows: 120 mg of leaf disc powder were placed in 1.5 mL of extraction buffer [1% (w/v) polyvinylpyrrolidone, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride and 50 mM HEPES buffer (pH 7.4)]. Then, this extract was centrifuged at 16,000×*g* at 4 °C for 20 min. The supernatant was recovered, thus constituting the protein extract.

TSP quantification was done using 20 µL of protein extract mixed with 1 mL 0.01% (w/v) Coomassie Brilliant Blue as previously described (Bradford 1976). TSP content was calculated using a BSA standard curve and expressed on the basis of DW.

Ascorbate peroxidase (APX) activity

Ascorbate peroxidase (EC 1.11.1.11) activity was estimated according to Klapheck et al. (1990). Twenty µL of protein extract used in the TSP quantification were mixed with 2 mL 50 mM phosphate buffer (pH 7) containing 0.5 nM ascorbic acid and 1 mM H_2O_2. Reaction absorbances were measured at 290 nm every 15 s for 15 min. APX activity used an extinction coefficient of 39.4 mM cm^{-1}, and expressed in µmol min^{-1} mg $protein^{-1}$.

Superoxide dismutase (SOD) activity

Superoxide dismutase (EC 1.15.1.1) activity was estimated as previously described by Beyer and Fridovich (1987) with minor modifications. Fifteen µL of protein extract used in the TSP quantification were mixed with 1 mL 50 mM phosphate buffer (pH 7.8) containing 57 µM nitro blue tetrazolium (NBT), 9.9 mM L-Methionine, 0.025% (w/v) Triton® X-100 and 2 mM riboflavin. Then, the reaction mixture was exposed to fluorescent light for 15 min. Absorbance was determined at 560 nm, and SOD activity was calculated and expressed as U_{SOD} µg $protein^{-1}$. One unit of SOD was the activity needed to inhibit the photoreduction of NBT by 50%.

Relative electrolyte leakage (EL%)

This assay was performed based on the protocol established by Guimarães (2015) with some modifications. After washing with ultrapure water, 5 leaf discs were transferred to plastic tubes containing 20 mL of ultrapure water. The tubes were kept under constant agitation for 2 h (20 rpm) at 25 °C. Then initial electrical conductivity (Ci) was measured with a portable conductivity meter (AZ model 8306, Taiwan). Next these tubes were stored for 24 h at − 20 °C. After 24 h of storage, the samples rested at room temperature until thawing. Then the tubes were shaken for 25 min to finally measure the final electrical conductivity (Cf). EL% was calculated according to the equation $(Ci/Cf) \times 100$.

Statistical analysis

Experiments were carried out in a totally randomized design and independently repeated twice whenever possible. Treatments of all experiments were performed at least in biological quintuplicates. Statistical analysis was performed using the GraphPad Prism® software, version 5.0. Normality tests and one-way Analysis of Variance (ANOVA) were applied followed by Tukey's test when appropriate. Statistical evaluation of changes in physiological parameters from the second to the fourth day was carried out using Mann Whitney test. Correlation analyzes were performed using Pearson's correlation index, applying the R Studio® software, version 4.0.3. Statistical significance level was set at $p \leq 0.05$. Dispersion statistics parameter was standard error of the means.

Results

Accumulation of brachycerine

Leaf disks exposed to T showed the highest accumulation of brachycerine, increasing alkaloid concentration compared to control by approximately 5.5 and 4.5 times after 2 and 4 days of exposure, respectively (Fig. 1), indicating attenuation of induction intensity over time. On the second day, PEG did not exhibit significant differences in relation to the control. In turn, T + PEG showed intermediate values between T and PEG, suggesting an antagonistic effect between the stresses in the early sampling time point.

On the fourth day of exposure to stress, disks on all treatments, including PEG only, showed an increase in brachycerine accumulation (Fig. 1) compared to those in control conditions. T continued with the highest accumulation of brachycerine, along with T + PEG.

Not only individual stresses, but also sequential ones (T-PEG and PEG-T) were statistically different. PEG-T had higher alkaloid yield compared to T-PEG, indicating that the order of applied stress affected brachycerine biosynthesis. In turn, T-PEG and PEG were equivalent in alkaloid accumulation (Fig. 1).

From the second to the fourth day of exposure, the sequential stresses had different profiles. Whereas the brachycerine level of T and T-PEG decreased, that of PEG-T increased (Fig. S1). The concentrations of alkaloid in the remaining treatments were relatively stable.

Pigment analysis

Total chlorophyll

Only T exhibited significant difference in total chloropyll compared to other treatments on the second day of stress, showing the highest levels (Fig. 2a).

After four days of stress, the same profile was observed. The treatment T-PEG had lower levels of total chlorophyll compared to PEG, T, and T + PEG (Fig. 2a). Over time, total chlorophyll concentration was relatively stable in the various treatments. From the second to the fourth day of exposure, all treatments showed a decrease in total chlorophyll content, except T (Fig. S2).

ChA/ChB ratio

Both at 2 and 4 days, an overall pattern of increased ratio of ChA/ChB in sequential and combined stress treatments can be observed (Fig. 2b).

On the second day of exposure, only PEG treated disks had significantly higher ratio compared to their control counterparts (Fig. 2b), whereas on the fourth day of treatment PEG-T and T + PEG surpassed control disks in this parameter (Fig. 2b). Over time, all treatments had an increase in the ChA/ChB ratio, except T (Fig. S3).

Carotenoid content

The highest carotenoid content on the second day of stress was observed in T and in T + PEG, which were statistically

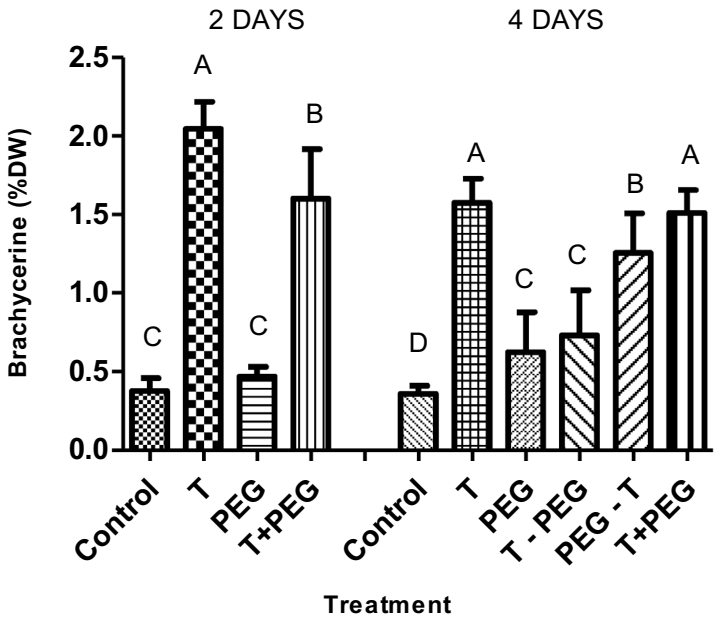

Fig. 1 Brachycerine accumulation in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). Bars not sharing a letter are significantly different ($p \leq 0.05$). Lines on top of bars are standard errors

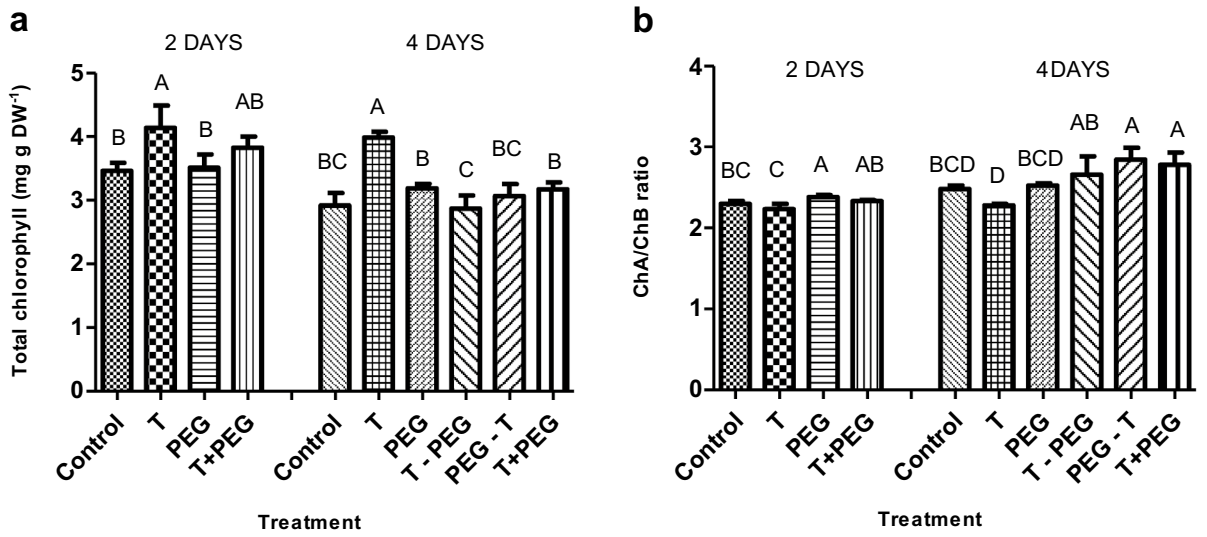

Fig. 2 Total chlorophyll concentration (**a**) and ChA/ChB ratio (**b**) in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). Bars not sharing a letter are significantly different ($p \leq 0.05$). Lines on top of bars are standard errors

equivalent. PEG and control disks yielded the lowest values (Fig. 3a).

On the fourth day of stress, disks of all treatments except those in PEG showed significantly higher carotenoid levels compared to control (Fig. 3a). T disks had the highest carotenoid content. PEG-T disks showed higher values than T-PEG ones, showing that the order of stress affects differently the content of this pigment. Disks in T + PEG exhibited intermediate values between those in T and PEG, suggesting an antagonistic effect between the stresses on carotenoid concentration (Fig. 3a).

With respect to changes over time, T treated disks had an increase in carotenoid concentration (Fig. S4). The application of heat in the sequential treatment PEG-T also yielded a trend for increase in carotenoids, whereas T-PEG showed an opposite response (Fig. S4).

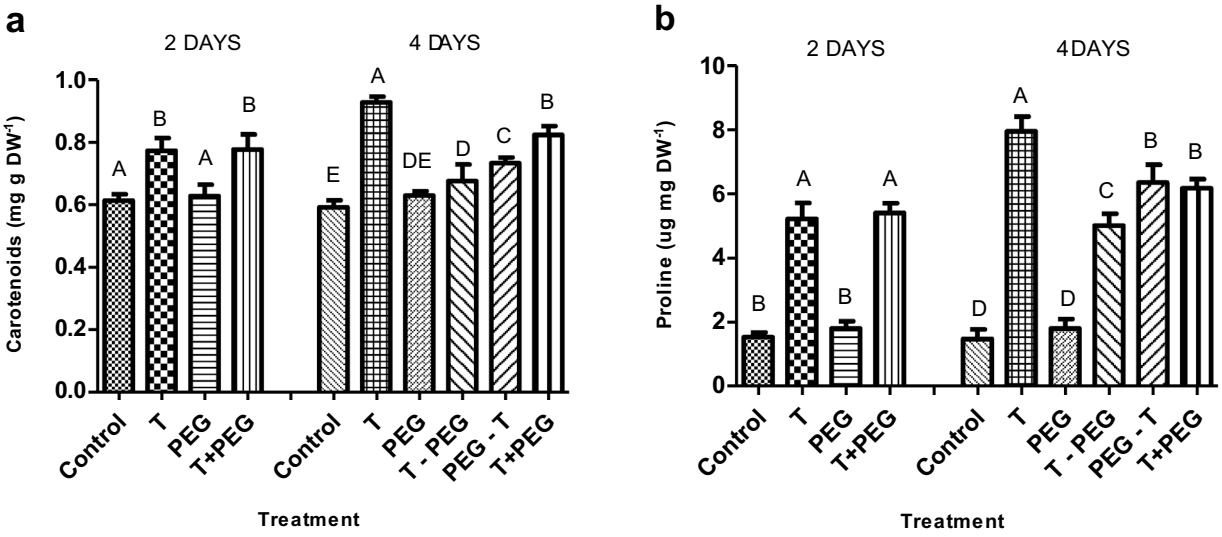

Fig. 3 Carotenoid concentrations (**a**) and Proline levels (**b**) in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). Bars not sharing a letter are significantly different ($p \leq 0.05$). Lines on top of bars are standard errors

Proline content

Proline concentration in disks of the different treatments and their time wise changes were very similar to those observed in brachycerine levels (Figs. 1 and 3b). On the second day of exposure, the highest accumulations of the osmolyte were seen in heat-treated disks, either alone or in simultaneous combination with PEG (T + PEG) (Fig. 3b).

On the fourth day of stress, T disks showed the highest accumulation of proline (Fig. 3b). Disks in PEG-T exhibited a higher value in relation to those in T-PEG, showing that the order of applied stress affected proline accumulation (Fig. 3b). T + PEG disks exhibited intermediate values between the corresponding ones in T and PEG, once again reinforcing an antagonistic effect between the stresses (Fig. 3b). After four days of stress, disks of all treatments showed significant differences when compared to the control, except those in PEG which exhibited the lowest proline content (Fig. 3b). From the second to the fourth day of exposure, the proline level of T, PEG-T and T + PEG disks increased (Fig. S5). The concentrations of proline in the remaining treatments were relatively stable.

Lipid peroxidation

On the second day of exposure to stress, T exposed disks showed lower MDA content when compared to the control (Fig. 4a). As recorded for several of the parameters analyzed, an antagonistic effect between treatments was apparent, MDA levels in T + PEG treated disks were intermediate between those of T and PEG conditions (Fig. 4a).

On the fourth day, all treatments showed significant differences in leaf disk MDA content in relation to the control, except for PEG (Fig. 4a). T + PEG and T disks had the lowest MDA content. Individual stresses (T and PEG) caused differences in disk MDA content, the former showing lower levels. In contrast, disks exposed to sequential stresses (T-PEG and PEG-T) had equivalent MDA content (Fig. 4a). With respect to changes over time, disks of all treatments had a decrease in MDA content, except the ones under T-PEG (Fig. S6).

H_2O_2 content

After two days of stress, no significant differences were observed among treatments in leaf disk H_2O_2 concentrations (Fig. 4b).

After four days of stress, all treatments showed significant differences in relation to disk H_2O_2 content when compared to the control, except for T (Fig. 4b). Neither disks exposed to the individual stresses (T and PEG) nor those submitted the sequential stresses (T-PEG and PEG-T) showed differences among them. T + PEG treated samples had higher peroxide levels than T and control ones (Fig. 4b). With respect to time changes, only the control disk group had a decrease in H_2O_2 content (Fig. S7).

Total soluble protein (TSP) content

On the second day of exposure to stress, there were no significant differences in TSP content in disks of the treatments compared to control (Fig. 5). However, T and PEG

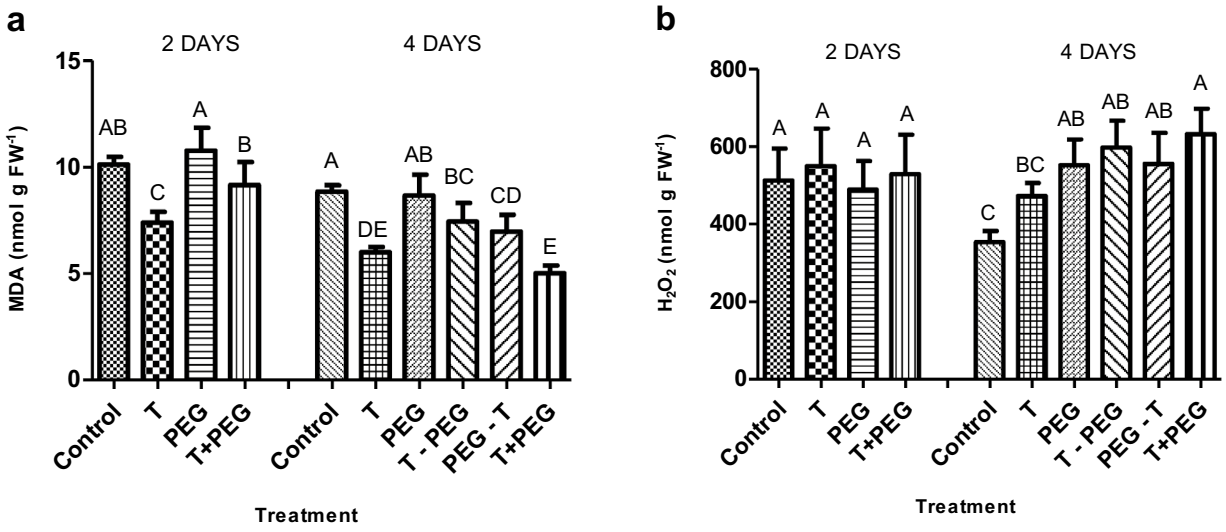

Fig. 4 MDA levels (**a**) and H_2O_2 concentration (**b**) in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). Bars not sharing a letter are significantly different ($p \leq 0.05$). Lines on top of bars are standard errors

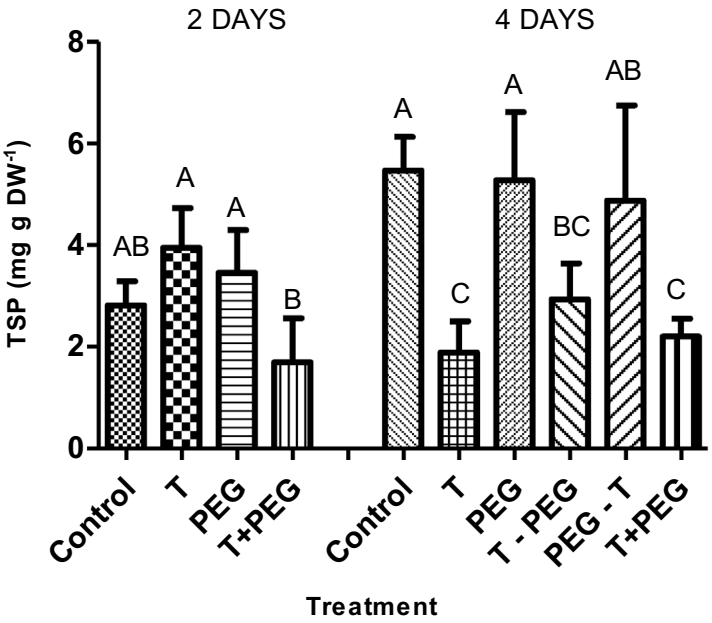

Fig. 5 Total soluble proteins (TSP) concentration in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). Bars not sharing a letter are significantly different ($p \leq 0.05$). Lines on top of bars are standard errors

samples showed higher values when compared to T + PEG ones (Fig. 5).

On the fourth day of stress, T, PEG-T and T + PEG incubated disks had significantly lower amounts of TSP compared to control ones (Fig. 5) albeit not differing among themselves. TSP were present in lower amounts in disks exposed to T compared to those in PEG, whereas sequential stresses had equivalent levels (Fig. 5). From the second to the fourth day, disks in the control group had an increase in TSP content, while those in T had a decrease (Fig. S8).

Ascorbate peroxidase (APX) enzyme activity

After two days of stress, T treated disks showed lower APX activity when compared to the control (Fig. 6a). The same disks also had less APX activity compared to PEG (Fig. 6a).

After four days of stress, disks of all treatments except those exposed to PEG showed significant differences in relation to APX activity when compared to control disks (Fig. 6a). Samples in T and T-PEG showed a higher activity of APX, followed by those in PEG and control. PEG-T

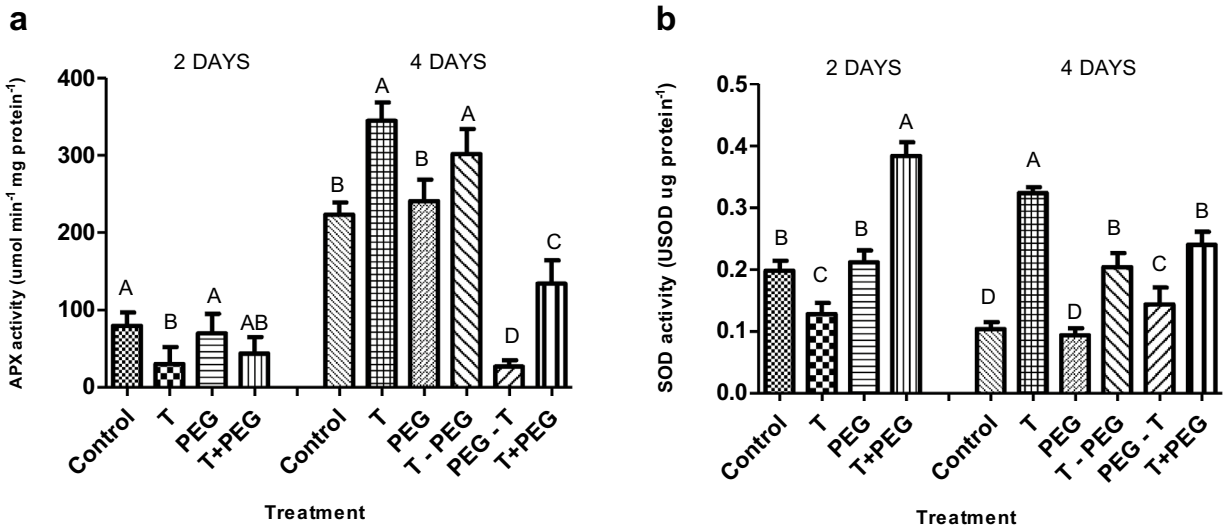

Fig. 6 Ascorbate peroxidase (APX) activity (**a**) and Superoxide dismutase (SOD) activity (**b**) in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). Bars not sharing a letter are significantly different ($p \leq 0.05$). Lines on top of bars are standard errors

disks exhibited the lowest activity value among treatments, followed by T + PEG. PEG-T treated disks exhibited significantly lower enzyme activity in relation to T-PEG, showing the importance of the order of stress application in APX activity (Fig. 6a). T + PEG samples had lower activity values when compared to individual stresses (Fig. 6a). Over time, samples in all treatments increased APX activity, except those of PEG-T (Fig. S9).

Superoxide dismutase (SOD) enzyme activity

On the second day of exposure to stress, disks under T + PEG had the highest SOD activity of all treatments, suggesting a synergic effect between T and PEG (Fig. 6b).

On the fourth day of stress, disks exposed to all the treatments showed significant increases in SOD activity when compared to the control, except for PEG, which exhibited the lowest SOD enzyme activity (Fig. 6b). T exposed disks had the highest SOD activity in relation to the other treatments, followed by those submitted to T + PEG. Disks from treatments of individual stresses (T and PEG) were statistically different, the former ones depicting much higher SOD activity than the latter (Fig. 6b). Sequential stresses also affected SOD activity in disks in a different fashion, providing evidence of the relevance of stress sequence on enzyme activity. T-PEG samples showed a higher value in relation to PEG-T. SOD activity in T + PEG treated disks exhibited intermediate values between those exposed to T and PEG, indicating an antagonistic effect between the stresses (Fig. 6b). From the second to the fourth day, SOD activity increased in T and T-PEG samples, whereas the rest of the treatments had a significant decrease in the same parameter (Fig. S10).

Relative electrolyte leakage (EL%)

After two days of stress, no significant differences were observed between treatments in terms of EL% (Fig. 7).

At four days of stress, T, T-PEG, and T + PEG exposed disks had significantly higher EL% compared to the control (Fig. 7). Samples submitted to individual stresses (T and PEG) had different EL%, being higher in those under T. On the other hand, sequential stresses (T-PEG and PEG-T) caused equivalent EL% in leaf disks (Fig. 7). From 2 to 4 days of exposure, T, T-PEG and T + PEG treated disks showed a significant increase in EL% (Fig. S11).

In order to provide an integrated overview of the data sets, evaluated parameters were also presented as spider plots, as follows: 1. protective systems against stress (brachycerine, carotenoids, proline, TSP, SOD and APX) on the second (Fig. 8a) and fourth (Fig. 8c) day of stress; 2. stress indicator parameters (total chlorophyll, ChA/ChB ratio, lipid peroxidation, H_2O_2 and EL%) on the second (Fig. 8b) and fourth day (Fig. 8d) of stress. To facilitate data trend visualization, APX values were divided by one hundred, and H_2O_2 values were divided by one thousand. In addition, correlation analyzes were also performed between the physiological parameters studied (Fig. 9).

On the second day, disks under T and T-PEG were submitted to the same conditions since osmotic stress had not yet been applied (see Table 1). Likewise, on the second day, samples under PEG and PEG-T were subjected to the same conditions because heat stress had not yet been applied (see Table 1). For this reason, on the second day of stress, T parameter values were the same in T-PEG,

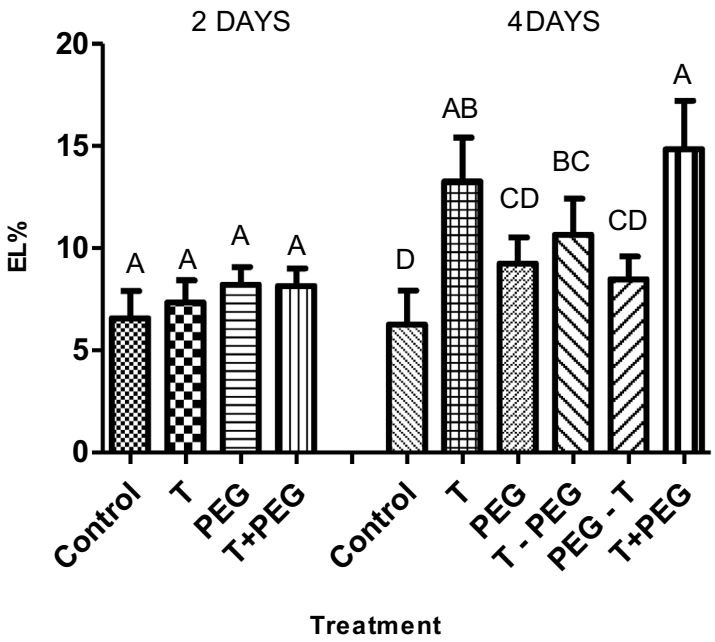

Fig. 7 Relative electrolyte leakage (EL%) in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). Bars not sharing a letter are significantly different ($p \leq 0.05$). Lines on top of bars are standard errors

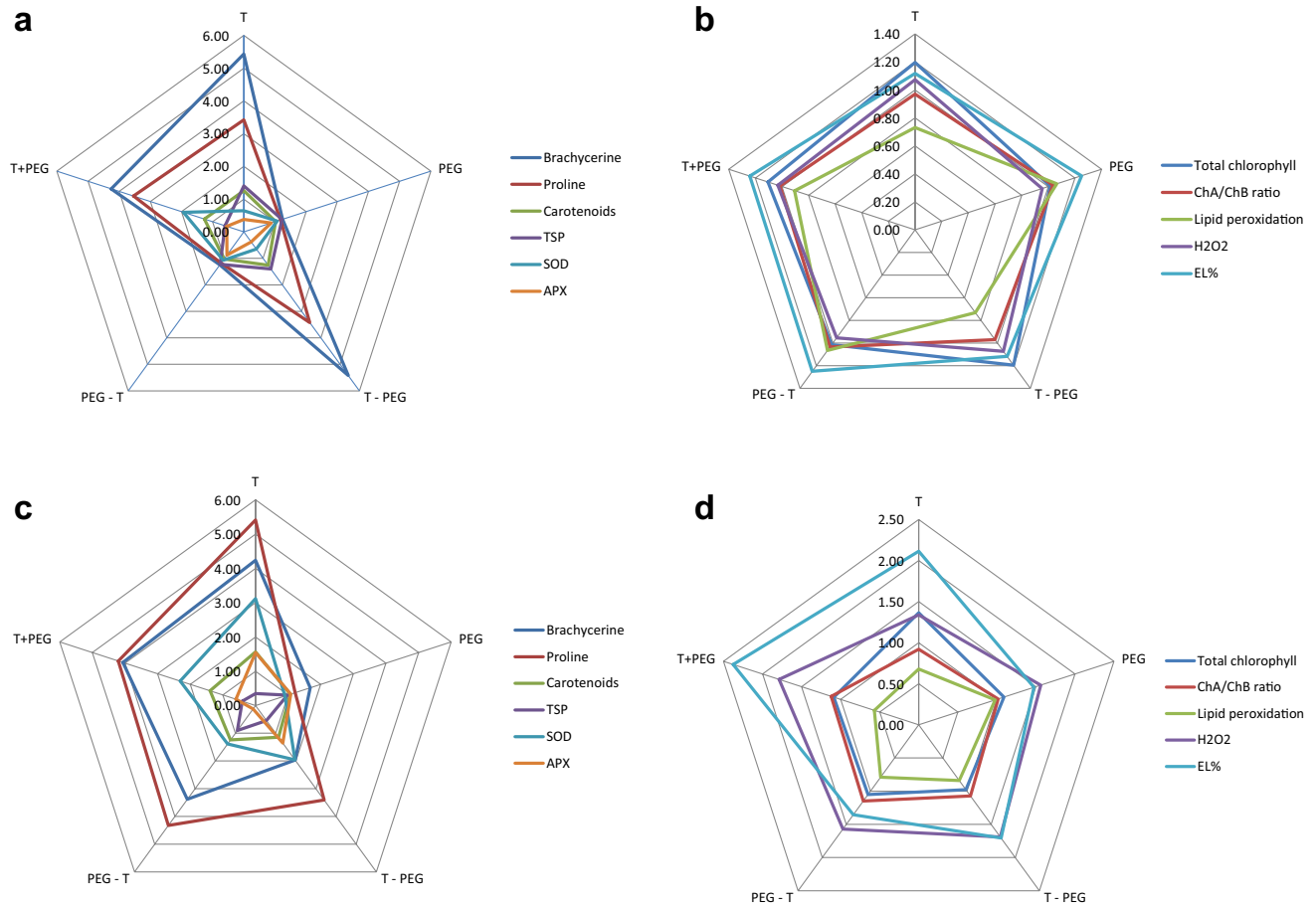

Fig. 8 **a** Protective systems against stress in *P. brachyceras* leaf disks on the second day of treatment—heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). On the first two days of stress, T-PEG was submitted to the same conditions of T and PEG-T was submitted to the same conditions of PEG (see Table 1). In this way, the parameter values were extrapolated, so the graphs acquired a certain symmetry. All values were normalized in relation to the control group (value = 1). **b** Stress indicator parameters in *P. brachyceras* leaf disks on the second day of treatment—heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). On the first two days of stress, T-PEG was submitted to the same conditions of T and PEG-T was submitted to the same conditions of PEG (see Table 1). In this way, the parameter values were extrapolated, so the graphs acquired a certain symmetry. All values were normalized in relation to the control group (value = 1). **c** Protective systems against stress in *P. brachyceras* leaf disks on the fourth day of treatment—heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). All values were normalized in relation to the control group (value = 1). **d** Stress indicator parameters in *P. brachyceras* leaf disks on the fourth day of treatment—heat stress (T) (40 °C), osmotic stress (PEG), T and PEG simultaneously (T + PEG) or sequentially (T-PEG or PEG-T). All values were normalized in relation to the control group (value = 1)

and PEG parameter values were also used for PEG-T. This caused the spider charts to show a certain symmetry.

The overall analysis of the spider plots shows complex profiles, which, as expected, changed with treatment and time of stress exposure (Fig. 8). Nonetheless, there is consistency among groups of stress indicator parameters and defense response factors.

At 2 days, TSP is prominent in disks submitted to individual (T and PEG) and sequential treatments (T-PEG and PEG-T) compared to those under simultaneous stress conditions, which had higher SOD activity (Fig. 8a). On the second day of stress, TSP and SOD activity in disks were inversely correlated (Fig. 9a). Samples exposed to temperature stress throughout the incubation time (T and T + PEG) or during the first 48 h (T-PEG) showed higher accumulation of PRO, brachycerine and carotenoids (Fig. 8a). As expected, these three non-enzymatic stress defense systems showed positive correlation (Fig. 9a). At the same time, T and T-PEG disks showed lower lipid peroxidation and higher total chlorophyll concentration compared to control ones (Figs. 8b and 9a). In contrast, disks under PEG alone in the first 2 days (PEG and PEG-T) yielded higher lipid peroxidation and lower total chlorophyll relative to their control counterparts (Fig. 8b).

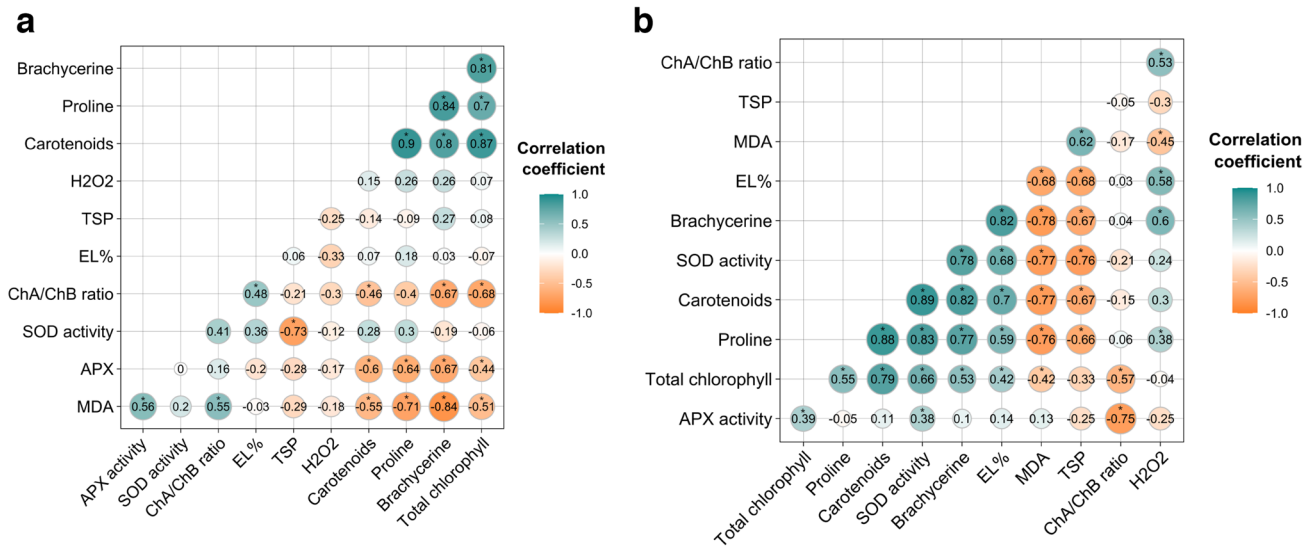

Fig. 9 **a** Correlations between the physiological parameters evaluated in *P. brachyceras* leaf disks on the second day of stress. TSP = Total Soluble Protein, EL% = Electrolyte Leakage, Ch = Chlorophyll, SOD = Superoxide Dismutase, APX = Ascorbate Peroxidase, MDA = Malondialdehyde. Asterisks indicate significant correlation ($p \leq 0.05$). **b** Correlations between the physiological parameters evaluated in *P. brachyceras* leaf disks on the fourth day of stress. TSP = Total Soluble Protein, EL% = Electrolyte Leakage, Ch = Chlorophyll, SOD = Superoxide Dismutase, APX = Ascorbate Peroxidase, MDA = Malondialdehyde. Asterisks indicate significant correlation ($p \leq 0.05$)

As stress progressed to 4 days, profiles changed (Fig. 8c, d). The content of TSP became similar among control, PEG and PEG-T disks, while reducing in other treatments (with T stress present in the first 2 days, i.e., T-PEG, or T stress during the four days, i.e., T and T + PEG). TSP and SOD activity continued to exhibit inverse correlation as in the second day of stress (Fig. 9b). The disk levels of PRO, brachycerine, and carotenoids remained high in the same treatments, as was the case at 2 days of stress, a behavior that was shared by PEG and PEG-T samples at 4 days (Fig. 8c). At this time PRO, brachycerine, and carotenoids still showed a positive correlation (Fig. 9b). Increased APX activity was also recorded in disks submitted to T and T-PEG. Leaf disks under T, T-PEG, PEG-T and T + PEG had lower lipid peroxidation compared to those in control condition. T incubated disks also had higher levels of total chlorophyll than in all other treatments (Fig. 8d).

Discussion

Protective systems against stress

Among the various individual, sequential, and combined stress treatments applied to leaf disks, heat alone was most stimulating to brachycerine accumulation, increasing alkaloid concentration compared to control by approximately 5.5 and 4.5 times after 2 and 4 days of exposure, respectively. In agreement, previous work recorded 4.5-fold increase in brachycerine compared to control after 3 days at 40 °C (Magedans et al. 2017). The fold induction decrease of brachycerine accumulation upon heat stress over time is probably due to the activation of other antioxidant systems. Brachycerine has been shown to mitigate several ROS, such as hydroxyl, peroxide, singlet oxygen and superoxide (Gregianini et al. 2003; Nascimento et al. 2013) and can therefore constitute a non-enzymatic antioxidant agent.

The leaf disk accumulation of brachycerine during individual osmotic stress by PEG was only higher on the fourth day when compared to the control, with no difference recorded on the second day. Working with twice the concentration of PEG used in the present experiments (0.05 rather than 0.025 M), Nascimento et al. (2013) observed significant increase in brachycerine in leaf disks only on the second, but not on the fourth day of exposure. This difference in response kinetics may be related to the early and more intense stress effect of the higher concentration of PEG on the tissues. Similar concentration-dependent time of brachycerine accumulation response were recorded for NaCl and sorbitol exposure (Nascimento et al. 2013).

After two days of stress, T + PEG had an antagonistic effect on leaf disk brachycerin accumulation in relation to individual stresses (T and PEG). The combination of drought and high temperature stress often results in a potential negative interaction, triggering shared and unique responses due to changes in multiple pathways and the crosstalk between different sensors and signal transduction pathways (Mittler 2006). On the fourth day, brachycerine levels in T + PEG

samples reached the values comparable to those in T. The higher accumulation of brachycerine on the fourth day of stress observed in PEG-T disks compared to those in T-PEG showed that the order of application of stresses affects differently the content of alkaloid. This is probably due to differences between the impact intensity of the stresses at the cellular level, as well as to the specific capacity of leaf discs to adapt to the subsequent (second) stress examined.

There are multiple events that take place in plant cells under stress conditions. These include perception by receptors, multilevel transduction pathway networks, responses and, ultimately, adaptation. The overt complexity of these various steps is easily realized by the layers of molecular players involved. This is illustrated by the dissection of heat stress responses. Heat is at least partly perceived in membranes due to changes in their fluidity, as well as by the generation of ROS. Calcium, calmodulin, and different phytohormones (nitric oxide-NO, methyl jasmonate-MeJA, salicylic acid–SA, ethylene-ET, and abscisic acid–ABA) may participate in signaling. Moreover, changes in RNA and protein folding and stability, substance transport, and triggering of antioxidant defenses also occur (Huang et al. 2022). Crosstalk among the different components of this chain of events under various stresses adds to complexity and provides a means of response specificity.

Leaf disk brachycerine level increases were generally accompanied by higher proline and carotenoid concentrations at two and four days of exposure, suggesting complementary activities of these non-enzymatic antioxidants in mitigating oxidative imbalance. Under stress conditions, the osmolyte proline is involved in a wide variety of functions such as stabilizing membranes, subcellular structures, and proteins, thereby generating cellular protection by scavenging reactive oxygen species (ROS). Proline accumulation may be a product of de novo synthesis or protein hydrolysis (Kaur and Asthir 2015). In fact, at four days of stress, the relationship between TSP and proline suggests a possible contribution of protein hydrolysis as a source of the amino acid (Figs. 8c and 9b). Previous studies have shown that exogenous application of proline in plants under stress decreased lipid peroxidation (relationship also verified in this work with the endogenous osmolyte; Fig. 9a, b), H_2O_2 and $^1O_2$ concentrations, and increased the chlorophyll content (relationship also verified in this work with the endogenous amino acid; Fig. 9a, b).

Proline exposure was also shown to enhance the activity of antioxidant enzymes (Aggarwal et al. 2011; Yan et al. 2011). Combined freeze–thaw, acidic and salt stress in alfalfa seedlings increased proline levels compared to control conditions, apparently having an important role in stress mitigation (Bao et al. 2020). Rizhsky et al. (2004) reported that the combination of drought and heat stress in Arabidopsis led to the accumulation of sucrose as the main osmoprotectant rather than proline. The fact that proline accumulation was only induced by heat and not by PEG in our experiments was somewhat unexpected. This may be a result of the sampling time choice that could have missed a transient increase in this metabolite, degradation, or conversion of free proline to other amino acids, and participation of other osmolytes in osmotic stress responses of *P. brachyceras*.

Accumulation of carotenoids in samples exhibited a similar profile of that of brachycerine and proline, supporting the presence of a complementary triad of antioxidants (Fig. 9). The interaction between these metabolites of different origin and chemical nature could yield enhanced protection against oxidative stress. Carotenoids are known to be effective in protecting against photooxidative damage by sequestering O_2^- and peroxyl radicals (Stahl and Sies 2003).

TSP changes in disks under the different treatments and over the sampling times likely resulted from a combination of factors that include protein synthesis and degradation, increased stability, inhibition of biosynthesis, or oxidation by ROS. Ashoub et al. (2015) reported that during drought stress, upregulation of detoxifying proteins, chaperones, heat shock proteins (HSPs), and proteins related to amino acid synthesis, lipid metabolism and water homeostasis took place, while proteins associated with nitrogen metabolism were negatively regulated. Heat stress induces upregulation of several HSPs. The responses in the combination of the two stresses include upregulation of additional forms of HSPs and downregulation of enzymes associated with photosynthetic machinery and chlorophyll-binding proteins (Ashoub et al. 2015).

On the second day of stress, APX activity in T disks was significantly lower than that of those in PEG (Fig. 8a). On the other hand, after two days of stress, disks in PEG showed no differences in APX activity compared to the control, as previously recorded by Nascimento et al. (2013). In contrast, after four days of stress, APX activity was higher in T than in PEG-exposed disks (Fig. 8c). PEG-T showed the lowest APX activity among all treatments, and, surprisingly, T + PEG samples had lower values than samples in individual stresses (Fig. 8c). Studies in Arabidopsis showed that gene expression and protein accumulation of cytosolic ascorbate peroxidase 1 (APX1) was significantly higher in combined stress (drought and heat) when compared to individual stresses. Mutants in *APX1* were more susceptible to combined stress, showing cell damage and death (Koussevitzky et al. 2008). The present data in *Psychotria* suggests the involvement of other antioxidant defenses in controlling H_2O_2 levels, such as brachycerine (Fig. 9b).

Similar to the APX profile, it was observed that after two days of stress, SOD activity was lower in T disks compared to those in control and PEG (Fig. 8a). However, in the case of SOD, T + PEG disks indicated a synergistic effect

between T and PEG (Fig. 8a). At 4 days, just like APX, SOD activity became higher in T samples compared to PEG ones (Figs. 8c and 9b). Leaf disks under T-PEG showed higher SOD activity than those exposed to PEG-T (Fig. 8c). In several conditions, brachycerine and SOD activity levels in disks appeared to have an inverse relationship on the second day of stress (Fig. 9a) (*e.g.,* T-PEG and PEG-T relative values, T at 2 days, PEG at 4 days). Such observation agrees with a previous suggestion that brachycerine has a SOD-like action (Nascimento et al. 2013). Nonetheless, this situation was not maintained for samples in T at 4 days and in T + PEG at both time points, in which a positive relationship was apparent. This suggests that it is also possible that the combined action of the alkaloid and the enzyme may help mitigate oxidative stress more effectively. The prevalence of one or another kind of interaction likely depends on stress type, length, and intensity. A direct relationship between SOD and APX activities, as well as between brachycerine and SOD activity levels, was observed in disks on the fourth day of stress (Fig. 9b). In contrast to the synergistic effect observed in SOD activity at 2 days, samples in T + PEG evidenced an antagonistic interaction between T and PEG at 4 days (Figs. 6 and 8a, c). These changes in profile may also reflect the production of varying levels of $O_2^{\cdot-}$ under distinct conditions which can differently affect SOD activity.

Stress indicator parameters

On the second day of stress, there were no differences among disks in the various treatments regarding H_2O_2 content and electrolyte leakage. This was probably due to the fact that the antioxidant systems were able to maintain the H_2O_2 content at basal levels in combination with the possibility that stress consequences were not intense enough at 2 days. H_2O_2 exhibits a dual role, acting as a signaling molecule at low concentrations and as a toxic metabolite at higher ones (Dumanović et al. 2021). Likewise, these same defense systems were probably capable of preserving membrane integrity, thereby containing electrolyte leakage (EL). However, it must be pointed out that EL is not always related to membrane integrity and loss of cell viability. It has been shown that EL involves efflux of ions, mainly K^+, but also Ca^{2+}, and various counterions, such as Cl^-, HPO_4^{2-}, NO^{3-}, $citrate^{3-}$, and $malate^{2-}$ (Demidchik et al. 2014). The same authors have proposed that, despite also being caused by lipid peroxidation, EL is mostly dependent on K^+ channel activation triggered by the production of ROS ($O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$). This may partly explain the lack of direct relationships between response profiles of MDA and EL in disks exposed to T at days 2 and 4, and to T + PEG at 4 days (Figs. 4 and 7). On the fourth day of stress, there is an inverse correlation between MDA and EL. Perhaps the increase in ion efflux, a product of the increase in ROS (H_2O_2) and consequent increase in EL, occurs as a previous homestasis mechanism to avoid lipid peroxidation (Fig. 9b). It has been proposed that under moderate stresses, potassium efflux may act as a metabolic switch triggering catabolism to provide energy for repair responses (Demidchik et al. 2014). This may help explain the inverse relationship between TSP and EL levels in disks under T, T-PEG, and T + PEG on the fourth day (Figs. 5, 7, and 9b).

After two days of stress, leaf disks in T had the highest total chlorophyll content and the lowest lipid peroxidation when compared to the control ones (Figs. 8b and 9a). Lower formation of MDA as an end-product of lipid peroxidation in leaf disks was also observed by Magedans et al. (2017) after three days of heat stress compared to the control group. At first this may be surprising but is probably the result of the high efficiency of protective systems. The fact that at 2 days disks exposed to T exhibited low levels of APX and SOD enzymatic activity but also the highest content of brachycerine, proline and carotenoids when compared to the control (Figs. 8a and 9a) is suggestive of a compensatory or time of stress application dependent response. Similar behavior was recorded at 4 days, when T disks displayed the highest total chlorophyll and the lowest lipid peroxidation levels when compared to their control counterparts (Fig. 8d), which corresponded to the highest levels of antioxidant enzyme activities, brachycerine, proline and carotenoid concentrations when compared to the control samples (Figs. 8c and 9b). Exogenous application of brachycerine in leaves of the heat-sensitive species *Brugmansia suaveolens* caused an increase in total chlorophyll content under heat shock (Magedans et al. 2017). On the other hand, SOD acts as the first biochemical defense barrier against ROS, transforming $O_2^{\cdot-}$ into H_2O_2, which is later detoxified by APX (Dumanović et al. 2021). In addition, on the fourth day, the H_2O_2 content in T was not different from the control as previously observed by Magedans et al. (2017) after three days of stress; however, the electrolyte leakage was much higher (Fig. 8d).

PEG disks on the second day of stress showed high ChA/ChB ratio compared to the control (Fig. 8b). This increase in ChA/ChB ratio is probably a result of higher sensitivity of ChB to osmotic stress relative to ChA (Jain et al. 2010). Other evaluated parameters (protective systems against stress, and stress indicators) did not show significant differences when compared with the control disks, although there were differences among stress treatments (Fig. 8). This could indicate that osmotic stress was not sufficiently acute to produce fast changes in physiological parameters. On the other hand, it has been shown that the most intense regulation of some of these physiological defense mechanisms takes place in the first few hours. APX activity, for instance, was higher in leaf disks submitted to PEG 0.05 M after 12 h of exposure when compared to the control, thus avoiding H_2O_2 accumulation (Nascimento et al. 2013).

On the fourth day, PEG exposed disks differed from the control by exibiting higher brachycerine (Fig. 8c) and H_2O_2 content (Fig. 8d). As expected, the accumulation of H_2O_2 stimulated the biosynthesis of brachycerine (Fig. 9b). On the second day of stress, T + PEG disks showed the same total chlorophyll content as the individual stresses and the same ChA/ChB ratio as those in PEG (Fig. 8b). Lipid peroxidation in T + PEG disks showed an antagonistic response of T and PEG ones (Fig. 8b), which interestingly coincides with the antagonistic effect seen in brachycerine accumulation and the synergistic effect seen in SOD enzyme activity in leaf disks of these same treatments (Fig. 8a). On the second day of stress, brachycerine exhibited an inverse correlation with MDA (Fig. 9a). On the fourth day of stress, not only brachycerine but also SOD, proline and carotenoids exhibited an inverse correlation with MDA (Fig. 9b). These observations further support the existence of effective complementation of antioxidant systems.

On the fourth day of stress, the disks in sequential stresses (T-PEG and PEG-T) showed no significant differences in relation to total chlorophyll, ChA/ChB ratio, lipid peroxidation, H_2O_2 content and EL% (Fig. 8d). However, although samples of these treatments did not show differences regarding the stress indicator parameters, there were differences between them regarding the stress protection systems. Thus, for example, PEG-T disks had a higher content of carotenoids, proline and brachycerine when compared to those in T-PEG (Fig. 8c). Conversely, samples in PEG-T had lower SOD and APX activities than T-PEG ones (Fig. 8c). Hence, although disks in the two treatments managed to mitigate stress to the same magnitude, the protective defense mechanisms against stress were different in terms of intensity. Such profiles may reflect a combination of specific sets of defenses against the different stresses and cross-protection (Mittler 2006), *i.e.,* improved response to a second stress as a result of the biochemical changes induced by the first that remain in cells (*e.g.,* osmoprotectants, antioxidant enzymes, chaperones). When two stresses are applied sequentially, plants can exhibit a transcriptome profile very similar to the second applied stress, regardless of the nature of the first stress to which it was exposed, even though some mRNA signatures of the first stress also remain (Coolen et al. 2016).

After four days of stress, T + PEG disks showed levels of H_2O_2 and total chlorophyll equivalent to those under PEG (Fig. 8d). At the same time, T + PEG samples had lipid peroxidation and EL% similar to those in T (Fig. 8d), coinciding with the similarity observed between these treatments in the profile of brachycerine and TSP (Fig. 8c). The ChA/ChB ratio in T + PEG samples was higher than of disks in individual stresses T and PEG (Fig. 8d). Taken together, this indicates that disks exposed to simultaneous stress T + PEG present a behavior that combines characteristics of those in individual treatments, encompassing shared and unique responses (Mittler 2006). Interestingly, on the fourth day of stress, leaf disks in the T + PEG treatment showed the most variable behavior, with an antagonistic effect in relation to the content of proline and carotenoids compared to disks in individual stresses (Fig. 8c). Another noteworthy fact is that the activity of the SOD enzyme in T + PEG samples showed a synergistic effect on the second day of stress (Figs. 6b and 8a), and an antagonistic effect on the fourth day of stress (Figs. 6B and 8c) relative to the response of T and PEG disks.

The unique and shared profiles of combined treatments compared to those of individual ones have been observed in detailed studies with simultaneous abiotic stresses in tomato. In one of these investigations, transcript and metabolite data indicated that proline and ascorbate pathways acted in conjunction to re-establish cellular redox homeostasis, with a unique reprogramming profile of metabolic pathways and expression of transcription factor genes (Lopez-Delacalle et al. 2021). Another study revealed that the combination of salt and heat caused more Na accumulation, nutrient depletion, and growth reduction than salinity alone. However, the former condition did not yield higher oxidative damage or ROS accumulation, presumably as a function of stronger antioxidant defenses (Sousa et al 2022). A physiological evaluation of tomato cultivars differing in heat tolerance submitted to combined drought and heat stress did not find a correlation between combined and single stress tolerance, whereas it recorded a predominant effect of drought over heat stress on the plants (Zhou et al. 2017).

Leaf disks exposed to individual stresses presented clear and somewhat predictable patterns of response. This was often not the case of samples under sequential or combined stresses, probably as result of the complex interactions of metabolic pathways under these conditions. In fact, the definition of single major indexes of stress damage and mitigation is a difficult task, often requiring the use of several parameters (Xiong et al. 2022). Although each type of individual stress can produce characteristic responses, the combination of these may show positive or negative interactions of synergistic, antagonistic, and additive nature, which also could vary over time.

High efficiency and complementarity of the antioxidant defense systems for the recovery of cellular homeostasis is an essential component to successfully overcome stress damage. The data herein may provide clues to follow in the search for key components in stress responses and their appropriate balance to modulate tolerance and metabolic fluxes. Future studies of the physiological mechanisms at various levels of regulation will improve the current knowledge about sequential and combined stresses, which, in turn, may contribute to the management of environmental stresses in crops, the genetic improvement of plants, and more effective production of bioactive metabolites of interest.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10265-023-01441-z.

Acknowledgements This work was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES—Finance code 001), the Research Support Foundation of the State of Rio Grande do Sul (FAPERGS—Project 19/2551-0001709-0), National Council for Scientific and Technological Development (CNPq—Project 310775/2021-3) and the Organization of American States (OAS).

Data Availability Statement All data generated or analysed during this study are included in this published article (and its supplementary information files).

Declarations

Conflict of interest The authors of this article declare no conflicts of interest.

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Electronic supplementary materials

Title:

Biochemical responses in leaf tissues of alkaloid producing *Psychotria brachyceras* under multiple stresses

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Journal of Plant Research

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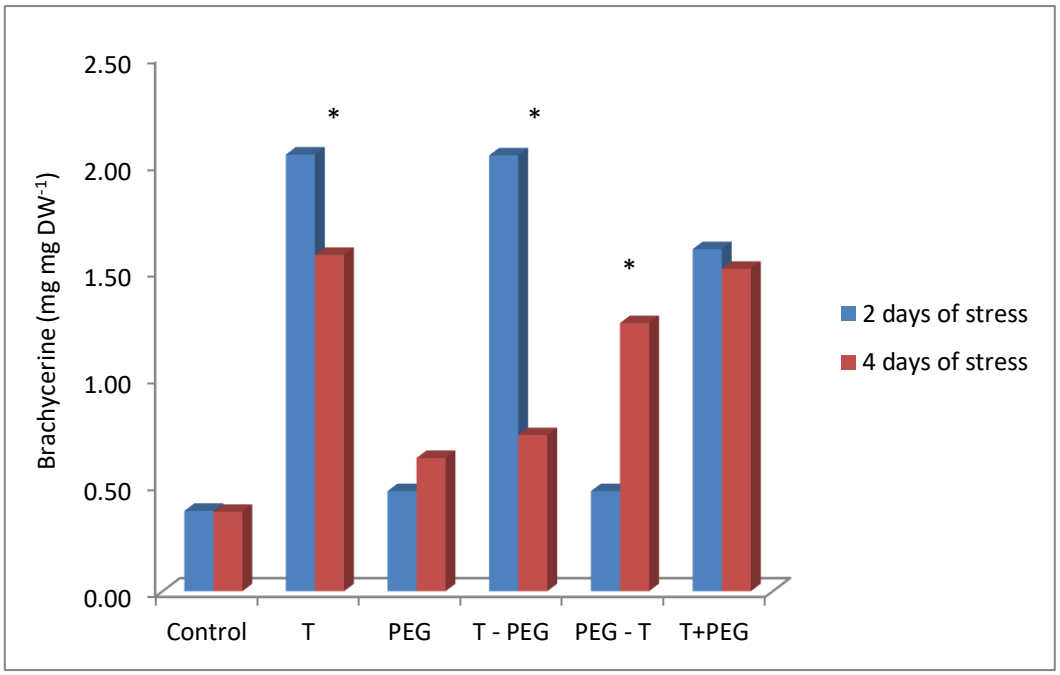

Fig. S1. Brachycerine accumulation in *P. brachyceras* leaf disks on the second and the fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

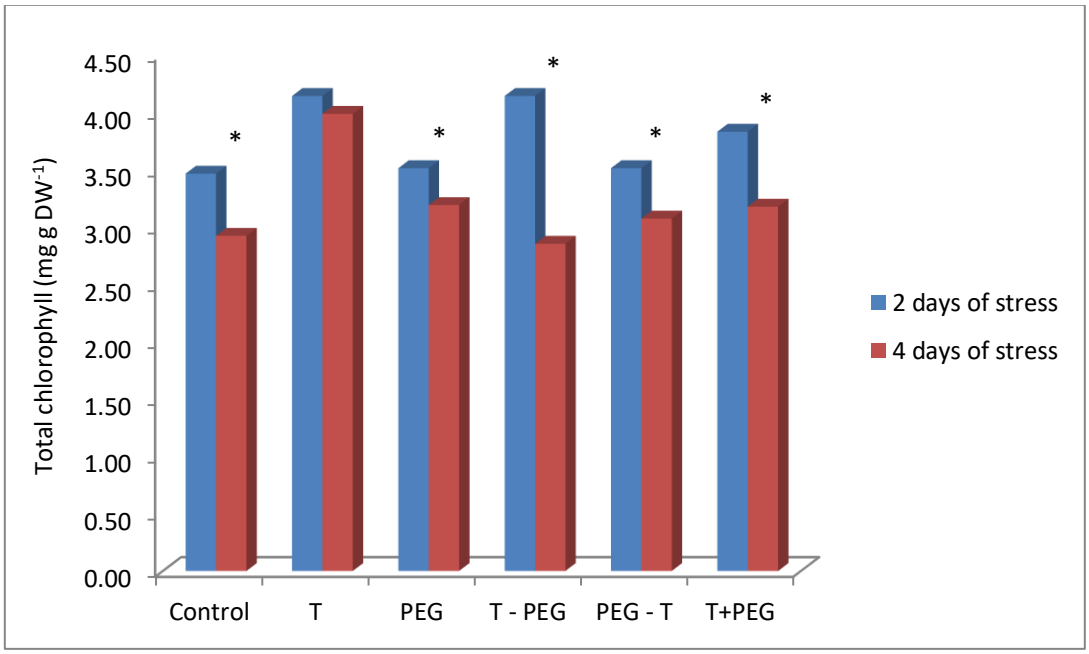

Fig. S2. Total chlorophyll concentration in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

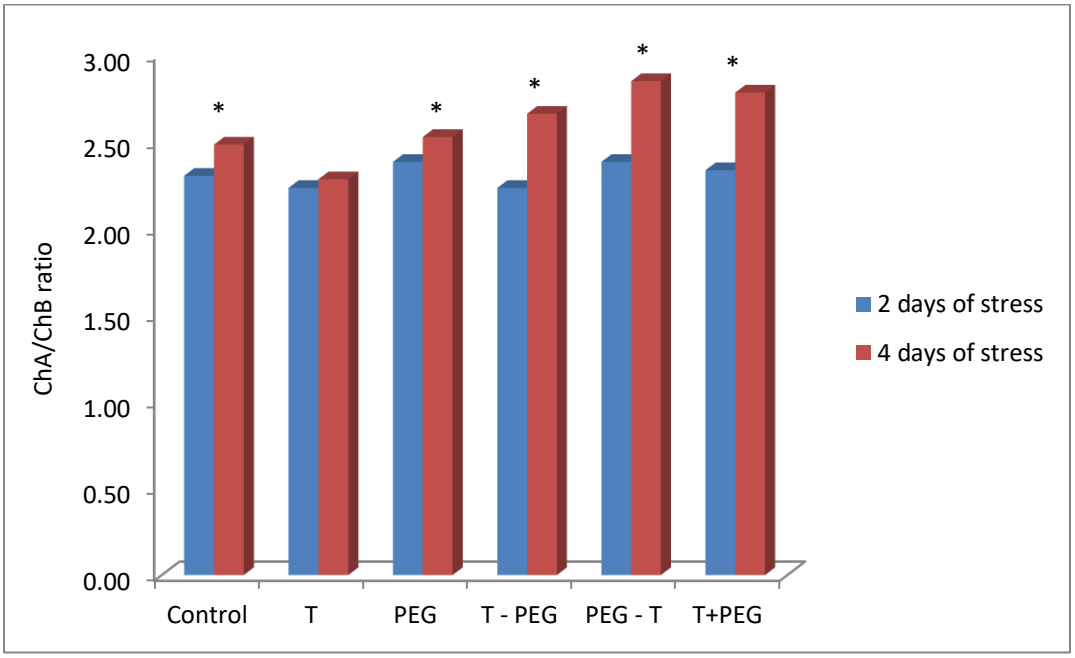

Fig. S3. ChA/ChB ratio in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

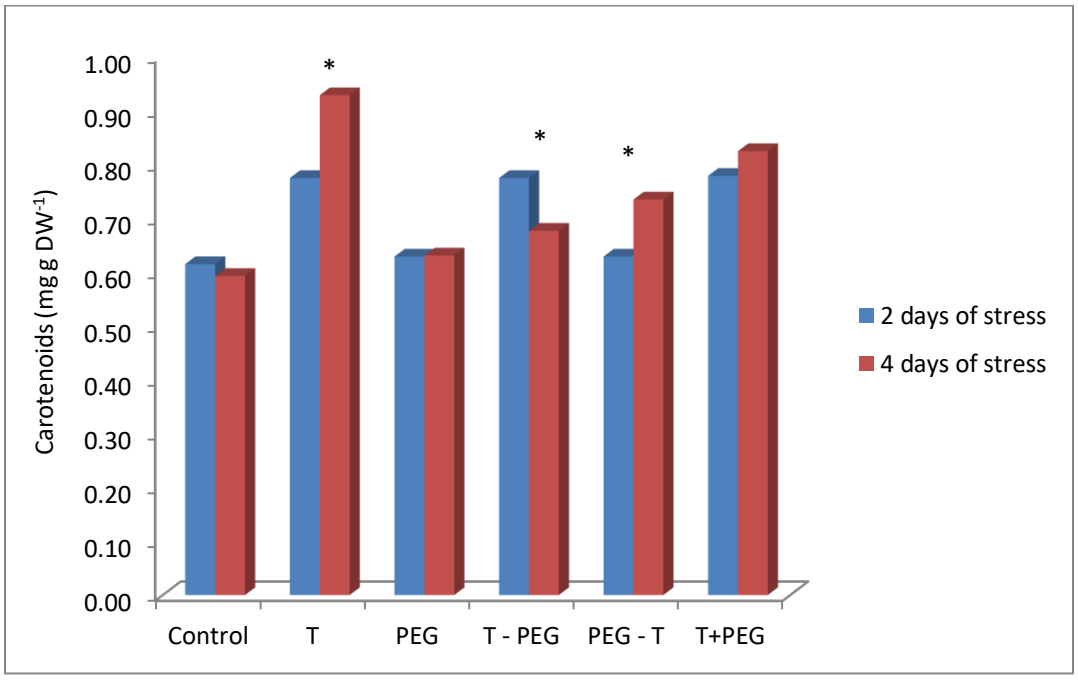

Fig. S4. Carotenoid concentrations in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

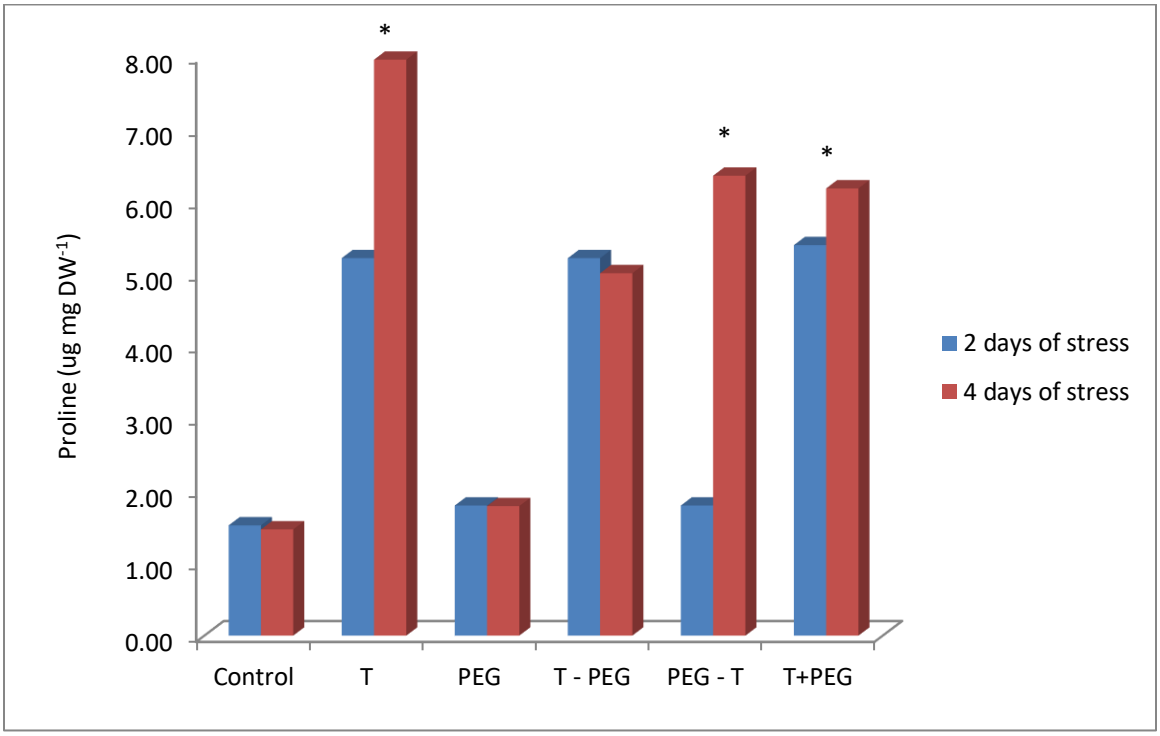

Fig. S5. Proline concentration in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

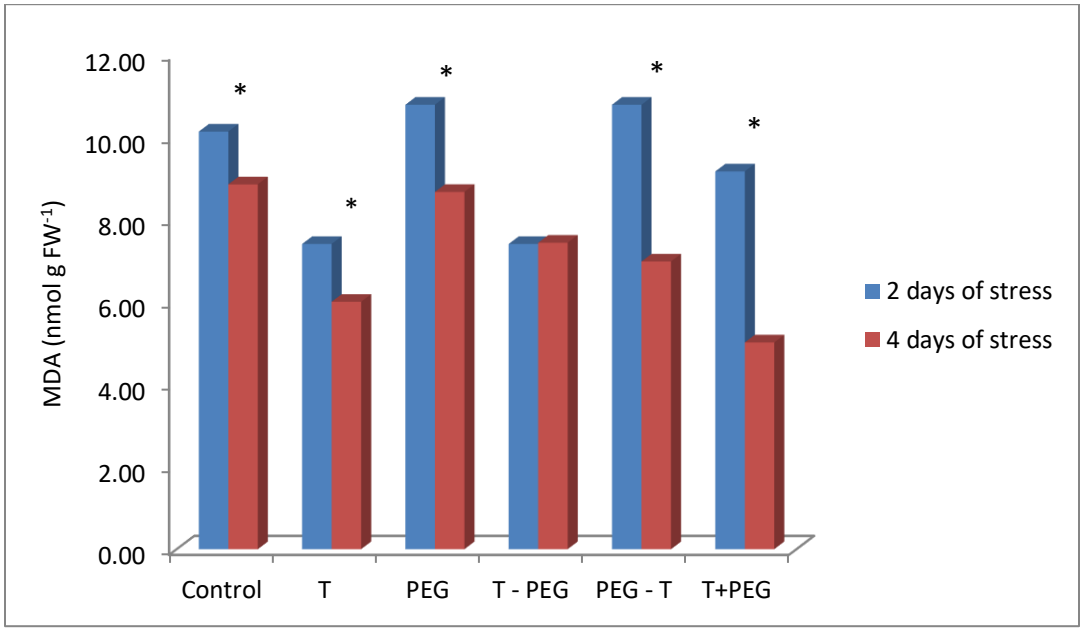

Fig. S6. MDA levels in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

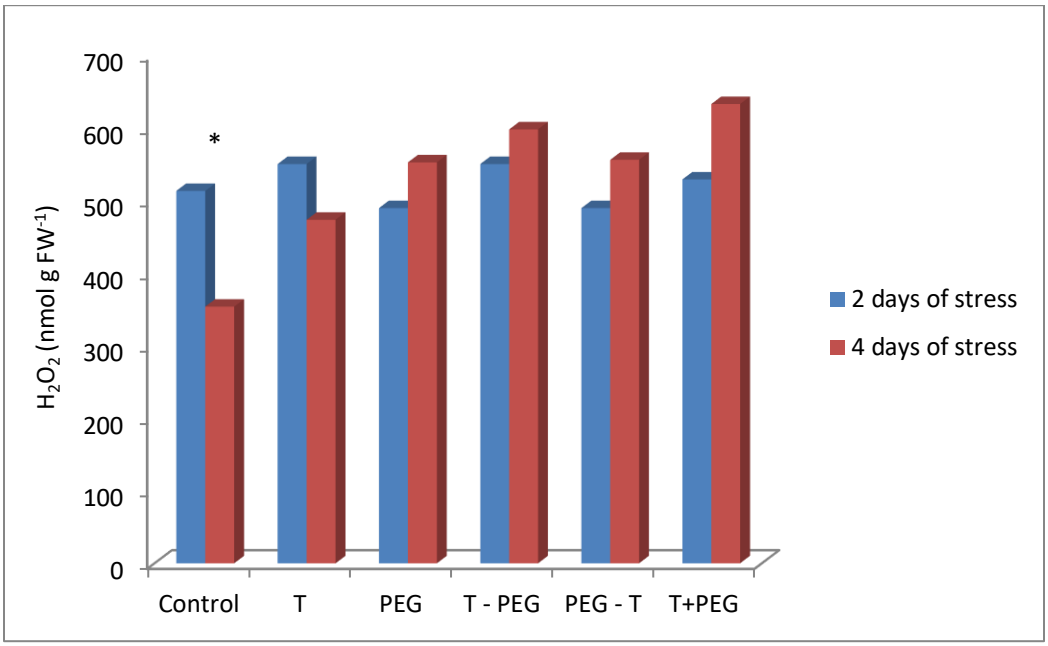

Fig. S7. H_2O_2 concentration in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

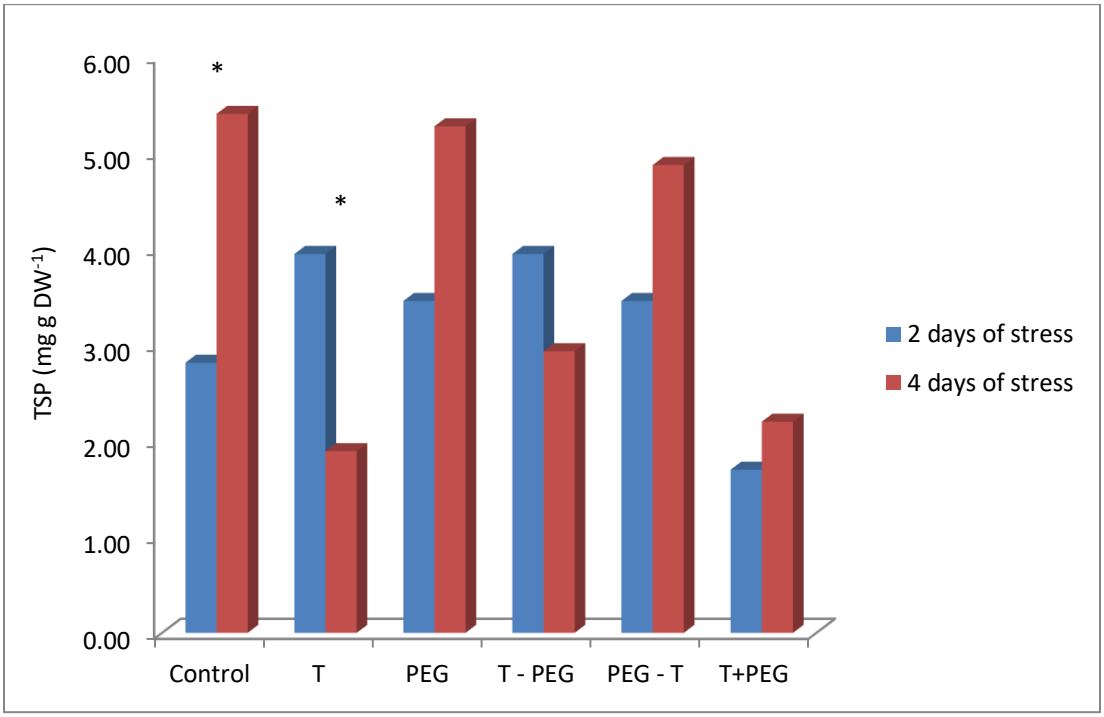

Fig. S8. Total soluble proteins (TSP) concentration in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

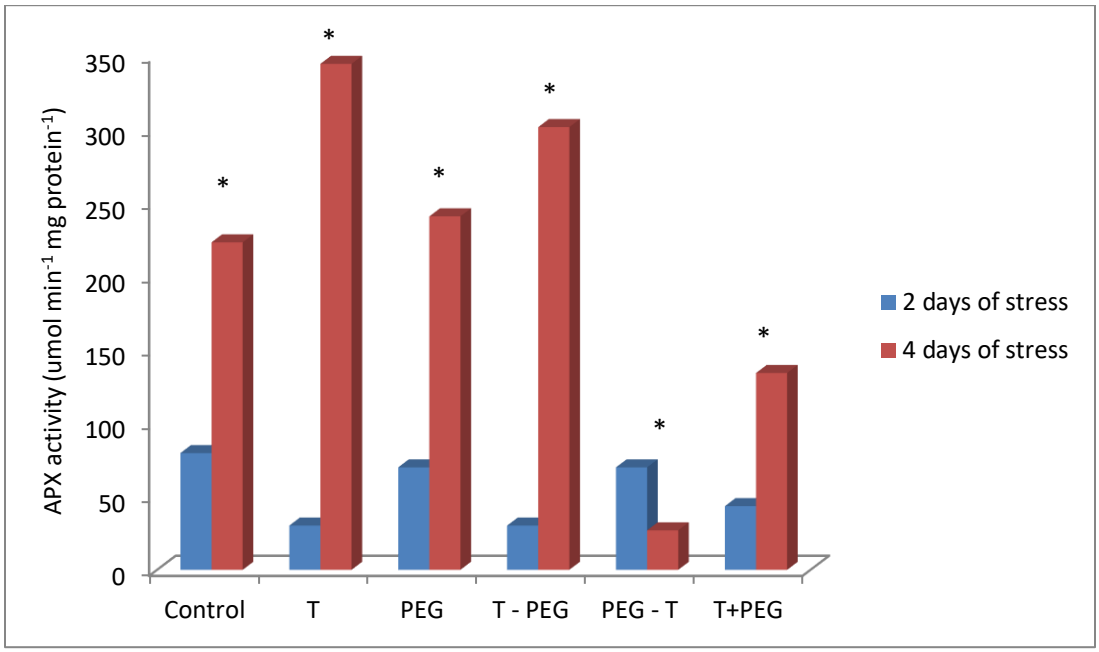

Fig. S9. Ascorbate peroxidase (APX) activity in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

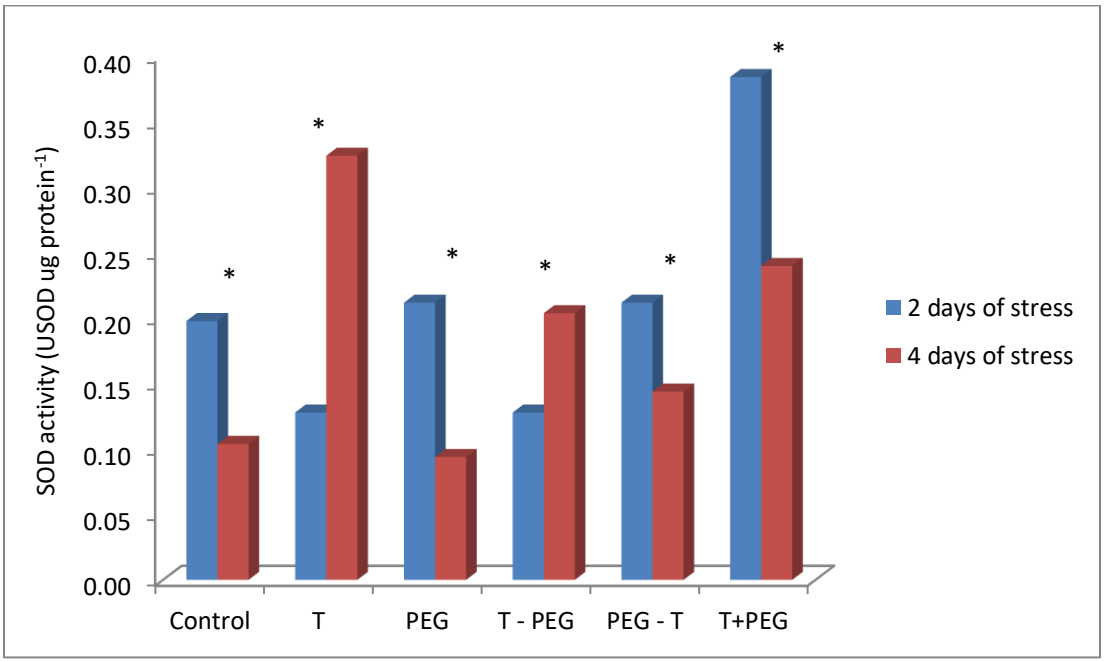

Fig. S10. Superoxide dismutase (SOD) activity in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).

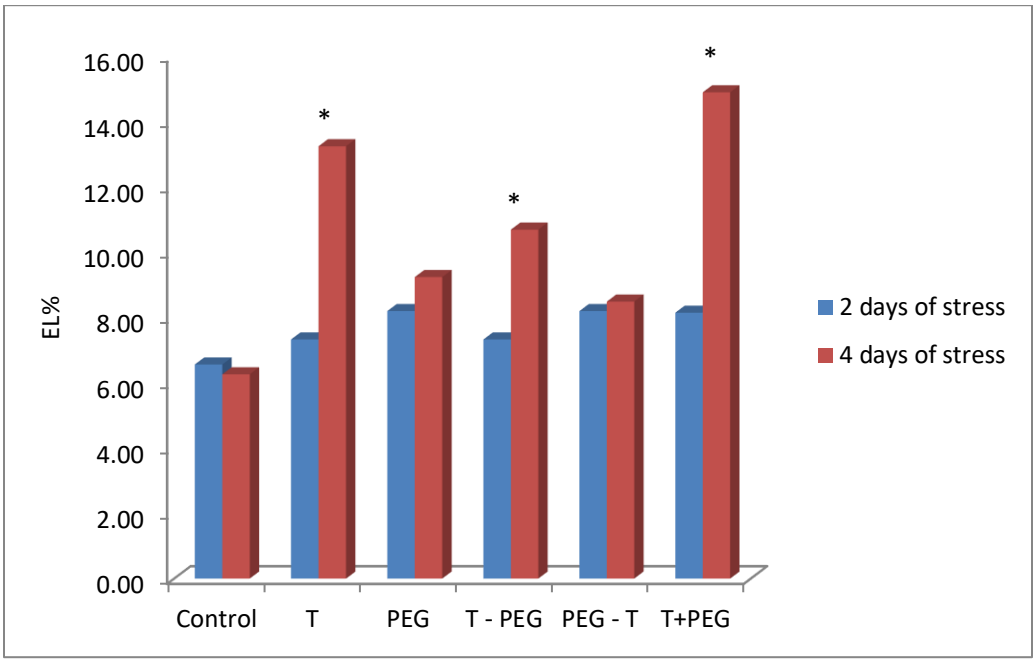

Fig. S11. Relative electrolyte leakage (EL%) in *P. brachyceras* leaf disks on the second and fourth day of exposure to heat stress (T) (40 °C) and osmotic stress (PEG). Asterisks indicate significant difference within treatment and between days of stress ($p \leq 0.05$).