RESEARCH ARTICLE

Level and distribution of 20-hydroxyecdysone during *Pfaffia glomerata* development

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The ecdysteroid 20-hydroxyecdysone (20E) is a steroid hormone found in insects and few plants. To analyze its temporal and spatial distribution in *Pfaffia glomerata* (Amaranthaceae), we selected among 71 accessions, the accession 13 which showed the highest root 20E total quantity (1.48 g/plant root). The 20E was constantly detected in flowers, leaves, stems, and roots, but its percentage was variable throughout its development. The highest 20E percentage was found in flowers (0.82%), roots (0.66%), leaves (0.60%), and stems (0.24%). While stems showed the least variable 20E percentage (0.13% - 0.24%), followed by roots (0.42% - 0.66%), and flowers (0.47% - 0.82%). Leaves showed the greatest 20E percentage variation (0.21% - 0.60%). The stem and leaves 20E total amount remained constant, showing a little variation while root 20E total amount increased over time, suggesting that 20E may be accumulated in roots. These findings suggest that 20E has tissue specific functions in plant and may have ecological significance since uncoordinated pulses of 20E are often lethal to insects.

Key Words: *Pfaffia glomerata* (Spreng.) Pedersen; Amaranthaceae; Brazilian ginseng; phytochemical analysis; terpenes; phytoecdysteroid; 20-hydroxyecdysone.

INTRODUCTION

Ecdysteroids are steroid hormones found in arthropods (zooecdysteroids - ZEs) and plants (phytoecdysteroids - PEs). Their structures are available on Ecdybase (Lafont et al., 2002). Plants and insects only rarely have the same ecdysteroids. In insects, embryogenesis, larval development, metamorphosis, and molting are controlled or elicited by specific pulses of the ZE 20-hydroxyecdysone (20E), which is constantly required for embryonic development to be completed (Makka et al., 2002).

In plants, the function of PE is still unknown (Slama, 1993; Machackova et al., 1995; Dinan, 2001). However, it is suspected that PEs may have three different roles: 1) as physiologically active compounds (Dinan, 1998; Golovatskaya, 2004); 2) as compounds that protect plants from unadapted phytophagous insects (Lafont, 1997); and, 3) as compounds that supply the polyhydroxylated phytosterols that are required for growth and cell proliferation (Machackova et al., 1995). In addition, the PE 20E may have many pharmacological and medicinal effects on mammals (Slama and Lafont, 1995; Dinan, 2001; Lafont and Dinan, 2003; Bathori and Pongracz, 2005; Dinan and Lafont, 2006; Festucci-Buselli et al., 2008).

The occurrence of PEs has been examined in only about 2% of world's flora. In addition, only 5-6% of tested species have PEs and its percentage is typically only about 0.1% of the plant dry mass (Dinan, 2001). PEs are present in fruits, seeds, flowers, anthers, leaves, stems, and roots of few plants during their life cycle (Grebenok and Adler, 1991; Grebenok et al., 1991; Adler and Grebenok, 1995; Adler and Grebenok, 1999), but their precise ecological, physiological, and biochemical functions are unknown. In spinach, the distribution of 20E occurs in apical leaves and stems (Grebenok and Adler, 1991; Grebenok et al., 1991).

Pfaffia glomerata (Amaranthaceae), traditionally known as Brazilian ginseng, is a Brazilian medicinal plant that produces the PE 20E (Shiobara et al., 1993). Traded as an alternative for the Asian ginseng (*Panax* spp. - Araliaceae), *Pfaffia* roots have been popularly used to treat or prevent diseases (Lorenzi and Matos, 2002). Several papers focusing on its potential pharmacological and medicinal properties have been published (De-Paris et al., 2000; Freitas et al., 2004; Neto et al., 2004; Marques et al., 2004; Neto et al., 2005; Teixeira et al., 2006).

The presence of 20E in *P. glomerata* is interesting since this compound may have agrochemical, biotechnological, medicinal, and pharmaceutical uses as well as a role in plant physiology (Festucci-Buselli et al., 2008). However, 20E temporal and spatial distributions are still unknown in this species. This information is important to estimate the ecological, physiological, and biochemical functions of 20E. The *P. glomerata* accession 13, which showed the highest root 20E quantity among 71 available accessions in our germoplasm bank, was chosen for a temporal and spatial analysis of 20E percentage using HPLC.

MATERIALS AND METHODS

Germoplasm Bank Accessions: The 71 germoplasm bank accessions of *Pfaffia glomerata* (Amaranthaceae) were collected in Brazil as listed below: accession number 1 (Viçosa, State of Minas Gerais); 2 and 5 (Unknown origins); 3 (Vicent Pires, Capital); 4 (Fortaleza, State of Ceará); 6 (Olinda, State of Pernambuco); 7-24 and 48-49 (Ivaí River bank, State of Paraná); 25-36 and 50-55 (Mineiro Island, State of Paraná); 37-47 and 56-60 (Baitaporã, State of Mato Grosso do Sul); and, 61-71 (Vila Alta, State of Paraná).

Plant disinfection: To set up our *in vitro* germplasm bank, plants were vegetatively propagated by stem cuttings and grown under greenhouse conditions. They were sprayed two times, once per week, with a fungicide Captan® (2 g/L) (Captan 500 PM). After bud break, actively growing stem flushes (2-3 cm height) with 2-3 axillary dormant buds were removed from donor plants and washed with sterile water for 60 min. They were sequentially immersed in ethanol 70% (v/v) for 30 s, in Captan solution (2 g/L) for 30 min, in sodium hypochlorite 0.4% (w/v) (Globo® UFE, Brazil) plus 2 drops of Tween-20 for 15 min, and rinsed five times in sterile distilled water. Unless stated otherwise, all reagents were purchased from Sigma-Aldrich Co, St Louis, MO. All procedures were carried out under sterile conditions in a laminar flow hood (VECO®, Brazil).

Tissue culture establishment: To obtain axenic cultures, tissue culture medium was initially established to control funghi and bacteria by adding Captan (2 g/L) and chloranfenicol (15 mg/L) to the MS-based medium with vitamins (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose (Vetec®, Brazil) and myo-inositol (100 mg/L). To prevent oxidation of phenol, ascorbic acid (0.15 mg/L), citric acid (0.1 mg/L), activated charcoal (1 g/L), L-cystein (0.02 mg/L) and polyvinylpyrrolidone (1 g/L) were also incorporated into the establishment medium. Unless otherwise stated, all media were prepared with 0.7% (w/v) agar (Merck, Germany) and its pH was adjusted to 5.7 $\pm$ 0.1 before autoclaving at 120 °C, 1.1 kgf cm^{-2} for 20 min. Aliquots of 10-15 mL were poured into test tubes (25 X 150 mm), closed with polypropylene lids (Sigma Chem. Co, USA). Surface-sterilized nodal explants (with 1-2 axillary buds) were individually transferred to test tubes. Cultures were incubated in a growth room at 25 $\pm$ 2 °C, under a 16 h photoperiod and PFD (Photon Flow Density) of 35 μmol m^{-2} s^{-1} provided by two cool white fluorescent tubes (20 W, Phillips, Brazil).

Shoot proliferation and rooting: Shoot induction and rooting were achieved on a MS medium containing vitamins, and supplemented with 3% (w/v) sucrose and myo-inositol (100 mg/L) devoid of growth regulators. Elongated shoots rooted spontaneously. The cultures were maintained by subcultures every 25-30 days, using either nodal or apical explants.

Acclimatization: Rooted plants were individually transferred to plastic pots containing 30 mL of sterile distilled

water, covered with a transparent plastic bag, and kept under a 16 h photoperiod and PFD of 35 μmol m^{-2} s^{-1} in a growth room at 25 ± 2 °C for two days. They were transferred to plastic containers filled with horticulture organic substrate (Plantmax®, Brazil) in a shaded house (50% light interception). Plastic bags were progressively opened by lateral cuts throughout the first two weeks of the hardening-off process. At the end of the second week, the containers were completely uncovered. Plants were irrigated on a daily basis.

Transplanting: Acclimatized plants were transplanted into 20 L containers filled with substrate composed of soil:sand:manure (4:1:1). In each pot, macronutrients [N (6.40 g), P (9.78 g), K (5.31 g), Ca (6.41 g), Mg (1.15 g), Fe (0.07 g)] and micronutrients [B (14.44 mg), Mn (73.19 mg), Zn (26.08 mg), Mo (1.32 mg), Cu (0.08 mg), Co (0.08 mg)] were added before transplantation. Plants were watered daily.

The 20-hydroxyecdysone (20E) analysis: The quantification of 20E present in the methanolic extract was performed by HPLC. To obtain a methanolic extract, 200 mg of powdered flower, leaf, stem, and root were individually added in 20 mL of methanol and incubated at 25 °C for seven days under continuous agitation. The resulting solution was then centrifuged at 12.000 x *g* for 5 min. HPLC analyses were performed using a Shimadzu LC 10AD apparatus equipped with an SPD-10A UV detector set at 245 ηm, a Bomdesil C_{18} column (5.0 μm x 4.6 mm x 250 mm), and a slow flow (1.2 mL/min) of methanol: water solution (1:1) (v/v). A 50 μL volume of the methanolic extract was injected for 20E quantification. The 20E standard was purchased from Sigma-Aldrich Co. (St Louis, MO).

Determination of root 20-hydroxyecdysone (20E) distribution in the germplasm bank accessions: To examine root dry mass and 20E percentage, 71 germplasm bank accessions (three replicates of each plant, total of 213 plants) were acclimatized and transplanted into 20 L pots, as previously described. These values were evaluated about nine months after transplanting into pots. To obtain root dry mass, roots were individually sliced and dried at 50 °C until constant dry mass. Dried roots were weighed, powdered and stored at -80 °C until 20E percentage was determined. Values of root dry mass were multiplied by values of root 20E percentage (on a dry mass basis), resulting in values of root 20E total amount.

Determination of 20-hydroxyecdysone (20E) level and distribution in different organs of the accession 13: A total of 84 plants (accession 13) were acclimatized and transplanted into 20 L pots. They were set in four independent blocks, each containing 21 plants. Every month, 12 plants (three from each block) were randomly analyzed. Flowers, leaves, stems, and roots from these plants were individually sliced, dried at 50 °C until constant dry mass, weighed, and powered. They were stored at -80 °C until 20E percentage was evaluated independently in each organ. Values of dry mass were multiplied by values of 20E percentage (on a dry mass basis), resulting in values of 20E total amount.

RESULTS

Analysis of root 20E distribution in Pfaffia glomerata germplasm bank accessions: To choose a specific germplasm bank accession that combines both a high root dry mass and a high root 20E percentage (on a dry mass basis), 71 germplasm bank accessions of *P. glomerata* were analyzed. The root 20E percentage was accessed by HPLC. The 20E retention time in extractive solution was 4.5 minutes. Correlations between root dry mass and root 20E percentage in each accession revealed that 20E total quantity was above 1.0 g/plant root in only eight accessions (4, 13, 19, 40, 46, 47, 56, and 57) and below 0.5 g/plant root in 19 accessions (5, 6, 9, 27, 29, 31, 35, 49, 51, 54, 55, 58, 60, 63, 65, 66, 67, 68, and 70). In the remaining 44 accessions, 20E total quantity was between 0.5 – 1.0 g/plant root (Fig. 1). Because accession 13 had a combination of both a high root dry mass (230.75 g) and high root 20E percentage (0.64%), accumulating the highest root 20E total amount (1.48 g/plant root) (Fig. 1), it was chosen for further analysis.

Analysis of 20E level and distribution in different organs of the accession 13: To analyze 20E level and distribution in different organs during the development of *P. glomerata* (accession 13), dry mass accumulation (Fig. 2A) was analyzed in flowers, leaves, stems, and roots harvested on a monthly basis, and their 20E percentage (on a dry mass basis) was assessed by HPLC (Fig. 2B). The results indicated that 20E was consistently detected in all analyzed organs, but its percentage was variable throughout the development of *P. glomerata* (Fig. 2B).

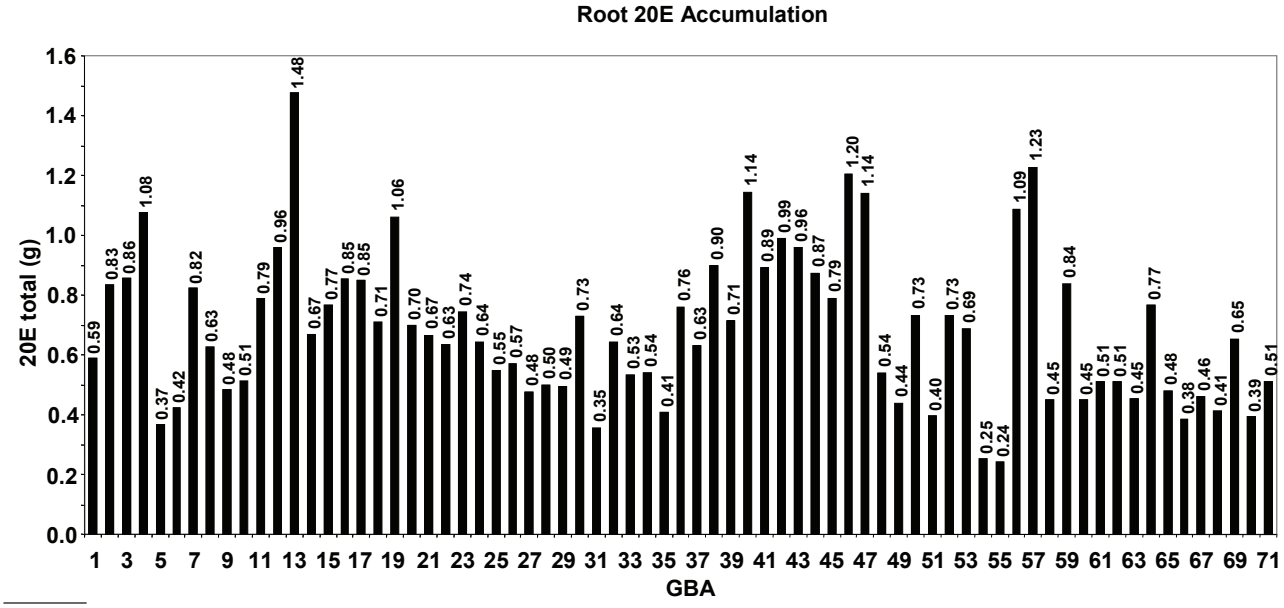

Figure 1. Root 20E analysis in 71 germplasm bank accessions (GBA) of *Pfaffia glomerata* by HPLC. To calculate root 20E total quantity, the average of the values of root dry mass was multiplied by the average of the values of root 20E percentage. As an example (accession 13): 230.75 g x 0.64% ≈ 1.48 g of 20E.

Coincidently with periods in which the temperature was cooler (16.2 – 16.5 °C), the lowest 20E percentage was found in flowers, leaves and roots at the same time of the development (90 - 120 days) (Fig. 2B). The decrease of root 20E percentage in the first month may be explained by the fact that plants were transplanted from 200 mL pots into 20 L pots. Overall, from 0 – 60 days, there was less intense dry mass accumulation (Fig. 2A), an increase in the flower, root and stem 20E percentages and a decrease in leaf 20E percentage (Fig. 2B). From 60 – 120 days, there was more intense dry mass accumulation (Fig. 2A), and although stem and root 20E percentages were initially reduced, it was maintained at constant levels, showing a little variation while flower and leaf 20E percentages decreased (Fig. 2B). From 120 – 210 days, there was less intense dry mass accumulation (Fig. 2A), an increase in flower, leaf and root 20E percentages and a decrease in stem 20E percentage (Fig. 2B).

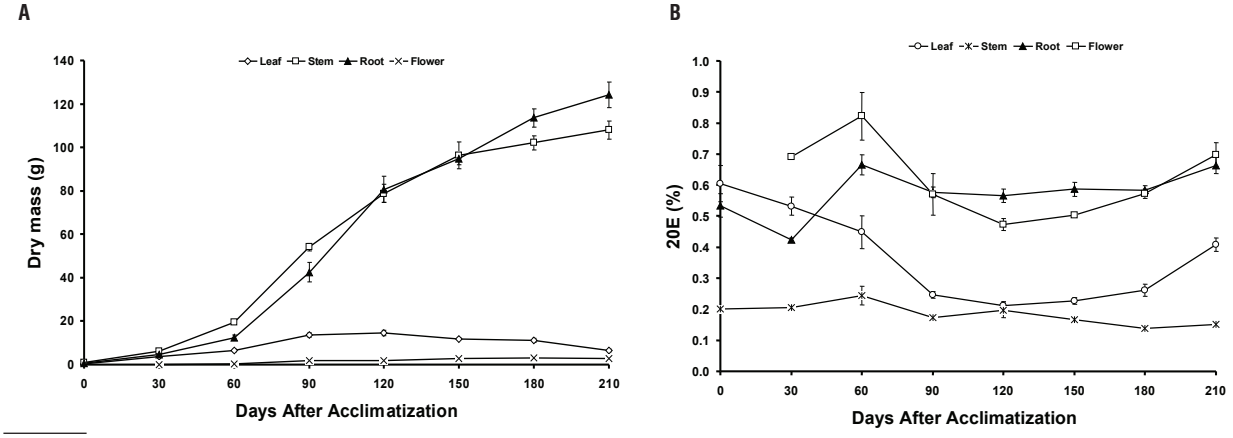

Figure 2. Dry mass accumulation (A) and 20E percentage analysis (B) in different organs of *Pfaffia glomerata* (accession 13) by HPLC. Average of monthly temperature: 0 (March: 22.3 °C), 30 (April: 21.2 °C), 60 (May: 17.5 °C), 90 (June: 16.2 °C), 120 (July: 16.5 °C), 150 (August: 17.7 °C), 180 (September: 19.6 °C), and 210 (October: 21.8 °C). The standard error (SE) (n=12) is represented by vertical bars. When not shown, the SE was smaller than the symbols.

The highest 20E percentage was found in flowers (0.82%), roots (0.66%), leaves (0.60%), and stems (0.24%) (Fig. 2B). In contrast, the lowest 20E percentage was found in stems (0.13%), leaves (0.21%), roots (0.42%), and flowers (0.47%). Stems showed the least variable 20E percentage (0.13% - 0.24%), followed by roots (0.42% - 0.66%), and flowers (0.47% - 0.82%). Leaves showed the greatest variation in 20E percentage (0.21% - 0.60%) (Fig. 2B).

The analysis of 20E total amount during *P. glomerata* development revealed that it was predominantly accumulated in roots (Fig. 3) whereas in leaves and stems, it remained constant, showing a little variation (Fig. 3).

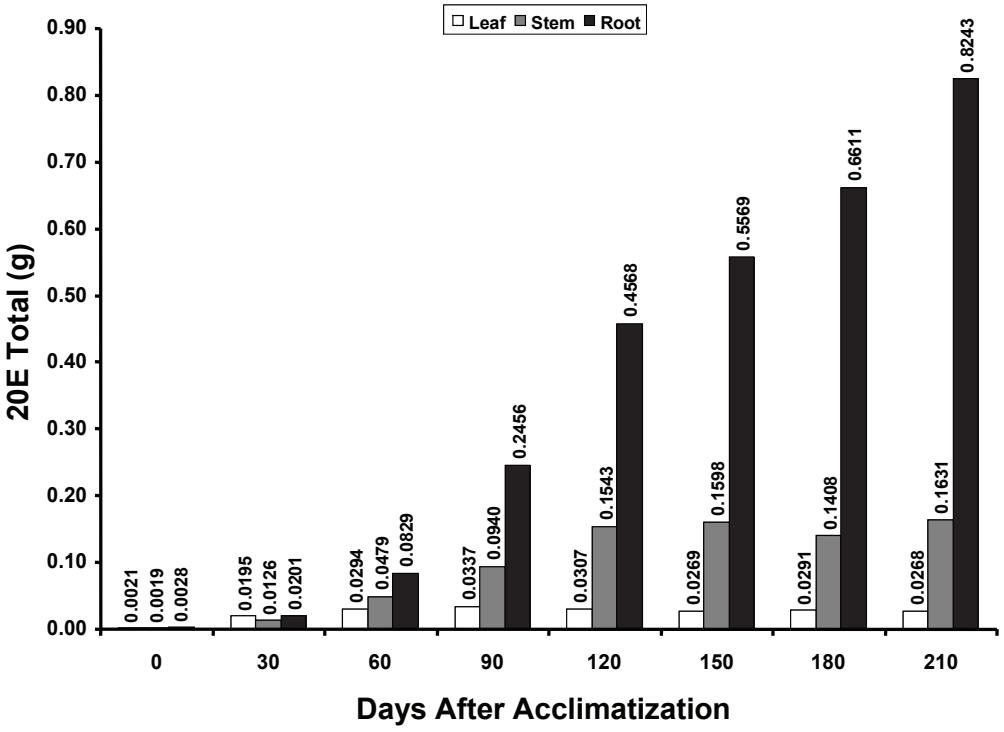

Figure 3. Analysis of 20E total quantity in leaf, stem, and root of *Pfaffia glomerata* (accession 13). The 20E total amount in each organ was estimated by multiplying the average of the monthly values of dry mass by the average of the monthly values of 20E percentage. As an example (leaf): 0.3483 g x 0.60% ≈ 0.0021 g of 20E.

DISCUSSION

Among the few plant species that are known to produce PEs, *P. glomerata,* a Brazilian perennial herbaceous plant, is a source of the PE 20E. It is therefore important to know if 20E is always detected in flowers, leaves, stems, and roots during its development to analyze 20E ecological, physiological and biochemical functions in this species. Between 71 analyzed accessions available in our germoplasm bank, accession 13 showed the highest root 20E total amounts (Fig. 1). It is possible that association of genetic background of each accession and several environmental factors (e.g. insect and funghi attacks) may control root dry mass and 20E production and they may explain this expected change found in between germplasm accessions (Fig. 1). Changes in root 20E percentage of *P. glomerata* were also reported by other research groups (Figueiredo et al., 2004; Corrêa Júnior et al., 2008).

The analysis of dry matter (Fig. 2A) and 20E percentage (Fig. 2B) revealed that stem and leaves 20E total amount showed a little variation while root 20E total amount increased over time (Fig. 3), indicating that 20E may be accumulated in *P. glomerata* roots. Although 20E was detected in all organs

(Fig. 2B), the precise sites of its biosynthesis (sources) and accumulation (sink) in *Pfaffia glomerata* remain unknown. In spinach, 20E may be biosynthesized in older leaves (sources) and translocated to young leaves (sink) where it may be accumulated (Bakrim et al., 2008).

The presence of high 20E percentage over several months in all analyzed organs (Fig. 2B) and its predominant accumulation in *P. glomerata* roots (Fig. 3) suggest that it can play an important role in plant life-cycle and plant-insect interactions and may confer enhanced resistance against below-ground herbivorous insects. In spinach, root phytoecdysteroid concentration increases in response to root injure while leaves phytoecdysteroid concentration is unchanged (Schmelz et al., 1998), suggesting that there may be an evolved defense mechanism against subterranean insect herbivores (Hunter, 2001). Plant defenses against herbivore insects involve the production of jasmonic acid (biosynthesized from the octadecanoid pathway), which activates defense responses against a large number of insects (Howe and Jander, 2008). There is an increment in 20E concentration after application of methyl jasmonate, suggesting involvement of jasmonate pathway in signaling the damage-induced accumulation of root 20E levels (Schmelz et al., 1998, 1999).

Since PEs have been found just in few plants, it may not be essential to plant's life, but it might confer some plant selective advantages. The potential dual functions of 20E in plants (physiologically active compound and protective compound against unadapted arthropods) (Festucci-Buselli et al., 2008) might suggest that the occurrence and increment of 20E in response to stresses may not only protect plants against unadapted insects, but may also be involved in other plant biochemical and physiological processes.

The potential ecological importance of 20E may be illustrated by several observations: *(i)* 20E deters oviposition and larval feeding (Calas et al., 2006); *(ii)* monophagous or oligophagous species feeding on host plants containing exogenous 20E were either deterred from feeding or showed abnormalities in growth and development (Blackford and Dinan, 1997); *(iii)* during sexual activity of the spider *Tegenaria atrica* 20E inhibits cannibalism and changes sex pheromone production (Trabalon et al., 2005); *(iv)* anomalous molting, immobility, reduced invasion, impaired development, and death were observed in nematodes exposed to 20E (Soriano et al., 2004); and, *(v)* 20E concentration increases in response to mechanical damage (Schmelz et al., 1998), insect herbivory (Schmelz et al., 1999), and application of methyl jasmonate (Schmelz et al., 1999). These findings suggest that the induction of PEs may protect plants from insect attack (Schmelz et al., 2002). It still needs to be investigated, if concentration of 20E in *P. glomerata* increases in response to these stresses, as well as if PEs may act as physiologically active compounds.

P. glomerata is an interesting plant to analyze 20E physiological and biochemical functions (Festucci-Buselli et al., 2008) since it may accumulate in its roots large quantities of 20E (Figs. 1, 2B and 3) as well as to study plant-insect interactions since 20E was always detected in all analyzed organs during its development (Figs. 2B and 3) and it may disrupt insect development. The isolation of genes involved in 20E biosynthesis is an important step into gaining insight into its ecological, physiological, and biochemical functions in plants. Once identified, it will be possible to examine the effect of knocking them out in *P. glomerata*.

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