

JOÃO PAULO DE OLIVEIRA CORRÊA

***IN VITRO PHOTOAUTOTROPHIC POTENTIAL,
PHOTOSYNTHETIC CAPACITY AND 20E ACCUMULATION OF
DIPLOID AND SYNTHETIC POLYPLOID ACCESSIONS OF
Pfaffia glomerata (SPRENG.) PEDERSEN (AMARANTHACEAE)***

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Botânica, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS - BRASIL
2014

**Ficha catalográfica preparada pela Biblioteca Central da Universidade
Federal de Viçosa - Câmpus Viçosa**

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C824i
2014

Corrêa, João Paulo de Oliveira, 1987-

In vitro photoautotrophic potential, photosynthetic capacity and 20E accumulation of diploid and synthetic polyploid accessions of *Pfaffia glomerata* (SPRENG.) Pedersen (Amaranthaceae) / João Paulo de Oliveira Corrêa. – Viçosa, MG, 2014.

vi, 52f. : il. (algumas color.) ; 29 cm.

Orientador: Wagner Campos Otoni.

Dissertação (mestrado) - Universidade Federal de Viçosa.

Inclui bibliografia.

1. *Pfaffia glomerata*. 2. Ginseng brasileiro.
3. Fitoecdisteróides. 4. Poliploidia. I. Universidade Federal de Viçosa. Departamento de Biologia Vegetal. Programa de Pós-graduação em Botânica. II. Título.

CDD 22. ed. 615.32384

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APROVADA: 14 de março de 2014.

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AGRADECIMENTOS

Agradeço aos meus pais, João Batista Lúcio Corrêa e Magda Carvalho de Oliveira Corrêa, por todo amor, confiança e apoio incondicional durante toda minha vida. Por me mostrarem que é possível se adaptar e se tornar maior diante das grandes mudanças que a vida nos apresenta.

À minha irmã Marina pelo amor e companheirismo sempre.

Aos meus grandes amigos que cultivei durante esses anos em Viçosa, por tudo que representam para mim e por terem sempre sido essenciais ao meu crescimento pessoal.

Ao Professor Wagner Campos Otoni, pela amizade, pela confiança depositada em mim, por todos os ensinamentos passados e por ter sempre se empenhado para que meus trabalhos fossem realizados da melhor forma possível.

Aos amigos e companheiros de trabalho Ana Cláudia, Marcos, Diego e Camilo, que foram essenciais na realização desse trabalho.

Às bolsistas de iniciação científica Jéssica, Débora e Maíra, por sempre me ajudarem com responsabilidade e boa vontade durante a execução dos trabalhos.

À Marcela, Joseila, Cléber, Léo, Ana, Andrea, Perácio, Itaina e todos que já passaram pelo grupo da fábia, com quem tive a oportunidade de aprender muito.

A todos os meus amigos do LCTII que, além de sempre terem me auxiliado em meus trabalhos, tornam o laboratório um ambiente de muito aprendizado, companheirismo e descontração, onde se ri nos bons e maus momentos. Com uma turma dessas nossas longas horas de trabalho se tornam imensamente mais prazerosas.

Ao Dr. Cleber Witt Saldanha e ao Prof. João Paulo Viana Leite por terem aceito participar da banca e por todos os conhecimentos passados.

À Universidade Federal de Viçosa, onde concluí o ensino médio, a graduação e estou concluindo o mestrado, por ter me proporcionado tantas oportunidades, tanto pessoais quanto acadêmicas. Acabou se tornando minha segunda casa ao longo desses 10 anos de tantas mudanças.

Ao Departamento de Biologia Vegetal e ao Programa de Pós-Graduação em Botânica pela oportunidade de realização do curso de mestrado.

À CAPES, pela concessão da bolsa de estudo.

E, finalmente, a Deus pelo dom da vida.

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RESUMO

CORRÊA, João Paulo de Oliveira, M. Sc., Universidade Federal de Viçosa, Março, 2014. **Potencial fotoautotrófico *in vitro*, capacidade fotossintética e produção de 20E em acessos diploides e poliploides de *Pfaffia glomerata* (Spreng.) Pedersen (Amaranthaceae).** Orientador: Wagner Campos Otoni.

Conhecida popularmente como ginseng brasileiro, *Pfaffia glomerata* (Amaranthaceae) é uma espécie medicinal nativa do Brasil, e produz o phytoecdysteroid 20 hydroxyecdisone (20E) como seu principal ingrediente ativo. Este estudo teve como objetivo investigar se o potencial fotoautotrófico *in vitro* e a produção de 20E de 6 acessos de *P. glomerata* estão relacionados com o desempenho fotossintético e acúmulo de biomassa sob condições de cultivo *ex vitro* e estudar como estes parâmetros são afetados pela poliploidização artificial. Acessos de *P. glomerata* demonstraram capacidades fotossintéticas significativamente diferentes, resultando em variações no acúmulo de biomassa tanto *in vitro* quanto *ex vitro*. Conforme hipotetizado, os acessos com maior potencial fotoautotrófico *in vitro* também apresentaram maiores desempenhos fotossintéticos e acúmulo de biomassa *ex vitro*. Esses mesmos acessos também apresentaram a maior massa de 20E por planta *ex vitro*. A produção de 20E *in vitro* também variou entre os acessos, com a maior massa total observada nos acessos 4 e 43, que também estão entre os melhores produtores *ex vitro*. A poliploidização sintética mostrou-se eficiente na produção de indivíduos tetraplóides com maior potencial fotoautotrófico *in vitro* e acúmulo de biomassa *ex vitro*, embora as taxas de fotossíntese por área foliar não variaram entre diplóides e tetraplóides. Entre os 5 tetraploides gerados independentemente (P28, P60, P68, P75 e P75), apenas P28 apresentou um aumento efetivo no crescimento, com maior acúmulo de biomassa em relação às plantas diplóides, tanto *in vitro* quanto *ex vitro*. A poliploidização não aumentou as taxas de fotossíntese por área foliar, mas aumentou a área foliar em P28, o que pode ter resultado em um aumento da fotossíntese líquida por planta. O acúmulo de 20E *in vitro* foi maior em plantas diplóides que em tetraplóides. A concentração de 20E *ex vitro* no P28 tetraploid foi 31% maior do que em diplóides. Em comparação com diplóides, P28 apresentou o dobro da massa total 20E produzidos por planta. Em relação ao potencial fotoautotrófico *in vitro*, acúmulo de biomassa e produção 20E *ex vitro*, a indução de poliploidia e seleção de genótipos mostraram-se estratégias eficazes para alcançar uma melhor produção de *P. glomerata*.

ABSTRACT

CORRÊA, João Paulo de Oliveira, M. Sc., Universidade Federal de Viçosa, March, 2014. ***In vitro* photoautotrophic potential, photosynthetic capacity and 20E accumulation of diploid and synthetic polyploid accessions of *Pfaffia glomerata* (Spreng.) Pedersen (Amaranthaceae).** Adviser: Wagner Campos Otoni.

Popularly known as Brazilian ginseng, *Pfaffia glomerata* (Amaranthaceae) is a medicinal species native to Brazil that produces the phytoecdysteroid 20-hydroxyecdysone (20E) as its main active ingredient. This study aimed to shed light if the *in vitro* photoautotrophic potential and 20E production of 6 *P. glomerata* accessions could be related to photosynthetic performance and biomass accumulation under *ex vitro* conditions and study how these parameters are affected by induced polyploidy. *P. glomerata* accessions have significantly different photosynthetic capacities, resulting in differential biomass accumulation both *in vitro* and *ex vitro*. As hypothesized, the accessions with the highest photoautotrophic potential also showed greater photosynthetic performances and biomass accumulation *ex vitro*. The same accessions also showed the highest 20E mass per plant *ex vitro*. 20E production *in vitro* also varied among accessions, with the highest total mass achieved by accessions 4 and 43, which are also among the best producers *ex vitro*. Synthetic polyploidization was efficient in producing tetraploid individuals with increased *in vitro* photoautotrophic potential and *ex vitro* biomass accumulation, although photosynthetic rates per leaf area did not vary between diploids and tetraploids. Among the 5 independently generated tetraploids tested (P28, P60, P68, P75 and P75), only P28 showed effectively increased growth performance, with higher biomass accumulation *in vitro* and *ex vitro* in comparison to diploid plants. Polyploidization did not increase the photosynthetic rates per leaf area, but increased leaf area in P28, what may have resulted in an increased net photosynthesis per plant. 20E accumulation *in vitro* was higher in diploid plants than in tetraploids. The 20E content *ex vitro* in the tetraploid P28 was 31% greater than in diploids. In comparison to diploids, P28 showed the double of the total 20E mass produced per plant. The induction of polyploidy and selection of genotypes showed to be effective strategies to achieve an improved *P. glomerata* production in terms of higher *in vitro* photoautotrophic potential, biomass accumulation and 20E production *ex vitro*.

INTRODUCTION

The genus *Pfaffia* (Amaranthaceae) comprises approximately 90 species that occur throughout South America, and 27 were described in Brazil. Among them, *P. glomerata*, *P. paniculata* and *P. iresinoides* are medicinal species popularly known as Brazilian ginseng as various properties in common to the Korean ginseng (*Panax ginseng*) are attributed to the them, such as adaptogen and aphrodisiac properties, also having effects over physical and mental stress relief (Vigo et al., 2003; Lorenzi and Matos, 2002). *Pfaffia glomerata*, a species of wide occurrence throughout Brazil, is effectively traded in the pharmaceutical market (Vigo et al., 2004). The economic interest for the species is mainly associated to its popularity as a medicinal plant in Brazil and its potential uses as herbal drugs (Nascimento et al., 2007).

P. glomerata produces a wide variety of saponins (about 11% of the root dry weight), including its main active ingredient: the phytoecdysteroid 20-hydroxyecdysone (20E and also known as β -ecdysone). Phytoecdysteroids are plant secondary metabolites that mimic insect hormones responsible for the regulation of the molting process. Weight loss, premature molt, metabolic disorders and death may be observed in insects that feed from phytoecdysteroid producing plants (Festucc-Buselli, 2008; Dinan 2001).

20E is used in many commercial anabolic preparations for athletes and receives a remarkable attention from the phytoterapeutic and pharmaceutical industries (Lafont and Dinan, 2003; Vigo et al., 2004). *P. glomerata* trade is destined to both Brazilian and international markets (mainly United States and Japan), and the low production of raw material constitutes a factor that limits *P. glomerata* exports (Nascimento et al., 2007). As part of this raw material is obtained by extractivism (Figueiredo et al., 2004), the material harvested generally have low quality and high heterogeneity. Furthermore, the overexploitation and unsustainable harvest constitute a threat to the genetic diversity of the species (Sarasan et al., 2011). A better understanding of the propagation, cultivation and genetic diversity of the species are essential in order to achieve an improved production, with the quality and quantity needed to fulfill the pharmaceutical industry demand (Magalhães, 2000), also reducing the genetic erosion caused by the extractivism.

P. glomerata already showed a remarkable potential for micropropagation in photoautotrophic systems (Iarema et al., 2012; Saldanha et al., 2012; Saldanha et al., 2013; Saldanha et al., 2014), as vitroplants accumulate more biomass and present higher photosynthetic rates when grown with permeable membranes in the flask cap and

increased CO₂ concentrations provide by forced ventilation systems, it is, under conditions that provided higher CO₂ input. Furthermore, 20E contents *in vitro* were higher when cultures were not supplemented with sucrose in the culture medium (Iarema et al., 2012). Considering this, photoautotrophic micropropagation is a promising issue concerning research on *P. glomerata* production and propagation aiming an improved agricultural production for industrial purposes. Understanding the response of different *P. glomerata* genotypes to optimized *in vitro* culture conditions such as photoautotrophic systems and its relations with *ex vitro* plant growth is needed to achieve a really optimized and uniform production of high yield selected genotypes. Previous studies already showed that 20E accumulation pattern varies among *P. glomerata* accessions *ex vitro*. (Kamada, 2006; Festucci-Buselli, 2008)

Synthetic polyploidization has also been shown as a promising tool to obtain highly productive plants in terms of secondary metabolite accumulation and biomass production, and previous studies already achieved similar results with medicinal plants (Gao et al., 1996; Jesus-Gonzalez and Weathers, 2003; Kim et al., 2004; Dehgan et al., 2012, Xu et al., 2014). Gomes et al. (2014) produced 5 polyploid plants generated from *P. glomerata* accession 22 on independent polyploidization events and one of them showed a 50% higher 20E content in the roots in comparison to diploid plants, although the other 4 polyploids were not characterized. Nevertheless, phenotypic variations among the independently generated polyploids may be expected, as previous studies reported different DNA methylation patterns among polyploids of independent origins in other species (Mecchia et al. 2006).

In this context, the goal of the present study was to use six *P. glomerata* accessions (Chapter I) and the diploid accession 22 jointly with the 5 tetraploids generated independently from this accession (Chapter II) in order to test the following hypothesis:

- *P. glomerata* photoautotrophic potential *in vitro* is genotype dependent and accessions grow differently under this propagation system.
- Genotypes with higher photoautotrophic potentials *in vitro* also show higher biomass accumulation and photosynthetic rates *ex vitro*.
- 20E accumulation *in vitro* varies among accessions.
- Synthetic polyploidization increases *P. glomerata* photoautotrophic potential *in vitro*.

- Synthetic polyploidization increases *P. glomerata* biomass accumulation and photosynthetic rates *ex vitro*.
- Synthetic polyploids show higher 20E accumulation both *in vitro* and *ex vitro*

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CHAPTER 1

***In vitro* photoautotrophic potential and *ex vitro* photosynthetic competence of *Pfaffia glomerata* (Spreng.) Pedersen accessions**

Abstract

Pfaffia glomerata is an endangered medicinal species native to Brazil, and its main active compound is the phytoecdysteroid 20-hydroxyecdysone (20E). It has been demonstrated in previous studies that this species displays higher photosynthetic rates and 20E production when propagated under *in vitro* photoautotrophic conditions. Although a germplasm collection has been assembled there are still no reports on the screening for genotypes that may respond better to *in vitro* photoautotrophic conditions and their relations to *ex vitro* growth performance. This study aimed to investigate whether the *in vitro* photoautotrophic potential and 20E production of six *P. glomerata* accessions could be related to photosynthetic performance and biomass accumulation under *ex vitro* conditions in a greenhouse environment. Our results indicated that *P. glomerata* accessions displayed varying photosynthetic rates which might have resulted in differential biomass accumulation both *in vitro* and *ex vitro*. Accessions 4, 13 and 43 showed the highest dry weight under *in vitro* photoautotrophic conditions, coinciding with the higher CO₂ assimilation rates of the same accessions in greenhouse. They also showed the highest 20E mass per plant *ex vitro*. 20E production *in vitro* also varied among accessions, with the highest total mass achieved by accessions 4 and 43. Besides having the potential of optimizing propagation by reducing the duration of *in vitro* culture stage and lessening death rate during the acclimatization, the use of these genotypes with higher *in vitro* photoautotrophic potential can guarantee highly productive plants *ex vitro*, as the biomass accumulation pattern observed *in vitro* matched that under greenhouse conditions.

Key words: Photoautotrophic micropropagation, Brazilian ginseng, germplasm, chlorophyll *a* fluorescence, photosynthetic gas exchange.

Introduction

The genus *Pfaffia* (Amaranthaceae) comprises species known for their medicinal properties, and much of them are used by the medical and food industries (Corrêa-Júnior et al., 2006). Among these species, *Pfaffia glomerata* is widely distributed throughout Brazil and effectively traded in the pharmaceutical market (Vigo et al., 2004). The economic interest for the species has mainly been associated with its popularity as a medicinal plant in Brazil and its potential use as a phytotherapeutic (Nascimento et al., 2007).

The species, also called as ‘fáfia’ or Brazilian ginseng, produces a wide variety of triterpenic saponins, including the phytoecdysteroid 20-hydroxyecdysone (20E and also known as β -ecdysone), considered its main active ingredient (Vigo et al., 2004). This compound is also used in many commercial anabolic preparations for athletes (Lafont and Dinan, 2003). In the popular medicine various properties in common to the Korean ginseng (*Panax ginseng*) are attributed to the Brazilian ginseng, such as adaptogen, aphrodisiac, and effects over physical and mental stress relief (Vigo et al., 2003; Lorenzi and Matos, 2002).

The Brazilian exports of the species are mainly destined to the American and Japanese markets, and the low production of raw material constitutes a bottleneck to the *P. glomerata* trade (Nascimento et al., 2007). Furthermore, this raw material is mainly obtained by extractivism (Figueiredo et al., 2004), what brings up two main consequences: low quality and high heterogeneity of the material harvested; and overexploitation and unsustainable harvest, threatening natural populations and leading to the genetic erosion of the species (Sarasan et al., 2011). Therefore, studies aiming at better understanding the genetic variability, propagation and cultivation methods are essential to the achievement of an improved and homogeneous production of *P. glomerata*, in addition to reducing the harvesting pressure over the species in its natural ecosystems. Previous studies of our research team focused on the optimization of *P. glomerata* *in vitro* growth conditions and demonstrated that the species has a remarkable potential for micropropagation in photoautotrophic systems (Iarema et al., 2012, Saldanha et al., 2012; Saldanha et al., 2013; Saldanha et al., 2014). In these studies *P. glomerata* vitroplants displayed higher biomass accumulation and higher photosynthetic rates when grown under conditions that provided a higher CO₂ input, such as permeable membranes that allowed gas exchange in the flask cap and increased

CO_2 concentrations through a forced ventilation system. Furthermore, *P. glomerata* shows higher 20E contents *in vitro* when grown in the absence of sucrose (Iarema et al., 2012). These findings suggest that photoautotrophic micropropagation is a promising issue concerning research on *P. glomerata* production and propagation.

A *P. glomerata* germplasm *in vitro* collection of nearly 71 accessions was previously assembled (Kamada, 2006; Kamada et al., 2009). Recently, a karyotype analysis and DNA content of our *Pfaffia* germplasm bank has been assessed by flow cytometry (Gomes et al., 2014). The metaphases showed a constant chromosome number ($2n = 34$) and the DNA content (2C) observed for all accessions varied from 2.37 to 2.60 pg (average = 2.41 pg). Despite this genetic uniformity, screening for genotypes that may respond better to *in vitro* photoautotrophic conditions remains to be accomplished. In this regard, testing the hypothesis whether *P. glomerata* accessions grow differently under photoautotrophic conditions would be an insightful beginning. The confirmation of a possible genotype-dependent response to an *in vitro* photoautotrophic reality would bring another question: how does the *in vitro* response to photoautotrophic conditions relate to the actual *ex vitro* growth and photosynthetic performance of each accession?

Understanding the response of different *P. glomerata* genotypes to optimized *in vitro* culture conditions and their relations with *ex vitro* plant growth is needed to achieve a really optimized and uniform production of high yield selected genotypes. In this context, the goal of the present study was to use six *P. glomerata* accessions to: (1) verify whether accessions respond differently to *in vitro* photoautotrophic culture conditions; (2) test whether *in vitro* photoautotrophic growth is related to *ex vitro* growth, and photosynthetic of each accession; and (3) evaluate the 20E production of the accessions under *in vitro* photoautotrophic conditions and *ex vitro*.

Materials and Methods

Plant material and treatments

Two experiments were carried out using six *Pfaffia glomerata* accessions:.Experiment I aimed at evaluating the *in vitro* photoautotrophic growth and 20E production, whereas Experiment II aimed at assessing the *ex vitro* growth, leaf gas exchange, chlorophyll *a* fluorescence and 20E production. On both experiments, the same six accessions (which constituted the single source of variation) were used.

P. glomerata plants used in the experiments were taken from the Plant Tissue Culture Laboratory (LCT, BIOAGRO, UFV) germplasm bank, where 71 accessions are maintained *in vitro* by means of monthly subcultures using either apical or single-node in MS semi-solid medium plus vitamins (Murashige and Skoog, 1962), supplemented with 3% (w/v) sucrose and myo-inositol (100 mg L⁻¹) (Sigma-Aldrich Co, St Louis, MO, USA) and solidified with 7 g L⁻¹ of granulated agar (Merck®, Germany). For both experiments, plants (accessions 4, 13, 22, 43, 57 and 58) were subcultured for 30 days in the same conditions already described for the germplasm bank plants. These accessions were chosen because they already showed varying biomass accumulation patterns when grown under field conditions (Kamada et al., 2009)

Nodal segments without leaves excised from these subcultured plants were then used as explants in experiment I. Four explants were inoculated in 750 mL-flasks containing 100 mL of liquid basal MS medium with myo-inositol (100 mg L⁻¹), without sucrose and in place of gelling agents, a mixture of vermiculite and ground cellulose pulp 2:1 (w/w) developed in our lab was used as supporting material. The 750 mL-flasks were obtained by using couplers (Sigma Chemical Company, EUA) to join two Magenta® vessels (Figure 1). Two 10 mm-diameter holes were made in the top of the flasks and covered with a fluoropore hydrophobic membranes (PFTE; MilliSeal® Air Vent, Tokyo, Japan) in order to provide gas exchange between the flask and the outside environments. The cultures were incubated for 40 days in growth room with 25 ± 2 °C air temperature and s photon irradiance of 60 µmol m⁻² s⁻¹ provided by two tubular fluorescent lamps (cool white) with a 16-h day⁻¹ photoperiod.

Subcultured plants were acclimatized by using hydroponics with macronutrient solution [101,10 mg L⁻¹ KNO₃, 27,11 mg L⁻¹ MgSO₄·7H₂O, 188,93 mg L⁻¹ Ca(NO₃)₂·4H₂O and 42,56 mg L⁻¹ NH₄H₂PO₄] for 15 days in order to assemble experiment II. Plants were then transferred to 5 L-pots containing substrate (Tropstrato

Florestal Vida Verde, Mogi-Mirim, SP, Brazil), made of pinus bark, vermiculite, charcoal, calcium nitrate and coconut fiber, and kept in greenhouse conditions for 90 days.

Growth parameters

The plant height (cm), number of nodal segments and dry weight (grams per experimental unit) were assessed. Plant tissues were separated into root, stem and leaves and then oven-dried at 45 °C for 48 h, after which their dry weights were determined. Based on these data, leaf mass ratios (leaf mass per total mass), stem mass ratios (stem mass per total mass) and root mass ratios (root mass per total mass) were obtained.

Leaf gas exchange and chlorophyll a fluorescence

The net CO₂ assimilation rate (*A*), stomatal conductance to water vapor (*g_s*) and internal-to-ambient CO₂ concentration ratio (*C_i/C_a*) were determined simultaneously with measurements of chlorophyll *a* fluorescence using the open gas exchange system Li-6400XT (Li-Cor, Lincoln, NE, USA) equipped with an integrated fluorescence chamber head (Li-6400-40, Li-Cor). Measurements were taken between 0800 h and 1300 h at 25°C ambient temperature, 55 to 65% humidity and approximately 39 Pa CO₂ partial pressure under artificial photosynthetically active radiation of 1,000 μmol photons m⁻² s⁻¹.

Previously dark-adapted (8 h) leaf tissues were illuminated with weak modulated measuring beams (0.03 μmol m⁻² s⁻¹) to obtain the initial fluorescence (*F₀*). Saturating white light pulses of 8000 μmol photons m⁻² s⁻¹ were applied for 0.8 s to ensure for maximum fluorescence emissions (*F_m*), from which the variable-to-maximum chlorophyll fluorescence ratio, *F_v/F_m* = [(*F_m* - *F₀*)/*F_m*], was calculated. This ratio expresses the maximum photosystem II photochemical efficiency. In light-adapted leaves, the steady-state fluorescence yield (*F_s*) was measured following a saturating white light pulse (8,000 μmol m⁻² s⁻¹; 0.8 s) that was applied to achieve the light-adapted maximum fluorescence (*F_{m'}*). The actinic light was then turned off and far-red illumination was applied (2 μmol m⁻² s⁻¹) to measure the light-adapted initial fluorescence (*F_{0'}*). Using these parameters, the coefficient for photochemical quenching

(q_P) was calculated as $q_P = (F_m' - F_s)/(F_m' - F_0')$, while that for non-photochemical quenching (NPQ) was calculated as $NPQ = (F_m/F_m') - 1$. The actual quantum yield of PSII electron transport (Φ_{PSII}) was computed as $\Phi_{PSII} = (F_m' - F_s)/F_m'$, from which the apparent electron transport rate (ETR) was calculated as $ETR = \Phi_{PSII} * PPF D * f * \alpha$, where f is a factor that accounts for the partitioning of energy between PSII and PSI and is assumed to be 0.5, which indicates that the excitation energy is distributed equally between the two photosystems, and α is the leaf absorptance by the photosynthetic tissues and is assumed to be 0.84 (Maxwell and Johnson, 2000).

Stomatal density calculation

Whole leaves (second and third fully expanded leaves from the shoot tip) were processed by diaphanization with 10% (w/v) sodium hydroxide, bleached with 10% (v/v) sodium hypochlorite, stained with 0.001% (w/v) basic alcoholic fuchsin, and mounted in glycerinated gelatin. Slides were sealed with colorless nail polish. Images of the abaxial epidermis of each leaf were captured with an Olympus AX70TRF microscope (Olympus Optical, Tokyo, Japan) with a U-Photo Camera System (Spot Insight Color 3.2.0, Diagnostic Instruments Inc., USA). Stomatal density was calculated by using the software ANATI QUANTI (Aguiar et al., 2007).

Total chlorophyll concentration estimation

Total chlorophyll was estimated using a SPAD chlorophyll meter (SPAD-502, Minolta, Osaka, Japan). Measurements were taken from the second and third fully expanded leaves from the shoot tip.

High Performance Liquid Chromatography (HPLC) determination of 20E concentration

Methanolic extracts were prepared in 15 mL Falcon tubes with 100 mg of powdered dried plant material in 10 mL of methanol, and incubated at room temperature under agitation for 7 days. The extracts were then centrifuged at 5000 rpm for 20 minutes in 10 mL-falcon tubes, the supernatant was transferred to 1.5 mL-Eppendorf tubes and centrifuged again in the same conditions. After the second centrifugation the supernatant was transferred to 1.5 mL-vials and the quantification of 20E in the methanolic extract was performed by HPLC in a Shimadzu LC-10AI equipment,

coupled to a SPD-10AI detector and a Bomdesil C18 (5.0 µm x 4.6 mm x 250 mm) column. The HPLC detection was performed at 245 nm and the mobile phase used was a water/methanol 1:1 (v/v) mixture, in a 0.7 mL min⁻¹ flow rate. The line equation regarding the calibration curve was obtained by adding the 20E standard (Sigma-Aldrich) to methanol at 10, 20, 40, 60 and 80 mg L⁻¹ and performing a linear regression. 20E data were expressed both in concentration (percentage by mass) and in total mass per plant/organ.

Experimental design and statistical analysis

Two experiments were assembled separately. Experiment I, which consisted in an *in vitro* experiment aiming at assessing the *in vitro* photoautotrophic potential of the accessions, was assembled with 9 replicates per treatment, each represented by one flask containing 4 plants. Experiment II was assembled in greenhouse conditions and each of its 6 replicates consisted on a pot containing one plant. The experiments were arranged in a completely randomized design, and the accession was the only source of variation on both. Each of the 6 accessions represented a treatment. All variables were examined by ANOVA and means were compared using the Tukey's test at 5% probability. Prior to ANOVA, data were tested for normality and homogeneity of variances. Data on 20E content were analyzed after adopting a square root transformation procedure. The Pearson linear correlation technique was used for examining the relationships among variables. All of the statistical analyses were performed using the software SISVAR (Ferreira, 2003).

Results

*Photoautotrophic potential in in vitro cultured *P. glomerata* accessions is genotype-dependent*

The growth parameters from the various accessions of *in vitro* plants grown photoautotrophically differed significantly ($p < 0.05$) (Figures 1 and 2). Accessions 4, 13 and 43 accumulated more biomass (0.39 g on average) followed by accession 57 (0.27 g) and accessions 22 and 58 (approximately 0.16 g) (Figure 2D). This tendency was also observed for the other growth parameters (Figure 2), with the exception of the number of internodal segments (Figure 2B). The relatively low values for biomass accumulation in accession 22 (Figure 1) were likely a result of the low explant survival rate, which ultimately led to low dry weight values per replicate.



Figure 1. *Pfaffia glomerata* plantlets of different accessions propagated *in vitro* under photoautotrophic conditions, at 40 days of culture. From the left to the right: accessions 4, 13, 22, 43, 57 and 58. Bar = 6 cm

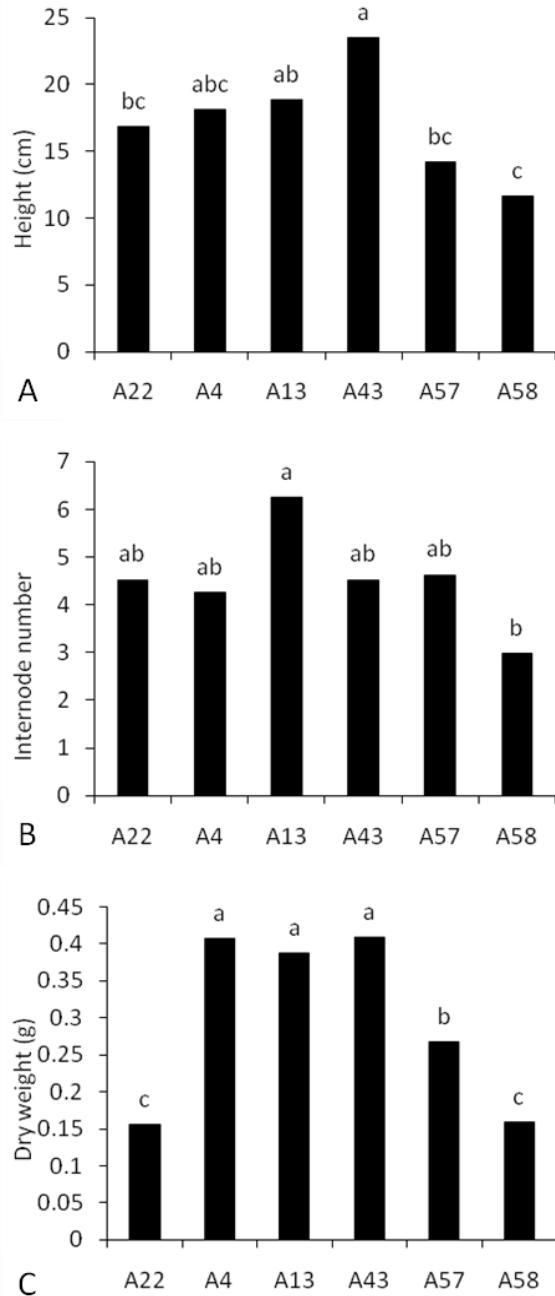


Figure 2. Growth parameters of *Pfaffia glomerata* plantlets of different accessions propagated *in vitro* under photoautotrophic conditions (after 40 days of culture). **A:** Plant height (cm). **B:** Number of internodal segments per plant. **C:** Dry weight (g) per replicate. Means followed by a same letter do not differ significantly from each other ($P > 0.05$, Tukey's test).

Genotypes with higher in vitro photoautotrophic potential also displayed better photosynthetic performance and growth ex vitro

After 90 days under greenhouse conditions (Figure 3), gas exchange, chlorophyll *a* fluorescence and growth traits of *P. glomerata* plants were assessed.. The net CO₂ assimilation rate (*A*) and stomatal conductance (*g_s*), but not the internal-to-ambient CO₂

concentration ratio (C_i/C_a), differed significantly among accessions (Figure 4). The highest A values were found in accession 4, followed by accessions 43, 13 and 57 (Figure 4A), whereas their counterparts 22 and 58 showed the lowest A values. A similar pattern was found for g_s , with the highest value observed in accession 4 and the lowest one in accession 22 (Figure 4B). Both A and g_s were positively correlated to one another ($r = 0.922$, Figure XX), and these traits in turn did not correlate significantly with C_i/C_a (data not shown).

All of the chlorophyll *a* fluorescence parameters varied significantly among accessions (Figure 5). In the case of the PSII maximum quantum yield (evaluated through F_v/F_m), the differences among accessions, despite significant, were quite narrow and, most importantly; the F_v/F_m values were maintained at 0.82 and above (Figure 5A), indicating that no type of photoinhibition took place (Maxwell and Johnson, 2000). Overall, both the apparent electron transport rate (ETR) and the coefficient for photochemical quenching (q_p) displayed a quite similar pattern of variation, with the highest values found in accessions 4, 13 and 43, and the lowest ones displayed by the accessions 13, 43 and 22 (Figure 5B and D). Indeed both q_p and ETR were strongly related to one another ($r = 0.966$, $n = 6$, $P < 0.001$). The higher values for the non-photochemical quenching (NPQ) were observed in accessions 13, 43 and 22 (Figure 4C), whereas the accessions 4, 57 and 58 showed lower values for NPQ.

Total chlorophyll concentration *in vitro* differed significantly among the accessions, and the higher concentrations were observed in the accessions that accumulated more biomass, namely 4, 13, 43 and 57 (Figure 5E). Similarly, the highest stomatal densities (Figure 5 F) were also observed on the accessions with higher biomass accumulation, with the exception of accession 58. Plants grown *ex vitro* showed higher leaf chlorophyll concentrations in comparison with their *in vitro* counterparts, and the variations among accessions *ex vitro* were similar to the pattern observed *in vitro*, with higher concentrations on accessions 4, 13, 43 and 57 (Figure 5E). The same pattern was also observed for the stomatal density, except by the fact that the stomata density in accession 57 was low *ex vitro* and relatively high *in vitro* (Figure 5F).



Figure 3.*Pfaffia glomerata* plants of different accessions grown in greenhouse for 90 days after acclimatization from *in vitro* conditions. From the left to the right: accessions 43, 13, 4, 57, 58 and 22.

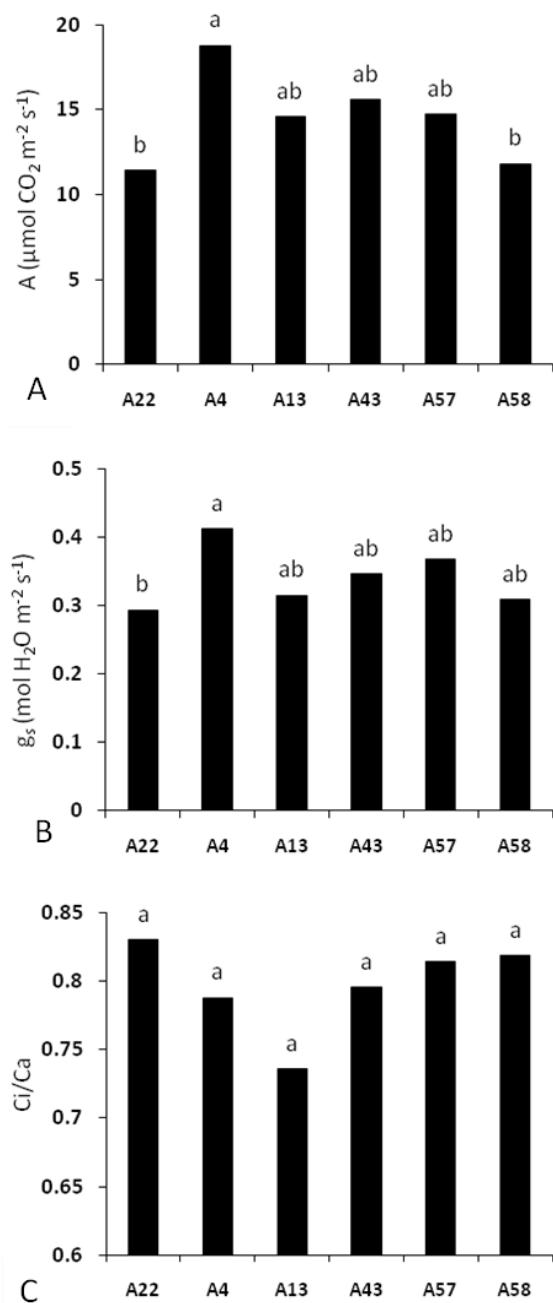


Figure 4. Gas exchange parameters from 90-day-old *P. glomerata* plants of different accessions grown under greenhouse conditions. **A:** CO₂assimilation rate (A). **B:** Stomatal conductance to water vapor (g_s). **C:** internal/external CO₂ concentration ratio (Ci/Ca). Means followed by different letters denote statistical differences calculated with Tukey's test at 5% significance.

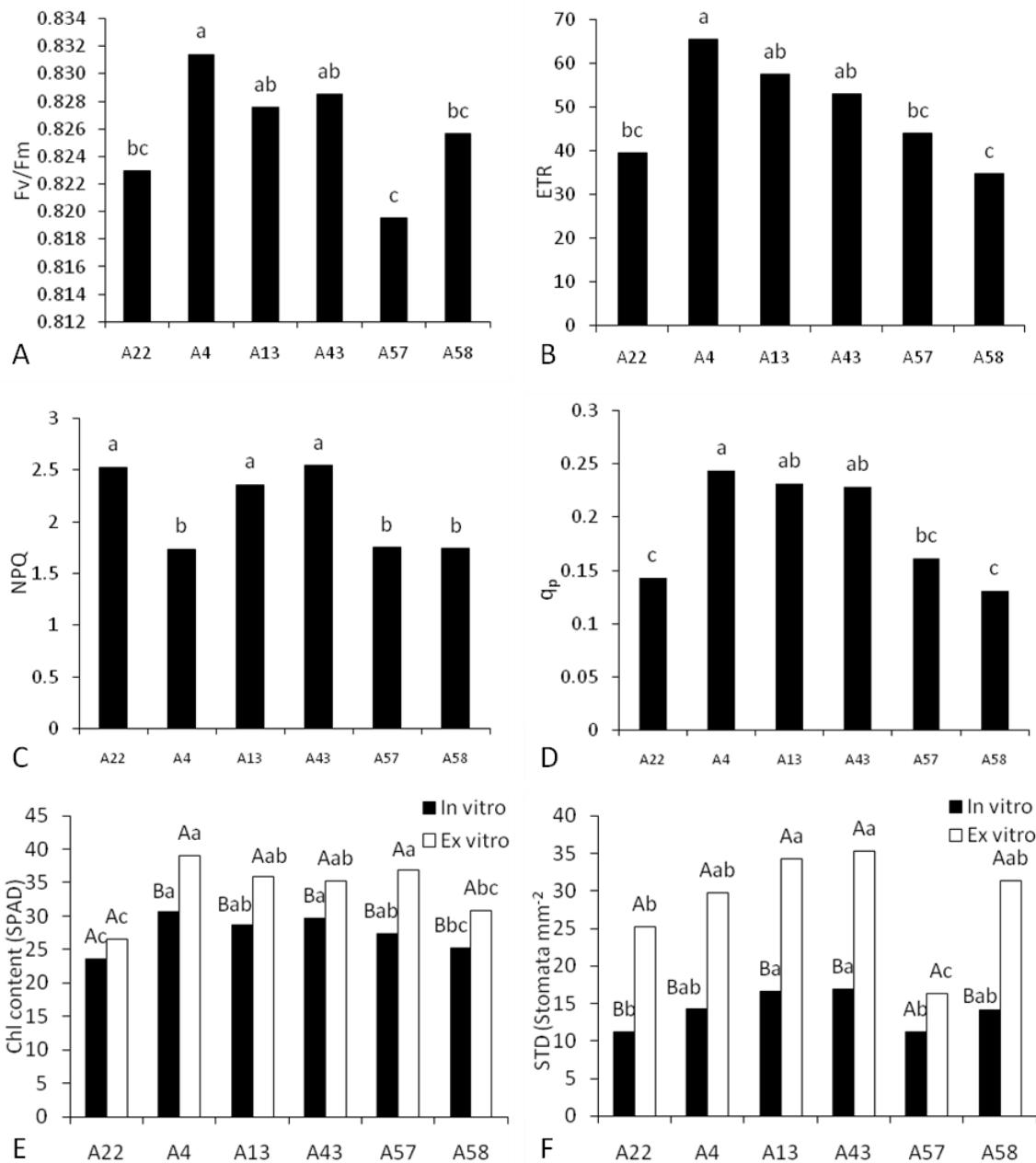


Figure 4. Chlorophyll fluorescence parameters, from 90-day-old *Pfaffia glomerata* plants of different accessions grown under greenhouse conditions (A to D). **A:** PSII maximum quantum yield (F_v/F_m). **B:** electron transport rate (ETR). **C:** Non-photochemical quenching (NPQ). **D:** Coefficient of photochemical quenching (q_p). **E** and **F:** Total chlorophyll content (E) and stomatal density (F) of both *in vitro* and *ex vitro* *P. glomerata* plants grown for 40 and 90 days respectively. Lower case letters denote differences among accessions and upper case letters denote differences between *in vitro* and *ex vitro* conditions ($P < 0.05$, Tukey's test). ΦPSII

Variations in height (Figure 6A) and internode number (Figure 6B) did not match those in biomass accumulation (Figure 6C). Two distinct groups could be identified with respect to biomass accumulation, with accessions 4, 13 and 43

producing, on average, 60% more biomass than their 22, 57 and 58 counterparts, similarly to what took place under *in vitro* conditions (*cf.* Fig. 2C). Most importantly, changes in biomass were significantly correlated with photosynthetic traits ($P \leq 0.05$), as denoted by the positive correlations between biomass with A ($r = 0.793$), q_P ($r = 0.937$), ETR ($r = 0.902$) and chlorophyll concentration ($r = 0.716$) (Figure XX). Furthermore, within each accession, biomass was always positively and significantly correlated with A (data not shown). Overall, biomass was preferentially allocated into stems, followed by leaves and roots (Figure 6D). The root mass ratio did not differ significantly regardless of the plant material, whereas the leaf mass ratio was significantly lower in accession 43 than in the other accessions which did not differ from each other. The stem mass ratio was higher in accession 43 followed by accession 4 compared with the other accessions (Figure 6D).

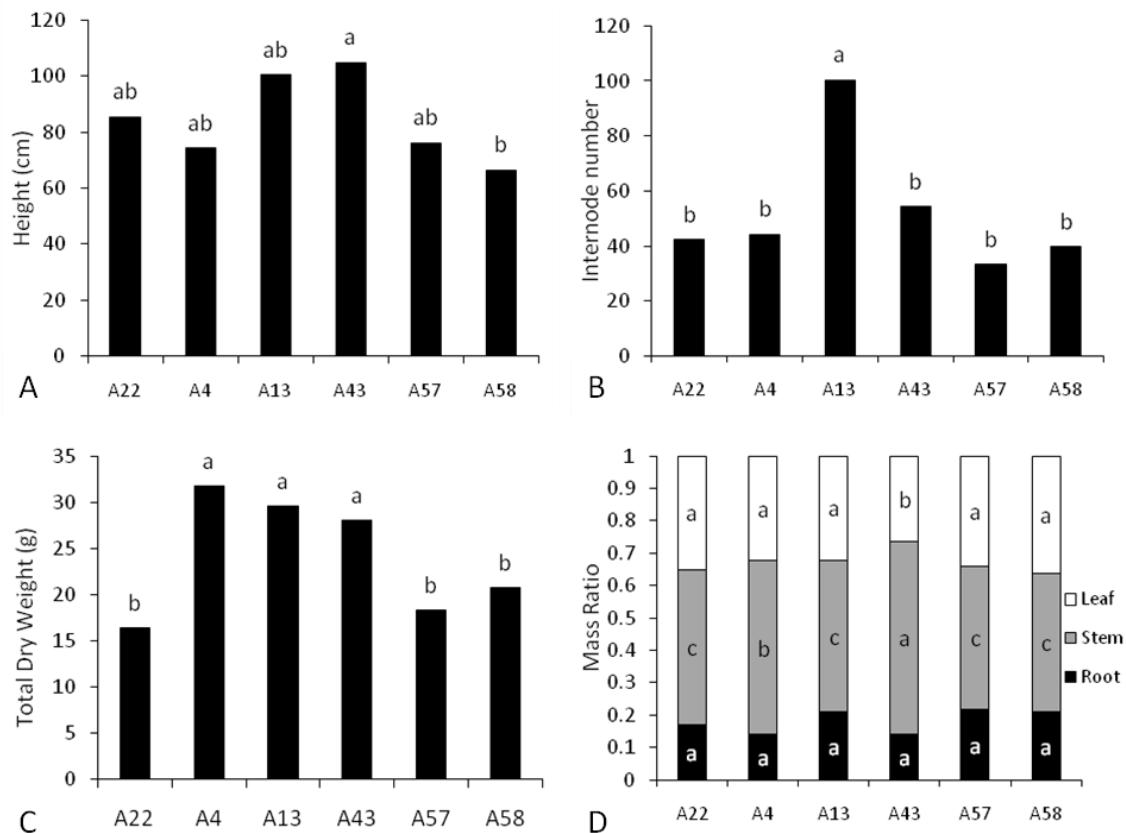


Figure 6. Growth parameters from 90-day-old *Pfaffia glomerata* plants of different accessions grown under greenhouse conditions. **A:** Plant height (cm). **B:** Number of internodal segments per plant. **C:** Dry weight per plant (g). **D:** Mass ratio per plant organ. Different letters denote statistical differences calculated with Tukey's test at 5% probability. Uppercase letters compare means among organs within the same accession, whereas lowercase letters compare means among accessions.

*20E production varied among accessions both *in vitro* and *ex vitro**

Both total 20E mass produced per replicate (mg) and 20E concentration (% m/m) varied significantly ($p < 0.05$) among accessions *in vitro* (Figures 7E-F). Vitroplants from accessions 4, 13 and 57 accumulated the highest 20E percentages. Accession 57 showed a relatively high 20E concentration, but due to its relatively low biomass production, the final 20E mass obtained per plant is not as high as the amount produced by accessions 4 and 43 (Figure 7C). In contrast, accession 13, which is among the three greater biomass producers, accumulated a low percentage of 20E, also resulting in a low final weight of this metabolite accumulated per plant *in vitro*.

Values of 20E concentrations per whole plants varied significantly among accessions, ranging from 0.24% to 0.59% (in accessions 22 and 43, respectively) in vitroplants and from 0.29 to 0.42% (in accessions 58 and 43, respectively) in plants grown *ex vitro*. Ninety-day-old plants grown in green house accumulated more 20E in roots and leaves in comparison with stems (Figure 7B). Accession 43 was the genotype that showed the higher 20E concentrations both *in vitro* and *ex vitro*, and the *ex vitro* concentrations in roots, stems and leaves were 0.59, 0.26 and 0.7% respectively (Figure 7B).

Due both to high biomass and 20E accumulation, accessions 4, 13 and 43 showed the highest 20E mass per plant *ex vitro*, and the values for this parameter ranged from 56.4 to 129.8 mg in accessions 57 and 4, respectively. The highest 20E masses per plant organ were found in leaves.

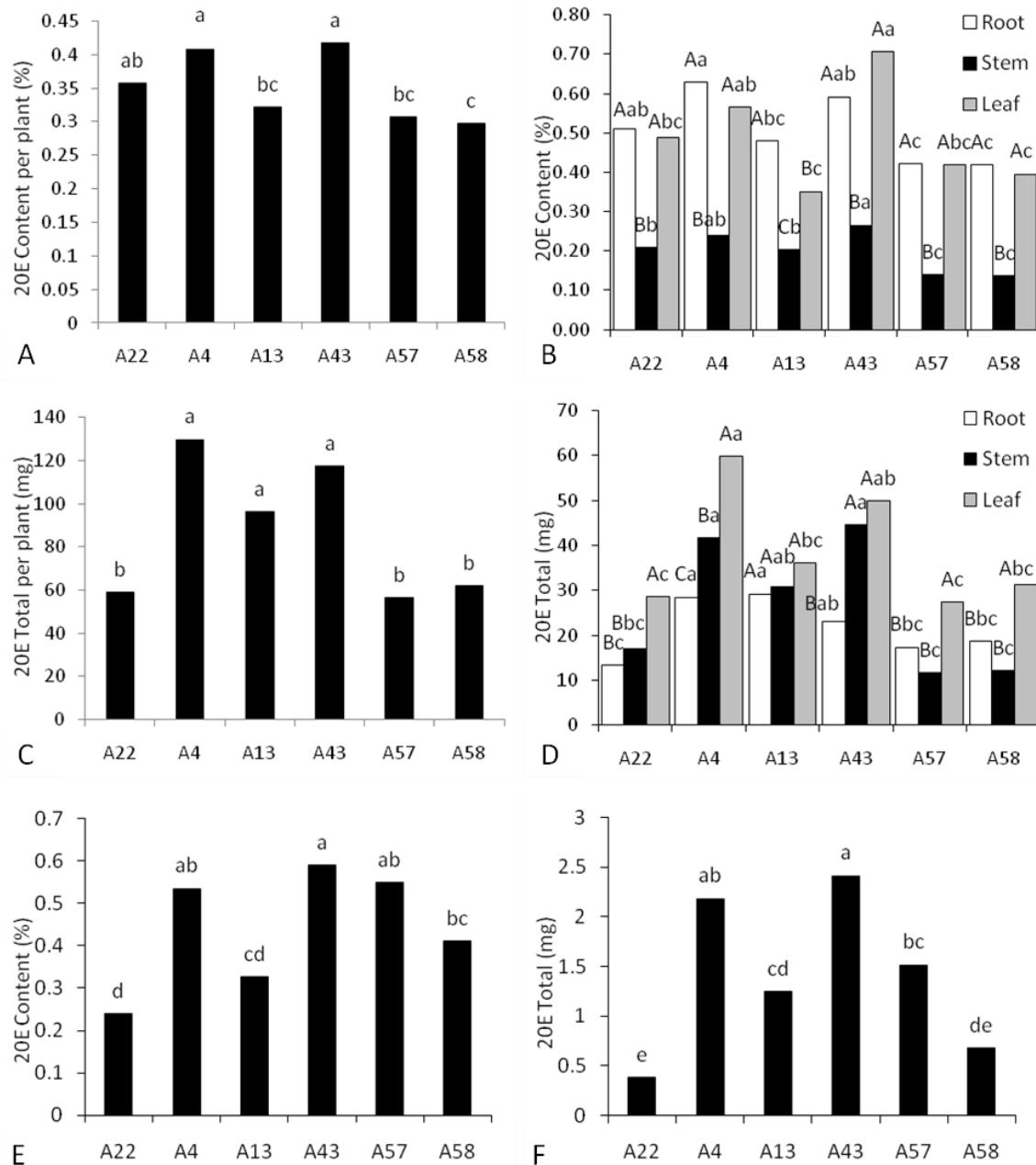


Figure 7. 20E concentrations per plant (**A**) and per plant organ (**B**) of *P. glomerata* grown in greenhouse for 90 days and total 20E mass per plant (**C**) and per plant organ (**D**). 20E content per plantlet (**E**) and total 20E mass per plantlet (**F**) grown *in vitro* for 40 days. . Different letters denote statistical differences calculated with Tukey's test at 5% probability. Uppercase letters compare means among organs within the same accession, whereas lowercase letters compare means among accessions.

Discussion

Previous studies have focused on photoautotrophic propagation as an optimization of *in vitro* cultivation in many species and its impacts over *ex vitro* acclimatization (Aldeberg et al., 1999; Kirdmanee et al., 2005; Valero-Aracama et al., 2006; Valero-Aracama et al., 2008) and *P. glomerata* also showed a three-fold increase on photosynthetic rates in an *in vitro* photoautotrophic system in comparison to plants grown in a photomixotrophic system (Iarema et al., 2012). Nevertheless, to the best of the authors' knowledge, the present study is the first report of the genotype-dependent variation in the *in vitro* photoautotrophic potential and its relations with *P. glomerata* growth *ex vitro*.

Evaluation of photosynthetic efficiency is critical for studies on plant responses to environmental conditions as well as for genotype selection. It was previously verified that *in vitro* photoautotrophic growth capacity is highly variable among *P. glomerata* accessions, and the low biomass produced per replicate by accession 22 was due mainly to the high explant loss. The high death rate observed in this accession when explants were cultivated in a photoautotrophic system is probably a consequence of the low *in vitro* photoautotrophic growth capacity. Valero-Aracama et al. (2006) examined the photosynthetic rates and the carbohydrate status of two *Uniola paniculata* genotypes with different acclimatization capacities and found a correlation between the high death rate in the difficult-to-acclimatize genotype and its lower photosynthetic capacity during the transference to acclimatization stage. As the plants used in this present study were previously cultured in a photomixotrophic system, the change imposed by the transference of the explants to a photoautotrophic system is analogue to a mild acclimatization stage, and the high death rate observed in accession 22 may also be related to an intrinsic photosynthetic capacity that was not high enough to overcome the environmental changes imposed by the photoautotrophic system.

The observation that genotypes with higher *in vitro* photoautotrophic growth also showed better growth and photosynthetic performance *ex vitro* suggests that the accessions have different intrinsic photosynthetic capacities related to their genetic backgrounds, and this could play a decisive role in determining the *in vitro* photoautotrophic potential of each accession. When vitroplants are cultured photoautotrophically instead of in a photomixotrophic system, the culture conditions are closer to the natural photoautotrophic reality of the plants (Kozai, 2001), and it is the

possible reason why the growth of plants of different accessions *ex vitro* were proportional to their growth *in vitro*.

Even though *in vitro* photoautotrophic cultivation may not increase the plant photosynthetic capacity *ex vitro* for a long period after acclimatization (Aldeberg et al., 1999; Van Huylenbroeck and Debergh, 1996), our data suggest that it is possible to use the simple assessment of 40-day-old *in vitro* photoautotrophic cultures of *P. glomerata* as a parameter to infer which genotypes show the highest growth rates in greenhouse conditions and select the most productive ones. Our data additionally indicate that differences in biomass accumulation *ex vitro* among the *P. glomerata* accessions were to a large extent explained by differences in photosynthetic rates per unit leaf area, as could be deduced by the significant correlations between *A* and biomass while the leaf mass ratio was indistinguishable among accessions with varying biomass accumulation patterns. The close association between both q_P (a measure of the trapped energy that is used in photochemical events such as carbon fixation) and ETR with *A* means that some selected chlorophyll *a* fluorescence traits may be used as a surrogate for photosynthetic rates, at least under the conditions of this experiment. Taking all of the above information together, it follows that growth of *P. glomerata* accessions under given conditions might be easily estimated by measuring those fluorescence parameters, highlighting the importance of chlorophyll *a* fluorescence as a good practical tool that can be used to perform a screening in the whole germplasm bank for accessions with improved growth under specific conditions.

Variations in *A* were highly correlated with g_s , suggesting at a first glance that stomatal limitations might play a role in constraining *A*. However, the lack of significant correlation between *A* (and g_s) and the C_i/C_a ratio can be interpreted as whether the stomatal limitations had little, if any, impact on *A*. Therefore, photochemical and/or biochemical factors are expected to be the major constraints on the photosynthetic performance. The close correlation between ETR (and q_P) with *A* suggests that, at least in part, photochemical limitations played a significant role in constraining *A*. Additional evidence for this statement comes from the fact that ETR, q_P and *A* all were well correlated with chlorophyll concentration, suggesting that there was insufficient photon capture/use to fuel carbon fixation with decreasing chlorophyll concentration. It remains to be demonstrated if varying photosynthetic capacities among the *P. glomerata* accessions were also related to biochemical factors at the chloroplast level.

Kamada et al. (2009) studied the genetic diversity of populations that constitute our *P. glomerata* germplasm bank. Except by accession 4, all of the accessions included in the present study were characterized after 271 days of growth in field conditions. In agreement with Kamada et al. (2009), accessions 13 and 43 accumulated much more biomass in the stems and roots than accessions 58 and 57. Nevertheless, accession 22 showed to be a high biomass accumulator in the study cited. This divergence is probably due to the response of the accessions to different growth conditions (field and greenhouse), and may also be related to the differential growth dynamics of this accession, as the data of the present trial was collected after 90 days.

This is also the first report on the variation of 20E production among accessions *in vitro*. Considering that the initial steps of the main research programs for the production of secondary metabolites from plant cell cultures include the collection of a large genetic pool of the species followed by the screening of hyper producing genotypes that present the most valuable secondary metabolites (Borgaud et al., 2001), the observation of this *in vitro* genotype-dependent variation in 20E production is an interesting finding to consider when planning future research aiming to produce secondary metabolites from *P. glomerata* cell cultures or any other *in vitro* system. Screening the whole germplasm bank for the most productive accessions in terms of 20E and biomass *in vitro* would be an interesting start for the establishment of *in vitro* cultures for secondary metabolites mass production.

Iarema et al. (2012) had already showed that *P. glomerata* *in vitro* 20E production is higher under photoautotrophic conditions, and the present study found that the concentrations of this metabolite on 40-day-old photoautotrophically-grown vitroplants were comparable to the percentages found in 90-day-old plants grown in greenhouse, suggesting an enormous potential for the *in vitro* commercial production of 20E from *P. glomerata* plants.

In agreement with previous studies (Festucci-Buselli et al., 2008), the 20E concentrations in general did not differ greatly between roots and leaves, and the lowest concentrations were found in stems. However, the highest total 20E mass in the study cited was found in roots, and root total 20E mass showed the highest increases along the time. This response was due to the high investment in tuberization that *P. glomerata* roots show along with the time, having a higher portion of the total biomass represented by roots (Festucci-Buselli et al., 2008), in contrast with our data in which biomass was to a greater extent allocated into stems (higher stem mass ratio). In any case, the present

study showed the leaves as the organs that accumulate the higher portion of total 2OE mass, but this pattern would probably change had the plants grew for a longer time before being assessed.

Our results show that *P. glomerata* accessions have different photosynthetic capacities, which are expressed even when plants are grown *in vitro* under photoautotrophic conditions. Therefore, in addition to the recent findings concerning the key role of photoautotrophic micropropagation on the optimization of *P. glomerata* *in vitro* under natural (Iarema et al., 2012; Saldanha et al., 2012) or forced ventilation (Saldanha et al., 2013; Saldanha et al., 2014), the growth performance under *in vitro* photoautotrophic conditions may be used as a trait to select highly productive accessions from the 71-accession germplasm collection, increasing even more the efficiency of this powerful propagation system. In addition to reducing the duration *in vitro* culture stage and the death rate during the acclimatization, the use of these selected genotypes will also provide highly productive plants *ex vitro*, as the biomass accumulation pattern observed *in vitro* was matched that under greenhouse conditions. Finally, it was demonstrated that, at least under the present experimental conditions, key traits such as A , q_P and ETR revealed to be very useful to assess the growth performance of *P. glomerata* accessions and, as such, these traits seems to constitute a promising future prospect for the species, giving many practical insights on the *P. glomerata* cultivation conditions.

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CHAPTER 2

Induced polyploidization increases *in vitro* photoautotrophic growth and *ex vitro* biomass and β -ecdysone accumulation in *Pfaffia glomerata* (Spreng.) Pedersen

Abstract

P. glomerata tetraploid plants were previously obtained from five independent polyploidization events induced with colchicine treatment. The present study verified the impacts of induced polyploidization over *P. glomerata* *in vitro* photoautotrophic potential, *ex vitro* biomass accumulation and photosynthetic rates and 20E production both *in vitro* and under greenhouse conditions. Synthetic polyploidization was efficient in producing individuals with increased *in vitro* photoautotrophic potential and *ex vitro* biomass accumulation, although photosynthetic rates per leaf area did not vary between diploids and tetraploids. Among the five tetraploids tested (P28, P60, P68, P75 and P75), P28 was the one that showed effectively increased growth performance both *in vitro* and *ex vitro* when compared to diploid plants, whereas the other tetraploids did not differ significantly from the diploids concerning biomass accumulation. Although photosynthetic rates per leaf area unit remained constant among all the plants tested, P28 showed a significantly greater total leaf area, what may have resulted in an increased net photosynthesis per plant. 20E accumulation *in vitro* did not follow the same pattern of variation observed among the plants *ex vitro*, being higher in diploid plants. The 20E content of the tetraploid P28 was 31% higher than the 20E contents of diploid plants in greenhouse conditions. The final 20E mass per plant produced by P28 *ex vitro* was approximately twice as much as the amount produced by diploid plants. The induction of polyploidy in *P. glomerata* showed to be a valid strategy to produce plants with higher biomass accumulation and 20E production *ex vitro*, besides higher *in vitro* photoautotrophic potential.

Key words: Photoautotrophic micropropagation, Brazilian ginseng, synthetic polyploidy, autoploidy, leaf gas exchanges

Introduction

Induced polyploidy in medicinal plants is generally a successful strategy aiming to obtain highly productive plants (Dhawan and Lavania, 1996) and, although the impacts of polyploidy over biomass accumulation and secondary metabolite production is variable, ploidy level manipulation in many species has been suggested as an interesting strategy for increasing plant biomass accumulation and the production of high-value secondary metabolites (Lavania, 2005; Xu et al., 2014). Increases in ploidy level often lead to structural changes such as leaf size, stomatal density, cell size and number of chloroplasts per cell (Dhawan and Lavania, 1996), which may affect physiological and biochemical activities such as net photosynthesis (Warner and Edwards, 1989).

Pfaffia glomerata (Amaranthaceae), also known as Brazilian ginseng, is a medicinal species native to South America that produces a wide variety of triterpenic saponins, including the phytoecdysteroid 20-hydroxyecdysone (20E and also known as β -ecdysone), considered its main active compound (Vigo et al., 2004). This compound is also used in many commercial anabolic preparations for athletes (Lafont and Dinan 2003), and various properties in common to the Korean ginseng (*Panax ginseng*) are attributed to the Brazilian ginseng, such as adaptogen, aphrodisiac, and effects over physical and mental stress relief (Vigo et al., 2003; Lorenzi and Matos, 2002). The species is effectively traded in both Brazilian and international markets (Nascimento et al., 2007). *P. glomerata* tetraploids were previously obtained from nodal bud thin-layer sections treated with colchicine. Five tetraploids were regenerated from independent polyploidization events, and the characterization of one of these polyploids (P68) showed that 90-day-old plants, although had reduced root and stem biomass in comparison to diploids, showed a 50% increase in the root 20E content (Gomes et al., 2014). Nevertheless, the other 4 *P. glomerata* tetraploids obtained by our research group remain uncharacterized.

Previous findings showed that photoautotrophic micropropagation is a very promising system for both *in vitro* production of secondary metabolites from *P. glomerata* and mass propagation of the species, as plants showed higher photosynthetic rates and produced more 20E *in vitro* when grown under photoautotrophic conditions (Iarema et al., 2012; Saldanha et al., 2012; Saldanha et al., 2013; Saldanha et al., 2014).

Considering that polyploidization may increase the plant net photosynthesis, it may also have positive impacts over the photoautotrophic potential of the species.

In this context, the goals of the present study were using the diploid accession 22 and the five autotetraploids independently generated from this accession in order to study the effects of *P. glomerata* induced polyploidization over: (1) *in vitro* photoautotrophic potential; (2) biomass accumulation and photosynthetic rates *ex vitro*; and (3) the 20E production by the species both in greenhouse and under *in vitro* photoautotrophic conditions.

Materials and Methods

Plant material and treatments

Two experiments were assembled, being the ploidy/polyplloidization event the only source of variation on both. The plants used were diploid plants from *Pfaffia glomerata* accession 22 and five synthetic polyploids (tetraploids) originated from this accession through independent events (named P28, P60, P68, P74 and P75). Experiment I aimed at evaluating the influence of ploidy level and polyplloidization event over *in vitro* photoautotrophic growth and 20E production in *P. glomerata*, whereas experiment II aimed at assessing the effects of chromosome doubling over *ex vitro* growth, leaf gas exchange, chlorophyll *a* fluorescence and 20E production.

P. glomerata plants used in the experiments were taken from the Plant Tissue Culture Laboratory (LCT – BIOAGRO - UFV) germplasm bank, where 71 diploid accessions and five synthetic polyploids originated independently from accession 22 are maintained *in vitro* by means of monthly subcultures using either apical or single-node in MS semi-solid medium plus vitamins (Murashige and Skoog, 1962), supplemented with 3% (w/v) sucrose and myo-inositol (100 mg L⁻¹) (Sigma-Aldrich Co, St Louis, MO) and solidified with 7 g L⁻¹ of granulated agar (Merck®, Germany). For both experiments plants (diploid accession 22 and the tetraploids P28, P60, P68, P74 and P75) were subcultured for 30 days in the same conditions already described for the germplasm bank plants.

Nodal segments without leaves excised from these subcultured plants were then used as explants in experiment I. Four explants were inoculated in 750 mL-flasks containing 100 mL of liquid basal MS medium with myo-inositol (100 mg L⁻¹), without sucrose and, instead of gelling agents, a mixture of vermiculite and ground cellulose pulp 2:1 (w/w) was used as supporting material. The 750 mL-flasks were obtained by using couplers (Sigma Chemical Company, EUA) to join two Magenta® vessels (Figure 1). Two 10 mm-diameter holes were made in the top of the flasks and covered with a fluoropore hydrophobic membranes (PTE; MilliSeal® Air Vent, Tokyo, Japan) in order to provide gas exchange between the flask and the outside environments. The cultures were incubated for 45 days in growth room with 25 ± 2 °C air temperature and 60 µmol m⁻² s⁻¹ irradiance provided by two tubular fluorescent lamps (cool white) with a 16-h day⁻¹ photoperiod.

Subcultured plants were acclimatized by using hydroponics with macronutrient solution [101,10 mg L⁻¹ KNO₃, 27,11 mg L⁻¹ MgSO₄·7H₂O, 188,93 mg L⁻¹ Ca(NO₃)₂·4H₂O and 42,56 mg L⁻¹ NH₄H₂PO₄] for 15 days in order to assemble experiment II. Plants were then transferred to 5 L-pots containing substrate (Tropstrato Florestal Vida Verde, Mogi-Mirim, SP), made of pinus bark, vermiculite, charcoal, calcium nitrate and coconut fiber, and kept in greenhouse conditions for 160 days.

Growth parameters

It was assessed plant height (cm), number of nodal segments, and fresh and dry weight (grams per experimental unit). Fresh weight was not assessed in experiment 2, and dry weight was measured separately for root, stem and leaves. Additionally, it was assessed leaf area (cm²), stem diameter (mm) and root volume (mL). Leaf area was assessed with Area Meter, model Li-Cor 3100 (LI-COR Biosciences, Inc Lincoln, Nebraska).

Total chlorophyll content

Total chlorophyll was calculated with a SPAD chlorophyll meter (SPAD-502, Minolta, Osaka, Japan). Measurements were taken from the second and third fully expanded leaves from the shoot tip.

High Performance Liquid Chromatography (HPLC) determination of 20E content

Methanolic extracts were prepared in 15 mL Falcon tubes with 100 mg of powdered dried plant material in 10 mL of methanol, and incubated at room temperature under agitation for 7 days. The extracts were then centrifuged at 5000 rpm for 20 minutes in 10 mL-falcon tubes, the supernatant was transferred to 1.5 mL-Eppendorf tubes and centrifuged again in the same conditions. After the second centrifugation the supernatant was transferred to 1.5 mL-vials and the quantification of 20E in the methanolic extract was performed by HPLC in a Shimadzu LC-10AI equipment, coupled to a SPD-10AI detector and a Bomdesil C18 (5.0 µm x 4.6 mm x 250 mm) column. The HPLC detection was performed at 245 nm and the mobile phase used was

a water/methanol 1:1 (v/v) mixture, in a 0.7 mL min⁻¹ flow rate. The line equation regarding the calibration curve was obtained by adding the 20E standard (Sigma-Aldrich) to methanol at 10, 20, 40, 60 and 80 mg L⁻¹ and performing a linear regression. 20E data were expressed both in concentration (percentage by mass) and in total mass per plant/organ.

Stomatal density calculation

Whole leaves (second and third fully expanded leaves from the shoot tip) were processed by diaphanization with 10% w/v sodium hydroxide, bleached with 10% v/v sodium hypochlorite, stained with 0.001% w/v basic alcoholic fuchsin, and mounted in glycerinated gelatin. Slides were sealed with colorless nail polish. Images of both abaxial and adaxial epidermis of each leaf were captured with an Olympus AX70TRF microscope (Olympus Optical, Tokyo, Japan) with a U-Photo Camera System (Spot Insight Color 3.2.0, Diagnostic Instruments Inc., USA). Stomatal density was calculated by using the software ANATI QUANTI (Aguiar, 2007).

Leaf gas exchange and chlorophyll a fluorescence

Gas exchange was assessed by using a infra red gas analyzer (IRGA; LI-6400xt, LI-COR Biosciences Inc., Lincoln, Nebraska, USA), and the parameters were net CO₂ assimilation rate (*A*), stomatal conductance to water vapor (*g_s*), internal/external CO₂ concentration ratio (*C_i/C_a*) and transpiration rate (*E*). Measurements were taken from fully expanded leaves from the shoot tip, from 8 am to 13 pm in greenhouse, with constant PAR of 1,000 µmol photons m⁻² s⁻¹, ~390 µmol mol⁻¹ atmospheric CO₂ concentration, 25 °C atmospheric temperature and 55 to 65% humidity.

Chlorophyll *a* fluorescence parameters were also assessed by using the IRGA. Leaves of previously dark-adapted plants were illuminated with weak measuring beams in order to obtain the minimal fluorescence (*F*₀). A saturating white light pulse were applied in order to measure maximum fluorescence (*F*_m), from which the photosystem II maximum quantum yield was calculated: $F_v/F_m = (F_m - F_0)/F_m$. In light-adapted leaves, the steady-state fluorescence (*F*) was measured after the application of a

saturating white light pulse that in order to measure the light adapted maximum fluorescence (F_m'). Far-red illumination was applied to measure the light-adapted initial fluorescence (F_0'). The coefficient for non-photochemical quenching (NPQ) was calculated as $\text{NPQ} = (F_m/F_m') - 1$. The actual quantum yield of PSII photochemistry (Φ_{PSII}) was computed as $\Phi_{\text{PSII}} = (F_m' - F_s)/F_m'$, from which the electron transport rate (ETR) was calculated as $\text{ETR} = \Phi_{\text{PSII}} \times \text{PPFD} \times f \times \alpha$, where f , assumed to be 0.5, accounts for the partitioning of energy between PSII and PSI, and α is the leaf absorbance by the photosynthetic tissues and is assumed to be 0.84.

Experimental design and statistical analysis

The experiments were arranged in a completely randomized design, and the ploidy/polyplloidization event was the only source of variation on both, being analyzed as a single factor. The plants tested were diploid accession 22 and the polyploids P28, P60, P68, P74 and P75, each of them representing a treatment. Experiment I was assembled with 8 replicates per treatment, each represented by one flask containing 4 plants, whereas each of the 6 replicates on experiment II consisted on a pot containing 1 plant. All variables were examined by ANOVA and means were compared by Tukey's test at 5% probability with the software SISVAR (Ferreira, 2003). Data on 20E content were analyzed using square root transformation.

Results

Synthetic polyploidization increased in vitro photoautotrophic potential

In vitro photoautotrophic growth varied significantly both among polyploid plants and between diploids and polyploids (Figure 2). The highest *in vitro* dry weight was shown by the polyploid P28 (472 mg per replicate) but, as P75 showed the lowest value for this parameter (255 mg), high biomass accumulation was not a predominating characteristic among polyploid plants. P28 dry weight *in vitro* was 32% higher than accession 22, and the other polyploids (P60, P78 and P74), jointly with the diploid 22, showed intermediate biomass accumulation (Figure 2C and D). So, the only artificial polyploid that really showed higher dry weight in comparison to the diploid was P28. The number of internodes per plant did not vary significantly among different ploidies or polyploidization event (Figure 2B).

Total chlorophyll also varied significantly among different plants *in vitro*, ranging from 22.5 to 29 SPAD (in P75 and P60, respectively), and accession 22 showed an intermediate content (26.3 SPAD) (Figure 5).



Figure 1. *Pfaffia glomerata* diploid and tetraploid plantlets from independent polyploidization events of different propagated *in vitro*, at 45 days of culture. From the left to the right: accession 22 diploid, P28, P60, P68, P74 and P75. Bar = 6 cm.

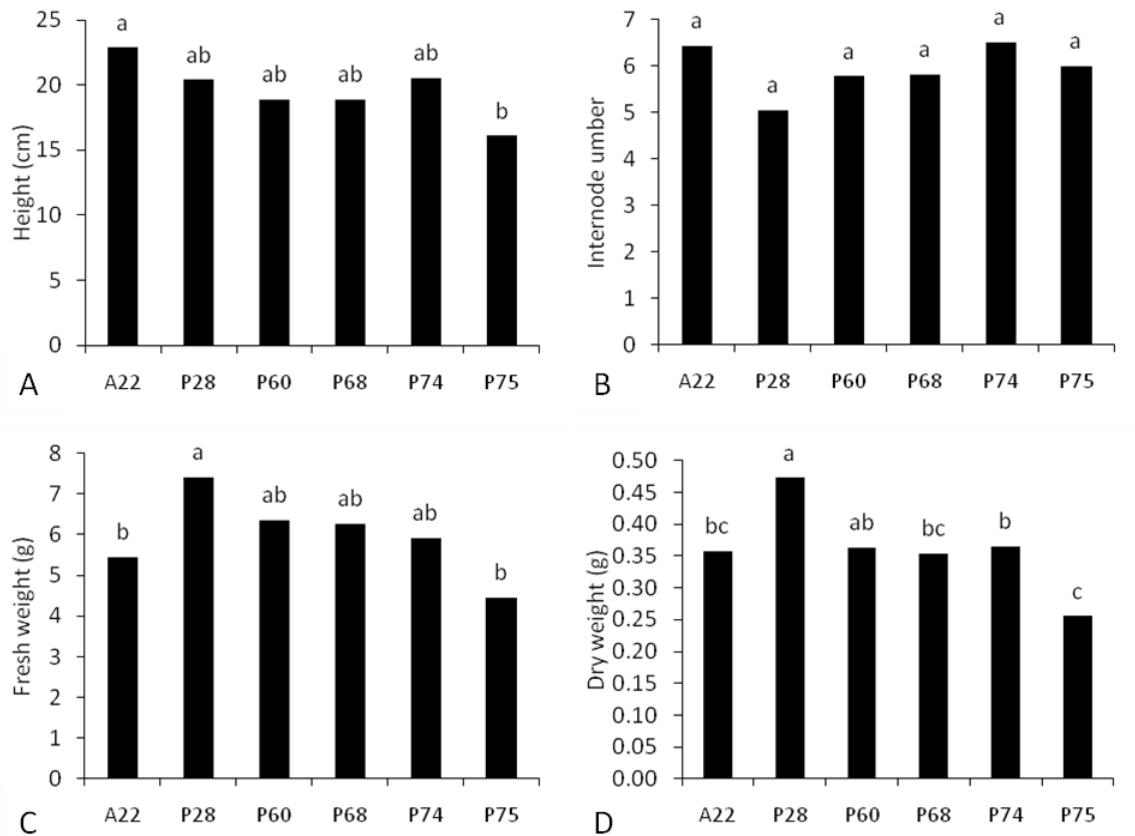


Figure 2. Growth parameters of *Pfaffia glomerata* of different ploidies/polyploidization events propagated *in vitro* under photoautotrophic conditions, after 45 days of culture. **A:** Plant height (cm). **B:** Number of internodal segments per plant. **C:** Fresh weight (g) per replicate. **D:** Dry weight (g) per replicate. Different letters denote statistical differences calculated with Tukey's test at 5% probability.

Synthetic polyploids showed higher biomass accumulation ex vitro, but photosynthesis did not increase

Among all plants, the lower biomass accumulation was found in diploid plants, although the only polyploid that differed significantly from accession 22 was P28 (Figure 5). This tetraploid showed a total dry weight of 44.1 g, which represents a 72% increase in comparison to diploid counterpart. Polyploids P60, P68, P74 and P75 showed intermediate dry weight values, not differing significantly from either the diploid 22 or P28 (Figure 5F). The same variation pattern was also observed in root volume and leaf area (Figure 5D and E). Leaf area was considerably higher in P28, and leaf shape also varied. The width/length ratio of leaves was greater in polyploids in comparison to diploids. Although this parameter was not measured, Figure 3 illustrates this variation in leaf shape and size. Additionally, it was observed abnormal leaf

development in P75, in which curly leaves were observed both *in vitro* and *ex vitro* (Figures 3 and 4).



Figure 3. *Pfaffia glomerata* leaves from the fourth lower nodas segment from plants of different ploidies/polyploidization events grown in greenhouse for 150 days after acclimatization from *in vitro* conditions. From the left to the right: P28, P60, P68, P74, P75 and accession 22 diploid. Bar= 5cm.



Figure 4. Aspect of curly leaves of tetraploid P75 plants grown *in vitro* for 45 days (A) and grown in greenhouse for 150 days after acclimatization from *in vitro* conditions (B). A - Bar =1.5 cm. B – Bar=5 cm.

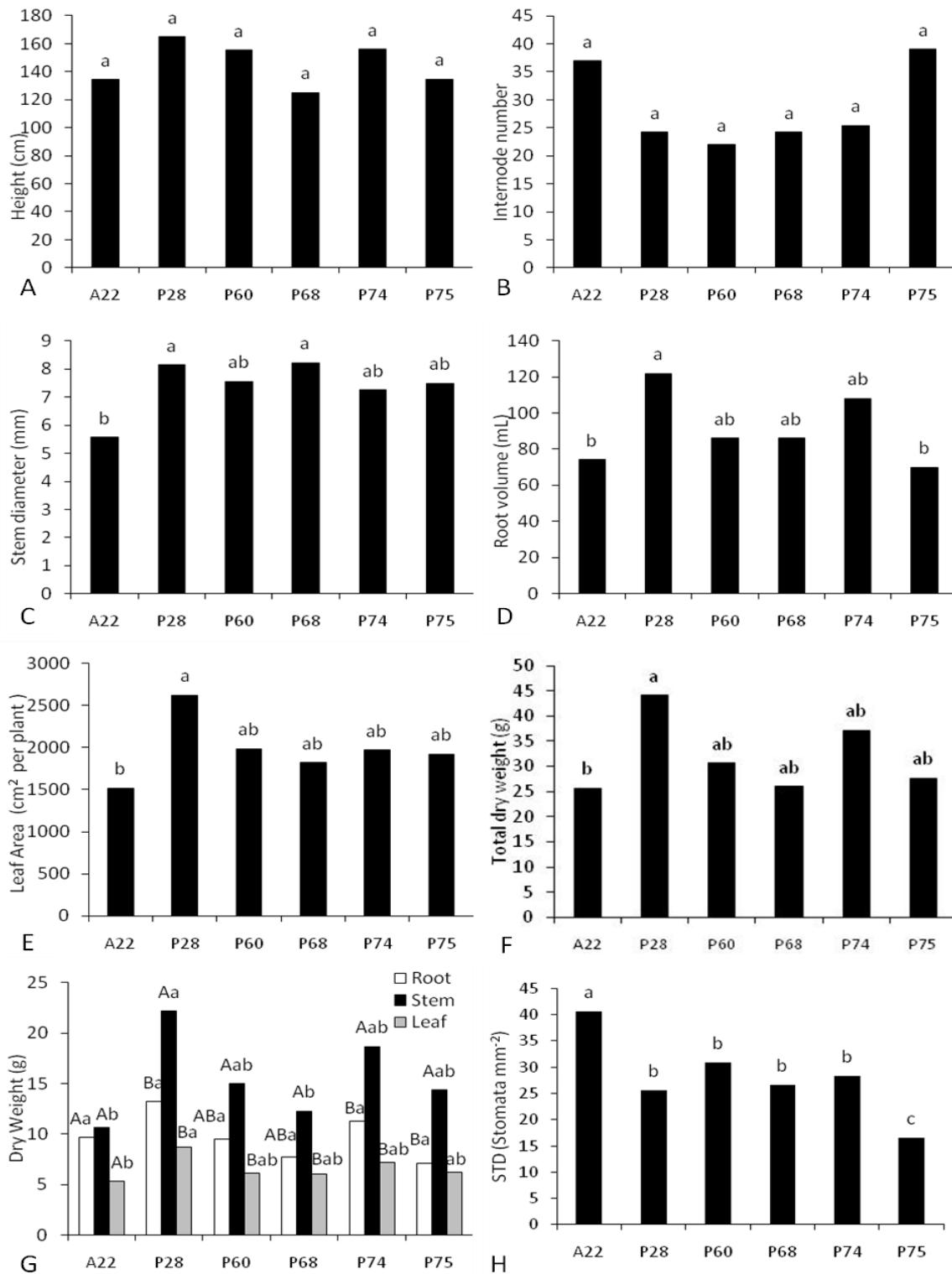


Figure 5. Growth parameters from 150-day-old *Pfaffia glomerata* plants of different ploidies/polypliodization events grown under greenhouse conditions. **A:** Plant height (cm). **B:** Number of internodal segments per plant. **C:** Stem diameter (mm). **D:** Root volume (mL). **E:** Leaf area (cm²). **F:** Dry weight per plant (g). **G:** Dry weight by plant organ (g). **H:** Stomatal density (stomata mm⁻²) Different letters denote statistical differences calculated with Tukey's test at 5% probability. Uppercase letters compare means among organs within the same accession, whereas lowercase letters compare means among treatments.

Diploid plants did not show significantly different amounts of biomass allocated to roots, stems and leaves, whereas polyploids tended to accumulate more biomass on stems (Figure 5G). Root biomass did not vary significantly among different ploidies/polyploidization origins, although stem and leaf biomass in P28 was significantly higher than in accession 22

Although the averages for internode number and plant height varied among genotypes, no significant difference was observed for any of these parameters (Figure 5B) due to the high variance observed within treatments. In general, polyploids showed larger stem diameters, mainly P28 and P68 (8.2 mm for both, Figure C), whereas average stem diameter of diploid plants was 5.6 mm. As already observed by Gomes et al. (2014), the stomatal density decreased due to the polyploidization process, showing the highest value on diploid plants (Figure 5H).



Figure 6. *Pfaffia glomerata* plants of different ploidies/polyplloidization events grown in greenhouse for 150 days after acclimatization from *in vitro* conditions. From the left to the right: P28, P60, P68, P74, P75 and accession 22 diploid. Bar= 25cm.

Despite biomass accumulation was higher in the tetraploid P28 when compared to accession 22 diploid plants, no significant variation was found on either chlorophyll *a* fluorescence (namely F_v/F_m , Φ_{PSII} , NPQ and ETR) or gas exchange parameters (A , g_s , C_i/C_a and E) among plants of different ploidies/polyplloidization events (Figures 7 A-D

and 8 A-D). Diploid plants showed slightly lower values for photosystem II maximum quantum yield (F_v/F_m), but this difference was not significant (Figure 7A). Chlorophyll contents *ex vitro* were higher in P28, intermediate in the other diploids and lower in diploid plants (Figure 7F).

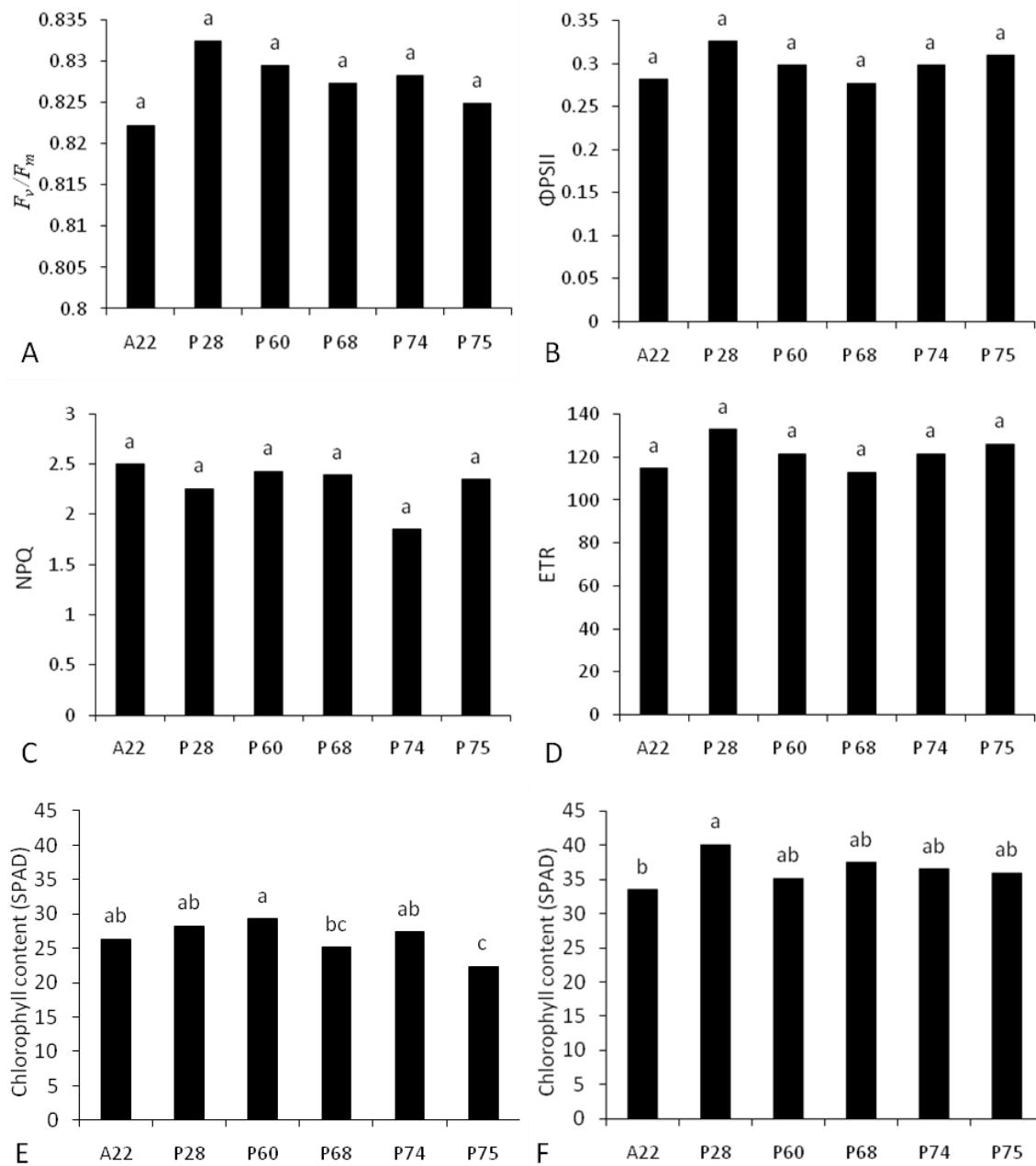


Figure 7. Chlorophyll fluorescence parameters and chlorophyll contents from 150-day-old *Pfaffia glomerata* plants of different ploidies/polyplloidization events grown under greenhouse conditions, and chlorophyll contents of 45 day old vitroplants. **A:** PSII maximum quantum yield (F_v/F_m). **B:** actual quantum yield of PSII photochemistry (Φ_{PSII}). **C:** Non-photochemical quenching (NPQ). **D:** Electron transport rate (ETR). **E** and **F:** Total chlorophyll content of *in vitro* (**E**) and *ex vitro* (**F**) *P. glomerata* plants grown for 45 and 15 days respectively. Different letters denote statistical differences calculated with Tukey's test at 5% probability

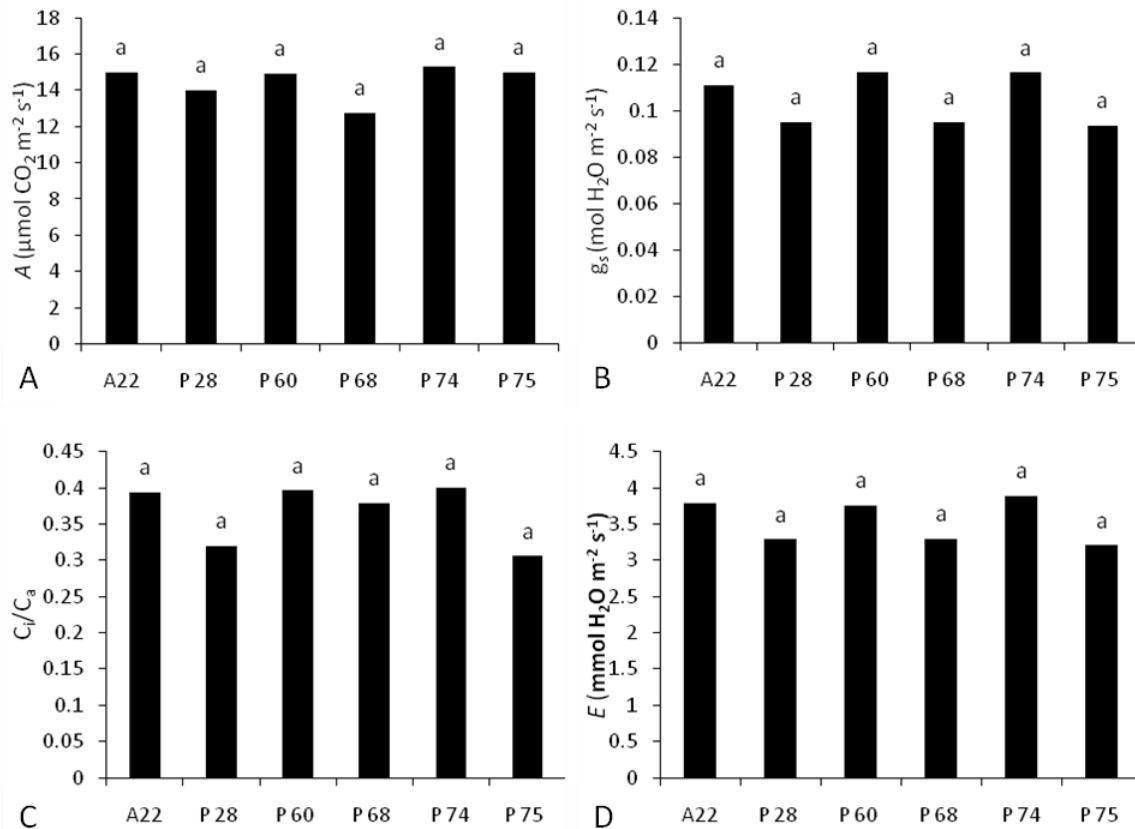


Figure 8. Gas exchange parameters from 150-day-old *P. glomerata* plants of different ploidies/polyploidization events grown under greenhouse conditions. **A:** CO₂ assimilation rate (A). **B:** Stomatal conductance to water vapor (g_s). **C:** internal/external CO₂ concentration ratio (C_i/C_a). **D:** Transpiration rate (E). Means followed by different letters denote statistical differences calculated with Tukey's test at 5% significance.

Synthetic polyploidization increased 20E production ex vitro

In greenhouse conditions, the highest 20E content per plant was found on polyploid P28 (0.8%), while diploid plants produced 0.61% (Figure 9A), which represents an increase of 31% in 20E production considering the whole plant. The lowest 20E content was found on P68 (0.56%) (Figure 9A). Leaves were the organs with the highest 20E contents in all plants, and the highest content in this organ was found in P28 (1.5%) and the lowest in P68 and diploid accession 22 (1.1 and 1.06%, respectively) (Figure 9B). Root and stem 20E contents did not differ significantly in polyploid plants, but, between these two organs, diploids showed higher contents on roots (Figure 9B).

Polyplloid P28, together with P74, also showed the highest 20E total mass per plant (293 mg), approximately twice as much the total amount produced by diploid

plants (146 mg) (Figure 9D). 20E mass did not differ among organs in any plant, except by P68, which showed a lower 20E mass in the roots in comparison to the other organs. The 20E mass in roots, stem and leaves showed the highest values in P28 (Figure 9D).

20E production *in vitro* did not follow the same pattern observed *ex vitro* (Figure 9E and F). The highest content *in vitro* was showed by diploid plants (1.24%), in opposition to what was observed *ex vitro* (Figure 9E). P75 showed the lowest values for 20E content and 20E total *in vitro*. The highest 20E masses were found in diploid accession 22, P74 and P28 (1.37, 1.45 and 1.17 mg, respectively) (Figure 9F).

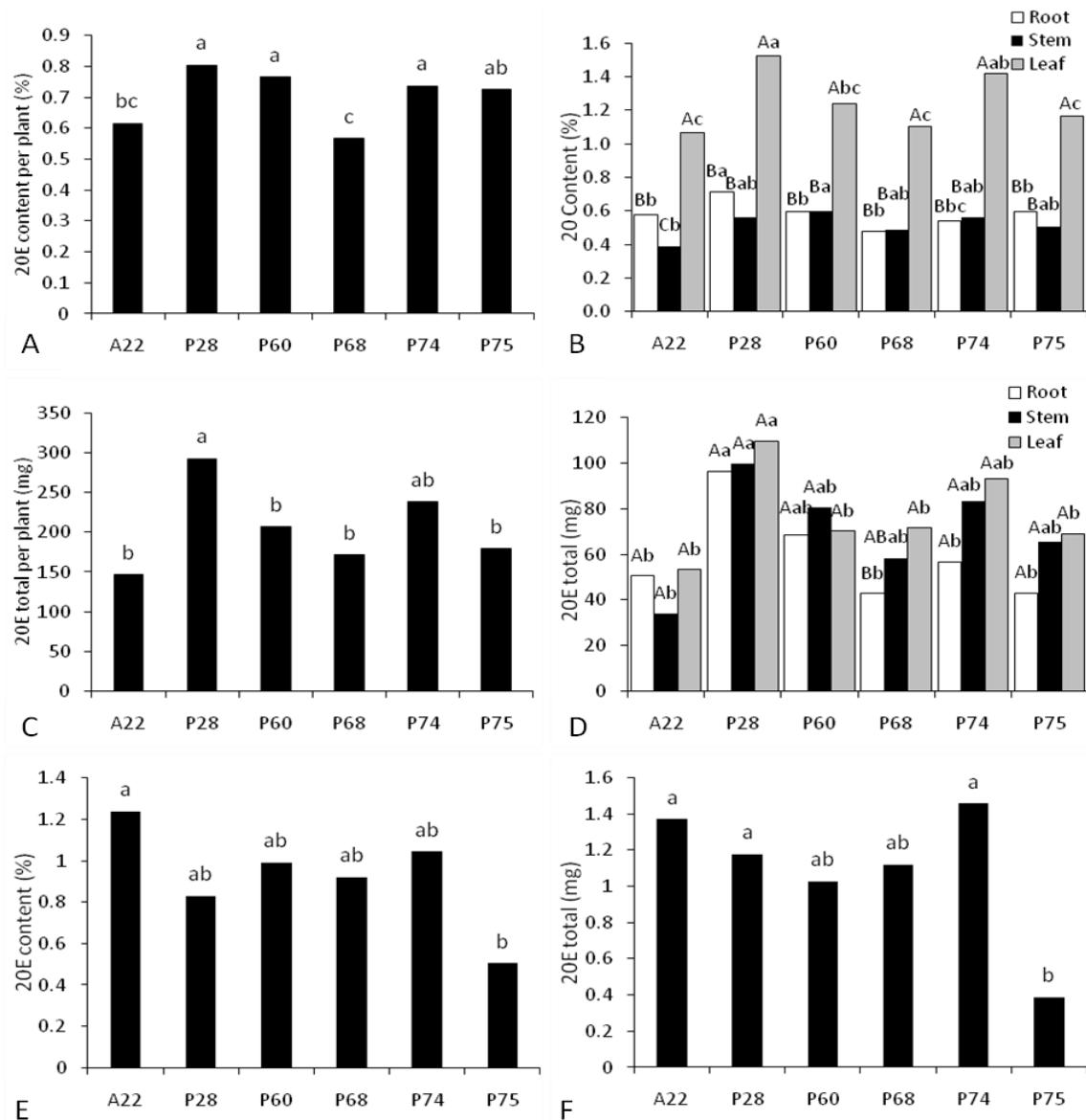


Figure 9. 20E contents per plant (A) and per plant organ (B) of *P. glomerata* grown in greenhouse for 150 days and total 20E mass per plant (C) and per plant organ (D). 20E content per plantlet (E) and total 20E mass per plantlet (F) grown *in vitro* for 45 days. Different letters denote statistical differences calculated with Tukey's test at 5% probability. Uppercase letters compare means among organs within the same accession, whereas lowercase letters compare means among treatments

Discussion

The results of the present study show that the synthetic polyploidization of *P. glomerata* was effective in producing individuals with increased biomass accumulation and secondary metabolite production, what is favorable to the commercial production of the species. Similar results were previously reported for other medicinal species (Gao et al., 1996; Jesus-Gonzalez and Weathers, 2003; Kim et al., 2004 Dehgan et al., 2012). Furthermore it is the first report on the improvement of *in vitro* photoautotrophic growth through induced polyploidization.

Chapter I showed that different accessions had different *in vitro* photoautotrophic potentials, and the accessions that accumulated more biomass *in vitro* also showed higher biomass accumulation and higher photosynthetic capacities per leaf area *ex vitro*. Variations in the *in vitro* photoautotrophic potential were detected between diploid and polyploid plants in the present study, and the tetraploid P28, which showed the highest *in vitro* biomass accumulation, also showed the highest dry weight *ex vitro*. Nevertheless, in opposition to the findings of chapter I, this increased biomass accumulation was not related to higher CO₂ assimilation per leaf area, and no significant variation was observed in any of the photosynthesis parameters among the plants (Figures 7 and 8). So, it is reasonable to conclude that the factors that are responsible for the variation in the photosynthesis and biomass accumulation among diploid accessions (chapter 1) are not the same factors involved in this difference observed between the diploid accession 22 and the tetraploid P28.

Polyploidy affects three factors that interact among them and determine the photosynthetic rate per leaf area: DNA content per cell, cell size and number of cells per leaf area unit (Warner and Edwards, 1989, 1993). Photosynthetic rate per cell is positively correlated with DNA content per cell, however, when the number of chromosomes doubles, cell size also increases, thus reducing the number of cells per leaf area unit (Werner and Edwards, 1993). So, the changes due to polyploidy on the photosynthetic rate per leaf area unit depend on the cell packing in the leaves and the ratio DNA content/cell volume. If cell volume also doubles when DNA amount is doubled, the number of cells per leaf area decreases to the half and although the photosynthetic rate per cell doubles, carbon assimilation per leaf area will remain the same. This occurs with induced polyploids of *Medicago sativa* (Molin et al., 1982), *Pennisetum americanum* (Warner and Edwards, 1988), and the constant photosynthetic

rates per leaf area observed between diploids and polyploids in the present study suggests that this is the case of *P. glomerata*. The higher biomass produced by some *P. glomerata* polyploids seems to have resulted simply from the increased organ size frequently caused by chromosome duplication (Osborn et al., 2003), as depicted on Figures 3 and 5. So, even though the photosynthesis per leaf area unit did not increase, the total leaf area was higher in some polyploids, resulting in increased plant productivity.

Although polyploids P28, P60 and P74 showed the highest 20E contents *ex vitro*, the same was not observed *in vitro*. This is most likely to be related to the fact that secondary metabolic pathways are induced routes, generally triggered by external factors, such as microorganisms, herbivores, heat stress, among others, as plant secondary metabolites are related to the plant-environment interaction (Bennett and Wallsgroove, 1994). None of these environment-related factors are normally present in an *in vitro* cultivation system, and one of the most successfully strategies that are used in order to increase *in vitro* secondary metabolite production is elicitation, which consists in applying stresses (e.g. autoclaved mycelium of pathogenic fungi, protein extracts, temperature, UV light, heavy metal salts, altered pH, etc.) to the *in vitro* cultures, triggering the production of secondary metabolites that are normally not produced (Borgaud et al., 2001).

Considering that *P. glomerata* presents better growth, higher photosynthetic rates and higher 20E yield *in vitro* when grown in photoautotrophic systems (Iarema et al., 2012; Saldanha et al., 2013; Saldanha et al., 2014), the optimization of photoautotrophic culture conditions, jointly with studies with elicitation may be a promising start in order to achieve a viable *in vitro* 20E production from the species. Although P28 plants did not produce more 20E than the diploids *in vitro*, they showed the highest *in vitro* dry weight and the highest 20E content *ex vitro*, and still may be a promising polyploid to invest in studies of *in vitro* 20E production with elicitation.

As already related for other species, the induced polyploidization of *P. glomerata* showed to be a successful approach to obtain more productive varieties concerning secondary metabolite and biomass, but not all of the polyploids produced independently from accession 22 were as productive as P28, and some of them did not differ significantly from the diploid 22. This difference observed among the polyploids, including the curly leaves that resulted from abnormal tissue growth in P75, may be a result of epigenetic changes during the genome duplication. Other studies provided

evidence for frequent epigenetic changes in new polyploids, such as DNA methylation, histone modification and chromatin packaging (Osborn et al., 2003). Polyploids of *Eragrostis curvula* obtained with colchicine in independent polyploidization events presented different methylation patterns, which can lead to dramatic changes in gene expression (Mecchia et al., 2007; Martelotto et al., 2007). This species also showed polymorphisms in 28% of the detected *loci* when compared to the diploid plants, but tetraploids obtained from parallel polyploidization events did not show any genetic polymorphism among them. Furthermore, wheat synthetic allopolyploids generated spontaneously showed the same pattern of sequence modifications of the ones generated by tissue culture or colchicine treatment (Ozkan et al., 2001). So, in summary, the phenotypic changes observed among the independently generated polyploid plants probably resulted only from epigenetic changes, whereas the changes between diploids and polyploids may have resulted from both genetic and epigenetic changes.

The present study showed that induced polyploidization of *P. glomerata* resulted in plants (in the case of P28) that present higher *in vitro* photoautotrophic potential, besides higher biomass accumulation and 20E production *ex vitro*. These characteristics have the potential to improve *P. glomerata* production from the propagation to the *ex vitro* cultivation, and the final result are plants that show a highly increased final 20E mass produced per plant. Although growth and 20E production showed to be variable among different polyploid produced from the same accession, at least one of the polyploids showed these desired characteristics.

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GENERAL CONCLUSIONS

- *P. glomerata* *in vitro* photoautotrophic potential is genotype-dependent, and the accessions with the best growth performance in an *in vitro* photoautotrophic system also showed high biomass accumulation and CO₂ assimilation rates *ex vitro*. So, these accessions have different photosynthetic capacities, which are expressed even when plants are grown *in vitro* under photoautotrophic conditions.
- 20E production *in vitro* is also genotype dependent, and comparable to the production by different accessions *ex vitro*, as accessions 4 and 43 showed the highest 20E accumulation both *in vitro* and *ex vitro*.
- Induced polyploidization produced plants with higher photoautotrophic potentials *in vitro* and higher biomass accumulation *ex vitro*.
- The increased biomass accumulation showed by some polyploids is not related to increases on photosynthetic rates per leaf area. It was probably related to the increased leaf area per plant on polyploids.
- Although polyploids generated from independent events showed different growth and secondary metabolite accumulation patterns, induced polyploidization was an efficient tool to produce plants with higher biomass and 20E accumulation.