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Stress induction of valuable secondary metabolites in *Hypericum polyanthemum* acclimatized plants

J. de Matos Nunes [a,*], L.O.O. Bertodo [a], L.M.G. da Rosa [b], G.L. Von Poser [a], S.B. Rech [a]

[a] Faculdade de Farmácia, Departamento de Produção de Matéria-Prima, UFRGS, Av. Ipiranga 2752, 90610-000 Porto Alegre, RS Brazil
[b] Faculdade de Agronomia, Departamento de Plantas Forrageiras e Agrometeorologia, UFRGS, Av. Bento Gonçalves 7712, 91509-900 Porto Alegre, RS Brazil

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ABSTRACT

Uliginosin B, an antidepressant-like phloroglucinol derivative, three benzopyrans and total phenolic compounds (TPC) of flowering *Hypericum polyanthemum* acclimatized plants were quantitatively analyzed in response to stress factors. Drought stress alone or combined with mild fertilization; continuous salicylic acid (SA) application alone or combined with mild fertilization and time course effects of mechanical damage and SA exposure alone and combined were analyzed. Drought stress did not affect final plant biomass, promoted significant increase of uliginosin B (40-fold in the leaves and 6-fold in the reproductive parts), HP1, HP3 and TPC, whereas the stress applied on mild fertilized plants increased biomass and uliginosin B (7.5-fold and 1.5-fold in the leaves and reproductive parts, respectively) and HP3 (4.4-fold in the leaves) concentrations, unaffecting TPC. Continuous application of 2 mM SA alone or combined with mild fertilization resulted in similar increase of uliginosin B and TPC. Time course experiments of mechanical damage with and without SA (10 mM) as well as the treatment with SA induced the biosynthesis of uliginosin B in the leaves, with higher levels verified in wounded plants after 2 days (5-fold) and 7 days (3-folds) of treatment, reducing or not affecting benzopyrans accumulation. The compiled results suggest that a substantial increase of the most important pharmacological active compounds uliginosin B and HP1, associated with higher plant biomass yield can be achieved by modulating drought stress response in fertilized plants.

1. Introduction

Uliginosin B, a valuable dimeric phloroglucinol derivative produced by *Hypericum* species from Trigynobrathys section in south Brazil, including *Hypericum polyanthemum*, showed antichemotactic activity (Barros et al., 2013a), antinociceptive effect (Stolz et al., 2012) and antidepressant-like activity being considered a promising new molecular pattern for the development of antidepressant drugs (Stein et al., 2012). In addition, *H. polyanthemum* accumulates three benzopyrans named 6-isobutyryl-5,7-dimethoxy-2,2-dimethyl-benzopyran (HP1), 7-hydroxy-6-isobutyryl-5-methoxy-2,2-dimethylbenzopyran (HP2) and 5-hydroxy-6-isobutyryl-7-methoxy-2,2-dimethyl-benzopyran (HP3) (Ferraz et al., 2001), with bioactivities (von Poser et al., 2006) while HP1 is highlighted for its antinociceptive effect (Haas et al., 2010). Chlorogenic acid and flavonoids such as hyperoside, quercitrin, isoquercitrin and guaijaverin are also described for the species (Nunes et al., 2010). The absence of compounds such as rutin (Nunes et al., 2010), hypericins (Ferraz et al., 2001) and hyperforins (Ccana-Capatinta et al., 2014) as well as dark glands structures are also reported for *H. polyanthemum* and endorses a hypothesis of chemotaxonomic importance for dimeric phloroglucinol synthesis in species from Trigynobrathys section (Ferraz et al., 2002; Crockett, 2012, Barros et al., 2013b; Ccana-Ccapatinta et al., 2014). Considering the importance of *H. polyanthemum* and its natural compounds likewise the possibility of phytochemical increase and plant preservation, in vitro (Bernardi et al., 2007) and ex vitro (Bernardi et al., 2008) cultivation protocols were established for the species.

Plants secondary metabolites may respond to treatment with various elicitors, signal compounds and abiotic stresses (Zhao et al., 2005; Vasconsuelo and Boland, 2007). Moreover, it is well known that plant response on the synthesis and the accumulation of a certain natural product are influenced and determined by numerous factors acting together (Vasconsuelo and Boland, 2007; Zhu et al., 2009; Selmar and Kleinwächter, 2013). In order to improve crop quality of medicinal plants, it is necessary to understand which kind of stress condition enhances the accumulation of the target compounds without causing losses in biomass or general plant health, as well as analyze these responses in the different plant parts of interest (Selmar and Kleinwächter, 2013).

Strategies to improve the yield production in the genus *Hypericum* demonstrated that drought stress increased the concentration of betulinic acid, quercetin and rutin in *Hypericum brasiliense* (de Abreu and Mazzafera, 2005), while 8 of the 10 metabolites examined in a

* Corresponding author. Tel.: +55 51 33085686; fax: +55 51 33085437.
E-mail address: jessica.nunes@ufrgs.br (J. de Matos Nunes).

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study with *Hypericum perforatum* showed that besides the stress, plants phytochemistry and dry weight were also influenced by the time of harvest (Gray et al., 2003). In another study performed with leaf tissues of *H. perforatum* grown under water stress condition, both hypericin and pseudohypericin concentrations reduced with time, while the hyperforin concentration increased significantly (Zobayed et al., 2007).

Studies on the salicylic acid application in *H. perforatum* plantlets demonstrated to be effective in the induction of hypericins and hyperforin (Sirvent and Gibson, 2002) and in stimulating the production of hypericins in shoot cultures of *Hypericum maculatum* and *Hypericum hirsutum* (Coste et al., 2011). Furthermore, positive results in inducing the accumulation of phenolic compounds on *H. perforatum* plantlets and cells treated with *Colletotrichum gloeosporioides*, a plant pathogen that causes anthracnose, have also been reported (Conceição et al., 2006), while *Diploceras hypericinum* fungus and the plant growth promoting bacterium *Pseudonomas putida* were able to induce the synthesis of chlorogenic acid, rutin, hyperoside, isoquercetin, quercitrin and quercetin in *H. perforatum* and *Hypericum triquetrifolium* (Çirak et al., 2014).

The study of *H. polyanthemum* propagation (Bernardi et al., 2007, 2008; Nunes et al., 2009a) and long-term mild fertilization of acclimatized plants improved plant yields with the increase of HP3 concentration and maintenance of the aforesaid metabolites contents (Nunes et al., 2009b). Moreover, the continuous treatment of acclimatized plants with dried autoclaved cell powder of the fungus *Nomuraea rileyi* resulted in biomass and major secondary metabolite increase (Meirelles et al., 2013). Thereby, the current study was attempted to evaluate uliginosin B, benzopyrans and total phenolic compounds (TPC) yields of *H. polyanthemum* acclimatized plants submitted to drought stress alone and combined with mild fertilization; to continuous salicylic acid (SA) application alone and combined with mild fertilization. The time course effects of mechanical damage and SA alone and combined were also analyzed.

2. Materials and methods

2.1. Plant material and culture conditions

A pot experiment with clonal propagated *H. polyanthemum* was cultured and acclimatized as previously described (Bernardi et al., 2008). Plantlets cultured in vitro for 8 weeks were transferred to plastic pots containing a sterile mixture of non-fertilized commercial soil and vermiculite (1:2, v/v), covered with transparent plastic sheets and kept under controlled environmental conditions (25 ± 1 °C, 16 h photoperiod, irradiance of 70 μmol m^{-2} s^{-1} and irrigation with sterile distilled water once a week) for 30 days. Plants were transplanted to pots (18 cm diameter × 14 cm high) containing commercial soil, daily irrigated with water and grown on open field with irradiance of 2000 μmol m^{-2} s^{-1} at plant level, measured in the middle of sunny days without clouds with a sensor Quantum Li-cor. Induction treatments were applied from the beginning until the end of the acclimatization period (18 weeks) or just in the last 14 days of cultivation, before harvest.

2.2. Induction treatments

Acclimatized plants were subjected to stress treatments as follows: 1 — Drought stress: after 16 weeks of acclimatization plants were subjected to 2 weeks of drought stress by daily irrigation with 20 mL of water; 2 — Drought stress + fertilization: acclimatizing plants were drought stressed (treatment 1) after week 16 and during all acclimatization period fertilized by weekly irrigation with 20 mL of 50% MS nutrient solution (Murashige and Skoog, 1962) without sucrose; 3 — Continuous SA application: plants were sprayed every week with 10 mL of 2 mM SA during all acclimatization period; 4 — Continuous SA application + fertilization: plants were weekly sprayed with 10 mL of 2 mM SA and received 20 mL of 50% MS nutrient solution without sucrose, during all the acclimatization period; 5 — Mechanical damage: leaves of 17 — weeks acclimatized plants were wounded with a fine sterile needle; 6 — Salicylic acid — the upper parts of 17-weeks acclimatized plants were sprayed with 10 mL of 10 mM SA; and 7 — Mechanical damage + SA: upper parts and mechanical damaged leaves (treatment 5) of 17-week acclimatized plants were sprayed with 10 mL of 10 mM SA. For the analysis of the treatments 5, 6 and 7, a time course experiment was also carried out to measure uliginosin B and TPC after 1, 2 and 7 days of treatment. After 18 weeks of growth, plants of all treatments were harvested, vegetative and total reproductive yields were recorded and bioactive metabolites were quantified. Three replicates were used per treatment, and each replicate consisted of six plants. The pots were randomly rearranged fortnightly to minimize possible positional effects.

2.3. Uliginosin B and benzopyran determination

The metabolites were quantified in the *n*-hexanic extract obtained from 0.05 g DW of freeze-dried plants. HPLC using a Shimadzu 600 pump (LC-6AD) and a Shimadzu SPD-10A dual absorbance detector was operated according to the methods previously described. The phloroglucinol derivative was separated under isocratic solvent condition (95% CH_3CN, 5% H_2O, 0.01% TFA) and identified at 220 nm by comparison with the retention time of an authentic sample and co-injection of isolated compound (Nunes et al., 2009b). Benzopyrans were eluted under isocratic solvent condition (60% CH_3CN, 40% H_2O) and identified at 270 nm by comparison with the retention time of authentic samples and co-injection of isolated compounds (Bernardi et al., 2007).

2.4. Determination of total phenol content

Freeze-dried powdered material (0.1 g DM) was extracted 5 times at room temperature with 5 mL of methanol, under 20 min sonication. Phenolic compounds were assayed by the method adapted from Singleton and Rossi (1965). Appropriate dilutions of the samples were oxidized with 0.2 N Folin Ciocalteu reagent (2 N, diluted 10-fold), neutralized with saturated sodium carbonate solution (75 g L^{-1}) and the resultant absorbance was measured at 765 nm with an ultraviolet–visible BIOESPECTRO SP-200 spectrophotometer after incubation for 40 min at room temperature. TPC was expressed as milligrams of quercetin equivalent (QE) per gram of dry plant weight through a calibration curve with quercetin (Ivanova et al., 2005), ranging from 50 to 500 μg mL^{-1} ($R^2 > 0.99$).

2.5. Statistical analysis

ANOVA was performed using the SPSS version 16 software to determine if the studied parameters were significantly different (Tukey's test with $P < 0.05$).

3. Results

3.1. Effects on plant growth

Flowering *H. polyanthemum* field grown acclimatized plants subjected to a 14-day drought stress period showed that the dry mass of vegetative and reproductive parts were not affected (Table 1), whereas mild fertilization plants subjected to drought conditions demonstrated that the combined effects resulted on 2-fold increment on dry biomass of vegetative and reproductive parts, compared to the control treatment. On the other hand, a reduction of 13% and 20% of vegetative and reproductive parts, respectively, were obtained in this treatment when compared to fertilization (Table 1).

Table 1
Dry weight (g) of *Hypericum polyanthemum* acclimatized plants in response to (A) water stress alone or combined with mild fertilization and to continuous salicylic acid application alone or combined with mild fertilization and (B) time course effect of salicylic acid and mechanical damage stress, alone or combined.

A

	Vegetative parts	Reproductive parts	Total biomass
Control	$1.23^{cd} \pm 0.20$	$0.59^{b} \pm 0.08$	$1.83^{b} \pm 0.18$
Drought	$1.24^{d} \pm 0.08$	$0.48^{b} \pm 0.03$	$1.72^{b} \pm 0.11$
Fertilization	$2.61^{a} \pm 0.01$	$1.21^{a} \pm 0.02$	$3.82^{a} \pm 0.18$
Drought + fertilization	$2.27^{ab} \pm 0.21$	$0.97^{a} \pm 0.04$	$3.24^{a} \pm 0.24$
Continuous SA treatment	$0.77^{bc} \pm 0.06$	$0.82^{c} \pm 0.15$	$1.59^{b} \pm 0.07$
Continuous SA + fertilization	$1.07^{ab} \pm 0.11$	$1.06^{bc} \pm 0.11$	$2.13^{b} \pm 0.07$

VP: Vegetative parts; RP: Reproductive parts. Different letters within a stress treatment indicate significant differences at Tukey's test

B

	Vegetative parts			Reproductive parts			Total biomass		
	Day 1	Day 2	Day 7	Day 1	Day 2	Day 7	Day 1	Day 2	Day 7
Control	1.15 ± 0.07	1.04 ± 0.24	1.18 ± 0.07	0.56 ± 0.06	0.52 ± 0.03	0.55 ± 0.06	1.71 ± 0.51	1.50 ± 0.11	1.61 ± 0.06
Mechanical damage	1.19 ± 0.23	1.17 ± 0.21	1.21 ± 0.23	0.50 ± 0.05	0.49 ± 0.03	0.52 ± 0.10	1.69 ± 0.21	1.71 ± 0.23	1.63 ± 0.13
SA	1.16 ± 0.17	1.19 ± 0.22	1.10 ± 0.11	0.50 ± 0.02	0.52 ± 0.02	0.53 ± 0.03	1.67 ± 0.17	1.68 ± 0.23	1.63 ± 0.13
Mechanical damage + SA	1.18 ± 0.23	1.21 ± 0.19	1.15 ± 0.15	0.51 ± 0.01	0.50 ± 0.02	0.50 ± 0.05	1.70 ± 0.23	1.71 ± 0.21	$1.65^{b} \pm 0.17$

Plants exposed to the continuous application of a 2 mM SA solution showed increase in the reproductive part dry matter (40% than control plants) and decrease in the vegetative part (40% than control plants). Moreover, the weekly application of a 2 mM SA solution to mild fertilized plants demonstrated a 2-fold increase on reproductive part dry matter and unchanged vegetative part dry matter compared to well-watered plants (Table 1). The time course experiments of mechanical damage with and without the application of salicylic acid (10 mM) unaffected plants dry biomass.

3.2. Effects on bioactive metabolites accumulation

Acclimatized plants subjected to drought stress showed significantly higher yields of uliginosin B (40-fold in the leaves and 6-fold in the

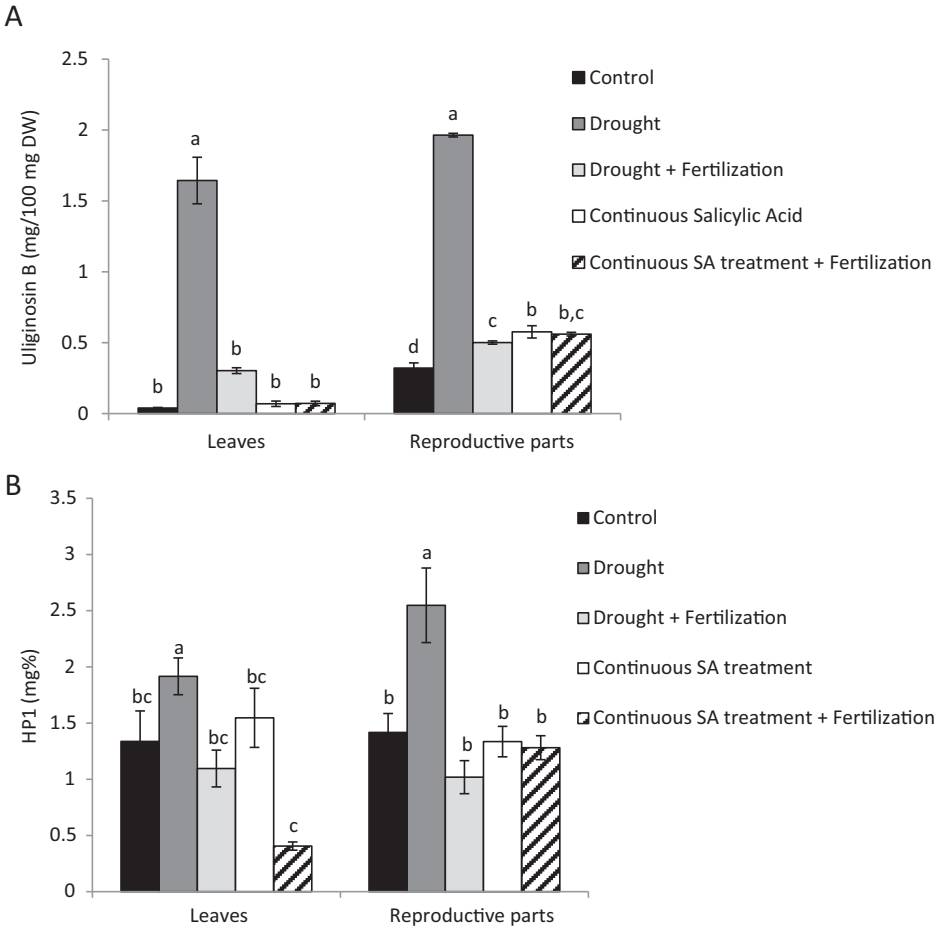

Fig. 1. Uliginosin B (A), HP1 (B), HP2 (C), HP3 (D) and TPC (E) production in leaves and reproductive parts of 18 week *H. polyanthemum* acclimatized plants submitted to water stress alone or combined with mild fertilization and to continuous salicylic acid application alone or combined with mild fertilization.

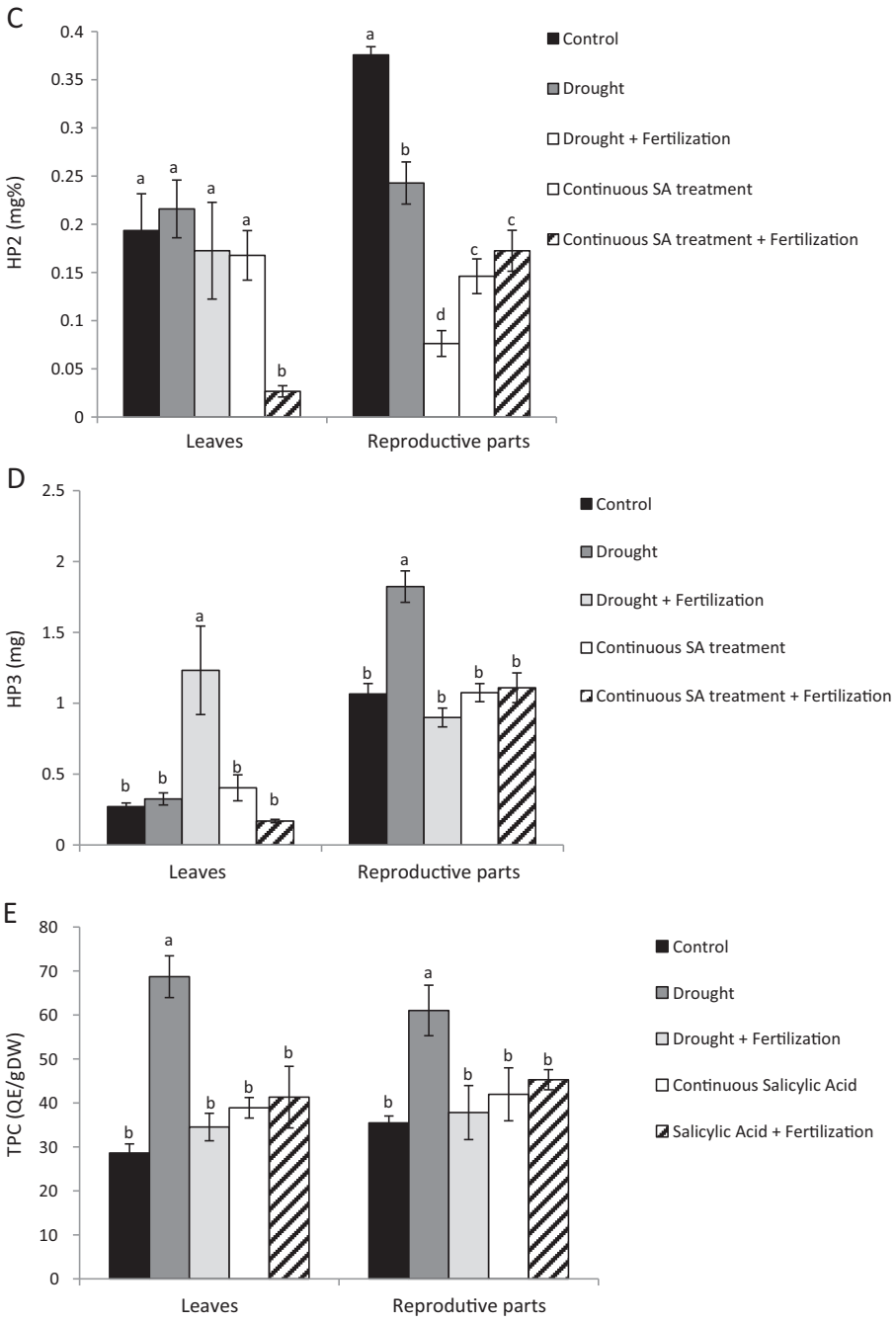

Fig. 1 (*continued*).

reproductive part, Fig. 1a), HP1 (1.4-fold in the leaves and 1.8-fold in the reproductive part, Fig. 1b), HP3 (1.6-fold in the reproductive part, Fig. 1d) and of TPC (2.4-fold and 1.7-fold increase, respectively, Fig. 1e), compared to levels accumulated in control plants.

The investigation of the combined effects of mild fertilization throughout the cultivation period and drought-stress demonstrated that the phloroglucinol derivative concentrations (7.5-fold and 1.5-fold in the leaves and reproductive part) and HP3 in leaves (4.4-fold) were higher than control plants, while HP1, HP2, HP3 in reproductive part and TPC were unaffected. Nevertheless, excepting HP3, the measured metabolite yields were found to be smaller than those found in plants submitted to drought only.

Continuous application of a 2 mM solution of salicylic acid alone or combined with mild fertilization resulted in similar contents of uliginosin B and TPC in treated plants, being 2-fold and 1.4 superior, respectively, than control plants in the analyzed plant parts (Fig. 1). Considering the three benzopyrans, salicylic acid application showed similar amount of all metabolites, excepting HP2 in reproductive parts that was approximately 2.5-fold smaller than control plants.

Time course experiments carried out to investigate the effect of mechanical damage with and without the application of salicylic acid (10 mM) as well as the treatment with salicylic acid only demonstrated that all treatments induced the biosynthesis of uliginosin B in the leaves of the plants, with higher levels verified in wounded plants after 2 days of treatment (5-fold of the control plants, Fig. 2a). Moreover, the accumulation of the metabolite in the reproductive parts of the plants increased (65% compared to control plants) in the treatment with SA only, after 7 days of application. Concerning TPC, increased levels were

quantified in the leaves after 1 and 2 days of mechanical damage and mechanical damage + SA treated plants and after 7 days of SA treated plants (Fig 2e). On the other hand, smaller or similar accumulation of the three benzopyrans was quantified in treated plants compared to controls, excepting HP3 that reached maximum accumulation in leaves in the first day after mechanical damage decreasing afterwards, being also stepwise increased during the days after mechanical damage combined with SA application in reproductive parts (Fig. 2d).

4. Discussion

Large scale cultivation of medicinal plants aims biomass increase with predictable extracts profiles. Therefore, many agronomic factors should be considered when developing production strategies for medicinal plant cultivation. For *Hypericum* genus efforts have been made on several investigations with *H. perforatum* (Sirvent and Gibson, 2002; Çirak et al., 2007) while some other studies are directed to main phenolic compounds produced for the genus considering biotic (Conceição et al., 2006; Meirelles et al., 2013; Çirak et al., 2014) and abiotic factors (Zhao et al., 2005; Gadzovska et al., 2007).

The results from this study indicated that drought stress period imposed on flowering *H. polyanthemum* acclimatized plants did not limit the growth neither affected dry mass of vegetative and reproductive parts. Acute drought stress imposed on *H. perforatum* has also been reported to not adversely affect the vegetative parameters, while the flower dry weight decreased significantly (Gray et al., 2003). Moreover, the drought stress imposed on mild fertilized *H. polyanthemum* plants promoted a 2-fold increase on biomass, demonstrating that the fertilization could improve water-use efficiency, mitigating the stress effects on plant growth. The results of this experiment are in agreement with the report of Zhu and co-workers (2009), who demonstrated that the decrease in root and shoot dry weight due to water-stress was smaller in fertilized treatments compared to the unfertilized control. Similarly,

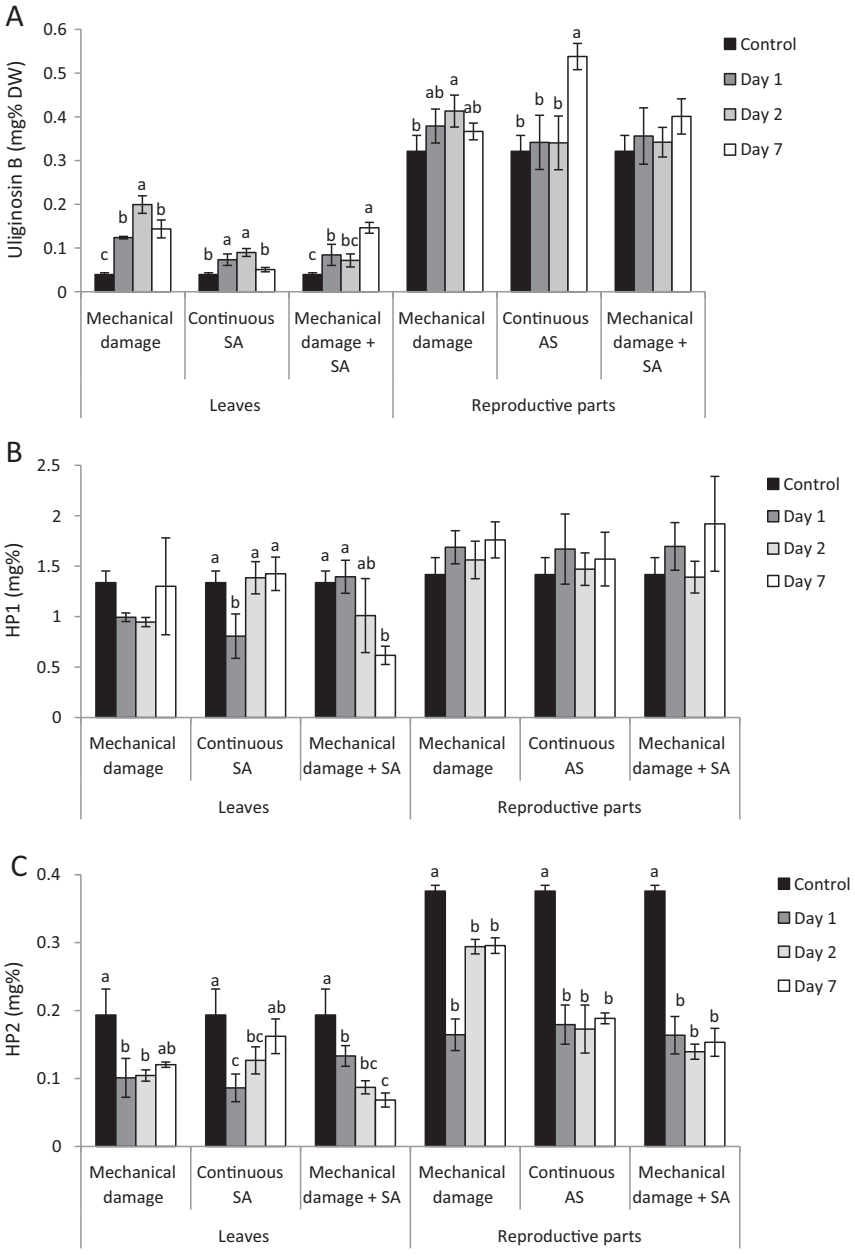

Fig. 2. Uliginosin B (A), HP1 (B), HP2 (C), HP3 (D) and TPC (E) accumulation in leaves and reproductive parts of 18 weeks *H. polyanthemum* acclimatized plants subjected to time course effect of salicylic acid and mechanical damage stress, alone or combined.

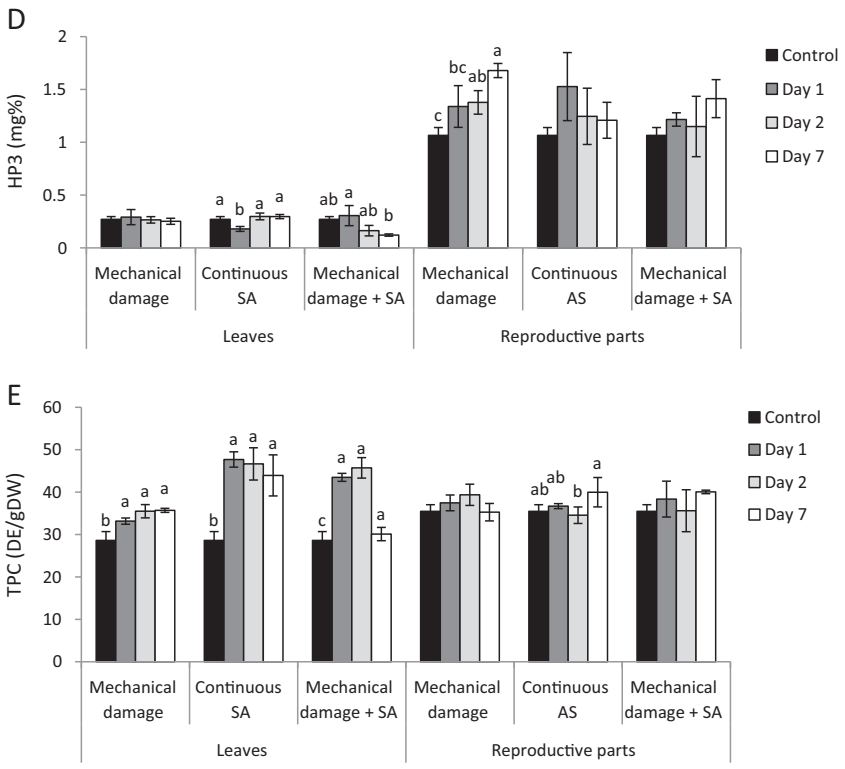

Fig. 2 (*continued*).

fertilization increased the growth of *Eucalyptus grandis* (Graciano et al., 2005) and *Mentha arvensis* (Ram et al., 2006).

Salicylic acid has been found to play a key role in the regulation of plant growth, development, interaction with pathogens and in the responses to environmental stresses (Zhao et al., 2005; Hayat et al., 2010). Nevertheless, the study of the effect of SA on biomass accumulation in cell suspension cultures of *H. perforatum* demonstrated that the elicitor failed to induce cell growth (Walker et al., 2002) and the treatment of in vitro cultured *H. perforatum* with the compound has been reported to negatively affect the growth of plantlets (Sirvent and Gibson, 2002). The weekly supplementation of 2 mM SA solution during all acclimatization of investigated plants in this study showed significant increase in the reproductive part dry matter and a decrease tendency in the vegetative parts. On the other hand, mild fertilized plants that received the same SA treatment demonstrated decreased dry matter biomass compared to fertilized plants only. Moreover, the time course experiment with a single 10 mM SA application on flowering plants, after mechanical damage with and without the application of 10 mM SA demonstrated not to affect plant dry biomass. The knowledge that different plant species, including ornamental plants, flowered earlier when they received an exogenous foliar spray of SA (Martin-Mex et al., 2005) and that the induction of flowering is one of the functions of this compound (Rivas-San Vicente and Plasencia, 2011) can be attributed to the increment in dry biomass of acclimatized plants continuously treated with 2 mM SA.

The ecological roles of plant secondary compounds range from protecting plants from environmental stresses to defense against the attack of insects, herbivores and pathogens, and a large number of elicitors of biotic and abiotic origin have been exploited to improve the yields of such plant metabolites (Zhao et al., 2005; Bruni and Sacchetti, 2009; Çirak et al., 2014). Plants show a great variety of responses to water deficit on a physiological, morphological and developmental basis. Water-stress can alter the oxidative balance of cells and acclimation to drought is generally correlated with keeping the level of active oxygen species (AOS) relatively low through the antioxidant system (Dat et al., 2000) and consequently activating the expression of genes encoding antioxidant enzymes (Torres-Franklin et al., 2008).

Investigations suggest that oxidative stress plays an important role in the synthesis of secondary metabolites in plants (Selmar and Kleinwächter, 2013) and the results of the present study demonstrated high increases on uliginosin B, HP1 and TPC contents in the leaves and reproductive parts, likewise HP3 in reproductive parts of stressed *H. polyanthemum* plants. These aforementioned findings are in agreement with the reports that water-stress effect on the content of active constituents of *H. brasiliense* increased phenolic compounds, betulinic acid and isouliginosin B in stressed plants (de Abreu and Mazzafera, 2005) and with the observation that leaves from greenhouse-grown *H. perforatum* plants under a water stress condition had a significantly higher capacity to detoxify oxygen radicals, with 2.5-fold increase over the antioxidant potential of the leaves of control plants, suggesting that the higher accumulation of the acylphloroglucinol hyperforin under water-stress conditions may be an important part of the complex antioxidant system (Zobayed et al., 2007). On the other hand, the combined effects of mild fertilization throughout the cultivation period and drought-stress demonstrated that the concentration of the phloroglucinol derivative in both analyzed plant parts and HP3 in leaves were higher than that in control plants, while HP2, HP3 and TPC were unaffected. Nevertheless, the measured metabolite yields were found to be smaller than those found in plants submitted to drought only. It is important to consider that drought did not decrease total plant biomass and dramatically enhanced uliginosin B and HP1, accumulating double yields of fertilized plants submitted to drought.

Mechanical damage in *H. polyanthemum* leaves significantly increased the uliginosin B concentration, whereas had only a small effect on TPC and decreased HP2 in leaves. Moreover, the intentionally imposed injury in the leaves did not influence uliginosin, HP1 and TPC yields of the reproductive parts of the plants, while reduced HP2 and increased HP3. Many responses occur as wounding stress is involved

in the activation of the expression of wound-inducible genes and such processes can start between a few minutes and several hours after wounding (Leon et al., 2001). In the present study, HP3 concentration on reproductive parts displayed increasing values throughout the days and the highest uliginosin B concentration accumulated after 2 days of imposed mechanical damage in the plant leaves, coinciding with the results obtained with the mechanical damage of *Psychotria brachyceras* leaves, where a twofold increase concentration of the bioactive indole alkaloid brachycerine was observed (Gregianini et al., 2004).

Several compounds have been proposed to play a role in wound signaling, such as phytohormones, reactive oxygen species, oligosaccharides and salicylic acid (Zhao et al., 2005). The latter, is a hormone-like substance that has important role in the regulation of plant growth and development (Hayat et al., 2010) and was also investigated in this study. The application of a 10 mM SA solution alone showed higher concentration of the uliginosin B in the leaves and reproductive parts of treated plants after 2 and 7 days of treatment, respectively, while the TPC contents were significantly higher in the leaves and unchanged in the reproductive parts. Moreover, plants subjected to a single (10 mM) or to the continuous (2 mM) application of SA accumulated similar levels of the analyzed metabolites. The work concerning the influence of SA (1 to 5 mM) in *H. perforatum* plantlets was also been reported to significantly increase the hyperforin biosynthesis after a week of application (Sirvent and Gibson, 2002). Regarding benzopyrans, it has noted a slightly decrease on compounds concentration in leaves one day after SA application which is partially or totally recovered until the seventh day after treatment. In flowers, SA reduced or did not affect metabolite concentrations. These observations allow to hypothesize that SA only is not a suitable strategy to induce benzopyrans synthesis.

Prior studies with the species described protocols capable to efficient increase plant biomass, benzopyrans and flavonoids production (Nunes et al., 2009b; Meirelles et al., 2013), but it is the first time that a massive increase is observed for HP1 and uliginosin B, valuable compounds of *H. polyanthemum*. In summary, the results indicate that environmental stress, particularly drought has a marked influence on *H. polyanthemum* secondary metabolites, especially on uliginosin B and HP1 contents, pharmacologically active molecules. These results also indicate the possibility of increasing the content of bioactive metabolites by manipulating cultivation techniques through the establishment of a production protocol consisting of drought combined to other stresses treatment of acclimatized plants.

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