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Short Paper

Accumulation of the antioxidant indole alkaloid brachycerine under high temperature and high irradiance in leaf disks of *Psychotria brachyceras*

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Abstract: *Psychotria brachyceras* Müll.Arg. (Rubiaceae) is an understorey shrub of the Atlantic Forest whose leaves accumulate brachycerine, an antioxidant monoterpene indole alkaloid (MIA). This work aimed at evaluating the response of this MIA in leaf disks upon exposure to different temperatures and irradiance, similar to those experienced by the plant in its environment. Leaf disks obtained from growth room-acclimated cuttings were submitted to three distinct series of treatments: 1) *Light* - two days under control condition (50 µmol.m^{-2}.s^{-1}) in a growth chamber followed by three days treatment of exposure to high irradiance (500 µmol.m^{-2}.s^{-1}); 2) *Temperature Cold/Heat* – three days under control conditions (25ºC) and then exposure for two days to 10°C or 40°C; 3) *Acclimation* – gradual heat increase from 25°C up to 40°C. Brachycerine, chlorophyll, and lipid peroxidation levels were not significantly affected by high light or cold exposure, although a decrease in chlorophyll a/b ratio was observed under high irradiance. In contrast, there was increased accumulation of brachycerine at elevated temperatures (where higher cell damage was expected) and significantly lower lipid peroxidation, indicating that this MIA is possibly playing a central role in preventing or mitigating cell damage. A threshold temperature to trigger alkaloid accumulation was identified at approximately 40ºC.

1 - Introduction:

Plant primary metabolism involves the production of substances essential for survival. Through various specific biosynthetic pathways, primary metabolites originate secondary metabolites, which, in general, have very complex chemical structures and several biological activities. Secondary metabolites are not essential for plant survival

under ideal growth conditions; however, they play major roles in the interaction of plants with the environment, such as in defense against herbivores and micro-organisms, protection from UV rays, high temperatures, attraction of pollinating insects or seed dispersing animals, contributing to plant adaptation to stresses and ensuring reproductive success (SANDES & BLASI, 2000).

As sessile organisms, plants have developed different evolutionary strategies for defense, association with other organisms and occupation of different habitats, being highly capable of facing environmental adversity. UV-B radiation, excess visible irradiance, and high temperature, for example, can be harmful to living organisms by damaging DNA, proteins, and/or membranes (DODGE, 1994; FOYER et al., 1997; PRASAD et al., 1999; TSUGANE et al., 1999; GEOFFROY et al., 2002; MITTLER, 2002). It is well known that irradiance and temperature extremes, which often take place simultaneously, may lead to increased content of reactive oxygen species (ROS) (GILL & TUTEJA, 2010). Plants have acquired strategies of using light effectively and protecting themselves from damage under light and temperature stresses.

Secondary metabolites, particularly flavonoids and monoterpene indole alkaloids (MIAs), may minimize the damage caused by stresses, such as UV-B, drought and wounding, by quenching ROS generated under these conditions (SUZUKI & MITTLER, 2006; MATSUURA et al., 2013). Recent studies with *Psychotria* species have shown the capacity of MIAs to protect against the effects of UV-B radiation (NASCIMENTO, 2011; MATSUURA et al., 2014).

Psychotria (Rubiaceae) is one of the largest genus within the angiosperms, with estimates between 1000 to 1650 species distributed worldwide (NEPKROEFF et al., 1999). In southern Brazil, 22 species have been recorded, and the main species are *P.*

brachyceras, P. carthagenensis, P. leiocarpa, P. myriantha, P. suterella e P. umbellata, all occurring in the understorey of the Atlantic Forest (Rio de Janeiro Botanical Garden Institute - REFLORA program http://reflora.jbrj.gov.br).

The major MIA of *P. brachyceras,* object of this study, is brachycerine. Some studies suggest that this indole alkaloid originates from the direct condensation of tryptamine (which originates from the decarboxylation of the amino acid tryptophan) with *epi*-loganin (from the plastidic mevalonate-independent pathway) (CONTIN et al., 1998; KERBER et al., 2001). It has been suggested that there is a close link between brachycerine induction by UV-B radiation and the possible role of this MIA in protection against ROS (NASCIMENTO et al., 2007; MATSUURA & FETT-NETO, 2013). Brachycerine has shown significant bioactivity as antioxidant, antimutagenic, and as UV-B protectant in heterologous systems (NASCIMENTO et al., 2007; NASCIMENTO et al., 2013; PORTO et al., 2014).

However, caution is advised since experiments were conducted with high rates of UV-B radiation, hardly found in the natural environment in which *P. brachyceras* thrives (tropical and subtropical forests). The roles of temperature extremes and high visible irradiance in the metabolism of brachycerine are unknown, although these factors can have significant effects on the redox equilibrium of the cell, apparently a predominant factor in controlling production of the alkaloid (MATSUURA et al., 2013).

The ecological roles of MIAs from *Psychotria* are not fully established, although studies to date indicate that these alkaloids and extracts from plants such as *P. brachyceras* have the ability to prevent damage to cells caused by excess ROS (common products of high light irradiance, temperature extremes, and herbivory damage to the plant). To better understand the function of these alkaloids *in planta*, it becomes necessary

to study the dynamics of these metabolites under conditions and stresses similar to those faced by plants in their environment.

Thus, this study aims to evaluate the role of brachycerine in response to thermal and high light stresses in *Psychotria brachyceras*, which are likely to be faced by individuals of the species in their natural habitat. The specific objectives are: 1) to examine whether high light irradiance induced photo-oxidative stress affects production of brachycerine; 2) to verify if extreme temperature shock changes the accumulation pattern of brachycerine; 3) to investigate whether alkaloid content changes occurs both in conditions of acute stress and as incremental steps, allowing acclimatization (to be tested only for the abiotic factor that most markedly changes brachycerine content); 4) gauge the level of oxidative stress generated in each of the conditions outlined in 3 to access its role as a trigger of alkaloid accumulation.

2 - Methods

2.1 - Sample source

Field-grown *Psychotria brachyceras* Müll. Arg. tip cuttings (with 6-8 leaves) were harvested (between April and June, 2014 at Morro Santana, Porto Alegre, RS, Brazil) and acclimated in 10% (v/v) MS (MURASHIGE & SKOOG, 1962) nutrient solution, pH 5.8 for a week [16 $h.day^{-1}$ photoperiod, 60 $\mu mol.m^{-2}.s^{-1}$ photosynthetically active radiation (P.A.R.), 25 ± 3° C] prior to use as leaf disk source. A voucher specimen (7899) is deposited at the ICN herbarium (UFRGS).

2.2 - Brachycerine extraction

Brachycerine was purified from *Psychotria brachyceras* leaves essentially following

a previously described method (KERBER et al., 2001). HPLC was used to evaluate purity index in comparison to authentic brachycerine. Chromatographic parameters were as described below for sample analysis (GREGIANINI et al., 2003).

2.3 - HPLC analysis of extracts

Methanolic extracts were obtained from leaf disk samples and analyzed by HPLC, as previously described (GREGIANINI et al., 2003). Shortly, 150 mg of fresh tissue were ground with mortar and pestle in liquid nitrogen, followed by 1 mL of methanol (Merck-HPLC grade) addition; the extract was then sonicated for 20 min. The extract was centrifuged at 13.000 x g at 4°C for 20 min and the methanolic layer recovered for HPLC analysis. Pellets were dried at 60°C until reaching constant weight for extracted dry weight measurement.

Samples were analyzed by HPLC (Thermo Scientific Surveyor) in a linear gradient starting with water:methanol (81:19), and ending with 100% methanol, both eluents containing trifluoroacetic acid (TFA) (Sigma) in a final concentration of 0.05%. Flow rate was 1 $mL.min^{-1}$ and column was a C8 Shimadzu equipped with respective guard column; an external standard curve was prepared with authentic brachycerine. The content of brachycerine was expressed on a leaf extracted dry weight basis.

2.4 - Chlorophyll analysis

Chlorophyll concentrations were obtained as previously described (ROSS, 1974). Leaf disks (1 cm diameter, 3 disks per sample) were homogenized in a tissue homogenizer (PowerGen 125, Fischer Scientific - USA) with 1.5 mL acetone 85% and then sonicated for 15 min. After centrifugation (10 000 x g for 10 min at 4°C), pellets were re-extracted twice and combined supernatants were brought to a final volume of 10 mL.

The absorbances were recorded at 645 and 663 nm; pellet was dried at 60°C until constant weight for extracted dry weight determination.

2.5 - Lipid peroxidation assay

Determination of TBARS for lipid oxidative damage measurement was assayed essentially as previously described (VELIKOVA et al., 2000). Samples (150 mg) were ground with liquid nitrogen and homogenized with trichloroacetic acid (TCA) 0.1%. The homogenate was centrifuged for 20 min at 10 000 x g, 4°C, and the supernatant (0.5 mL) reacted for 30 min at 100°C with 1 mL of thiobarbituric acid (TBA) 0.5% in TCA 20%; the reaction was stopped by immediate chilling and the absorbance was taken at 532 nm. Non-specific absorption values at 600 nm were subtracted. TBARS contents were calculated using an extinction coefficient of 155 $mM^{-1}.cm^{-1}$.

2.6 - Light assay

Leaf disks (1cm diameter) were prepared from growth chamber-acclimated shoots. In a Petri dish, forty disks were arranged on filter paper and 20 mL of distilled water, and exposed for two days to control condition (50 $\mu mol.m^{-2}.s^{-1}$ of P.A.R. and 25 ± 3° C) in a growth chamber before three days treatment exposure to high irradiance (500 $\mu mol.m^{-2}.s^{-1}$ PAR and 25 ± 3°C); control samples remained at 50 $\mu mol.m^{-2}.s^{-1}$ PAR condition. After 5 days assay, disks were harvested, immersed in liquid nitrogen and stored at -20° C until brachycerine, chlorophyll and TBARS analyses were carried out.

2.7 - Temperature assay

For temperature assays, leaf disks (1 cm diameter) were prepared from growth chamber-acclimated shoots. In each Petri dish, forty disks were arranged on a filter paper moistened with 20 mL of MS solution (MURASHIGE & SKOOG, 1962) at 10% strength, pH

5.8, and maintained two days under control conditions (25 ± 0.3°C) in a BOD (16 $h.day^{-1}$ photoperiod) and then exposed to treatments for three days (10°C or 40°C); control samples remained at 25°C. A second assay was performed to analyze brachycerine induction pattern by gradual heat increase from 25°C up to 40°C. During the control treatment condition, disks were gradually exposed to higher temperatures (increment of 5°C per day), reaching 40°C after three days; then the disks remained for two more days at 40°C. Control samples were maintained at 25°C. After 5 days, samples were harvested, immersed in liquid nitrogen and frozen at -20°C until analysis (brachycerine, chlorophyll and TBARS).

2.8 - Experimental layout and statistics

All assays herein described were performed in totally randomized design, in biological quadruplicates, containing technical duplicates or more when possible, and each assay was independently repeated at least twice. The results were analyzed using the software SPSS 17.0, checked for normality and submitted to ANOVA followed by Tukey, or a *t*-test, $P \leq 0.05$, whenever appropriate.

3 - Results

3.1 - Light assay

Exposure to light at 500 $\mu mol.m^{-2}.s^{-1}$ caused photosynthetic damage, mainly due to partial impairment of photosystem II (significant chlorophyll a:b ratio reduction – 50 $\mu mol.m^{-2}.s^{-1}$ = 1,988502 ± 0,116061; 500 $\mu mol.m^{-2}.s^{-1}$ = 1,672826 ± 0,185485 - mean ± sd; t-test $P \leq 0.05$). Brachycerine, total chlorophyll and TBARS contents remained at levels similar to those of the control disks (50 $\mu mol.m^{-2}.s^{-1}$) (Fig. 1).

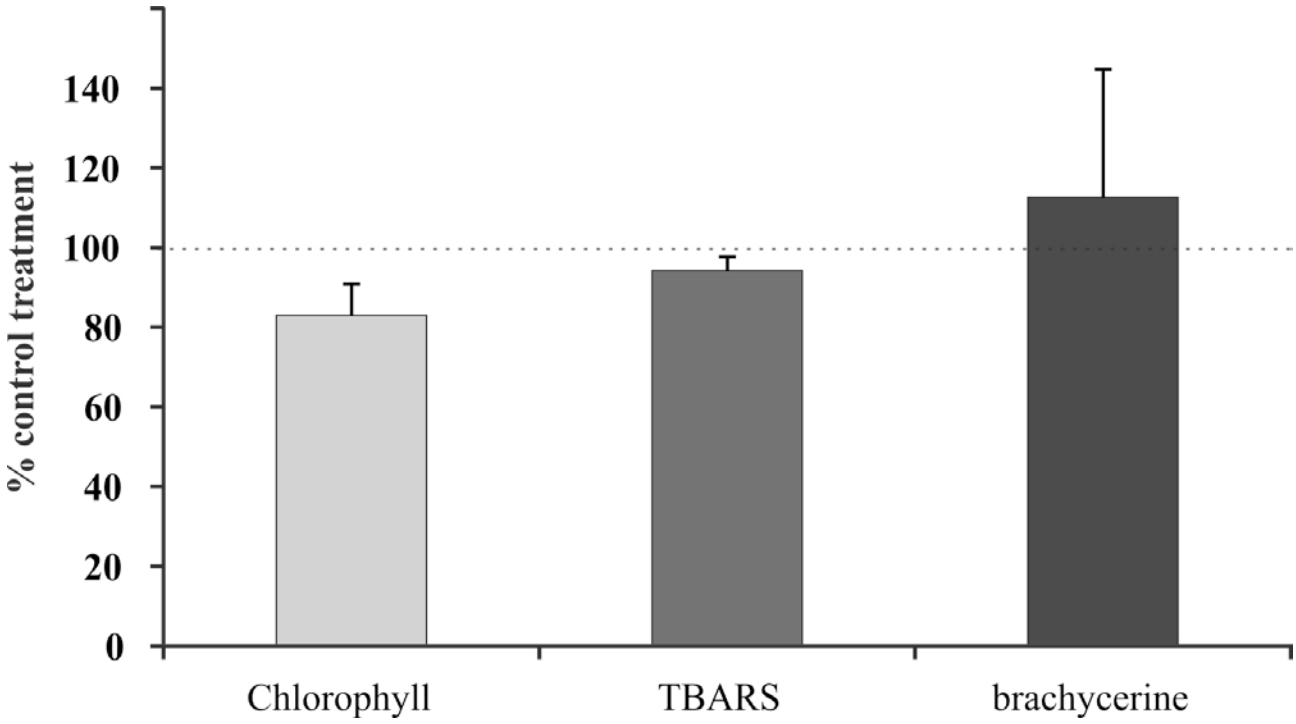

Figure 1 - Chlorophyll content, lipid peroxidation – estimated by TBARS concentration and brachycerine content in leaf disks of Psychotria brachyceras after high irradiance exposure 500µmol.m^{-2}.s^{-1} expressed as percentage of control levels (50 µmol.m^{-2}.s^{-1}). Parameters evaluated were not significantly different between treatments (ANOVA, P≤0.05).

3.2 - Temperature cold/heat assays

In the first temperature assay, cold treatment (10ºC) did not change brachycerine content. High temperature (40ºC) caused an approximately 20-fold brachycerine induction (Figure 2). Basal and induced levels are in good agreement with previously published results (GREGIANINI et al., 2003, 2004; PORTO et al., 2014).

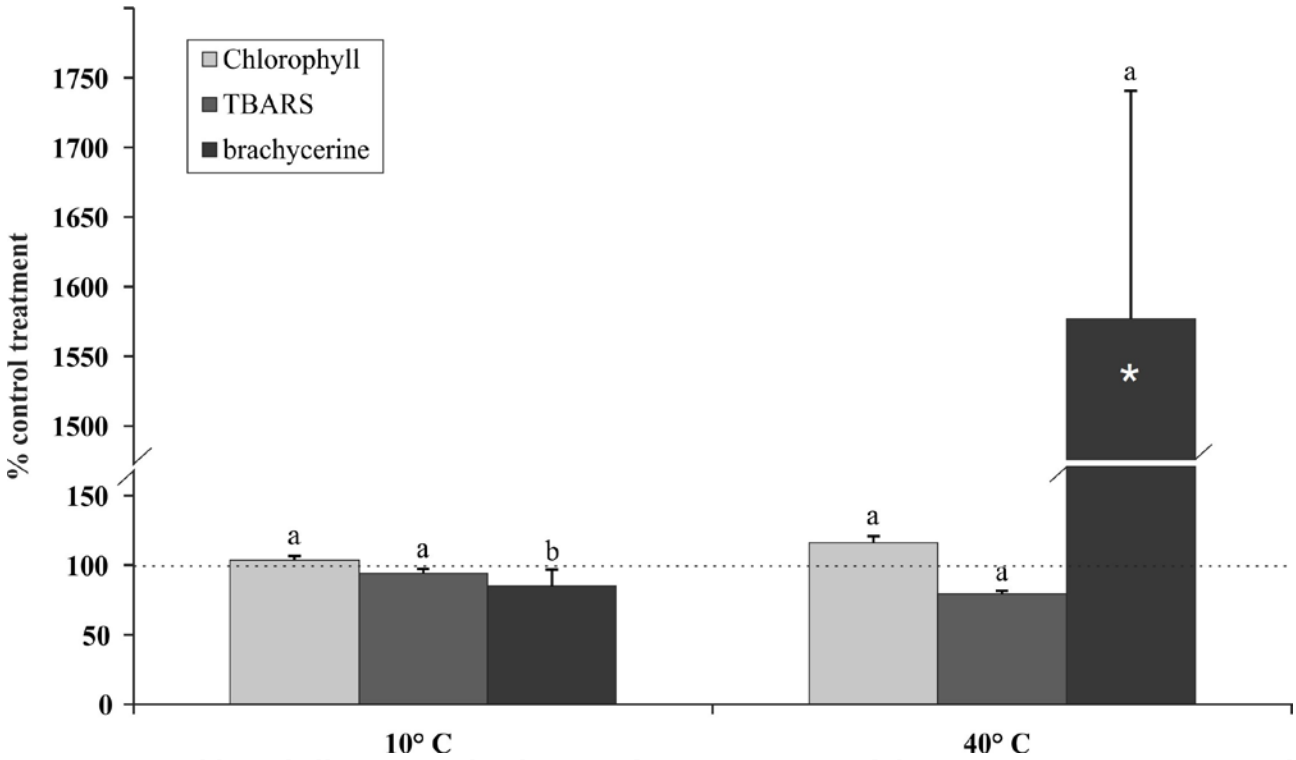

Figure 2 – Chlorophyll content, lipid peroxidation – estimated by TBARS concentration and brachycerine content in leaf disks of Psychotria brachyceras after low (10ºC) and high (40ºC) temperature treatment expressed as percentage of control levels (25ºC). Parameters not sharing a letter are significantly different between conditions. Bars marked with asterisk are significantly different from control values (ANOVA, P < 0.05).

3.3 - Acclimation treatment

When temperature was gradually increased, brachycerine content was higher upon reaching 40ºC than in disks that were suddenly exposed to same temperature. Interestingly, at 35ºC there was no brachycerine induction (Fig. 3).

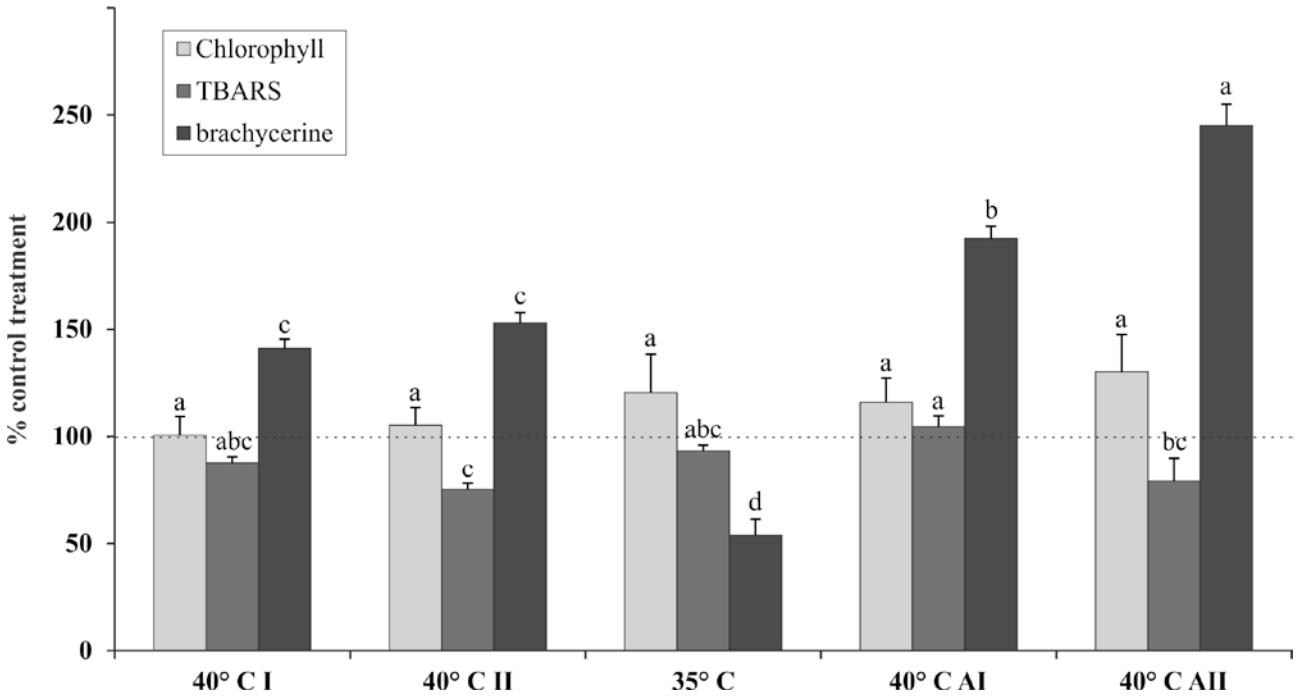

Figure 3 - Chlorophyll content, lipid peroxidation – estimated by TBARS concentration and brachycerine content in leaf disks of Psychotria brachyceras after 35ºC (condition that preceded the transfer to 40ºC in the stepwise increase of temperature) and 40ºC treatment expressed as percentage of control levels (25ºC). C I and C II – content after 24 and 48h of abrupt transfer to 40ºC, respectively. C AI and C AII – content after 24 and 48h of stepwise transfer to 40ºC, respectively. Parameters not sharing a letter are significantly different between conditions (ANOVA, $P < 0.05$). Bars marked with asterisk are significantly different from control values (t-test, $P < 0.05$).

3.4 - Lipid peroxidation

The results obtained in the analysis of TBARS showed that at 40ºC, both for abrupt or stepwise transfer, cell membranes showed less damage after 48 h of high temperature treatment (Fig. 3), the same time points when brachycerine concentration was significantly higher. Other conditions had membrane integrity comparable to that of control disks. Similarly, based on the same parameter, cellular damage under high irradiance did not differ statistically from the control condition (Fig. 1).

4 - Discussion

Irradiance conditions in the understorey of the forest where *P. brachyceras* leaves were harvested, at the transition from Fall to Winter 2014, varied considerably (around 10 $\mu mol.m^{-2}.s^{-1}$in a cloudy day to between 45 and 210 $\mu mol.m^{-2}.s^{-1}$in a sunny day). Therefore, the amounts of irradiance used in the experiments can be considered as basal and high taking into account the understorey values. From previous studies involving brachycerine accumulation (GREGIANINI et al., 2004), it was expected that exposure to high irradiance could reduce the photosynthetic capacity, mainly by interfering with photosystem II function due to ROS production. Indeed, damage to PSII, estimated by chlorophyll a:b ratio, was also apparent in our results, although total chlorophyll content and TBARS were not significantly affected (Fig. 1). It was also expected that brachycerine levels would increase due to oxidative stress, as a counteractive measure to modulate it (NASCIMENTO & FETT-NETO, 2010). However, under the high irradiance conditions used in the experiment, this alkaloid response did not take place.

There are no previous studies involving temperature and brachycerine, and there are few studies that demonstrate the relation between temperature and MIAs. It is known, however, that cold stress increases transcription, protein levels and activity of different ROS-scavenging enzymes, as well as the induction of H_2O_2 accumulation in cells (SUZUKI & MITTLER, 2006). In *Catharanthus roseus*, MIA biosynthetic pathway has been shown to be downregulated by cold (DUTTA et al., 2007). Our data for low temperature (10ºC) showed no change in the concentrations of brachycerine. This observation may be the result of brachycerine stability, at least within the time frame of the experiment.

Clearly, high temperature induced brachycerine accumulation. When acutely exposed to 40°C, for example, leaf disks accumulated approximately 16 times more

brachycerine than under control conditions (Fig. 2). Although significantly higher than the respective control treatment, the content of alkaloid upon acute exposure to 40°C in the second group of experiments had a lower fold increase (1.5 times) compared to the first group of experiments (brachycerine fold increase of this treatment in relation to control in Fig 2 and Fig 3, respectively). This discrepancy is possibly due to a prior induction in the field of the leaves harvested to prepare disks in the second group of experiments, which is indeed apparent based on the large difference in basal control content between these experiments (experiments Fig. 2 = 0.051 ± 0.026 versus experiments Fig. 3 = 0.767 ± 0.083, % d.w).

Besides accelerating metabolism, heat can cause malfunction in mitochondria and chloroplasts, often leading to oxidative damage and lipid peroxidation (SUZUKI & MITTLER, 2006; MANSOOR & NAVQI, 2013). Nevertheless, none of the conditions tested caused significant lipid peroxidation. At the highest temperature, cell damage was lower than in control conditions. However, under these circumstances large increases in brachycerine accumulation were also observed (Fig. 2 and Fig. 3). This suggests a possible relation between brachycerine accumulation and protection against the damage that commonly occurs at elevated temperatures, possibly as part of a strategy to prevent or mitigate cell damage. This potential protective function of brachycerine was also reported under other stresses, such as treatment with abscisic acid, heavy metals, and osmotic agents (NASCIMENTO et al., 2013).

Brachycerine accumulation was higher when temperature was progressively raised than when abruptly increased (Fig. 3). However, the acclimation assay did not show progressive accumulation of brachycerine as part of an adaptation response to deal with the increase in temperature. Surprisingly, we found that up to 35ºC there was no

significant difference in the production of this MIA, which accumulated expressively only when the disks were submitted to a temperature of 40°C (Fig. 3). This indicates that there is a threshold for the induction of brachycerine by heat, apparently reached when the cells are under a temperature of approximately 40°C.

5 – Conclusion

It is noteworthy that in stressful conditions of 40ºC, brachycerine levels in leaf disks are high, TBARS levels are low and chlorophyll levels remain constant. This indicates that under high temperature conditions, cell integrity is maintained and alkaloid metabolism is strongly stimulated. The relation between high levels of brachycerine and low cellular damage at high temperature suggests that this MIA takes part in the defense of *P. brachyceras* against oxidative stress caused by high temperature.

Contributions

AGFN designed the experiments and finalized the manuscript. HNM contributed in laying out the experimental design and carrying out HPLC, lipid peroxidation, and statistical analyses. RAJCT participated in all parts of the experiments and drafted the manuscript.

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