REGINALDO ALVES FESTUCCI BUSELLI

ACCUMULATION PATTERN OF THE PHYTOECDYSTEROID 20-HYDROXYECDYSONE IN DIFFERENT ORGANS OF *Pfaffia glomerata*

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Pos-Graduate Program in Genetic and Plant Breeding for the obtention of the degree of *Doctor Scientiae*.

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APPROVED: January 22nd, 2008.

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I dedicate it to my beloved Ana Amélia and to my entire family. I would like to express my gratitude to my friend and advisor Dr. Wagner Campos Otoni. I would also like to acknowledge the awarded doctoral scholarship by FAPEMIG (Minas Gerais State Research Foundation, Brazil) and the Universidade Federal de Viçosa. I thank the Professors Dr. Jeff J. Stuart (Purdue University - USA) and Dr. Chandrashekhar P. Joshi (Michigan Technological University - USA) for their advice and helpful discussions. I also thank my friends Aloisio Stein Carvalho Dias, Eric Workman, Ismael Moustafa, Daniel Wiggins, Travis Hampshire, and Luis Antônio Serrão Contim for their support.

BIOGRAPHY

REGINALDO ALVES FESTUCCI BUSELLI, son of José Festucci Buselli and Elza Alves Buselli, was born on September 21, 1973, in the city of Morro Agudo (São Paulo State, Brazil). As a requirement to obtain his Italian Citizenship, his family name (Buzeli) was rectified (Festucci Buselli) in 2002.

He graduated in Agronomy from the Universidade Federal de Viçosa (UFV) in 1999. As an undergraduate student, he was a teaching assistant in Cell Biochemistry and was awarded scholarships from the Brazilian government for the entire four years. Throughout this time, he worked on the plant molecular chaperone called BiP (*binding protein*) and published two papers, in *The Journal of Biological Chemistry* and in *Plant Science*.

He was awarded a first place scholarship from the Brazilian government to work on his M.Sc. Degree (2000-2001) in Plant Physiology at UFV, finishing it in a year and a half. As a Master's student, he continued working on BiP and published a paper in *Plant Molecular Biology*. While pursuing his Master's degree, he was invited to work in the Entomology Department at Purdue University (USA), supported by Purdue Research Foundation.

As a researcher at Purdue University (2001-2003), he conducted research in biochemistry, genetics, and the molecular biology of insects using *D. melanogaster* as a model to study structure, organization, regulation, and function of P450 genes. Additionally, he advised undergraduate students. During this time, he collaborated with Dr. Jeff Stuart (Purdue University, USA). The data obtained resulted in the publication of two scientific articles, in the journals *Insect Molecular Biology* and in *Proteomics*.

He was also awarded a third place scholarship from the Brazilian government to work on his D.Sc. Degree (2005-2008) in Genetic and Plant Breeding at UFV, finishing it in two years and ten months. As a doctoral student, he was a teaching assistant in Cell Biology and worked with two distinct research

areas. One was about biosynthesis and regulation of cellulose synthase genes in higher plants. In collaboration with Dr. Chandrashekhar P. Joshi from Michigan Technological University (USA), he published a paper about this research in the *Brazilian Journal of Plant Physiology*. The second research area concerned biosynthesis, regulation, and functions of the phytoecdysteroid 20-hydroxyecdysone. Two scientific papers entitled "Biosynthesis and potential functions of the ecdysteroid 20-hydroxyecdysone" and "Accumulation pattern of the 20E in different organs of *Pfaffia glomerata"* were submitted for publication.

Since 1995, he has collaborated with his D.Sc. advisor, Dr. Wagner Campos Otoni, and he has performed research in plant physiology, genetics, biochemistry and the molecular biology of plants and insects. During this time, he has improved his skills in many aspects relating to scientific research including planning, execution, analysis, interpretation of data, and writing of scientific reports, proposals, and manuscripts as well as he had advised undergraduate students.

Published or developed scientific articles:

D.Sc.

1. Festucci-Buselli RA, Contim LAS, Barbosa LCA, Stuart JJ, Otoni WC (2008) Accumulation pattern of the 20E in different organs of *Pfaffia glomerata*. Submitted.

2. Festucci-Buselli RA, Contim LAS, Barbosa LCA, Stuart JJ, Otoni WC (2008) Biosynthesis and potential functions of the ecdysteroid 20-hydroxyecdysone – a review. Submitted.

3. Festucci-Buselli RA, Otoni WC, Joshi CP (2007) Structure, organization, and functions of cellulose synthase complexes in higher plants. Review. Brazilian Journal of Plant Physiology. 19:1-13.

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4. Pedra JHF, **Festucci-Buselli RA**, Sun W, Muir W, Scharf ME, Pittendrigh BR (2005) Profiling of abundant proteins associated with dichlorodiphenyltrichloroethane resistance in *Drosophila melanogaster*. Proteomics. 5:258-269.

5. Festucci-Buselli RA, Carvalho-Dias AS, Andrade MO, Caixeta-Nunes C, Li HM, Stuart JJ, Muir W, Scharf ME, Pittendrigh BR (2005) Two P450s Cyp6g1 and Cyp12d1 may be associated with DDT resistance in both laboratory and field selected strains of *Drosophila melanogaster*. Insect Molecular Biology. 14:69-77.

M.Sc.

6. Buzeli RAA, Cascardo JCM, Rodrigues LAZ, Andrade MO, Almeida RS, Loureiro ME, Otoni WC, Fontes EPB (2002) Tissue-specific regulation of BiP genes: a cis-acting regulatory domain is required for BiP promoter activity in plant meristems. Plant Molecular Biology. 50:757-771.

B.Sc.

7. Cascardo JCM, **Buzeli RAA**, Almeida RS, Otoni WC, Fontes EPB (2001) Differential expression of the soybean BiP gene family. Plant Science (Limerick). 160:273-281.

8. Cascardo JCM, Almeida RS, **Buzeli RAA**, Carolino SMB, Otoni WC, Fontes EPB (2000) The phosphorylation state and expression of soybean BiP isoforms are differentially regulated following abiotc stresses. The Journal of Biological Chemistry. 275:14494-14500.

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CHAPTER 2

RESUMO

FESTUCCI-BUSELLI, Reginaldo Alves, D. Sc., Universidade Federal de Viçosa, janeiro de 2008. **Padrão do acúmulo do fitoecdisteróide 20-hidroxiecdisona em diferentes órgãos de *Pfaffia glomerata***. Orientador: Wagner Campos Otoni. Co-Orientadores: Sérgio Yoshimitsu Motoike e Luiz Cláudio de Almeida Barbosa.

Os ecdisteróides são hormônios esteróides encontrados em insetos e plantas. Em insetos, vários processos que ocorrem durante o seu desenvolvimento são iniciados ou controlados por pulsos específicos do zooecdisteróide 20-hidroxiecdisona (20E). Em plantas, a biossíntese, a regulação e a função precisa do fitoecdisteróide 20E permanecem desconhecidas e três hipóteses sobre suas funções potenciais têm sido propostas: i) compostos fitohormonais; ii) compostos protetores contra insetos fitófagos não adaptados; e iii) fonte de fitoesteróides polihidroxilados requeridos no crescimento e proliferação celular. A 20E foi detectada nas raízes de *Pfaffia glomerata*, que foi selecionada para a condução de outros experimentos. Entre os 71 acessos de *P. glomerata* analisados, o maior acúmulo de 20E (1,48 g) foi encontrado nas raízes do acesso 13, que foi escolhido para a realização das análises de altura, massa seca, detecção, concentração e acúmulo de 20E. Durante o desenvolvimento do acesso 13, foram identificadas três fases distintas. Na primeira fase (0 – 60 dias), ocorreu intenso aumento da altura em combinação com um baixo acúmulo de matéria seca. Em contraste, durante a segunda fase (60 – 120 dias), ocorreu intenso acúmulo de matéria seca e um pequeno aumento da altura. Durante a terceira fase (120 – 210 dias), ocorreu acúmulo de matéria seca menos intenso em combinação com um pequeno aumento da altura. A 20E foi constantemente detectada em todos os órgãos analisados, mas sua concentração variou durante o desenvolvimento de *P. glomerata*. As maiores concentrações de 20E foram seqüencialmente encontradas em flores (0.8221%), raízes (0,6658%), folhas (0,6042%), e caules (0,2445%). Em contraste, as menores concentrações de 20E

foram consecutivamente encontradas em caules (0,1379%), folhas (0,2118%), raízes (0,4224%), e flores (0,4731%). Enquanto os caules apresentaram as menores variações nas concentrações de 20E (0,1379% - 0,2445%), seguidos pelas raízes (0,4224% - 0,6658%) e flores (0,4731% - 0,8221%), as folhas apresentaram as maiores variações nas concentrações de 20E (0,2118% - 0,6042%). Apesar da existência de variação na porcentagem de 20E em cada órgão analisado, o seu acúmulo apresentou um padrão específico. O padrão do acúmulo de 20E revelou que este foi predominantemente acumulado nas raízes (após 60 dias), enquanto nas folhas (após 60 dias) e nos caules (após 120 dias) o conteúdo total de 20E foi mantido constante, apresentando pequena variação.

ABSTRACT

FESTUCCI-BUSELLI, Reginaldo Alves, D. Sc., Universidade Federal de Viçosa, January 2008. **Accumulation pattern of the phytoecdysteroid 20-hydroxyecdysone in different organs of *Pfaffia glomerata***. Advisor: Wagner Campos Otoni. Co-Advisors: Sérgio Yoshimitsu Motoike and Luiz Cláudio de Almeida Barbosa.

The ecdysteroids are steroid hormones found in insects and plants. In insects, several developmental processes are controlled or elicited by specific pulses of the zooecdysteroid 20-hydroxyecdysone (20E). In plants, the biosynthesis, regulation, and precise functions of the phytoecdysteroid 20E remain unknown and three major hypotheses for their potential roles have been proposed: i) phytohormonal compounds; ii) protective compounds against unadapted phytophagous insects; and iii) supply of polyhydroxylated phytosterols required for growth and cell proliferation. The 20E was detected in the roots of *P. glomerata* that was chosen to carry out further investigations. Among the 71 analyzed accessions of *P. glomerata*, the highest root 20E accumulation (1.48 g) was found in the accession 13, which was selected to perform analysis of height, dry matter, 20E occurrence, concentration and accumulation. During the development of the accession 13, it was identified three distinct phases. In the first (0 – 60 days), an intense increase of height occurred in combination with a lower dry matter accumulation. In contrast, during the second (60 – 120 days), an intense dry matter accumulation occurred with a lower increase of height. During the third (120 – 210 days), a less intense dry matter accumulation occurred in combination with a lower increase of height. The 20E was constantly detected in all analyzed organs, but its concentration was variable throughout the development of *P. glomerata*. The highest 20E concentration was sequentially found in flowers (0.8221%), roots (0.6658%), leaves (0.6042%), and stems (0.2445%). In contrast, the lowest 20E concentration was consecutively found in stems (0.1379%), leaves (0.2118%), roots (0.4224%), and flowers (0.4731%). While stems showed the less

variable 20E concentration (0.1379% - 0.2445%), followed by roots (0.4224% - 0.6658%), and flowers (0.4731% - 0.8221%), leaves showed the more variable 20E concentration (0.2118% - 0.6042%). There is a variation of 20E percentage in each analyzed organ, but its accumulation followed a specific pattern. The accumulation pattern of 20E revealed that 20E was predominantly accumulated in roots (after 60 days) whereas in leaves (after 60 days) and stems (after 120 days), the 20E total content was kept constant, showing a little variation.

INTRODUCTION

The ecdysteroids (ECDs) are steroid hormones found in insects (zooecdysteroids, ZEs) and plants (phytoecdysteroids, PEs). In mammals, they do not seem to be produced, but they may have pharmacological and medicinal effects (Slama and Lafont, 1995; Dinan, 2001; Lafont and Dinan, 2003; Bathori and Pongracz, 2005; Dinan and Lafont, 2006). In insects, embryogenesis, larval development, metamorphosis, and molting are controlled or elicited by specific pulses of the ZE 20E which are constantly required for embryonic development to be completed (Makka et al., 2002). Each of the major developmental transitions in the life cycle of *D. melanogaster* is elicited by pulses of 20E (Thummel, 2001). Pulses of 20E precede each of the major developmental transitions (see Figure 7 of Chapter 1). The consecutive conversions of ketodiol, ketotriol, 2-deoxyecdysone, ecdysone, and 20-hydroxyecdysone are catalyzed respectively by the P450s CYP306A1 (*phantom*), CYP302A1 (*disembodied*), CYP315A1 (*shadow*), and CYP314A1 (*shade*). All these genes are involved in 20E biosynthesis (see Figure 8 of Chapter 1). The disruption of these genes causes disruption of embryonic development due to low levels of 20E (Gilbert, 2004; Petryk et al., 2003). Since the P450s have been found in all kingdoms (Feyereisen, 1999; Werck-Reichhart and Feyereisen, 2000), it would be possible to find P450s related to 20E biosynthesis in *P. glomerata*. In plants, three major hypotheses for their putative roles have been proposed: i) phytohormonal compounds (Dinan, 1998; Golovatskaya, 2004); ii) protective compounds against unadapted phytophagous insects (Lafont, 1997); and iii) supply of polyhydroxylated phytosterols required for growth and cell proliferation (Machackova et al., 1995).

The presence of PEs has been examined in about 2% of world's flora. Only 5-6% of the tested species have PEs and their content is around 0.1% of the plant dry matter (Dinan, 2001). The 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). As an illustration, the herbaceous perennial *Ajuga* produces and accumulates high levels of PEs in its

roots. There are others herbaceous perennial plants that accumulate PEs in their roots and distribute them to other organs during its ontogeny. The total PE content in herbaceous perennial plants is increased over the years (Adler and Grebenok, 1995). In contrast, for instance, spinach (*Spinacia oleracea*) which is an annual plant produces PEs in its aerial parts. Annual plants have to flower and produce their seeds which carry PEs that may be used to support the development of seedlings, allowing perpetuation of their species (Adler and Grebenok, 1995).

The PE 20E is an attractive phytochemical to perform further studies since it is likely that it might have agrochemical, biotechnological, medicinal, and pharmaceutical uses as well as it may affect in plants morphological and physiological process. The 20E was detected in the roots of *P. glomerata* (Shiobara et al., 1993). Our previous studies showed that 20E was present in the roots of all 71 analyzed accessions of *Pfaffia glomerata* germplasm bank (Kamada, 2006; unpublished data); however, in this specie, it is unknown i) its occurrence, concentration and accumulation in differents organs over time; ii) its biosynthesis, regulation, and precise functions; iii) if this metabolite is biosynthesized and accumulated in roots or if it is biosynthesized in leaves and translocated to roots; and iv) the exactly time in the plant development in which 20E shows its highest levels.

To investigate the occurrence, concentration and accumulation of 20E in flowers, leaves, stems, and roots of *P. glomerata*, it was selected one accession among the 71 available accessions which showed the highest 20E accumulation. On a monthly basis it was analyzed: a) the height of *P. glomerata* plants; b) the dry matter accumulation in flowers, leaves, stems, and roots; c) the 20E concentration in different organs; and d) the 20E accumulation in different organs. Here, we will show i) a review about biosynthesis and potential functions of the ecdysteroid 20E (Chapter 1) and ii) an article about accumulation pattern of the 20E in different organs of *Pfaffia glomerata* (Chapter 2).

REFERENCES

Adler JH, Grebenok RJ (1995) Biosynthesis and distribution of insect-molting hormones in plants - a review. Lipids 30:257-262.

Bathori M, Pongracz Z (2005) Phytoecdysteroids – from isolation to their effects on humans. Curr. Med. Chem. 12:153-172.

Dinan L, Lafont R (2006) Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. J. Endocrinol. 191:1-8.

Dinan L (2001) Phytoecdysteroids: biological aspects. Phytochemistry 57:325-339.

Dinan L (1998) A strategy towards the elucidation of the contribution made by phytoecdysteroids to the deterrence of invertebrate predators on plants. Russ. J. Plant Physiol. 45:296-305.

Feyereisen R (1999) Insect P450 enzymes. Annu. Rev. Entomol. 44:507-533.

Gilbert LI (2004) *Halloween* genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. Mol. Cell. Endocrinol. 215:1-10.

Golovatskaya IF (2004) Effect of ecdysterone on morphological and physiological processes in plants. Russ. J. Plant Physiol. 51: 407-413.

Grebenok RJ, Ripa PV, Adler JH (1991) Occurrence and levels of ecdysteroids in spinach. Lipids 26:666-668.

Grebenok RJ, Adler JH (1991) Ecdysteroids distribution during development of spinach. Phytochemistry 30:2905-2910.

Kamada T (2006) Avaliação da diversidade genética de populações de fáfia (*Pfaffia glomerata* (SPRENG.) PEDERSEN) por RAPD, caracteres morfológicos e teor de βeta-ecdisona. Tese de Doutorado, Universidade Federal de Viçosa, Viçosa, MG, Brazil.

Lafont R (1997) Ecdysteroids and related molecules in animals and plants. Arch. Insect Biochem. Physiol. 35:3-20.

Lafont R, Dinan L (2003) Practical uses for ecdysteroids in mammals including humans: an update. J. Insect Sci. 3:1-30.

Machackova I, Vagner M, Slama K (1995) Comparison between the effects of 20-hydroxyecdysone and phytohormones on growth and development in plants. Eur. J. Entomol. 92:309-316.

Makka T, Seino A, Tomita S, Fujuwara H, Sonobe H (2002) A possible role of 20-hydroxyecdysone in embryonic development of the silkworm *Bombyx mori*. Arch. Insect Biochem. Physiol. 51:111-120.

Petryk A, Warren JT, Marques G, Jarcho MP, Gilbert LI, Kahler J, Parvy JP, Li Y, Dauphin-Villemant C, O'Connor MB (2003) Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc. Natl. Acad. Sci. USA 100:13773-13778.

Shiobara Y, Inoue SS, Kato K, Nishiguchi Y, Oishi Y, Nishimoto N, De Oliveira F, Akisue G, Akisue MK, Hashimot G (1993). A nortriterpenoid, triterpenoids and ecdysteroids from *Pfaffia glomerata*. Phytochemistry 32:1527-1530.

Slama K, Lafont R (1995) Insect homones - ecdysteroids: their presence and actions in vertebrates. Eur. J. Entomol. 92:355-377.

Thummel CS (2001) Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. Dev. Cell. 1:453-465.

Werck-Reichhart D, Feyereisen R (2000) Cytochromes P450: a success story. Genome Biol. 1 (Reviews 3003).

CHAPTER 1

Biosynthesis and potential functions of the ecdysteroid 20-hydroxyecdysone – a review

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ABSTRACT

Ecdysteroids (ECDs) are steroid hormones found in arthropods (zooecdysteroids - ZEs) and plants (phytoecdysteroids - PEs). A vast structural diversity of PEs is found in several plant and insect species. Ecdysone (E) and 20-hydroxyecdysone (20E) are ECDs present in both insects and plants. In insects, 20E controls or elicits molting and other developmental processes. Several characterized P450 enzymes are involved in 20E biosynthesis. The disruption of the gene called *shade* which encodes the P450 enzyme CYP314A1 responsible for the conversion of E into 20E causes embryonic lethality due to low levels of 20E. In plants, 20E may act as a defensive substance against unadapted insects or nematodes and may be used in crop protection strategies. It has also been proposed that 20E, being a physiologically active compound, may affect morphological and physiological processes in plants. It is proposed that C_{27} phytosterols (PS) are the precursors of the PE 20E. However, both the enzymes

and the 20E intermediates are unknown. In spinach, it has been shown that lathosterol may be the 20E precursor. Usually, the content of PEs is about 0.1% of the plant's dry matter, sometimes achieving 0.5 – 3.2%. The root 20E concentration in *Pfaffia glomerata* is about 0.65%. The 20E might have agrochemical, biotechnological, medicinal, and pharmaceutical applicability. The biosynthetic pathways as well as the precise biochemical and physiological functions of 20E in plants are unknown.

Key words: Ecdysone (E), 20-hydroxyecdysone (20E), Ecdysteroid (ECD), Zooecdysteroid (ZE), Phytoecdysteroid (PE), Phytosterol (PS), *Pfaffia glomerata*.

INTRODUCTION

The ecdysteroids (ECDs) are steroid hormones found in insects (zooecdysteroids, ZEs) and plants (phytoecdysteroid, PEs). For example, Ecdysone (E) and 20-hydroxyecdysone (20E) are ECDs present in both. In mammals, they do not seem to be produced, but they may have pharmacological and medicinal effects (Slama and Lafont, 1995; Dinan, 2001; Lafont and Dinan, 2003; Bathori and Pongracz, 2005; Dinan and Lafont, 2006). Several ECDs have been isolated from animal and plant sources and their structures are available on Ecdybase (Lafont et al., 2002). In plants, PEs may act as defensive substances (Dinan, 2001) and may be used in crop protection strategies (Dinan, 1995b). It has been also proposed that 20E may affect morphological and physiological processes in plants, being a physiologically active compound (Golovatskaya, 2004).

Several plant species including ferns, gymnosperms and angiosperms produce PEs (Dinan et al., 2001). The occurrence of PEs has been analyzed in about 2% of world's flora. Only 5-6% of the tested species have PEs and their content is around 0.1% of the plant dry matter (Dinan, 2001). High levels of PEs (3.2% of the bark dry matter) were found in *Diploclisia glaucescens* (Bandara et al., 1989). PEs have also been detected in *Pfaffia glomerata* (Shiobara et al., 1993)*, Pfaffia paniculata, Pfaffia stenophylla, Pfaffia iresinoides, Polypodium aureum, Polypodium lepidopteris, and Polypodium vulgare,* amid others (Lafont and Dinan, 2003). Among crop species, just few have been shown to produce detectable levels of PEs such as spinach (*Spinacia oleracea*) (Grebenok and Adler, 1991; Grebenok et al., 1991) and quinoa (*Chenopodium quinoa*) (Dinan, 1995a).

In insects, molting and other developmental processes (embryogenesis, larval development, and metamorphosis) are controlled or elicited by the PE 20E (see bellow biosynthesis and metabolism of 20E in insects). These developmental processes are synchronized by specific pulses of 20E which are constantly required for embryonic development to be completed (Makka et al., 2002). Uncoordinated pulses of 20E might lead insects to death. Several characterized

P450 enzymes are involved in 20E biosynthesis and its intermediates are known. In plants, 20E biosynthesis, its intermediates, and its precise functions are unknown. The comparison between both insect and plant 20E biosynthesis pathways may provide clues about the regulation of 20E biosynthesis and its mode of action. Furthermore the identification of compounds which interfere with the mode of action of ECDs or with ECD biosynthesis may provide new strategies to control insects which damage several crop species.

The *Pfaffia glomerata* (SPRENG.) PEDERSEN, a Brazilian medicinal plant, produces 20E (Shiobara et al., 1993). Its presence in this plant might explain why its roots are widely used in Brazilian traditional medicine to cure or prevent several diseases (Lorenzi and Abreu Matos, 2002). Given 20E pharmacological and medicinal properties and *P. glomerata* medicinal uses, it would be of considerable relevance to perform detailed clinical trials to evaluate its importance as a potential medicine as well as to understand the 20E biosynthesis and its biochemical and physiological functions on this species. It might be a promising plant for studying 20E biosynthesis, given that it produces large quantities of 20E as well as for studying plant-insect interactions. The understanding of the biochemical mechanisms and pathways by which plants produce 20E is crucial to quickly produce larger quantities at lower costs.

In this review, we will highlight the importance, structure, and potential functions of the PEs. The 20E biosynthesis in plants and in insects as well as *P. glomerata* relevant information will also be discussed.

IMPORTANCE OF PHYTOECDYSTEROIDS

The PEs might have agrochemical, biotechnological, medicinal, and pharmaceutical uses, and may potentially be involved in biochemical and physiological processes in plants. It has been suspected that PEs deter invertebrate predators, being effective toxins which cause hormonal disruption upon ingestion or acting as antifeedants towards unadapted insect species (Dinan, 1998). Crop species producing high levels of PEs might control unadapted invertebrate predators. It has also been proposed that the PE 20E may affect morphological and physiological processes in plants. It appears to be a physiologically active compound being involved in coleoptile elongation, α-amylase activation, and leaf yellowing retardation (Golovatskaya, 2004).

Several interesting features which would allow their use as medicine are displayed by PEs. They show low toxicity to mammals (LD_{50} > 6g/kg). They are rapidly eliminated, showing an effective half-life of elimination of 4 hours for E and 9 hours for 20E. The PEs are not hypertensive and they do not have androgenic, oestrogenic, or antioestrogenic effects and they do not induce virilization (Lafont and Dinan, 2003). Besides these features, it is likely that 20E possesses many pharmacological and medicinal effects on mammals or humans. It seems that 20E stimulates growth, accelerates bone fracture healing, wound healing and skin regenerating properties, increases milk production, enhances sexual functions, stimulates protein biosynthesis (anabolism), stimulates the incorporation of glucose into glycogen and protein (glycoprotein), enhances glucose utilization, reduces cholesterol biosynthesis and increases its catabolism, improves nervous functions, stimulates hepatic functions, improves heart and lung functions, improves renal functions, and antioxidant properties (Slama and Lafont, 1995; Dinan, 2001; Lafont and Dinan, 2003; Bathori and Pongracz, 2005; Dinan and Lafont, 2006). Given the broad PE pharmacological and medicinal properties, it has been reported that PEs might be a universal medicine (Lafont and Dinan, 2003).

The PEs may be used as inducers for gene switch systems based on insect ECD receptor proteins and genes of interest placed under the control of ECD

response elements. Indeed, PEs show important features of gene switch systems: i) they do not seem to bind to vertebrate steroid receptors; ii) they show low toxicity to mammalian cells; iii) they show specificity to ECD receptor complex; iv) they do not seem to be synthesized by mammalian cells; v) they are relatively easily distributed within cells; and vi) they are available in plants in relatively large quantities. Thus, it may be possible to have a spatial and temporal control of heterologous gene expression. It would be of considerable relevance to basic and applied medical research, involving gene therapy and functional genomics (Lafont and Dinan, 2003; Bathori and Pongracz, 2005; Dinan and Lafont, 2006).

Given the importance of PEs, it is essential to understand their precise biochemical and physiological functions in plants and their role in plant-insect interactions as well as to evaluate their use as a potential medicine.

STRUCTURE AND TYPES OF PHYTOECDYSTEROIDS

The ECDs comprise a class of steroids (cyclopentano-perhydro-phenanthrene) with a beta side chain at C_{17} (Figure 1) (Bathori and Pongracz, 2005).

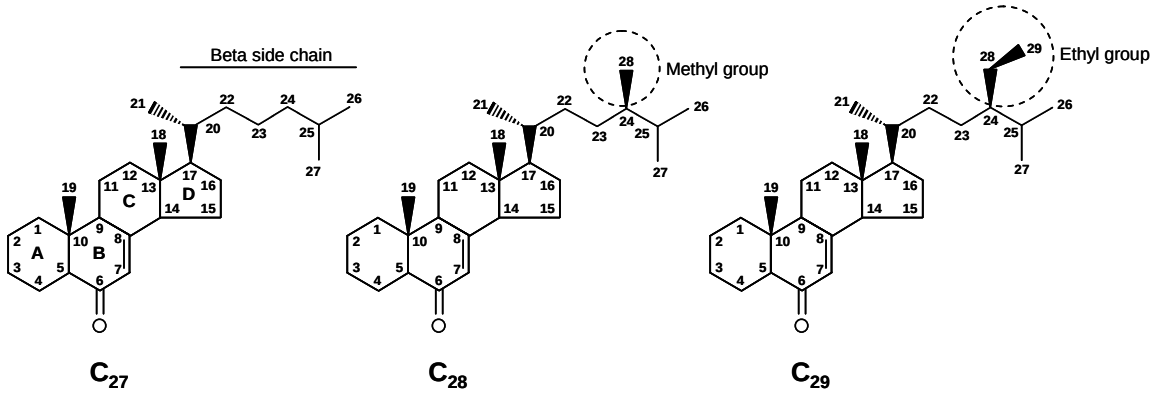

Figure 1. General structure of C_{27}, C_{28}, and C_{29} ECDs.

The ECDs are structurally different from mammalian steroids. PEs and ZEs have a polyhydroxylated side chain that mammalian steroid hormones lack (Figure 2). Consequently, they are less lipophilic than mammalian steroid hormones, being soluble in the aqueous cell environment and in lipophilic cell membranes. Thus, they are relatively easily distributed within cells (Gilbert et al., 2002).

PEs seem to be synthesized from C_{27}, C_{28}, or C_{29} PSs. Most C_{27} PEs are derived from cholesterol. Some C_{28} or C_{29} PEs are derived from PSs, possessing an alkyl group at C_{24} (Figure 1). The cleavage of the side chain generates C_{19}, C_{21}, and C_{24} ECDs. Major alterations on the sterol structure may be necessary to produce most of the ECDs: i) conversion of the trans A/B ring junction in sterol to the cis A/B ring junction in the ECD; ii) addition of a 7-en-6-one chromophore in the

B ring; iii) addition of α-hydroxyl group at C_{14}; and iv) addition of β-hydroxyl group at C_3 (Figure 3 A and B).

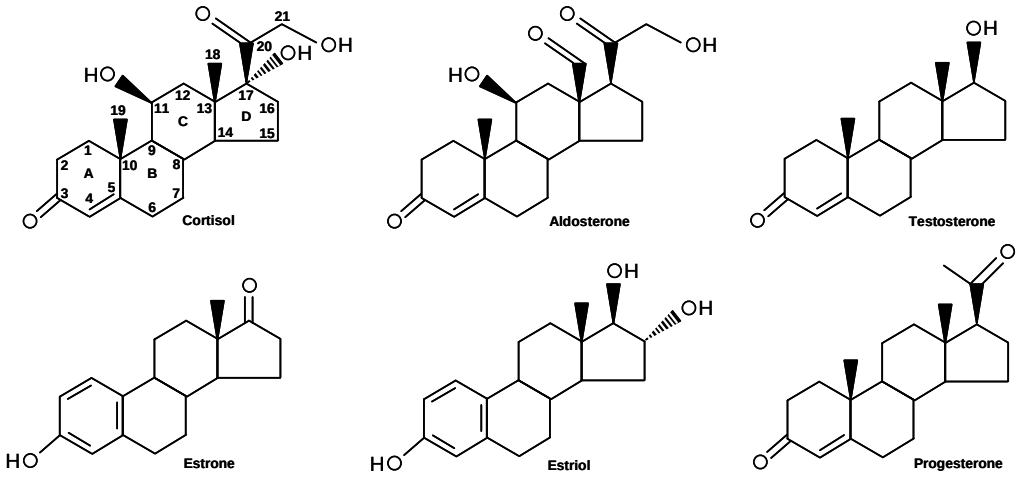

Figure 2. Major human steroid hormones.

The A/B ring junction is usually a *cis* (5β-H) conformation, except in few cases the conformation is *trans* (5α-H). The B/C and C/D ring junctions are always *trans*. The methyl groups at C_{10} and C_{13} have a β-configuration (Figure 1) (Adler and Grebenok, 1999; Dinan, 2001; Bathori and Pongracz, 2005). The 14α-hydroxy-7-en-6-one chromophore is responsible for ultraviolet (UV) absorption with λ_{max} at 242 nm in methanol (Dinan, 2001). To increase ECDs detection specificity, HPLC combined with UV detection at 254 nm, instead of 242 nm, has been used in ECDs analysis (Bathori et al., 2000).

Usually, two to eight hydroxyl groups are present on ECDs. Except for C_7, C_{15}, and C_{18}, any other carbon atom may be hydroxylated. Additional or alternative double bond may be present at one of these locations $C_{4\text{-}5}$, $C_{8\text{-}9}$, $C_{9\text{-}11}$, $C_{12\text{-}13}$, $C_{14\text{-}15}$, $C_{24\text{-}25}$, $C_{25\text{-}26}$, or $C_{24\text{-}28}$. In one of the following positions C_2, C_3, C_{12}, C_{17}, C_{20}, or

C_{22} a second oxo group may be found. Almost all ECDs which show hydroxylations at the positions C_1, C_5, and C_{11} are PEs. Only PEs possess hydroxylations in the following positions C_9, C_{12}, C_{17}, C_{19}, C_{21}, C_{24}, C_{28}, and C_{29} (Figure 1). The PEs may be found conjugated with both organic and inorganic acids, organic alcohols, and glycosides (Bathori and Pongracz, 2005).

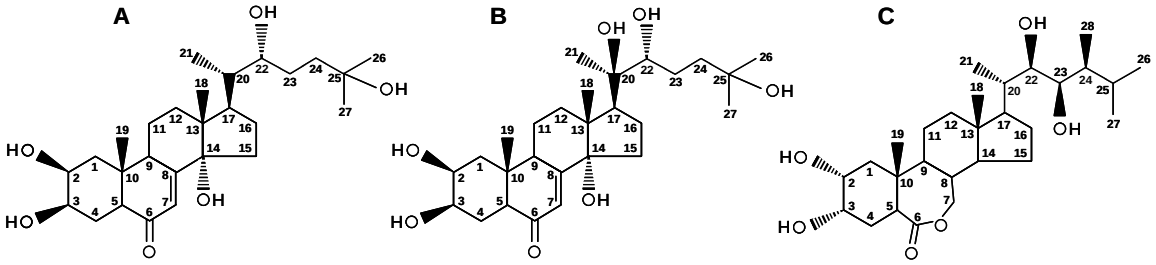

Figure 3. Structural representation of ecdysone (A), 20-hydroxyecdysone (B), and 24-epibrassinolide (C).

The changes in the number, position, orientation of the hydroxyl groups, and the conjugating moieties linked to the hydroxyl groups would explain the huge structural diversity of ECDs (Dinan, 2001; Bathori and Pongracz, 2005). While PEs have 7-8 hydroxyl groups, ZEs only have 5-6 hydroxyl groups, except four ZEs which have at most 7 hydroxyl groups (Bathori and Pongracz, 2005). For example, the hydroxylations at the positions 2β, 3β, 14α, 22R, and 25 produce E (2β,3β,14α,22(R),25-pentahydroxy-7-cholesten-6-one) (Figure 3A). An additional hydroxylation at the position 20R produces 20E (2β,3β,14α,20β,22,25-hexahydroxy-7-cholesten-6-one) (Figure 3B). The E and 20E are present in insects and plants.

As an illustration of the structural diversity of ECDs, the following PEs were found on Pfaffia glomerata: i) 20E and ii) rubrosterone (Figure 4A) (Shiobara et al., 1993) as well as on Pfaffia iresinoides i) 20E 25-o-β-D-glucoside; ii) podecdysone B; iii) pterosterone 24-o-β-D-glucoside; iv) pterosterone; v) polypodine B; and vi) podecdysone B 25-o-β-D-glucoside (Figure 4B) (Lafont et al., 2002).

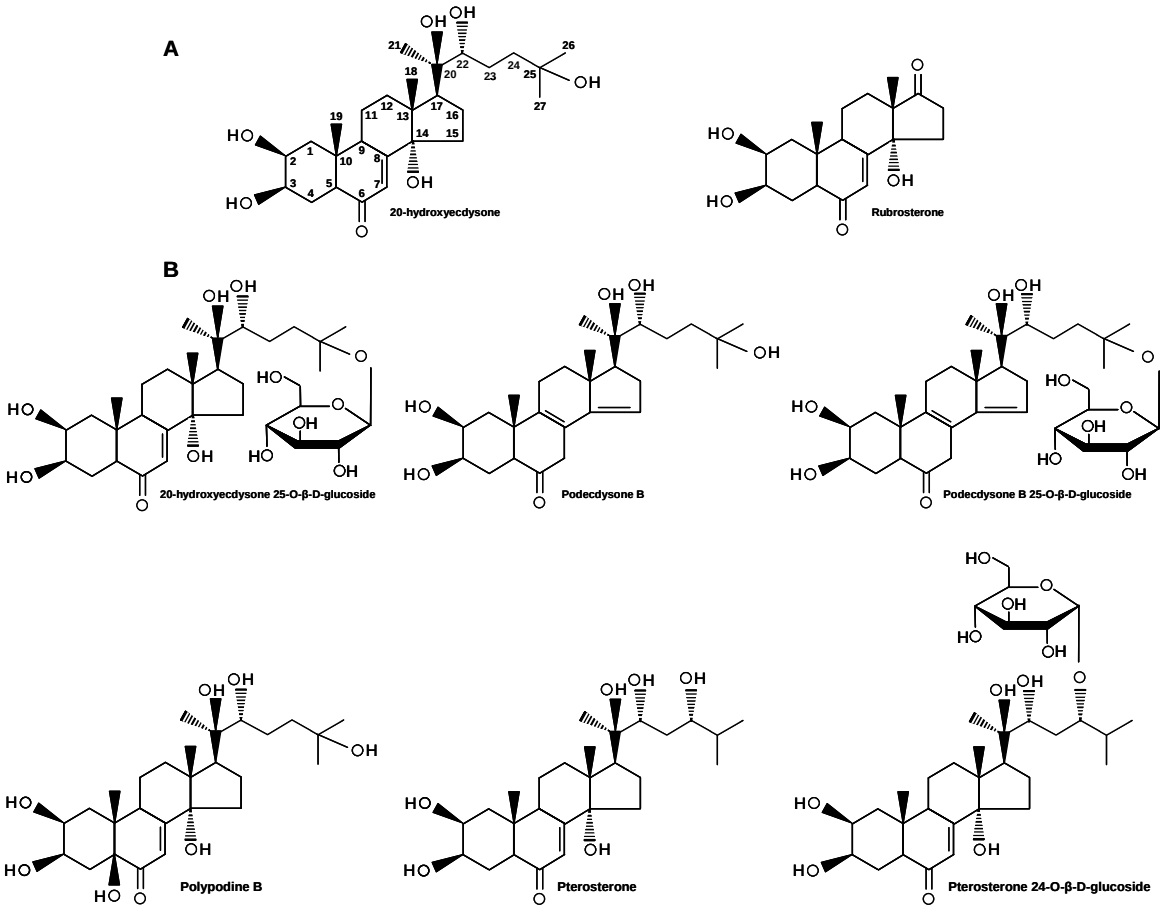

Figure 4. PEs found on *Pfaffia glomerata* (A) and *Pfaffia iresinoides* (B).

FUNCTIONS OF PHYTOECDYSTEROIDS IN PLANTS

The precise function of PEs in the life cycle of plants is still unknown (Slama, 1993; Machackova et al., 1995; Dinan, 2001). Three major hypotheses for their potential roles have been proposed: i) phytohormonal compounds (Dinan, 1998; Golovatskaya, 2004); ii) protective compounds against unadapted phytophagous insects (Lafont, 1997); and iii) supply of polyhydroxylated phytosterols required for growth and cell proliferation (Machackova et al., 1995). The most acceptable one is that PEs are plant defense substances. It has been reported that 20E affects differentiation of somatic embryos in tissue cultures of alfalfa and that 20E has no biological activity, except for slight gibberellin-like activity in rice (Machackova et al., 1995). However, it has been also reported that 20E may be a biologically active compound, affecting morphological and physiological processes in plants such as coleoptile elongation (10^{-13} – 10^{-5} M), α-amylase activation (10^{-9} – 10^{-7} M), and leaf yellowing retardation (10^{-9} – 10^{-8} M). It is likely that distinct processes are regulated at different 20E concentrations (Golovatskaya, 2004). Synergetic effects of 20E and indol-3-il acetic acid (IAA) are observed on coleoptile elongation. This effect might be explained by the existence of two different action mechanisms of 20E and IAA. A synergetic effect might be mediated by a 20E induced increase in the sensitivity of plant tissues to auxin (Golovatskaya, 2004). The antagonistic effects of 20E and 24-epibrassinolide are also observed in coleoptile elongation. Since 20E and 24-epibrassinolide have structural similarity (Figure 3, compare B and C), they may compete for a common receptor (Golovatskaya, 2004). In addition, antagonistic effects of 20E and gibberellic acid (GA_3) are also observed on coleoptile elongation and α-amylase activity. It may also be possible that 20E and GA_3 compete for GA_3 receptors (Golovatskaya, 2004).

Evidence supporting a protective function is also available. However, this does not exclude the possibility that 20E may also have phytohormonal functions. The 20E is a major PE present in plants (Schmelz et al., 1999; Dinan, 2001), being stable (Schmelz et al., 2000) and inducible (Schmelz et al., 1999). PEs stimulate anomalous molting in several arthropods, causing death (Dinan, 2001). The

following effects of PEs are observed in unadapted insects: deterrent (Blackford and Dinan, 1997), abnormalities in growth and development (Camps and Coll, 1993; Tanaka and Takeda, 1993; Savolainen et al., 1995; Mondy et al., 1997; Blackford and Dinan, 1997), and antifeedant (Blackford and Dinan, 1997; Descoins and Marion-Poll, 1999; Marion-Poll and Descoins, 2002). Nematodes exposed either directly or indirectly to 20E displayed the following symptoms: anomalous molting, immobility, reduced invasion, impaired development, and death (Soriano et al., 2004).

In spinach, there is an increment on the PE content in response to mechanical damage (Schmelz et al., 1998), insect herbivory (Schmelz et al., 1999), and application of methyl jasmonate (wound hormone) (Schmelz et al., 1999). Collectively, these observations led to the conclusion that the induction of PEs may protect plants from insect attack (Schmelz et al., 2002). The biosynthesis of 20E in plants remains unknown. It seems that C_{27}, C_{28}, and C_{29} PSs are the PE precursors; however, the intermediates and the enzymes involved in this process are uncharacterized.

BIOSYNTHESIS OF PHYTOECDYSTEROIDS

The plant sterols (PSs) are triterpenes, generally including a methyl (C_{28} PS) or ethyl group (C_{29} PS) at C_{24} and a double bond at C_{22} (Goodwin, 1971). However, some plants also contain amounts of cholesterol (Schaeffer et al., 2001; Schaller, 2003; Schaller, 2004), lanosterol (Ramadan et al., 2007), lathosterol (Grebenok and Adler, 1993; Adler and Grebenok, 1999), and 7-dehydrocholesterol (Skliar et al., 2000) which do not have a methyl or ethyl group at C_{24} (C_{27} PS). A typical PS profile in *A. thaliana* is characterized by cholesterol (2%), cycloartenol (3%), 24-methylene cycloartanol (4%), obtusifoliol (1%), 24-methylene lophenol (1%), campesterol (11%), brassicasterol (2%), 24-ethylidene lophenol (1%), Δ7-sitosterol (1%), Δ7-avenasterol (1%), isofucosterol (3%), sitosterol (64%), and stigmasterol (6%) (Schaeffer et al., 2001).

In *A. thaliana*, 24 genes which encode 12 distinct enzymes catalyze eleven steps necessary to synthesize the PS backbone (Suzuki and Muranaka, 2007) (Figure 5). The biosynthesis of plant sterol backbones starts with two molecules of acetyl-CoA which are converted into acetoacetyl-CoA by acetoacetyl-CoA thiolase (1). From acetoacetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA is synthesized by 3-hydroxy-3-methylglutaryl-CoA synthase (2). The 3-hydroxy-3-methylglutaryl-CoA reductase (3) transforms 3-hydroxy-3-methylglutaryl-CoA into mevalonate, which is converted into 5-phosphomevalonate by mevalonate kinase (4). The 5-phosphomevalonate is phosphorylated by phosphomevalonate kinase (5), producing 5-diphosphomevalonate, which is decarboxylated by mevalonate diphosphate decarboxylase (6) making isopentenyl diphosphate. The isomerization of isopentenyl diphosphate by isopentenyl diphosphate isomerase (7) yields dimethylallyl diphosphate. The farnesyl diphosphate is synthesized from the condensation of isopentenyl diphosphate with dimethylallyl diphosphate by farnesyl diphosphate synthase (8). The squalene synthase (9) catalyzes the condensation of two farnesyl diphosphate molecules producing squalene, which is then oxidized by squalene epoxidase (10) generating 2,3-oxidesqualene.

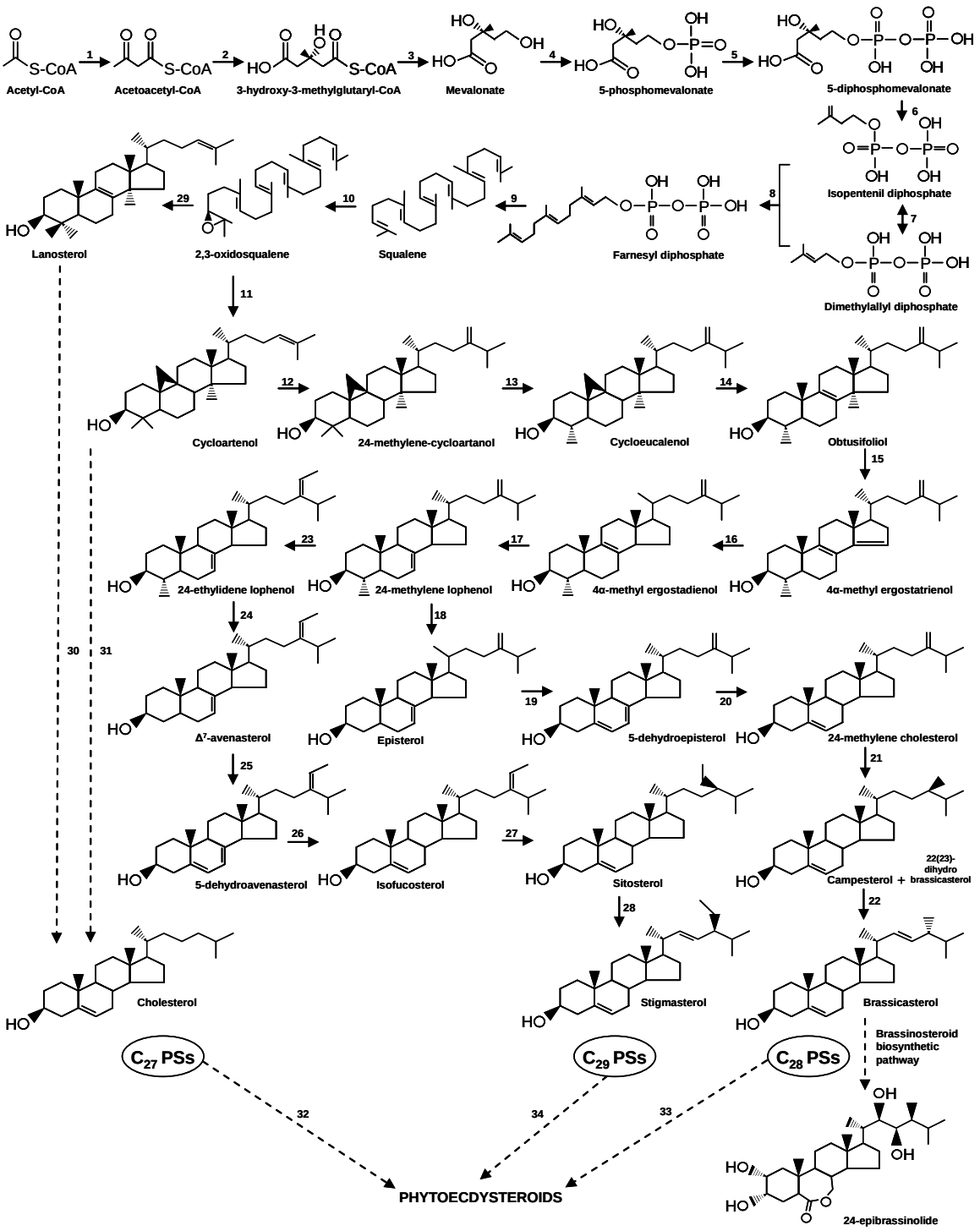

Figure 5. Biosynthesis of C_{27}, C_{28}, and C_{29} PSs and PEs. The numbers are representing the enzymes. See text for further details.

The cycloartenol and lanosterol molecules are synthesized respectively by cycloartenol synthase (11) and lanosterol synthase (29), which catalyze the cyclization of 2,3-oxidesqualene (Sawai et al., 2006; Suzuki and Muranaka, 2007). Cycloartenol is the major cyclic triterpene intermediate for C_{28} and C_{29} PS biosynthesis such as campesterol, brassicasterol, sitosterol, and stigmasterol. Lanosterol may be an alternative intermediate for the C_{27} PSs (lathosterol, 7-dehydrocholesterol, and cholesterol) or other metabolites (Kolesnikova et al., 2006; Sawai et al., 2006). In animals, lanosterol is the precursor of lathosterol, 7-dehydrocholesterol, and cholesterol. It still needs to be determined if lanosterol could be the precursor of PS biosynthesis (Suzuki and Muranaka, 2007). The lanosterol or cycloartenol might be the precursor of cholesterol or other C_{27} PS. Whether there are intermediates is still unknown. In addition, the enzymes involved in these conversions are unknown (Figure 5: 30 and 31).

Two major independent pathways which start with cycloartenol are responsible for the biosynthesis of brassicasterol and stigmasterol. There are six common steps from cycloartenol to 24-methylene lophenol, involving six different enzymes. The sequential conversion of cycloartenol into 24-methylene-cycloartanol, cycloeucalenol, obtusifoliol, 4α-methyl ergostatrienol, 4α-methyl ergostadienol, and 24-methylene lophenol is respectively catalyzed by C_{24}-methyltransferase (12), C_4-demethylase (13), cyclopropyl-sterol isomerase (14), C_{14}-demethylase (15), Δ^{14}-reductase (16), and Δ^{8}-isomerase (17). The 24-methylene lophenol is converted by $C_{24}{}^{1}$-methyltransferase (23) into 24-ethylidene lophenol. The brassicasterol and stigmasterol are synthesized respectively from 24-methylene lophenol and 24-ethylidene lophenol. The 24-methylene lophenol is sequentially converted into episterol, 5-dehydroepisterol, 24-methylene cholesterol, campesterol, and brassicasterol respectively by C_4-demethylase (18), C_5-desaturase (19), Δ^{7}-reductase (20), Δ^{24}-isomerase/reductase (21), and C_{22}-desaturase (22). The 24-ethylidene lophenol is also sequentially converted into Δ7-avenasterol, 5-dehydroavenasterol, isofucosterol, sitosterol, and stigmasterol respectively by the same set of enzymes C_4-demethylase (24), C_5-desaturase (25),

Δ^7-reductase (26), Δ^{24}-isomerase/reductase (27), and C_{22}-desaturase (28) (Schaller, 2004) (Figure 5).

PEs seem to be synthesized from C_{27} (e.g. cholesterol), C_{28} (e.g. campesterol), or C_{29} PSs (e.g. stigmasterol) (Figure 5: 32, 33 and 34). The precise biosynthetic pathway involved in PEs production is unknown. In spinach, it has been proposed that lathosterol may be the direct precursor of 20E (Grebenok and Adler, 1993; Adler and Grebenok, 1995; Adler and Grebenok, 1999). However, the lathosterol precursor is unknown. The lathosterol seems to be converted into E which is phosphorylated producing E phosphate. The 20E is then produced from E phosphate (Grebenok et al., 1994; Adler and Grebenok, 1999) (Figure 6). If there is any intermediate, it is still unrevealed as well as the enzymes (Figure 6: 35, 36 and 37) involved in these conversions.

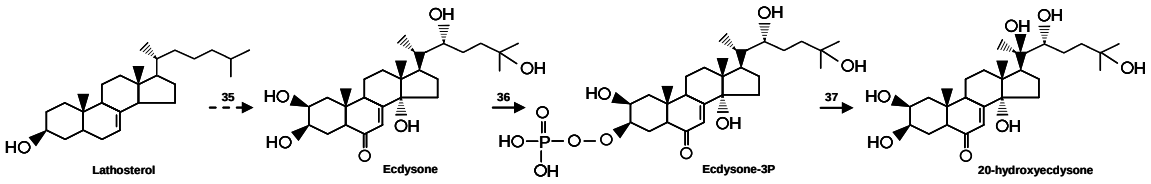

Figure 6. Biosynthesis of E and 20E in spinach. The numbers are representing the enzymes. See text for further details.

Cholesterol (Goodwin, 1971), lathosterol (Adler and Grebenok, 1999), or other C_{27} PS may act as precursors of C_{27} PEs such as E and 20E (Figure 5). If independent biosynthetic routes may be involved in the 20E production from cholesterol, lathosterol, or another PS are unidentified. Several plants produce PEs that are alkylated at C_{24}, indicating that C_{24} methyl or ethyl PSs may be their

precursors (Figure 5). Clerosterol (C_{29} PS) is the precursor of cyasterone (C_{29} PE), isocyasterone (C_{29} PE), and 29-norcyasterone (C_{28} PE). The conversion of clerosterol into 29-norcyasterone may happen via fission of the C_{28} - C_{29} bond (Okuzumi et al., 2003). In insects, the function and the enzymes involved in 20E biosynthesis are known.

BIOSYNTHESIS AND METABOLISM OF ZOOECDYSTEROIDS

Insects have a rigid exoskeleton (cuticle), which protects them from desiccation and predators. It provides substrate for development of jointed legs and wings. To grow and develop, insects must sporadically replace its exoskeleton. The entire cyclical process is called molting (Gilbert, 2004). Every molt corresponds to the end of one growth stage (instar) and the beginning of another (Figure 7).

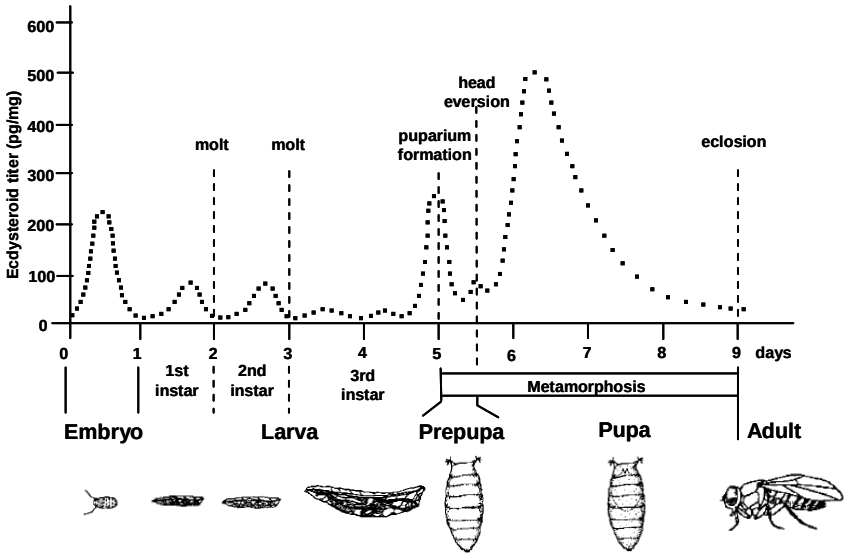

Figure 7. Correlations in between the major developmental transitions and 20E pulses in *D. melanogaster*. Many developmental processes (embryogenesis, larval development, and metamorphosis) are synchronized by pulses of ECDs. The major developmental transitions are marked by dashed lines. Reprinted with permission from Thummel, 2001. Copyright (2007) Developmental Cell.

In several insect species, the number of instars is invariable, but in others it may change in response to environmental factors, such as temperature and food availability. The molting process is elicited by ZEs 20E and E which is the precursor of 20E and it stops once that the insect becomes fully developed (Gilbert et al., 2002; Gilbert, 2004). The 20E is the major molting hormone; however, E has morphogenetic functions (Gilbert et al., 2002). Each of the major developmental transitions in the life cycle of *D. melanogaster* is elicited by pulses of 20E (Thummel, 2001). Pulses of 20E precede each of the major developmental transitions (Figure 7). The disruption of the gene called *shade* which encodes the enzyme CYP314A1 responsible for the conversion of E into 20E causes embryonic lethality due to low levels of 20E (Petryk et al., 2003). In addition to *shade*, other genes that disrupt embryonic development have been identified - *shadow*, *phantom*, and *disembodied* (Gilbert, 2004) - and each of these is involved in 20E biosynthesis (Figure 8). It has been reported that 20E is constantly required for embryonic development to be completed (Makka et al., 2002).

Molting and many other developmental processes in insects are controlled or elicited by the molting hormone 20E; however, the precise mechanism by which 20E accomplishes these functions is unknown (Warren et al., 2006). The diffusible factor called brain neuropeptide prothoracicotropic hormone (PTTH) is required to the biosynthesis of ZEs. The PTTH is produced at specific time interval by a couple of outsized lateral neurons in the brain. It is stored in neurohemal organs attached to the brain (corpora allata). It is under complex control in the brain, which integrates several factors such as time since last molting, nutritional status, and physical size. Once the corpus allatum releases PTTH into insect hemolymph (blood), the prothoracic gland is stimulated to synthesize ZEs (Gilbert et al., 2002; Gilbert, 2004).

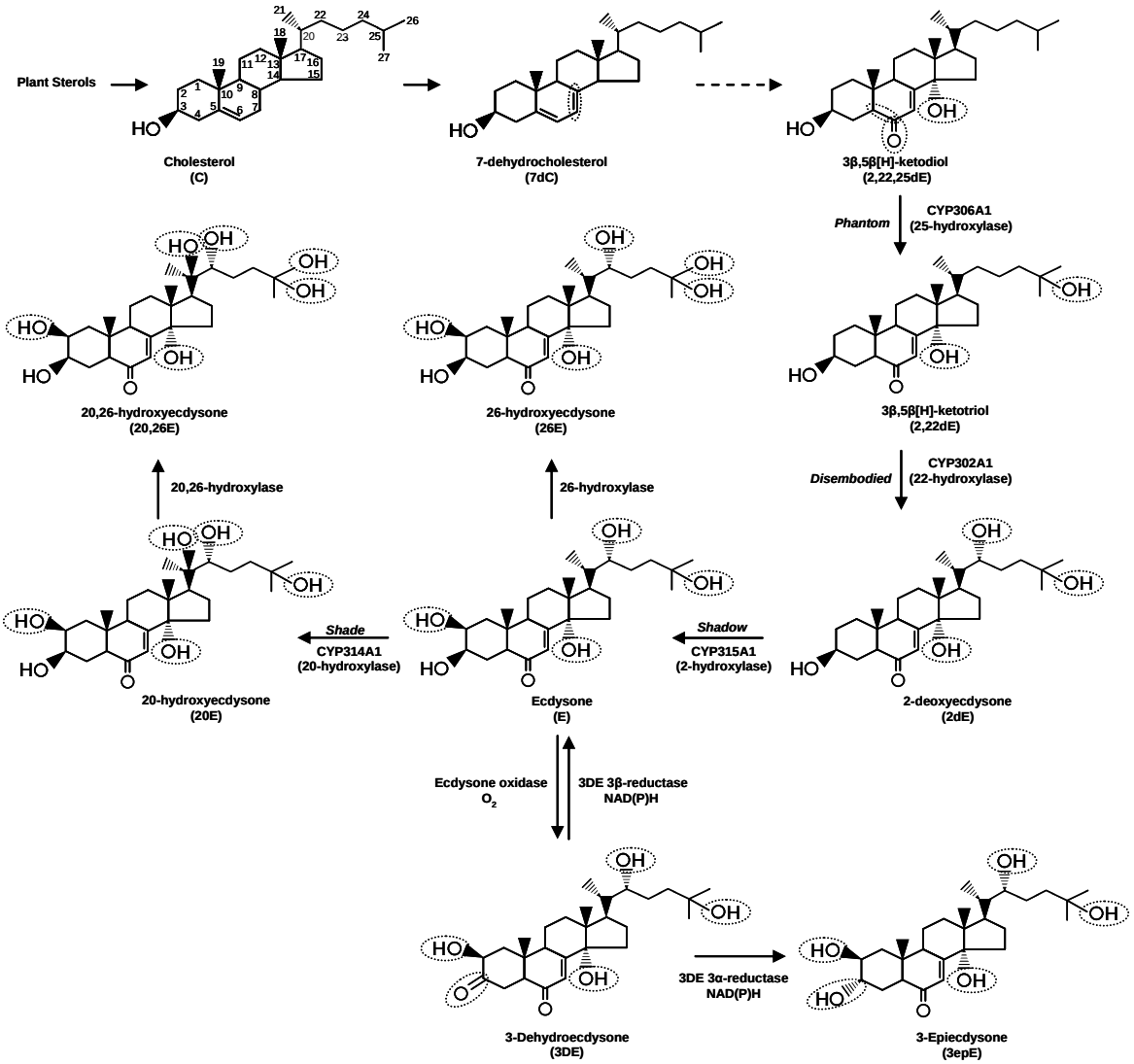

Figure 8. Biosynthesis and metabolism of ZEs. Insects obtain in their diet plant sterols which are converted into ketodiol. There are possibly many unknown steps in between 7dC and 2,22,25dE. Characterized cytochrome P450s are responsible for the sequential hydroxylations of 2,22,25dE (at carbon 25); 2,22dE (at carbon 22); 2dE (at carbon 2); E (at carbon 20). The major structural modifications are shown by a dotted circle.

In immature *D. melanogaster*, the prothoracic gland, corpus allatum, and corpus cardiacum compose the ring gland which is the source of E (Dai and Gilbert, 1991; Gilbert, 2004). In contrast, in many other immature insects, the prothoracic gland is a unique organ comprising a single steroidogenic cell type, which synthesizes 3-dehydroecdysone and its conversion to E occurs in the hemolymph. E is then 20-hydroxylated in peripheral tissues, yielding 20E (Gilbert et al., 2002). In some insects, during adult life, when the prothoracic glands have almost or completely disappeared due to programmed cell death during pupal adult development, epidermal and gonadal cells produce ZEs (Gilbert, 1962; Dai and Gilbert, 1997). ZEs may be inactivated in several tissues, including the fat body, midgut and Malpighian (Lafont and Koolman, 1984). Its inactivation by the midgut is important in protection of the adapted insect from ingested PEs (Zhang and Kubo, 1993; Blackford et al., 1997). It is also possible that phytophagous insects have a diversity of detoxification pathways that protect them against ingested PEs (Rharrabe et al., 2007).

Arthropods are not able to synthesize sterols (e.g. ECDs) from a simple precursor molecule such as acetate or pyruvate. Sterols are required (cholesterol or sitosterol) in their diet to accomplish this task (Gilbert et al., 2002). The plant eating insects consume mainly sitosterol in their diet while the meat eating insects acquire cholesterol directly in their diet. Cholesterol is the precursor of 20E in insects (Warren and Hetru, 1990). Plant eating insects dealkylate sitosterol to cholesterol (Ikekawa et al., 1993; Gilbert, 2004). Nematodes also require sterols in their diet. In these organisms, two processes are dependent on sterols, dauer larva formation and molting (Entchev and Kurzchalia, 2005).

In insects, conversion of sterols into 20E involves several P450 enzymes (Gilbert, 2004). Cytochromes P450 have been found in all kingdoms (eubacteria, archaebacteria, fungi, plants, insects, and vertebrates). Plant P450s play a role in the metabolism of a variety of secondary metabolites, in plant insect interaction, and in herbicide metabolism (Werck-Reichhart and Feyereisen, 2000). Insect P450s are involved in ZEs and juvenile hormone biosynthesis. These enzymes metabolize a great variety of substrates such as steroids, fatty acids, and

xenobiotics (Feyereisen, 1999). In *D. melanogaster*, two P450s called CYP6G1 and CYP12D1 may be involved with DDT resistance (Festucci-Buselli et al., 2005). Once those cytochrome P450s catalyze economic important reactions on a variety of substrates, they may have a biotechnological applicability (Bernhardt, 2006).

Plant sterols are converted into cholesterol (C) which may be further transformed into 7-dehydrocholesterol (7dC) (Figure 8). The pathway responsible for production of 3β,5β[H]-ketodiol (2,22,25dE) from 7dC is unknown. The P450 CYP306A1 (25-hydroxylase) converts 2,22,25dE into 3β,5β[H]-ketotriol (2,22dE) (Niwa et al., 2004; Warren et al., 2004) which is converted into 2-deoxyecdysone (2dE) by the catalytic activity of another P450 called CYP302A1 (22-hydroxylase) (Warren et al., 2002; Gilbert, 2004). The enzyme CYP315A1 (2-hydroxylase) is responsible for conversion of 2dE into E (Warren et al., 2002, Gilbert, 2004).

E is the precursor of 20E and the enzyme involved in this conversion is another P450 CYP314A1 (20-hydroxylase) (Petryk et al., 2003). E and 20E may be transformed, respectively, into 26-hydroxyecdysone (26E) and 20,26-hydroxyecdysone (20,26E) by an unidentified P450 enzyme (Warren et al., 2006). In *D. melanogaster* and *S. littoralis*, E may be oxidized by E oxidase producing 3-dehydroecdysone (Takeuchi et al., 2001; Takeuchi et al., 2005). In *S. littoralis*, 3-dehydroecdysone may be irreversibly reduced to 3-epiecdysone by 3-dehydroecdysone (3DE) 3α-reductase (Takeuchi et al., 2000). E may be regenerated back from 3-dehydroecdysone by 3DE 3β-reductase (Chen et al., 1999).

Pfaffia glomerata (SPRENG.) PEDERSEN – THE BRAZILIAN GINSENG

The genus *Pfaffia* spp. (Amaranthaceae) comprises several species, including *P. glomerata* which is commercialized in Brazil as a substitute for the Asian ginseng (*Panax* spp. Araliaceae). They are popularly known as Brazilian ginseng, “para-tudo”, “batata-do-mato”, “corango”, “corrente”, “sempre-viva”, and “suma”. *P. glomerata* produces several metabolites. The following compounds were isolated from *P. glomerata* roots: glomeric acid, pfameric acid, ecdysterone, rubrosterone, oleanolic acid, and β-glucopyranosyl oleanolate (Shiobara et al., 1993). In older works, 20E was referred to as ecdysterone (Adler and Grebenok, 1999). Among these compounds, 20E has attracted the attention of many researchers because of its potential pharmacological and medicinal effects on mammals or humans (Slama and Lafont, 1995; Lafont and Dinan, 2003; Bathori and Pongracz, 2005; Dinan and Lafont, 2006).

Since *P. glomerata* produces 20E (Shiobara et al., 1993), it might explain why it is widely used in Brazilian traditional medicine to cure or prevent several diseases. The medicinal properties of *P. glomerata* or other species of the genus *Pfaffia* spp. may also be attributed to other phytochemical or the combination of several compounds. *Pfaffia* roots have been popularly used to treat or prevent diseases such as diabetes, rheumatism, arthritis, mononucleosis, tumors, anemia, infertility, hormonal dysfunction, physical and mental stresses, high blood pressure, asthenia, and ulcer. In addition, it has been used as tonic, aphrodisiac, rejuvenator, tranquillizer, immunologic system stimulant, endocrine system balancer, toxin neutralizer, restorer of nervous and glandular functions, and stimulant of physical performance and body mass (Lorenzi and Abreu Matos, 2002). It has been reported that *P. glomerata* root extracts may have the following properties in rats: central nervous system depressant (De-Paris et al., 2000), gastro-protective function (Freitas et al., 2004), weak cytotoxic activity against *Leishmania braziliensis* (Neto et al., 2004), learning and memory stimulant (Marques et al., 2004), anti-inflammatory and analgesic effects (Neto et al., 2005),

and anti-oedematogenic action dependent on the stimulation of endogenous nitric oxide production (Teixeira et al., 2006).

Given its importance, several products based on purified PEs or PE containing plant powders or extracts of *Pfaffia* can be found on the market such as Adaptogen N (Muscle and Sport Science), Ecdy-Force (Peak Nutrition) (Lafont and Dinan, 2003), Ginseng Brasileiro (Herbarium), Lepti-trim (Total Body Research Labs), and On Cycle (Fizogen). Botanical extracts containing *Pfaffia* have been used to treat and prevent neuralgia (Dos Santos, 2007), skin disorders and diabetic lesions (Olalde, 2007), tumor and cancer (Oba and Baba, 2006), cellulite (Barrera-Arellano et al., 2006), aging (Imai et al., 2005), liver disease (Lee and Shinn, 2004a), ischemic heart disease, thrombus, and hyperlipidemia (Lee and Shinn, 2004b), skin wounds, decubitus and osteoporosis (Matsuura, 2003), insomnia (Gow, 2003), fever (Kagaku, 2002), and tonic effects in learning and memory (Marques, 2003).

Considering that 20E might have agrochemical, biotechnological, medicinal, and pharmaceutical uses as well as it may affect morphological and physiological process in plants, we decide to evaluate *P. glomerata* concentration. The content of 20E is about 0.64% of the root dry matter (unpublished data). It would be worth to understand its biosynthesis in plants where it is found in larger amounts when compared to insects. The understanding of 20E biosynthesis in plants may provide clues to investigate its functions and to produce 20E in crop species or even to increase 20E production by metabolic engineering in *Pfaffia*. In addition, the comparison of 20E biosynthesis in plants and insects may give insights to design strategies to control insects.

The metabolic engineering has been efficiently used to increase the content of many secondary metabolites in plants (Yun, 1992; Chen, 1999; Chen, 2000; Bouvier et al., 2003; Zhang, 2004). However, there are still several obstacles which need to be overcome to understand 20E biosynthesis and increase its concentration in *P. glomerata*: i) characterization of metabolic pathways involved in plant 20E biosynthesis; ii) determination of cellular and sub-cellular sites where

20E is biosynthesized; and iii) establishment of *in vitro* plant regeneration and genetic transformation protocols.

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REFERENCES

Adler JH, Grebenok RJ (1995) Biosynthesis and distribution of insect-molting hormones in plants - a review. Lipids 30:257-262.

Adler JH, Grebenok RJ (1999) Occurrence, biosynthesis, and putative role of ecdysteroids in plants. Crit. Rev. Biochem. Mol. Biol. 34:253-264.

Barrera-Arellano D, Polezel MA, Nogueira C, Del Carmem VM, Rodrigues SC (2006) Use of a vegetable extract based cosmetic to combat cellulite consists of a product incorporating flavonoids and saponins and activating micro circulation. Patent number: BR200403155-A.

Bandara BMR, Jayasinghe L, Karunaratne V, Wannigama GP, Bokel M, Kraus W, Sotheeswaran S (1989) Ecdysterone from stem of *Diploclisia glaucescens*. Phytochemistry 28:1073-1075.

Bathori M, Gergely A, Kalasz H, Nagy G, Dobos A, Mathe I (2000) Liquid chromatographic monitoring of phytoecdysteroids production of *Serratatula wolffii*. J. Liq. Chromatogr. Relat. Technol. 23:281-294.

Bathori M, Pongracz Z (2005) Phytoecdysteroids – from isolation to their effects on humans. Curr. Med. Chem. 12:153-172.

Bernhardt R (2006) Cytochromes P450 as versatile biocatalysts. J. Biotechnol. 124:128-145.

Blackford MJP, Clarke BS, Dinan L (1997) Distribution and metabolism of exogenous ecdysteroids in the Egyptian cotton leafworm *S. littoralis* (Lepidoptera: Noctuidae). Arch. Insect Biochem. Physiol. 34:329-346.

Blackford MJP, Dinan L (1997) The effects of ingested 20-hydroxyecdysone on the larvae of *Aglais urticae*, *Inachisio*, *Cynthiacardui* (Lepidoptera: Nymphalidae) and *Tyria jacobaeae* (Lepidoptera: Arctiidae). J. Insect Physiol. 43:315-327.

Bouvier F, Dogbo O, Camara B (2003) Biosynthesis of the food and cosmetic plant pigment bixin (annatto). Science 300:2089-2091.

Camps F, Coll J (1993) Insect allelochemicals from *Ajuga* plants. Phytochemistry 32:1361-1370.

Chen DH (1999) Ri-mediated transformation of *Artemisia annua* with a recombinant farnesyl diphosphate synthase gene for artemisinin production. Plant Cell Tiss. Organ Cult. 57:157-162.

Chen DH (2000) Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. Plant Sci. 155:179-185.

Chen JH, Turner PC, Rees HH (1999) Molecular cloning and characterization of hemolymph 3-dehydroecdysone 3β-reductase from the cotton leafworm, *Spodoptera littoralis*. A new member of the third superfamily of oxidoreductases. J. Biol. Chem. 274:10551-10556.

Dai JD, Gilbert LI (1991) Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal–adult transformation of *Drosophila melanogaster*: a cytophysiological analysis of the ring gland. Dev. Biol. 144:309-326.

Dai JD, Gilbert LI (1997) Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult metamorphosis. Insect Biochem. Mol. Biol. 27:69-78.

De-Paris F, Neves G, Salgueiro JB, Quevedo J, Izquierdo I, Rates SMK (2000) Psychopharmacological screening of *Pfaffia glomerata* Spreng. (Amaranthaceae) in rodents. J. Ethnopharmacol. 73:261-269.

Descoins Jr C, Marion-Poll F (1999) Electrophysiological responses of gustatory sensilla of *Mamestra brassicae* (Lepidoptera, Noctuidae) larvae to three ecdysteroids: ecdysone, 20-hydroxyecdysone and ponasterone A. J. Insect Physiol. 45:871-876.

Dinan L (1995a) Distribution and levels of phytoecdysteroids within individual plants of the species of the Chenopodiaceae. Eur. J. Entomol. 92:295-300.

Dinan L (1995b) A strategy for the identification of ecdysteroid receptor agonists and antagonists from plants. Eur. J. Entomol. 92:271-283.

Dinan L (1998) A strategy towards the elucidation of the contribution made by phytoecdysteroids to the deterrence of invertebrate predators on plants. Russ. J. Plant Physiol. 45:296-305.

Dinan L (2001) Phytoecdysteroids: biological aspects. Phytochemistry 57:325-339.

Dinan L, Savchenko T, Whiting P (2001) On the distribution of phytoecdysteroids in plants. Cell. Mol. Life Sci. 58:1121-1132.

Dinan L, Lafont R (2006) Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. J. Endocrinol. 191:1-8.

Dos Santos AS (2007) Composition, useful to treat/prevent e.g. neuralgia, comprises, as an active agent, a mixture of extracts from the plants of *Paullinia*

cupana H.B.K., *Trichilia catigua* Juss, *Ptychopetalum olacoides* Benth and *Pfaffia stenophylla* Sprena. Patent number: WO2007016756-A1.

Entchev EV, Kurzchalia TV (2005) Requirement of sterols in the life cycle of the nematode *Caenorhabditis elegans*. Semin. Cell Dev. Biol.16:175-182.

Festucci-Buselli RA, Carvalho-Dias AS, Oliveira-Andrade M, Caixeta-Nunes C, Li HM, Stuart JJ, Muir W, Scharf ME, Pittendrigh BR (2005) Expression of Cyp6g1 and Cyp12d1 in DDT resistant and susceptible strains of *Drosophila melanogaster*. Insect Mol. Biol. 14:69-77.

Feyereisen R (1999) Insect P450 enzymes. Annu. Rev. Entomol. 44:507-533.

Freitas CS, Baggio CH, Da Silva-Santos JE, Rieck L, De Moraes Santos CA, Correa Jr C, Ming LC, Cortez DAG, Marques MCA (2004) Involvement of nitric oxide in the gastroprotective effects of an aqueous extract of *Pfaffia glomerata* (Spreng.) Pedersen, Amaranthaceae, in rats. Life Sci. 74:1167-1179.

Gilbert LI (1962) Maintenance of the prothoracic gland by the juvenile hormone in insects. Nature 193:1205-1207.

Gilbert LI, Rybczynski R, Warren JT (2002) Control and biochemical nature of the ectysteroidogenic pathway. Annu. Rev. Entomol. 47:883-916.

Gilbert LI (2004) *Halloween* genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. Mol. Cell. Endocrinol. 215:1-10.

Goodwin TW (1971) Biosynthesis of carotenoids and plant triterpenes. Biochem. J. 123: 293-329.

Golovatskaya IF (2004) Effect of ecdysterone on morphological and physiological processes in plants. Russ. J. Plant Physiol. 51: 407-413.

Gow R (2003) Botanical extract useful in delivery of kavalactones for treating e.g. insomnia comprises extract of botanical, diluent, binder and sweetener (Eng). Patent number: WO2003084512-A1.

Grebenok RJ, Ripa PV, Adler JH (1991) Occurrence and levels of ecdysteroids in spinach. Lipids 26:666-668.

Grebenok RJ, Adler JH (1991) Ecdysteroids distribution during development of spinach. Phytochemistry 30:2905-2910.

Grebenok RJ, Adler JH (1993) Ecdysteroid biosynthesis during the ontogeny of spinach leaves. Phytochemistry 33:341-347.

Grebenok RJ, Venkatachari S, Adler JH (1994) Biosynthesis of ecdysone and ecdysone phosphates in spinach. Phytochemistry 36:1399-1408.

Ikekawa N, Morisaki M, Fujimoto Y (1993) Sterol metabolism in insects: dealkylation of phytosterol to cholesterol. Acc. Chem. Res. 26:139-146.

Imai S, Iwashima J, Matsura K (2005) Composition for promoting collagen synthesis useful in cosmetics for treating aging, contains extract of *Pfaffia*, animals-and-plants component e.g. linseed, and/or components derived from animals-and-plants e.g. methyl hesperidin. Patent number: JP2005255527-A.

Kagaku HS (2002) Antiallergic composition for treating hay fever symptoms, contains *Perilla* plant processed material and *Pfaffia* genus plant processed material, as active ingredients. Patent number: JP2002308795-A.

Kolesnikova MD, Xiong Q, Lodeiro S, Hua L, Matsuda SPT (2006) Lanosterol biosynthesis in plants. Arch. Biochem. Biophys. 447:87-95.

Lafont R, Koolman J (1984) Ecdysone metabolism. In: Hoffmann, Porchet, P. (Eds.), Biosynthesis, metabolism and mode of action of invertebrate hormones. Springer, Berlin, pp. 196-226.

Lafont R (1997) Ecdysteroids and related molecules in animals and plants. Arch. Insect Biochem. Physiol. 35:3-20.

Lafont R, Harmatha J, Marion-Poll F, Dinan L, Wilson ID (2002) The Ecdysone handbook, 3rd edition, on-line, http://ecdybase.org.

Lafont R, Dinan L (2003) Practical uses for ecdysteroids in mammals including humans: an update. J. Insect Sci. 3:1-30.

Lee BG, Shinn HS (2004a) Composition for treating liver disease such as liver failure or cirrhosis comprises *Carduus marianus*, *Echinacea*, *Taraxacum officinale*, *Uncaria* hooks and other medicinal plants. Patent number: KR2004030375-A.

Lee BG, Shinn HS (2004b) Composition for treating disease such as ischemic heart disease, thrombus, and hyperlipidemia comprises *Gingko biloba*, *Carduus marianus*, *Taraxacum officinale*, *Centella asiatica* and other medicinal plants. Patent number: KR2004030376-A.

Lorenzi H, Matos FJA (2002) Plantas medicinais no Brasil, nativas e exóticas. Instituto Plantarum de Estudos da Flora LTDA, pp. 45-46.

Machackova I, Vagner M, Slama K (1995) Comparison between the effects of 20-hydroxyecdysone and phytohormones on growth and development in plants. Eur. J. Entomol. 92:309-316.

Makka T, Seino A, Tomita S, Fujuwara H, Sonobe H (2002) A possible role of 20-hydroxyecdysone in embryonic development of the silkworm *Bombyx mori*. Arch. Insect Biochem. Physiol. 51:111-120.

Marion-Poll F, Descoins C (2002) Taste detection of phytoecdysteroids in larvae of *Bombyx mori*, *Spodoptera littoralis* and *Ostrinia nubilalis*. J. Insect Physiol. 48:467-476.

Marques LC (2003) Process for obtaining extract with tonic effects in learning and memory fortifies cognitive processes in individuals and is based on Brazilian ginseng. Patent number: BR200006645-A.

Marques LC, Galvão SMP, Espínola E, Dias RF, Mattei R, Oliveira MGM, Carlini ELA (2004) Psychopharmacological assessment of *Pfaffia glomerata* roots (Extract BNT-08) in Rodents. Phytother. Res. 18:566-572.

Matsuura K (2003) Living body collagen synthetic promoter for treating skin wounds, decubitus and osteoporosis, comprises powder extract or crude refined substance of extract belonging to *Pfaffia* genus and/or *Ajuga decumbens*. Patent number: JP2003277286-A.

Mondy N, Caıssa C, Pitoizet N, Delbecque JP, Corio-Costet MF (1997) Effects of the ingestion of *Serratula tinctoria* extracts, a plant containing phytoecdysteroids, on the development of the vineyard pest *Lobesia botrana* (Lepidoptera. Tortricidae). Arch. Insect Biochem. Physiol. 35:227-235.

Neto AG, Da Silva Filho AA, Costa JMLC, Vinholis AHC, Souza GHB, Cunha WR, Silva MLAE, Albuquerque S, Bastos JK (2004) Evaluation of the trypanocidal and leishmanicidal *in vitro* activity of the crude hydroalcoholic extract of *Pfaffia glomerata* (Amaranthaceae) roots. Phytomedicine 11:662-665.

Neto AG, Costa JMLC, Belati CC, Vinholis AHC, Possebom LS, Da Silva Filho AA, Cunha WR, Carvalho JCT, Bastos JK, Silva MLA (2005) Analgesic and anti-inflammatory activity of a crude root extract of *Pfaffia glomerata* (Spreng.) Pedersen. J. Ethnopharmacol. 96:87-91.

Niwa R, Matsuda T, Yoshiyama T, Namiki T, Mita K, Fujimoto Y, Kataoka H (2004) CYP306A1, a cytochrome P450 enzyme, is essential for ecdysteroid biosynthesis in the prothoracic glands of *Bombyx* and *Drosophila*. J. Biol. Chem. 279:35942-35949.

Oba H, Baba H (2006) Angiogenesis inhibitor for preventing and treating diseases such as tumor and cancer, contains extract of *Pfaffia*. Patent number: JP2006273739-A.

Okuzumi K, Hara N, Fujimoto Y, Yamada J, Nakamura A, Takahashi K, Morisaki M (2003) Biosynthesis of phytoecdysteroids in *Ajuga* hairy roots:clerosterol as a precursor of cyasterone, isocyasterone and 29-norcyasterone. Tetrahedron Lett. 44:323-326.

Olalde RJA (2007) Synergistic phytoceutical composition useful for treating e.g. skin disorder and diabetic lesions, comprises plant species selected from e.g. *Acantopanacis*, *Ajuga*, *Agaricus*, *Aloe*, *Angelica*, *Bupleurum*, and *Cimicifuga*; and excipients/carriers. Patent number: US2007104728-A1.

Petryk A, Warren JT, Marques G, Jarcho MP, Gilbert LI, Kahler J, Parvy JP, Li Y, Dauphin-Villemant C, O'Connor MB (2003) Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc. Natl. Acad. Sci. USA 100:13773-13778.

Ramadan MF, Zayed R, El-Shamy H (2007) Screening of bioactive lipids and radical scavenging potential of some solanaceae plants. Food Chem. 103:885-890.
Rharrabe K, Alla S, Maria A, Sayah F, Lafont R (2007) Diversity of detoxification pathways of ingested ecdysteroids among phytophagous insects. Arch. Insect Biochem. Physiol. 65:65-73.

Savolainen V, Wuest J, Lafont R, Connat JL (1995) Effects of ingested phytoecdysteroids in the female soft tick *Ornithodorus moubata*. Experientia 51:596-600.

Sawai S, Akashi T, Sakurai N, Suzuki H, Shibata D, Ayabe S, Aoki T (2006) Plant lanosterol synthase: divergence of the sterol and triterpene biosynthetic pathways in eukaryotes. Plant Cell Physiol. 47:673-677.

Schaller H (2003) The role of sterol in plant growth and development. Prog. Lipid Res. 422:163-175.

Schaller H (2004) New aspects of sterol biosynthesis in growth and development of higher plants. Plant Physiol. Biochem. 42:465-476.

Schaeffer A, Bronner R, Benveniste P, Schaller H (2001) The ratio of campesterol to sitosterol which modulates growth in *Arabidopsis* is controlled by sterol methyltransferase 2-1. Plant J. 25:605-615.

Schmelz EA, Grebenok RJ, Galbraith DW, Bowers WS (1998) Damage-induced accumulation of phytoecdysteroids in spinach: A rapid root response involving the octadecanoic acid pathway. J. Chem. Ecol. 24:339-360.

Schmelz EA, Grebenok RJ, Galbraith DW, Bowers WS (1999) Insect-induced synthesis of phytoecdysteroids in spinach, *Spinacia oleracea*. J. Chem. Ecol. 25:1739-1757.

Schmelz EA, Grebenok RJ, Ohnmeiss TE, Bowers WS (2000) Phytoecdysteroid turnover in spinach: Long-term stability supports a plant defense hypothesis. J. Chem. Ecol. 26:2883-2896.

Schmelz EA, Grebenok RJ, Ohnmeiss TE, Bowers WS (2002) Interactions between *Spinacia oleracea* and *Bradysia impatiens*: a role for phytoecdysteroids. Arch. Insect Biochem. Physiol. 51:204-221.

Shiobara Y, Inoue SS, Kato K, Nishiguchi Y, Oishi Y, Nishimoto N, De Oliveira F, Akisue G, Akisue MK, Hashimot G (1993) A nortriterpenoid, triterpenoids and ecdysteroids from *Pfaffia glomerata*. Phytochemistry 32:1527-1530.

Skliar M, Curino A, Milanesi L, Benassati S, Boland R (2000) *Nicotiana glauca*: another plant species containing vitamin D3 metabolites. Plant Sci. 156:193-199.

Slama K (1993) Ecdysteroids: insect hormones, plant defensive factors, or human medicine? Phytoparasitica 21:3-8.

Slama K, Lafont R (1995) Insect homones - ecdysteroids: their presence and actions in vertebrates. Eur. J. Entomol. 92:355-377.

Soriano IR, Riley IT, Potter MJ, Bowers WS (2004) Phytoecdysteroids: a novel defense against plant-parasitic nematodes. J. Chem. Ecol. 30:1885-1899.

Suzuki M, Muranaka T (2007) Molecular genetics of plant sterol backbone synthesis. Lipids 42:47-54.

Takeuchi H, Chen JH, O'Reilly DR, Rees HH, Turner PC (2000) Regulation of ecdysteroid signalling: molecular cloning, characterization and expression of 3-dehydroecdysone 3α-reductase, a novel eukaryotic member of the short-chain

dehydrogenases/reductases superfamily from the cotton leafworm, *Spodoptera littoralis*. Biochem. J. 349:239-245.

Takeuchi H, Chen JH, O'Reilly DR, Turner PC, Rees HH (2001) Regulation of ecdysteroid signaling: cloning and characterization of ecdysone oxidase: a novel steroid oxidase from the cotton leafworm, *Spodoptera littoralis*. J. Biol. Chem. 276:26819-26828.

Takeuchi H, Rigden DJ, Ebrahimi B, Turner PC, Rees HH (2005) Regulation of ecdysteroid signalling during *Drosophila* development: identification, characterization and modelling of ecdysone oxidase, an enzyme involved in control of ligand concentration. Biochem. J. 389:637-645.

Tanaka Y, Takeda S (1993) Ecdysone and 20-hydroxyecdysone supplements to the diet affect larval development in the silkworm, *Bombyx mori*, differently. J. Insect Physiol. 20:805-809.

Teixeira CGL, Piccoli A, Costa P, Soares L, Da Silva-Santos JE (2006) Involvement of the nitric oxide/soluble guanylate cyclase pathway in the anti-oedematogenic action of *Pfaffia glomerata* (Spreng.) Pedersen in mice. J. Pharm. Pharmacol. 58: 667-675.

Thummel CS (2001) Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. Dev. Cell. 1:453-465.

Warren JT, Hetru C (1990) Ecdysone biosynthesis: pathways, enzymes and the early steps problem. Invertebr. Reprod. Dev. 18:91-99.

Warren JT, Petryk A, Marques G, Jarcho M, Parvy J, Dauphin-Villemant C, O'Connor M, Gilbert LI (2002) Molecular and biological characterization of two

P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 99:11043-11048.

Warren JT, Petryk A, Marques G, Parvy JP, Shinoda T, Itoyama K, Kobayashi J, Jarcho M, Li Y, O'Connor MB, Dauphin-Villemant C, Gilbert LI (2004) *Phantom* encodes the 25-hydroxylase of *Drosophila melanogaster* and *Bombyx mori*: a P450 enzyme critical in ecdysone biosynthesis. Insect Biochem. Mol. Biol. 34:991-1010.

Warren JT, Yerushalmi Y, Shimell MJ, O'Connor MB, Restifo LL, Gilbert LI (2006) Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of *Drosophila melanogaster*: correlations with changes in gene activity. Dev. Dyn. 235:315-326.

Werck-Reichhart D, Feyereisen R (2000) Cytochromes P450: a success story. Genome Biol. 1 (Reviews 3003).

Yun DJ (1992) Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition. Proc. Natl. Acad. Sci. USA 89:11799-11803.

Zhang L (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc. Natl. Acad. Sci. USA 101:6786-6791.

Zhang ML, Kubo I (1993) Metabolic fate of ecdysteroids in larval *Bombyx mori* and *Heliothis virescens*. Insect Biochem. Mol. Biol. 23:831-843.

CHAPTER 2

Accumulation pattern of the 20-hydroxyecdysone in different organs of *Pfaffia glomerata*

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ABSTRACT

The ecdysteroid 20-hydroxyecdysone (20E) is a steroid hormone found in insects and plants. The 20E concentration and accumulation throughout *Pfaffia glomerata* development as well as the organs involved in 20E biosynthesis and accumulation are uncharacterized. Here, we show for the first time, the analyses of 20E concentration and accumulation in different organs over several months. This was accomplished using high performance liquid chromatography. Among 71 accessions, the highest root 20E accumulation (1.48 g) was found in the accession 13, which was selected to perform analysis of 20E concentration and accumulation. In the three distinct phases of *P. glomerata* development, 20E was constantly detected in flowers, leaves, stems, and roots, but its concentration was variable throughout its development. The highest 20E concentration was found in flowers (0.8221%), roots (0.6658%), leaves (0.6042%), and stems (0.2445%). In contrast, the lowest 20E concentration was found in stems (0.1379%), leaves

(0.2118%), roots (0.4224%), and flowers (0.4731%). While stems showed the less variable 20E concentration (0.1379% - 0.2445%), followed by roots (0.4224% - 0.6658%), and flowers (0.4731% - 0.8221%), leaves showed the more variable 20E percentage (0.2118% - 0.6042%). The analysis of 20E accumulation revealed that it was predominantly accumulated in roots whereas in leaves and stems, 20E total content remained constant, showing a little variation.

Keywords: ecdysteroid, 20-hydroxyecdysone, *P. glomerata*.

INTRODUCTION

The *Pfaffia glomerata* (SPRENG.) PEDERSEN, a Brazilian medicinal plant is traded as an alternative for the Asian ginseng (*Panax* spp. - Araliaceae). It is traditionally known as Brazilian ginseng, “para-tudo”, “batata-do-mato”, “corango”, “corrente”, “sempre-viva”, and “suma”. *Pfaffia* roots have been popularly used to treat or prevent diseases (Lorenzi and Abreu Matos, 2002). Recently, 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). In addition, there are available many patent applications, showing that botanical extracts containing *Pfaffia* have been used to treat or prevent diseases (Kagaku, 2002; Marques, 2003; Matsuura, 2003; Gow, 2003; Lee and Shinn, 2004a; Lee and Shinn, 2004b; Imai et al., 2005; Oba and Baba, 2006; Barrera-Arellano et al., 2006; Dos Santos, 2007; Olalde, 2007). The increasing number of both published papers and patent applications showing the potential pharmacological and medicinal properties of *P. glomerata* suggest that it may be worth to identify phytochemical compounds related to these properties and to study their biosynthesis.

The *P. glomerata* produces the ecdysteroid 20-hydroxyecdysone, 20E (Shiobara et al., 1993) and it is likely that 20E possesses many pharmacological and medicinal effects on mammals, including humans (Slama and Lafont, 1995; Dinan, 2001; Lafont and Dinan, 2003; Bathori and Pongracz, 2005; Dinan and Lafont, 2006; Festucci-Buselli et al., 2008). The ecdysteroids are steroid hormones found in arthropods (zooecdysteroids - ZEs) and in plants (phytoecdysteroids - PEs) and their structures are available on Ecdybase (Lafont et al., 2002). Many plants and insects share the same ecdysteroids, such as ecdysone, 20-hydroxyecdysone, among others. In mammals, they do not seem to be biosynthesized. In insects, embryogenesis, larval development, metamorphosis, and molting are controlled or elicited by specific pulses of the ZE 20E which is constantly required for embryonic development to be completed (Makka et al., 2002). In plants, the precise function of PEs in their life cycle is still

unknown (Slama, 1993; Machackova et al., 1995; Dinan, 2001). Three major hypotheses for their potential roles have been proposed: i) phytohormonal compounds (Dinan, 1998; Golovatskaya, 2004); ii) protective compounds against unadapted phytophagous insects (Lafont, 1997); and iii) supply of polyhydroxylated phytosterols required for growth and cell proliferation (Machackova et al., 1995). The PE 20E is an attractive phytochemical to perform further studies since it is likely that it might have agrochemical, biotechnological, medicinal, and pharmaceutical uses as well as it may affect in plants morphological and physiological process (Festucci-Buselli et al., 2008).

The presence of PEs has been examined in about 2% of world's flora. Only 5-6% of the tested species have PEs and their content is around 0.1% of the plant dry matter (Dinan, 2001). The 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). As an illustration, the herbaceous perennial *Ajuga* produces and accumulates high levels of PEs in its roots. There are others herbaceous perennial plants that accumulate PEs in their roots and distribute them to other organs during its ontogeny. The total PE content in herbaceous perennial plants is increased over the years (Adler and Grebenok, 1995). In contrast, for instance, spinach (*Spinacia oleracea*) which is an annual plant produces PEs in its aerial parts. Annual plants have to flower and produce their seeds which carry PEs that may be used to support the development of seedlings, allowing perpetuation of their species (Adler and Grebenok, 1995).

The PE 20E was detected in the roots of *P. glomerata* (Shiobara et al., 1993). Our previous studies showed that 20E was present in the roots of all 71 analyzed accessions of *Pfaffia glomerata* germplasm bank (Kamada, 2006; unpublished data); however, in this specie, it is unknown its occurrence, concentration and accumulation over time as well as 20E biosynthesis, regulation, and precise functions in plants (Festucci-Buselli et al., 2008). It is also unknown if this metabolite is biosynthesized and accumulated in roots or if it is biosynthesized in leaves and translocated to roots. In addition, it is unknown the exactly time in the plant development in which 20E shows its highest levels.

To investigate the occurrence, concentration and accumulation of 20E in flowers, leaves, stems, and roots of *P. glomerata*, it was selected one accession among the 71 available accessions which showed the highest 20E accumulation to evaluate the height, dry matter, 20E concentration and accumulation in the different organs over several months.

MATERIAL AND METHODS

Germplasm bank accessions

The *P. glomerata* germplasm bank accessions were kindly provided by both Dr. Roberto Fontes Vieria (Embrapa/Cenargen, Brasília, DF, Brazil) and Dr. Sílvio Lopes Teixeira (UENF, Campos dos Goytacazes, RJ, Brazil).

Plant disinfection

To set up our *in vitro* germplasm bank, plants were vegetatively propagated by shoot cuttings and grown under greenhouse conditions. They were sprayed two times, once per week, with a fungicide Captan® (2 mg L^{-1}) (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 mg L^{-1}) for 30 min, in sodium hypochlorite 0.4% (w/v) (Globo® UFE, Brazil) plus 2 drops of Tween-20 (Sigma-Aldrich Co, St Louis, MO) for 15 min, and rinsed five times in sterile distilled water. 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 fungus and bacteria by adding Captan (2 mg L^{-1}) and chloranfenicol (15 mg L^{-1}) to the MS-based medium with vitamins (Murashige and Skoog, 1962) (Phytotechnology Laboratories, Shawnee Mission, KS) supplemented with 3% (w/v) sucrose and myo-inositol (100 mg L^{-1}). To prevent phenol oxidation, ascorbic acid (0.15 mg L^{-1}), citric acid (0.1 mg L^{-1}), activated charcoal (1 g L^{-1}), L-cystein

(0.02 mg L^{-1}) and polyvinylpyrrolidone (1 g L^{-1}) were also incorporated to 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 ± 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 ± 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).

Shoot proliferation and rooting

Shoot induction and rooting were achieved on a MS medium plus vitamins (Murashige and Skoog, 1962), supplemented with 3% (w/v) sucrose (Sigma-Aldrich Co, St Louis, MO) and myo-inositol (100 mg L^{-1}) (Sigma-Aldrich Co, St Louis, MO) devoid of growth regulators. Elongated shoots rooted spontaneously. The cultures were maintained by subcutures every 25-30 days, using either nodal or apical explants.

Acclimatization

Rooted plants were individually transferred to 200-mL plastic pots containing 30 mL of sterile distilled water, covered with a transparent plastic bag, and kept for two days under a 16-h photoperiod and PFD of 35 μmol m^{-2} s^{-1} in a growth room at 25 ± 2 °C. 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 on a daily basis.

Determination of root 20-hydroxyecdysone (20E) accumulation in the germplasm bank accessions

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

Determination of 20-hydroxyecdysone (20E) concentration

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. This was then was centrifuged at 12.000 x *g* for 5 min. The quantification of 20E present on methanolic extract was performed by HPLC (High Performance Liquid Chromatography) as follows: We used 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^{-1}) of methanol: water solution (1:1) (v/v). The volume of 50 µL of the methanolic extract was injected allowing 20E quantification. The 20E standard was purchased from Sigma-Aldrich Co. (St Louis, MO).

Determination of 20-hydroxyecdysone (20E) accumulation 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, containing 21 plants each. Every month, 12 plants (three from each block) were randomly analyzed and their height measured. Flowers, leaves, stems, and roots from these plants were individually sliced, dried at 50 °C until constant dry matter, weighed, and powered. They were stored at -80 °C until 20E concentration was evaluated independently in each organ. Values of dry matter were multiplied by values of 20E concentration (on a dry matter basis), resulting in values of 20E accumulation.

RESULTS

Analysis of root 20E accumulation in *Pfaffia glomerata* germplasm bank accessions

To study 20E biosynthesis, regulation, and functions in plants, we have searched the literature for 20E occurrence in many Brazilian species. The 20E was detected in *P. glomerata* roots (Shiobara et al., 1993). The tuberous roots of *P. glomerata* are shown in Figure 1.

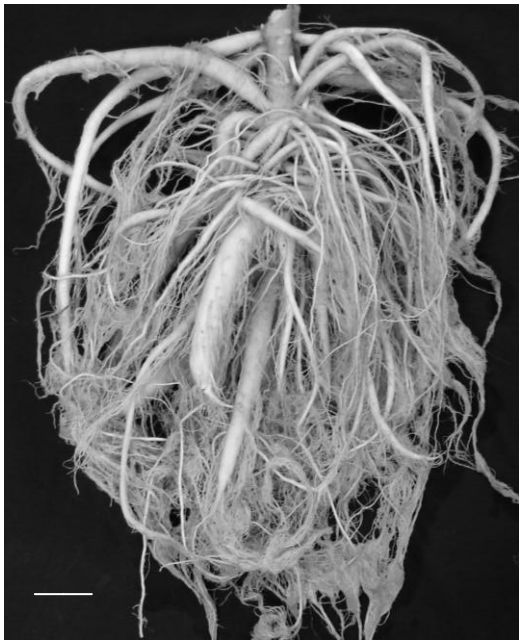

Figure 1. Detail of *Pfaffia glomerata* (accession 13) tuberous roots, harvested after 180 days. Barr = 3.5 cm.

Since other metabolites may be found adjacent to 20E, the analysis was performed a trial contrasting the resulting peaks from independent injections of the methanol solutions containing 20E, 20E plus root extract, and just the root extract (Figure 2).

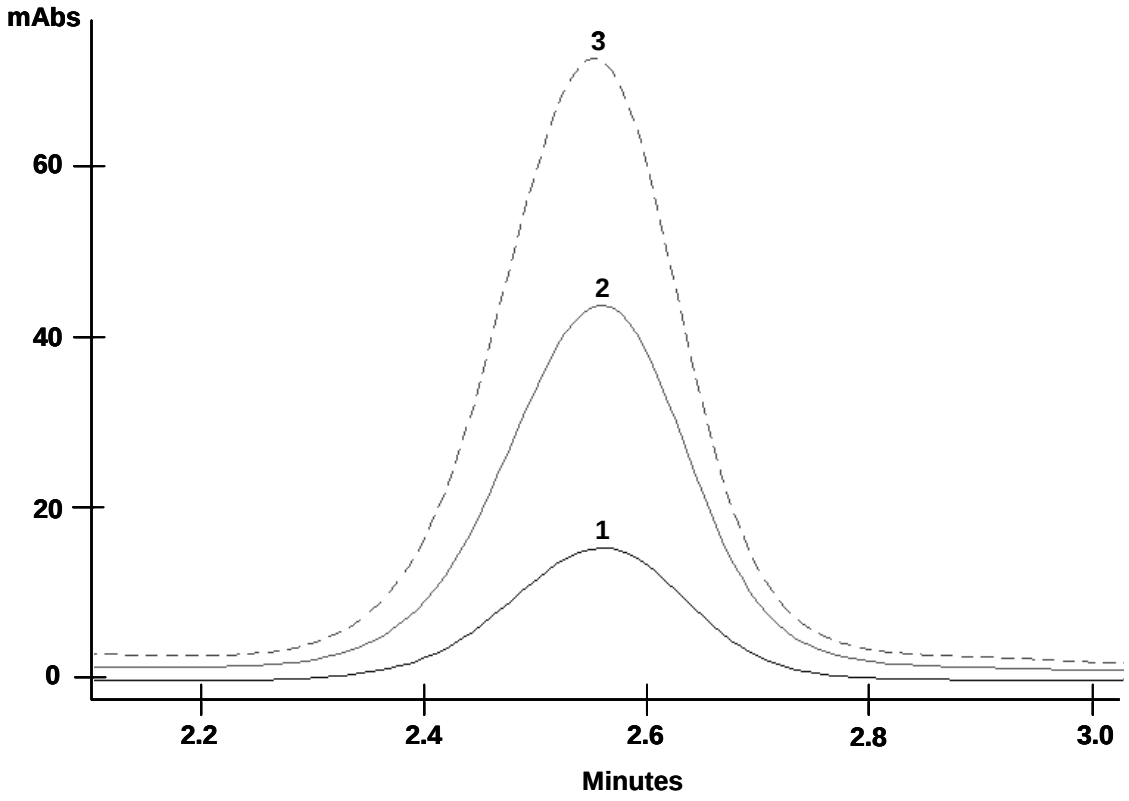

Figure 2. HPLC chromatogram. A volume of 50 µL of the solution containing 20-hydroxyecdysone (10 mg L^{-1}) (**1**)**,** 20-hydroxyecdysone (10 mg L^{-1}) plus root extract (1:1) (**2**), and root extract (**3**) was independently injected and their chromatograms were contrasted. A slow flow (1.4 mL min^{-1}) of methanol: water solution (60%:40%) (v/v) was used.

This allowed the identification of the 20E peak and it was possible to visualize that there is no adjacent peak to 20E since the sample having both 20E and root extract showed a unique peak. To choose a specific germplasm bank accession which combines both a high root dry matter and a high root 20E percentage (on a dry matter basis), 71 germplasm bank accessions of *P. glomerata* were analyzed. Values of root dry matter and 20E concentration above 220 g and 0.60% respectively were considered high. Analysis of root dry matter and root 20E concentration in each accession revealed that the accession 13 had a combination of both a high root dry matter (230.75 g) and a high root 20E concentration (0.64 %) (Figure 3).

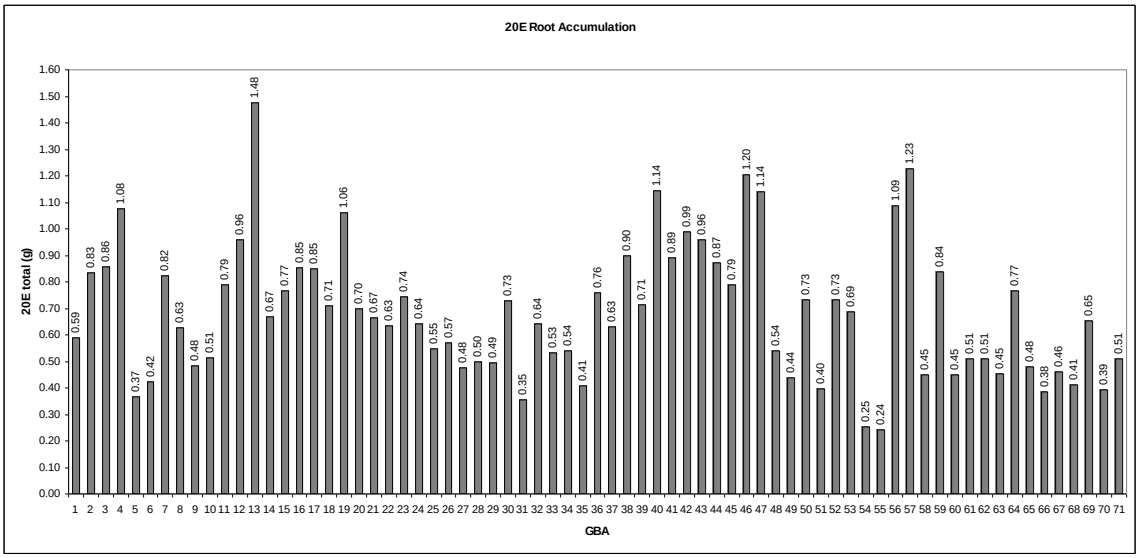

Figure 3. Root 20E accumulation in 71 Germplasm Bank accessions of *Pfaffia glomerata*. The clones of each access were obtained by micropropagation, acclimated, and transplanted into pots. Nine months after the transplantation, roots were individually sliced, dried, powdered, and weighed. The methanolic extract was prepared by adding 200 mg of the root powder in 20 mL of methanol and used in HPLC analysis after centrifugation. To calculate root 20E accumulation (20E total content), the average of the values of root dry matter were 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.

Regarding the most important economic quantitative traits (root dry matter and 20E concentration), the accession 13 showed the highest root 20E accumulation (1.48 g). For this reason, the accession 13 was chosen to perform further experiments.

Height and dry matter accumulation in different organs of the accession 13

Over the course of seven months, growth was analyzed through height and dry matter accumulation in different organs (flower, leaves, stems, and roots) (Figures 4 and 5)

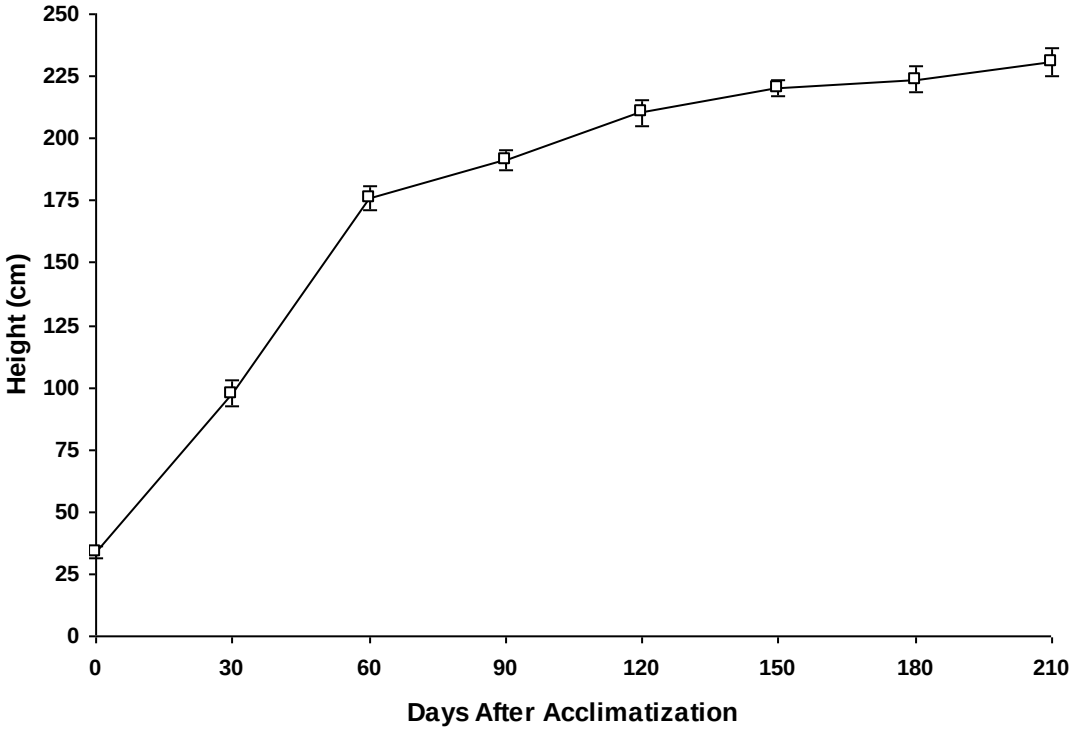

Figure 4. Height analysis of *Pfaffia glomerata* (accession 13) plants. The clones were obtained by *in vitro* propagation, acclimatized, and transplanted into pots. Every 30 days (± 3) after the transplantation, the height was measured. The standard error (SE) (n=12) is represented by vertical bars. When not shown, the SE was smaller than the symbols.

After acclimatization, plants were 33.75 cm high and transplanted into pots. In the first and second months plants grew 63.87 and 78.13 cm respectively and reached 175.75 cm high. In contrast, from the third to the seventh month plants grew 55.08 cm and reached 230.83 cm high (Figure 4).

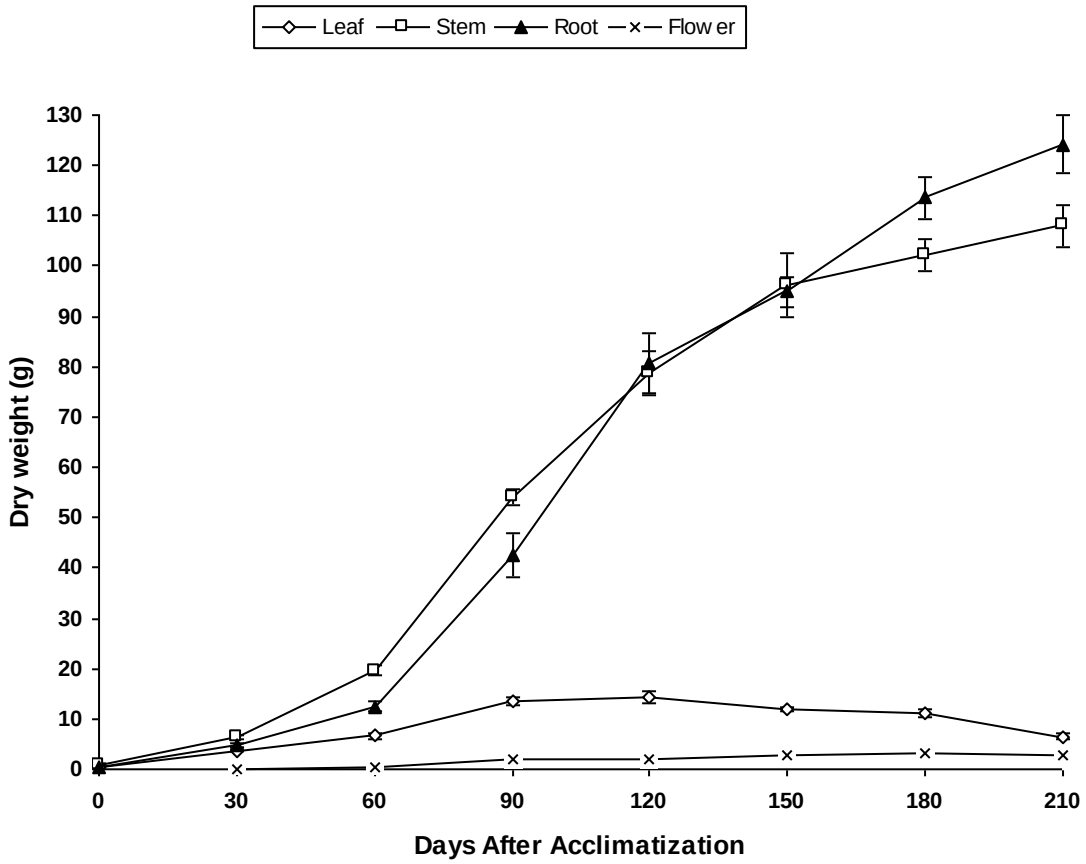

Figure 5. Dry matter accumulation analysis in *Pfaffia glomerata* (accession 13) plants. Every 30 days (± 3) after the transplantation, flowers, leaves, stems, and roots were individually sliced, dried, powdered, and weighed. The standard error (SE) (n=12) is represented by vertical bars. When not shown, the SE was smaller than the symbols.

The dry matter accumulation in leaves, stems, and roots was 0.34 g, 0.94 g, and 0.52 g respectively prior to transplantation into pots. In the first 60 days, the dry matter accumulation in leaves, stems, roots, and flowers was 6.22 g, 18.64 g, 11.93 g, and 0.36 g, respectively (Figure 5). The flowering started 30 days after transplantation. The dry matter accumulation was higher between 60 – 120 days in leaves (7.91 g), stems (59.21 g) and roots (68.23 g) and it was lower between 120 – 210 days in stems (23.34 g) and roots (32.86 g). The stems and roots kept accumulating dry matter through the fifth, sixth, and seventh month while leaves showed a decreasing of dry matter after the fourth month, due to gradual senescence. The flowers dry matter was increasing over the months, reaching in the sixth month 3.10 g and then it began to decrease in the seventh month (2.87 g). The intense dry matter accumulation phase in leaves, stems, and roots was between 60 - 120 days. From 120 – 210 days, there was a less intense dry matter accumulation (Figure 5) and a lower increase of height (Figure 4). Three distinct phases were observed in the development of *P. glomerata* (accession 13). In the first (0 – 60 days), an intense increase of height occurred in combination with a lower dry matter accumulation. In contrast, during the second (60 – 120 days), an intense dry matter accumulation occurred with a lower increase of height. During the third (120 – 210 days), a less intense dry matter accumulation occurred in combination with a lower increase of height. The 20E concentration and accumulation in these three phases, and its distribution in the various organs of *P. glomerata*, were still unknown.

Analysis of 20E occurrence, concentration and accumulation in the different organs of the accession 13

To analyze the occurrence, concentration and accumulation of 20E in different organs during the development of *P. glomerata* (accession 13), the 20E concentration (on a dry matter basis) in flowers, leaves, stems, and roots harvested on a monthly basis, was assessed by HPLC technique. The results indicated that 20E was consistently detected in all analyzed organs, but its concentration was variable throughout the development of *P. glomerata* (Figure 6). Coincidently with periods in which the temperature was cooler (16.2 – 16.5 ^{o}C), the lowest 20E concentrations were found in flowers, leaves and roots at the same time of the development (90 - 120 days) (Figure 6). The decrease of root 20E concentration in the first month may be explained by the fact that plants were transplanted from 200-mL pots into 20-L pots.

The highest 20E concentration was sequentially found in flowers (0.8221%), roots (0.6658%), leaves (0.6042), and stems (0.2445%) (Figure 6). In contrast, the lowest 20E concentration was consecutively found in stems (0.1379%), leaves (0.2118%), roots (0.4224%), and flowers (0.4731%). Stems showed the least variable 20E concentration (0.1379% - 0.2445%), followed by roots (0.4224% - 0.6658%), and flowers (0.4731% - 0.8221%). Leaves showed the greatest variation in 20E concentration (0.2118% - 0.6042%) (Figure 6).

Overall, during the first phase of development, there was less intense dry matter accumulation (Figure 5), a higher increase of height (Figure 4), an increase in the flower, root and stem 20E concentrations and a decrease in leaf 20E concentration (Figure 6). In the second phase of development, there was more intense dry matter accumulation (Figure 5), a lower increase of height (Figure 4), and although stem and root 20E concentrations were initially reduced, it was maintained at constant levels, showing a little variation while flower and leaf 20E concentrations decreased (Figure 6). In the third phase of development, there was less intense dry matter accumulation (Figure 5), a lower increase of height (Figure 4) an increase in flower, leaf and root 20E concentrations and decrease in stem 20E concentration (Figure 6). The analysis of 20E accumulation revealed that it

was predominantly accumulated in roots (Figure 7) whereas in leaves and stems, 20E total content remained constant, showing a little variation in the three phases of *P. glomera* development (Figure 7).

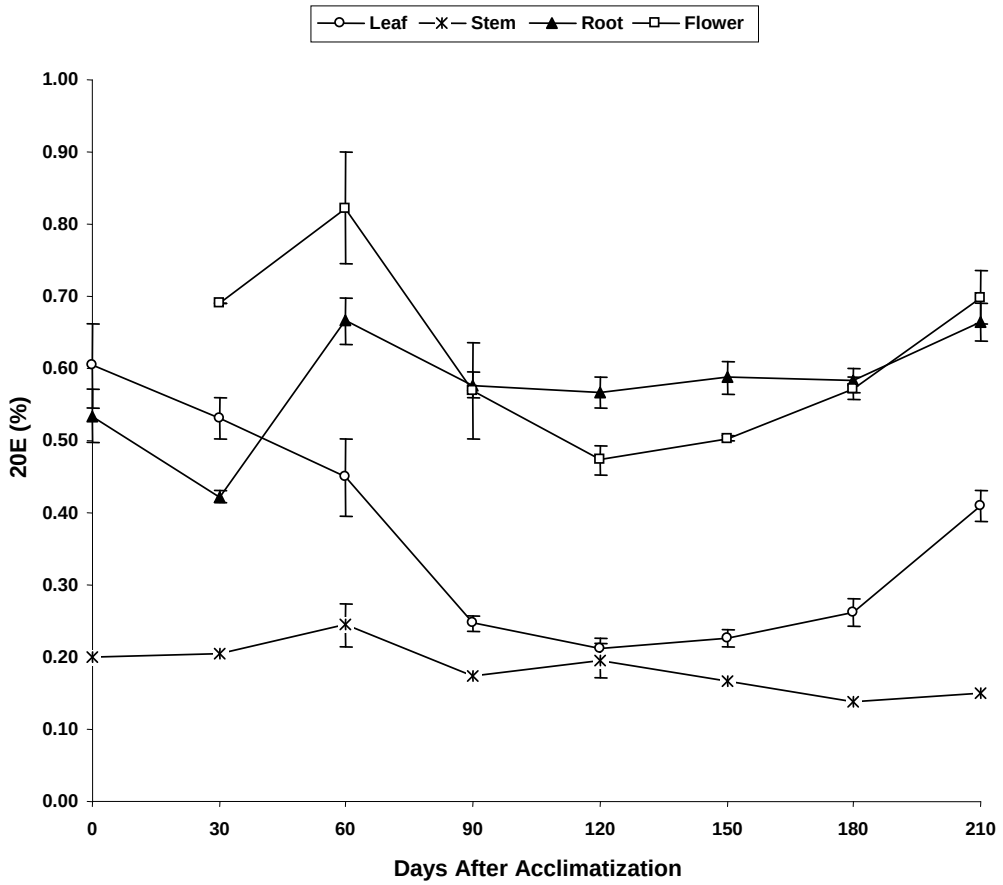

Figure 6. Analysis of 20E occurrence and concentration in different organs of *Pfaffia glomerata* (accession 13) by HPLC method. The flowers, leaves, stems, and roots of each clone were individually sliced, dried, powdered, and weighed. The methanolic extract was prepared by adding 200 mg of the powder in 20 mL of methanol and used in HPLC analysis after centrifugation. The standard error (SE) (n=12) is represented by vertical bars. When not shown, the SE was smaller than the symbols. Average of monthly temperature: 0 (March: 22.3 ^{o}C), 30 (April: 21.2 ^{o}C), 60 (May: 17.5 ^{o}C), 90 (June: 16.2 ^{o}C), 120 (July: 16.5 ^{o}C), 150 (August: 17.7 ^{o}C), 180 (September: 19.6 ^{o}C), and 210 (October: 21.8 ^{o}C).

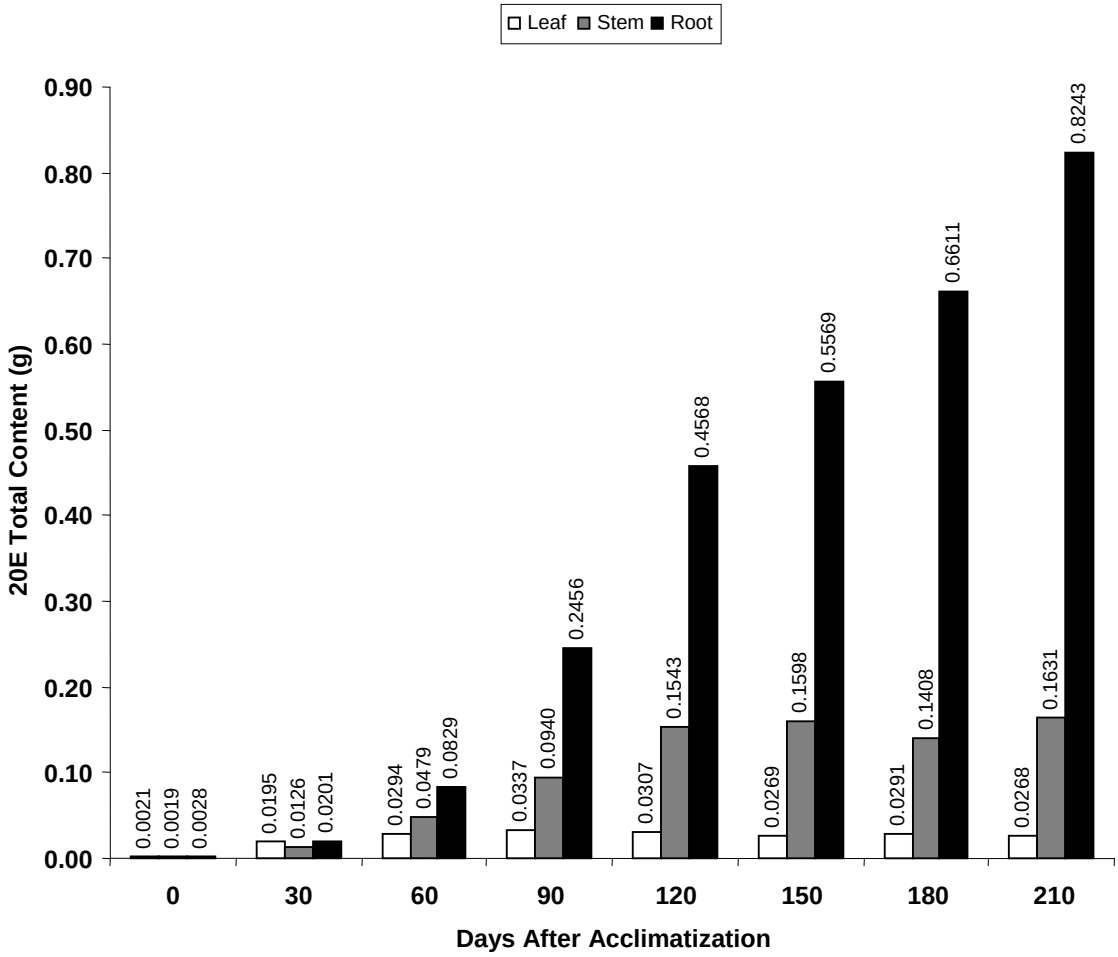

Figure 7. Analysis of 20E accumulation in leaf, stem, and root of *Pfaffia glomerata* (accession 13). The 20E accumulation (20E total content) in each organ was estimated by multiplying the average of the monthly values of dry matter by the average of the monthly values of 20E percentage. As an example (leaf): 0.3483 g x 0.6042 % ≈ 0.0021 g of 20E.

DISCUSSION

Initially, it was analyzed the root 20E accumulation in 71 accessions of the *P. glomerata* germoplasm bank. The accession 13 showed the highest root 20E accumulation and was chosen to perform further analyzes (Figure 3). The association between height and dry weight accumulation indicated that three distinct phases may be present in the development of this accession (Figures 4 and 5). The *P. glomerata* is an interesting plant to analyze 20E physiological and biochemical functions (Festucci-Buselli et al., 2008) since it produces and stores large quantities of 20E (Figures 3, 6 and 7) as well as to study plant-insect interactions since it produces continuously 20E during its development in all analyzed organs (Figures 6 and 7).

In *P. glomerata,* a perennial herbaceous plant, 20E was detected in flowers, leaves, stems, and roots during its development and accumulated mainly in its roots (Figures 3, 6 and 7), suggesting that it is playing an important function in plant's life. The high 20E accumulation found in roots (Figure 7) may explain why its roots (Figure 1) are widely used in Brazilian traditional medicine to cure or prevent several diseases (Lorenzi and Abreu Matos, 2002). Further research is necessary to validate this possibility. The medicinal properties of *P. glomerata* or other species of the genus *Pfaffia* spp. may also be attributed to other phytochemical or the combination of several compounds (Festucci-Buselli et al., 2008). In contrast, in spinach which is an annual plant, the biosynthesis and accumulation of 20E occurs in apical leaves and stems (Grebenok and Adler, 1991; Grebenok et al., 1991) and its content 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). If 20E content in *P. glomerata* increases in response to mechanical damage, insect herbivory, application of methyl jasmonate, and other stresses, it still needs to be determinated.

Several questions still need to be answered. Why do few plants including *P. glomerata* produce large amounts of 20E during its development and predominantly stock it in its roots? Which organ is responsible for 20E biosynthesis? Do plant 20E biosynthesis and accumulation concede some selective advantage? Were the genes involved in 20E biosynthesis in plants transferred from insect's genomes to plant's genomes? Is there high sequence identity between the genes involved in 20E biosynthesis in insects and plants? In plants, the genes related to 20E biosynthesis remain uncharacterized.

The genes related to 20E biosynthesis in insects have been isolated and characterized (Rewitz et al., 2007; Festucci-Buselli et al., 2008). The consecutive conversions of ketodiol, ketotriol, 2-deoxyecdysone, ecdysone, and 20-hydroxyecdysone are catalyzed by the P450s CYP306A1 (*phantom*), CYP302A1 (*disembodied*), CYP315A1 (*shadow*), and CYP314A1 (*shade*), respectively. All these genes are involved in 20E biosynthesis. The conversion of sterols into 20E involves many P450 enzymes (Gilbert, 2004). Since the P450s have been found in all kingdoms (Feyereisen, 1999; Werck-Reichhart and Feyereisen, 2000), it would be possible to find P450s related to 20E biosynthesis in other species, including *P. glomerata*. Indeed, the orthologs of *phantom*, *disembodied*, *shadow*, and *shade* are found in the genomes of the insects belonging to Hymenoptera, Coleoptera, Lepidoptera and Diptera (Rewitz et al., 2007). Sequence identity analysis between *P. glomerata* ESTs and sequences of characterized P450 genes involved in 20E biosynthesis in insects, may allow the identification of ESTs which may be used in designing strategies to isolate their orthologs in plants.

CONCLUSION

The presence of high concentrations of 20E over several months in all analyzed organs of *P. glomerata* and its predominant accumulation in roots suggest that it is playing an important role in plant's life. Its precise functions and the exact sites involved in plant 20E biosynthesis are unknown. The isolation and characterization of genes involved in 20E biosynthesis would provide evidences about its functions as well as their encoding proteins may be used to determine the precise sites responsible for 20E biosynthesis. Sequence identity analysis between genes involved in 20E biosynthesis in plants and insects woud provide evidences about its evolutionary relationship. In addition, the understanding of 20E biosynthesis in plants where it is found in larger amounts when compared to insects may provide clues to produce 20E in crop species or even to increase 20E production by metabolic engineering in *P. glomerata*. The evaluation of 20E biosynthesis in plants and insects may give insights to design strategies to control insects which damage crop species. The identification of compounds that interfere with the mode of action of ecdysteroids or with its biosynthesis would allow the designing of strategies that may control insects that damage several crop species, reducing consequently the use of insecticides.

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REFERENCES

Adler JH, Grebenok RJ (1995) Biosynthesis and distribution of insect-molting hormones in plants - a review. Lipids 30:257-262.

Barrera-Arellano D, Polezel MA, Nogueira C, Del Carmem VM, Rodrigues SC (2006). Use of a vegetable extract based cosmetic to combat cellulite consists of a product incorporating flavonoids and saponins and activating micro circulation. Patent number: BR200403155-A.

Bathori M, Pongracz Z (2005) Phytoecdysteroids – from isolation to their effects on humans. Curr. Med. Chem. 12:153-172.

De-Paris F, Neves G, Salgueiro JB, Quevedo J, Izquierdo I, Rates SMK (2000) Psychopharmacological screening of *Pfaffia glomerata* Spreng. (Amaranthaceae) in rodents. J. Ethnopharmacol. 73:261-269.

Dinan L (2001) Phytoecdysteroids: biological aspects. Phytochemistry 57:325-339.

Dinan L, Lafont R (2006) Effects and applications of arthropod steroid hormones (ecdysteroids) in mammals. J. Endocrinol. 191:1-8.

Dinan L (1998) A strategy towards the elucidation of the contribution made by phytoecdysteroids to the deterrence of invertebrate predators on plants. Russ. J. Plant Physiol. 45:296-305.

Dos Santos AS (2007) Composition, useful to treat/prevent e.g. neuralgia, comprises, as an active agent, a mixture of extracts from the plants of *Paullinia cupana* H.B.K, *Trichilia catigua* Juss, *Ptychopetalum olacoides* Benth and *Pfaffia stenophylla* Sprena. Patent number: WO2007016756-A1.

Festucci-Buselli RA, Contim LAS, Barbosa LCA, Stuart JJ, Otoni WC (2008) Biosynthesis and potential functions of the ecdysteroid 20-hydroxyecdysone – a review. Submitted.

Feyereisen, R (1999) Insect P450 enzymes. Annu. Rev. Entomol. 44:507-533.

Freitas CS, Baggio CH, Da Silva-Santos JE, Rieck L, De Moraes Santos CA, Correa Jr C, Ming LC, Cortez DAG, Marques MCA (2004) Involvement of nitric oxide in the gastroprotective effects of an aqueous extract of *Pfaffia glomerata* (Spreng.) Pedersen, Amaranthaceae, in rats. Life Sci. 74:1167-1179.

Gilbert, LI (2004) *Halloween* genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. Mol. Cell. Endocrinol. 215:1-10.

Golovatskaya IF (2004) Effect of ecdysterone on morphological and physiological processes in plants. Russ. J. Plant Physiol. 51:407-413.

Gow R (2003) Botanical extract useful in delivery of kavalactones for treating e.g. insomnia comprises extract of botanical, diluent, binder and sweetener (Eng). Patent number: WO2003084512-A1.

Grebenok RJ, Ripa PV, Adler JH (1991) Occurrence and levels of ecdysteroids in spinach. Lipids 26:666-668.

Grebenok RJ, Adler JH (1991) Ecdysteroids distribution during development of spinach. Phytochemistry 30:2905-2910.

Imai S, Iwashima J, Matsura K (2005) Composition for promoting collagen synthesis useful in cosmetics for treating aging, contains extract of *Pfaffia*, animals-and-plants component e.g. linseed, and/or components derived from animals-and-plants e.g. methyl hesperidin. Patent number: JP2005255527-A.

Kagaku HS (2002) Antiallergic composition for treating hay fever symptoms contains *Perilla* plant processed material and *Pfaffia* genus plant processed material, as active ingredients. Patent number: JP2002308795-A.

Kamada T (2006) Avaliação da diversidade genética de populações de fáfia (*Pfaffia glomerata* (SPRENG.) PEDERSEN) por RAPD, caracteres morfológicos e teor de βeta-ecdisona. Tese de Doutorado, Universidade Federal de Viçosa, Viçosa, MG, Brazil.

Lafont R, Dinan L (2003) Practical uses for ecdysteroids in mammals including humans: an update. J. Insect Sci. 3:1-30.

Lafont R, Harmatha J, Marion-Poll F, Dinan L, Wilson ID (2002) The Ecdysone handbook, 3rd edition, on-line, http://ecdybase.org.

Lafont R (1997) Ecdysteroids and related molecules in animals and plants. Arch. Insect Biochem. Physiol. 35:3-20.

Lee BG, Shinn HS (2004a) Composition for treating liver disease such as liver failure or cirrhosis comprises *Carduus marianus*, *Echinacea*, *Taraxacum officinale*, *Uncaria* hooks and other medicinal plants. Patent number: KR2004030375-A.

Lee BG, Shinn HS (2004b) Composition for treating disease such as ischemic heart disease, thrombus, and hyperlipidemia comprises *Gingko biloba*, *Carduus marianus*, *Taraxacum officinale*, *Centella asiatica* and other medicinal plants. Patent number: KR2004030376-A.

Lorenzi H, Matos FJA (2002) Plantas medicinais no Brasil, nativas e exóticas. Instituto Plantarum de Estudos da Flora LTDA, pp. 45-46.

Machackova I, Vagner M, Slama K (1995) Comparison between the effects of 20-hydroxyecdysone and phytohormones on growth and development in plants. Eur. J. Entomol. 92:309-316.

Makka T, Seino A, Tomita S, Fujuwara H, Sonobe H (2002) A possible role of 20-hydroxyecdysone in embryonic development of the silkworm *Bombyx mori*. Arch. Insect Biochem. Physiol. 51:111-120.

Marques LC, Galvão SMP, Espínola E, Dias RF, Mattei R, Oliveira MGM, Carlini ELA (2004) Psychopharmacological assessment of *Pfaffia glomerata* roots (Extract BNT-08) in Rodents. Phytother. Res. 18:566-572.

Marques LC (2003) Process for obtaining extract with tonic effects in learning and memory fortifies cognitive processes in individuals and is based on Brazilian ginseng. Patent number: BR200006645-A.

Matsuura K (2003) Living body collagen synthetic promoter for treating skin wounds, decubitus and osteoporosis, comprises powder extract or crude refined substance of extract belonging to *Pfaffia* genus and/or *Ajuga decumbens*. Patent number: JP2003277286-A.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol. Plant. 15:473-497.

Neto AG, Da Silva Filho AA, Costa JMLC, Vinholis AHC, Souza GHB, Cunha WR, Silva MLAE, Albuquerque S, Bastos JK (2004) Evaluation of the trypanocidal and leishmanicidal *in vitro* activity of the crude hydroalcoholic extract of *Pfaffia glomerata* (Amaranthaceae) roots. Phytomedicine 11:662-665.

Neto AG, Costa JMLC, Belati CC, Vinholis AHC, Possebom LS, Da Silva Filho AA, Cunha WR, Carvalho JCT, Bastos JK, Silva MLA (2005) Analgesic and anti-

inflammatory activity of a crude root extract of *Pfaffia glomerata* (Spreng.) Pedersen. J. Ethnopharmacol. 96:87-91.

Oba H, Baba H (2006) Angiogenesis inhibitor for preventing and treating diseases such as tumor and cancer, contains extract of *Pfaffia*. Patent number: JP2006273739-A.

Olalde RJA (2007) Synergistic phytoceutical composition useful for treating e.g. skin disorder and diabetic lesions, comprises plant species selected from e.g. *Acantopanacis*, *Ajuga*, *Agaricus*, *Aloe*, *Angelica*, *Bupleurum*, and *Cimicifuga*; and excipients/carriers. Patent number: US2007104728-A1.

Rewitz KF, O'Connor MB, Gilbert LI (2007) Molecular evolution of the insect Halloween family of cytochrome P450s: Phylogeny, gene organization and functional conservation. Insect Biochem. Mol. Biol. 37:741-753.

Schmelz EA, Grebenok RJ, Galbraith DW, Bowers, WS (1998) Damage-induced accumulation of phytoecdysteroids in spinach: A rapid root response involving the octadecanoic acid pathway. J. Chem. Ecol. 24: 339-360.

Schmelz, EA, Grebenok, RJ, Galbraith, DW, and Bowers, WS (1999) Insect-induced synthesis of phytoecdysteroids in spinach, *Spinacia oleracea*. J. Chem. Ecol. 25:1739-1757.

Schmelz EA, Grebenok RJ, Ohnmeiss TE, Bowers WS (2002) Interactions between *Spinacia oleracea* and *Bradysia impatiens*: a role for phytoecdysteroids. Arch. Insect Biochem. Physiol. 51:204-221.

Shiobara Y, Inoue SS, Kato K, Nishiguchi Y, Oishi Y, Nishimoto N, De Oliveira F, Akisue G, Akisue MK, Hashimot G (1993) A nortriterpenoid, triterpenoids and ecdysteroids from *Pfaffia glomerata*. Phytochemistry 32:1527-1530.

Slama K, Lafont R (1995) Insect homones - ecdysteroids: their presence and actions in vertebrates. Eur. J. Entomol. 92:355-377.

Slama K (1993) Ecdysteroids: insect hormones, plant defensive factors, or human medicine? Phytoparasitica 21:3-8.

Teixeira CGL, Piccoli A, Costa P, Soares L, Da Silva-Santos JE (2006) Involvement of the nitric oxide/soluble guanylate cyclase pathway in the anti-oedematogenic action of *Pfaffia glomerata* (Spreng.) Pedersen in mice. J. Pharm. Pharmacol. 58:667-675.

Werck-Reichhart D, Feyereisen R (2000) Cytochromes P450: a success story. Genome Biol. 1:Reviews 3003.

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