EVANDRO ALEXANDRE FORTINI

PHOTOPERIOD, SALINITY, AND ELICITORS MODULATE THE GROWTH, MORPHOPHYSIOLOGY, AND BIOSYNTHESIS OF 20-HYDROXYECDYSONE IN *Pfaffia glomerata* (SPRENG.) PEDERSEN

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Plant Physiology Graduate Program for the obtention of the degree of *Doctor Scientiae*.

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Co-advisor: Diego Silva Batista

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“There are no limits when you are surrounded by people who believe in you”

– Neil deGrasse Tysson

ABSTRACT

FORTINI, Evandro Alexandre, D.Sc., Universidade Federal de Viçosa, March 2021. **Photoperiod, salinity, and elicitors modulate the growth, morphophysiology, and biosynthesis of 20-hydroxyecdysone in *Pfaffia glomerata* (Spreng.) Pedersen.** Advisor: Wagner Campos Otoni. Co-Advisor: Diego Silva Batista.

Pfaffia glomerata is a plant widely used in traditional medicine, and a major producer of the secondary metabolite 20-hydroxyecdysone (20-E). Studies indicate that the biosynthesis of 20-E in *P. glomerata* can be regulated by abiotic stresses, but the mechanisms involved in this regulation are still unclear. Aiming to understand the physiological and molecular basis involved in the regulation of 20-E biosynthesis in *P. glomerata*, as affected by abiotic factors, ploidy level, and elicitation, three experiments were conducted. In the first experiment, plants of *P. glomerata* (accessions 22 and 43) were grown in vitro for 40 days under 4 different daily photoperiods: 4, 8, 16, and 24 h of light. We found that plants exhibited higher photosynthesis and biomass accumulation when grown in longer photoperiods. The plants also had anatomical and primary metabolism changes in response to the photoperiods tested, and the magnitude of these changes was accession-dependent. Furthermore, longer photoperiods promoted an increase in 20-E production, possibly as a result of higher carbon availability in the plants. In the second experiment, *P. glomerata* plants were grown in vitro and subjected to the following treatments: NaCl (50 mM), 5-azacytidine (5-azaC, 25 µM), and NaCl+5-azaC. After 40 days, we observed that the treatments promoted a significant reduction in growth and photosynthesis, as well as an increase in reactive oxygen species (ROS) and antioxidant enzyme production and an adjustment in primary metabolism. Through proteomic analyses, we identified differentially accumulated proteins mainly associated with the metabolism of carbohydrates, amino acids, and secondary metabolites, which may help us understand the salt stress and 5-azaC tolerance responses compared to control. Besides, we found that the 5-azaC and NaCl+5-azaC treatments reduced the accumulation of jasmonate biosynthesis-related protein, and this may contribute to reduced 20-E signaling and biosynthesis. In the third experiment, two different ploidies of *P. glomerata* (A22, diploid; and P28, a tetraploid derived from A22) were grown in vitro for 40 days, and the plants were elicited in the first 20 days with methyl jasmonate (methyl-JA) and methyl salicylate (methyl-SA). The elicitors induced contrasting anatomical modifications in *P. glomerata* leaves. Elicitation also reduced the photosynthetic rate of the plants, with a consequent change in primary metabolite content and reduction in growth. Treatments with methyl-JA and methyl-SA promoted oxidative stress, but also ROS mitigation by increasing antioxidant enzymes, and these changes were more significant in diploid plants. The production of 20-E was stimulated by elicitation with methyl-JA and was also higher in tetraploid plants. On the other hand, methyl-SA down-regulates *Phantom* gene expression, with a consequent reduction in 20-E biosynthesis. Taken together, our data provide important information on molecular, biochemical, and physiological mechanisms involved with the regulation of 20-E biosynthesis, as well as enable biotechnological strategies for increasing the production of this metabolite in *P. glomerata* plants grown in vitro.

Keywords: Phytoecdysteroids. Abiotic stress. Photosynthesis. Medicinal plant. Proteomics. Phytohormones. Polyploidy.

RESUMO

FORTINI, Evandro Alexandre, D.Sc., Universidade Federal de Viçosa, março de 2021. **Fotoperíodo, salinidade e elicitores modulam o crescimento, morfofisiologia e biossíntese de 20-hidroxiecdisona em *Pfaffia glomerata* (Spreng.) Pedersen.** Orientador: Wagner Campos Otoni. Coorientador: Diego Silva Batista.

Pfaffia glomerata é uma espécie amplamente utilizada na medicina tradicional, e uma das principais plantas produtoras do metabólito secundário 20-hidroxiecdisona (20-E). Estudos apontam que a biossíntese de 20-E em *P. glomerata* é regulada por estresses abióticos, mas os mecanismos envolvidos nessa regulação são pouco entendidos. Com o objetivo de compreender as bases fisiológicas e moleculares envolvidas na regulação da biossíntese da 20-E em *P. glomerata*, afetadas por fatores abióticos, pelo nível de ploidia e por elicitação, três experimentos foram conduzidos. No primeiro experimento, *P. glomerata* (acesso 22 e acesso 43) foram cultivadas in vitro por 40 dias em 4 diferentes fotoperíodos diários: 4, 8, 16 e 24 h de luz. As maiores taxas fotossintéticas e acúmulo de biomassa foram observadas em plantas cultivadas em fotoperíodos mais longos. As plantas também exibiram alterações anatômicas e no metabolismo primário em resposta aos fotoperíodos testados, sendo que a magnitude dessas variações foi acesso-dependente. Além disso, observamos que fotoperíodos mais longos levaram ao aumento na produção de 20-E, possivelmente como resultado da maior disponibilidade de carbono nas plantas. No segundo experimento, plantas de *P. glomerata* foram cultivadas in vitro submetidas aos seguintes tratamentos: NaCl (50 mM), 5-azacitidina (5-azaC, 25 µM), e NaCl+5-azaC. Após 40 dias, observou-se que os tratamentos promoveram redução significativa no crescimento e na fotossíntese, bem como incremento na produção de espécies reativas de oxigênio (ROS) e de enzimas antioxidantes e um ajustamento no metabolismo primário. Através de análises proteômicas, identificou-se proteínas diferencialmente acumuladas principalmente associadas ao metabolismo de carboidratos, aminoácidos e metabólitos secundários, que podem nos ajudar a entender as respostas de tolerância ao estresse salino e a 5-azaC, quando comparadas ao controle. Além disso, os tratamentos 5-azaC e NaCl+5-azaC reduziram o acúmulo de proteína relacionada à biossíntese de jasmonato, o que pode contribuir para uma menor sinalização e biossíntese de 20-E. No terceiro experimento, duas diferentes ploidias de *P. glomerata* (A22, diploide; e P28, um tetraploide derivado de A22) foram cultivadas in vitro por 40 dias, sendo que nos 20 primeiros dias foram elicitadas com metil jasmonato (methyl-JA) e metil salicilato (methyl-SA). Os elicitores induziram modificações anatômicas contrastantes em folhas de *P. glomerata*. A elicitação também reduziu a taxa fotossintética das plantas, com consequente alteração no conteúdo de metabólitos primários e redução no crescimento. Tratamentos com methyl-JA e methyl-SA promoveram não somente estresse oxidativo, mas também a mitigação de ROS por meio do incremento de enzimas antioxidantes, sendo que essas alterações foram mais significativas em plantas diploides. A produção de 20-E foi estimulada pela elicitação com metil-JA, bem como foi maior em plantas tetraploides. Por outro lado, metil-SA regula negativamente a expressão do gene *Phantom*, com consequente redução da biossíntese de 20-E. De modo geral, nossos dados fornecem informações importantes sobre mecanismos moleculares, bioquímicos e fisiológicos envolvidos com a regulação da biossíntese de 20-E, bem como provê estratégias biotecnológicas para o incremento da produção deste metabólito em plantas de *P. glomerata* cultivadas in vitro.

Palavras-chave: Fitoecdisteróides. Estresse abiótico. Fotossíntese. Planta medicinal. Proteômica. Hormônios vegetais. Poliploidia.

CONTENTS

GENERAL INTRODUCTION

Plants are a valuable source of bioactive compounds, and for millennia their pharmacological properties have been exploited by folk medicine in the prevention and cure of diseases. Currently, according to data from the World Health Organization, around 80% of the world's population essentially uses the knowledge of traditional medicine in basic health care (Singh et al. 2019). Thus, to carry out studies can validate the therapeutic uses of these species, as well as prospect new plants with medicinal potential. Moreover, efforts are needed to understand the biosynthesis pathways of secondary metabolites in plants, which may lead to an increase in the production of these compounds of pharmacological interest (Atanasov et al. 2015; Singh et al. 2019).

From an ecological point of view, secondary metabolites are key compounds in the interaction between plants and the environment. They act in the attraction of pollinators, defense against herbivores and pathogens, besides acting in the protection against abiotic stresses (Vasconsuelo and Boland 2007; Figueiredo et al. 2008). According to the metabolic pathway of origin and the chemical structure, they can be classified in some groups as phenolic compounds, alkaloids, and terpenes (Yang et al., 2018).

Among the terpenes, there is an important class of metabolites: the ecdysteroids, which can be found in a wide diversity of organisms, performing physiological functions essential to the development (Dinan and Lafont 2006; Hunyadi et al. 2016). In arthropods, ecdysteroids such as 20-hydroxyecdysone (20-E) act as essential hormones in the process of coordinating multiple developmental phases, such as the regulation of oogenesis and in signaling the stages of ecdysis (Yamanaka et al. 2012; Tsukagoshi et al. 2016; Swevers 2019). Plants are also able to synthesize and accumulate ecdysteroids, but usually in low concentrations (Dinan et al. 2009). The main ecdysteroid produced in plants is the 20-E, and the content of this compound varies according to the different organs and the climatic and geographical conditions in which they are grown (Dinan and Lafont 2006; Dinan et al. 2009; Ohnishi et al. 2009). The major function attributed to 20-E in plants is the protection against herbivore attack (Dinan et al. 2009; Wang et al. 2010). Furthermore, there are evidences that 20-E also regulates plant metabolism and affects growth and enzyme activity (Golovatskaya 2004; Festucci-Buselli et al. 2008; Chaubey 2017).

The conversion of esters to 20-E is mediated by enzymes of the P450 family, already identified in all classes of 20-E producing organisms (Festucci-Buselli et al. 2008), but the physiological and molecular mechanisms involved in the control of 20-E biosynthesis in plants are poorly understood when compared to insects (Tsukagoshi et al. 2016). However, recent studies have provided important insights into 20-E regulation through transcriptome analysis in several plant species (Li et al. 2016; Lei et al. 2018; Wang et al. 2018; Batista et al. 2019). Two genes of the P450 family previously described as regulators of 20-E biosynthesis in insects, *Spook* and *Phantom*, were identified in *Pfaffia glomerata*. Moreover, the expression of these two genes is regulated by changes in the culture conditions, such as salinity and increased rates of gas exchange (Batista et al. 2019; Felipe et al. 2019b).). From this, further studies are necessary for the understanding of 20-E biosynthesis at the molecular and physiological level, and *P. glomerata* is a great model species for the study of this metabolic pathway.

P. glomerata (Amaranthaceae) is a plant native to South America and popularly known as 'fáfia' and Brazilian-ginseng. It is widely used in Brazilian folk medicine, and its root extracts are attributed with tonic and fortifying effects (Mendes 2011; Caleffi et al. 2015). Several of its therapeutic properties have been confirmed (Freitas et al. 2004; Neto et al. 2005; Tóth et al. 2008; Caleffi et al. 2015; Dias et al. 2019; Franco et al. 2021), some of which are related to the biological action of 20-E (Franco et al. 2021).

Several studies have shown that *P. glomerata* presents great morpho-physiological plasticity to changes in abiotic conditions (Skrebsky et al. 2008; Iarema et al. 2012; Gomes et al. 2013; Saldanha et al. 2013; Corrêa et al. 2015; Felipe et al. 2019 a, b; Silva et al. 2020a, b; Louback et al. 2021). For instance, plants grown under mild salt stress showed no reduction in growth and no signs of photoinhibition, but showed an increase in 20-E content (Felipe et al. 2019a). Furthermore, an efficient protocol was also established for inducing artificial polyploidy in *P. glomerata*, and tetraploid plants showed higher biosynthesis of 20-E (Gomes et al., 2014; Corrêa et al., 2016). However, the molecular, biochemical, and epigenetic mechanisms involved in the regulation of 20-E biosynthesis as a function of ploidy level and under salt stress are not fully understood.

Light is one of the main environmental stimuli that affect plant development. Its influence on morphophysiological characteristics varies according to the light conditions to which the plants are exposed, and can be modulated by irradiance levels, spectral quality and photoperiod (Fankhauser and Chory 1997; de Wit et al. 2016). Light quality and irradiance directly affect the development and physiological responses of *P. glomerata*, as well as modulate secondary metabolism and 20-E production (Silva et al. 2020a, b), but little is known about the influence of photoperiod.

Furthermore, plants are continuously exposed to challenging environmental conditions, such as soil salinity, limitations in nutrient and water availability and are subject to attack by pathogens and herbivores. When exposed to these conditions, plants can recognize stress signals through internal and external receptors and activate complex defense mechanisms (Rojas et al. 2014; Gust et al. 2017). The signaling process involves the perception of environmental stimuli and their transmission from cell to cell or among organs, through signaling molecules such as hormones (Takahashi and Shinozaki 2019). Jasmonate and salicylate are two key hormones in triggering defense responses in plants (Huot et al. 2014). Studies that address the exposure of plants to elicitors (such as methyl jasmonate and methyl salicylate) can contribute to the understanding of the complex mechanisms involved in hormonal regulation during stress and how they affect the physiological and biochemical characteristics of plants (Arif et al. 2020), as well as be a biotechnological tool for the increment of secondary metabolites (Nabi et al. 2021).

Thus, here we aimed to understand the physiological and molecular basis involved in the regulation of 20-E biosynthesis in *P. glomerata*, as affected by abiotic stresses, ploidy level, and elicitation. In this vein, we hypothesized that: (*i*) changes in photoperiod affect the growth of *P. glomerata,* as well as regulate its primary and secondary metabolism; (*ii*) Salt stress and the demethylating agent 5-azacytidine induce proteomic changes in *P. glomerata,* also affecting the biosynthesis of 20-E; (*iii*) Jasmonate and salicylate act in the signaling pathway of 20-E biosynthesis; and (*iv*) the modulation of 20-E production is affected by the ploidy level of the plants.

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CHAPTER 1

Photoperiod modulates growth and pigments and 20-hydroxyecdysone accumulation in Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen] grown in vitro [1]

Abstract

Photoperiod regulates different morphophysiological processes in plants, directly impacting photosynthetic performance and, consequently, primary and secondary metabolism. To date, there are no studies reporting the influence of photoperiod on the biosynthesis of phytoecdysteroids, such as 20-hydroxyecdisone (20-E). Here, we evaluated the effects of photoperiod on the development and metabolism of in vitro grown *Pfaffia glomerata,* an important medicinal species and producer of 20-E. Two *P. glomerata* accessions (Ac22 and Ac43) were cultivated for 40 days under different photoperiods: 4, 8, 16, and 24 h. Then, growth, physiological performance, 20-E content and gene expression related to the synthesis of this compound were evaluated. Longer photoperiods resulted in higher photosynthetic rates, growth, and biomass accumulation in both accessions. *P. glomerata* showed great plasticity to the different photoperiods tested and no sign of photoinhibition (*Fv*/*Fm*). Primary metabolism was modulated by photoperiod, with marked differences in the production of soluble sugars, starch, and amino acids. Anthocyanin production was also affected by the photoperiod; However, the accessions showed contrasting responses, in which longer photoperiods led to the highest anthocyanin contents in Ac22 and the lowest in Ac43, reflecting different adaptive strategies the light conditions. As a result of better photosynthetic performance and higher carbon availability, *P. glomerata* accumulated more 20-E during longer photoperiods. In this way, growing *P. glomerata* plants for longer photoperiods may represent a strategy for obtaining plants with larger biomass and higher 20-E yields.

Keywords: Photoperiod, phytoecdysteroids, photosynthesis, medicinal plant.

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ORIGINAL ARTICLE

Photoperiod modulates growth and pigments and 20-hydroxyecdysone accumulation in Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen] grown in vitro

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Abstract

Photoperiod regulates different morphophysiological processes in plants, directly impacting photosynthetic performance and, consequently, primary and secondary metabolism. To date, there are no studies reporting the influence of photoperiod on the biosynthesis of phytoecdysteroids, such as 20-hydroxyecdisone (20-E). Here, we evaluated the effects of photoperiod on the development and metabolism of in vitro grown *Pfaffia glomerata*, an important medicinal species and producer of 20-E. Two *P. glomerata* accessions (Ac22 and Ac43) were cultivated for 40 days under different photoperiods: 4, 8, 16, and 24 h. Then, growth, physiological performance, 20-E content and gene expression related to the synthesis of this compound were evaluated. Longer photoperiods resulted in higher photosynthetic rates, growth, and biomass accumulation in both accessions. *P. glomerata* showed great plasticity to the different photoperiods tested and no sign of photoinhibition (Fv/Fm). Primary metabolism was modulated by photoperiod, with marked differences in the production of soluble sugars, starch, and amino acids. Anthocyanin production was also affected by the photoperiod. However, the accessions showed contrasting responses, in which longer photoperiods led to the highest anthocyanin contents in Ac22 and the lowest in Ac43, reflecting different adaptive strategies the light conditions. As a result of better photosynthetic performance and higher carbon availability, *P. glomerata* accumulated more 20-E during longer photoperiods. In this way, growing *P. glomerata* plants for longer photoperiods may represent a strategy for obtaining plants with larger biomass and higher 20-E yields.

Key Message

Photoperiod regulates the primary and secondary metabolism of *P. glomerata* plants grown in vitro, and longer photoperiods increase growth, photosynthetic performance, and accumulation of the phytoecdysteroid 20-hydroxyecdysone (20-E).

Keywords Photoperiod · Phytoecdysteroids · Photosynthesis · Medicinal plant

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Introduction

Light is one of the main environmental stimuli affecting the development, morphoanatomy, and physiology of plants (Galvão and Fankhauser 2015). Its influence varies according to the lighting conditions the plants are exposed to and can be modulated by irradiance, spectral quality, and photoperiod (Fankhauser and Chory 2002; de Wit et al. 2016). The photoperiod represents the foreseeable changes in the daily and yearly light regime, promoted by the Earth's rotation (Jackson 2009). Like other organisms, plants can perceive changes in photoperiod, based on a complex transcriptional and post-transcriptional mechanism that adjusts the circadian clock in response to changes in light conditions (Henriques et al. 2018), as well as photosensory proteins called photoreceptors (Kami et al. 2010; Galvão and Fankhauser 2015).

The photoperiod regulates various stages during plant development, such as germination (Munir et al. 2001), elongation of the hypocotyl (Martín et al. 2018), and flowering (Zheng et al. 2019). Photoperiod changes influence plant body structure, regulate its morphology and anatomy (Wake and Fennell 2000; Gibala et al. 2009), and impact on its metabolism through the coordination of essential physiological processes (Greenham and McClung 2015). Therefore, the photoperiod regulates plant growth and coordinates adaptive responses to these environmental changes (Serrano-Bueno et al. 2017).

Fluctuations in daily light periods directly affect photosynthesis and, as a result, plants need to adjust the allocation of energy between growth and defense processes against biotic and abiotic stresses (Cagnola et al. 2018). Despite several studies have looked at how photoperiod changes affect the production of secondary metabolites (Khan et al. 2018; Feng et al. 2019; Castro et al. 2019), little is known about its influence on the biosynthesis of phytoecdisteroids.

The main plant-produced ecdysteroid is 20-hydroxyecdisone (20-E). Its content varies between different plant organs and in relation to specific climatic and geographic conditions (Dinan and Lafont 2006; Dinan et al. 2009). The main function of 20-E in plants is to protect them against herbivores (Dinan et al. 2009; Wang et al. 2010). In addition, 20-E may act as a phytohormone and in regulating plant metabolism, thus affecting growth and enzymatic activity (Festucci-Buselli et al. 2008; Chaubey 2018).

The biosynthetic route of 20-E is best known in insects (Tsukagoshi et al. 2016), but recent studies have helped to elucidate its production mechanisms also in plants (Li et al. 2016; Lei et al. 2018; Batista et al. 2018). *Spook* and *Phantom*, two genes of the *Halloween* family previously described as regulators of 20-E biosynthesis in insects, were found to perform the same role also in the medicinal plant *Pfaffia glomerata.* Moreover, the expression of these two genes was regulated by changes in abiotic conditions (Batista et al. 2018). Thus, *P. glomerata* may aid in the understanding of 20-E biosynthesis pathway in plants.

Pfaffia glomerata is widely used in traditional medicine, owing to the tonic and fortifying effects of its root extracts (Mendes 2011; Caleffi et al. 2015). Furthermore, it can be used in the treatment of muscle atrophy (Tóth et al. 2008), and has been shown to possess prebiotic, anti-inflammatory, analgesic, antimicrobial, and aphrodisiac properties (Freitas et al. 2004; Neto et al. 2005; Caleffi et al. 2015; Dias et al. 2019). Ecdysteroids, and 20-E, are highly abundant in *P. glomerata*, which could explain its medicinal properties.

Several studies have shown that *P. glomerata* presents great plasticity and adaptability to changes in abiotic conditions. This species is tolerant to moderate amounts of cadmium and, as an accumulator species, can be used for phytoremediation (Skrebsky et al. 2009; Gomes et al. 2013). UV-B radiation (Felipe et al. 2019a) and salt stress (Felipe et al. 2019b) also affect growth and modulate its 20-E biosynthesis. When cultivated in vitro, *P. glomerata* responds to changes in light quality (Silva et al. 2020), carbon availability in the culture medium, increased gas exchange, and CO_2 enrichment, thus increasing growth and 20-E production (Iarema et al. 2012; Saldanha et al. 2014; Corrêa et al. 2015). Considering its medicinal importance and its plasticity in responding to abiotic variations, *P. glomerata* can be of great potential for studies aiming to understand the role of photoperiod on the biosynthesis of 20-E. To evaluate this, we grew in vitro two accessions of *P. glomerata* over four different photoperiods, analyzing the effect of this light regime variation on growth, anatomy, photosynthesis, sugar, amino acids and pigments accumulation, oxidative stress enzymes, 20-E production, as well as the expression of 20-E pathway genes.

Materials and methods

Plant material and experimental design

Pfaffia glomerata accessions (Ac22 and Ac43) were obtained from the Germplasm Bank of the Plant Tissue Culture Laboratory, Federal University of Viçosa, Brazil. Voucher material was deposited at the Leopoldo Krieger Herbarium (UFJF, Juiz de Fora, MG, Brazil) under code number CESJ 63,317. Plants were maintained in vitro under monthly subcultures in MS medium (Murashige and Skoog 1962), supplemented with MS vitamins, myo-inositol (100 mg L^{-1}), and 3% (w/v) sucrose (Supplementary Fig. 1). The medium was adjusted to pH 5.8 ± 0.1, solidified with 5.5 g L^{-1} agar (PhytoTechnology Laboratories®, Overland Park, KS, USA), and autoclaved at 121 °C and 108 kPa for 20 min.

Nodal explants (approximately 2 cm) of accessions 22 and 43 were transferred to glass flasks of 62 mm diameter × 15.5 mm height (AZ 200; Embalagens Rio, Nova Friburgo, RJ, Brazil) containing 100 mL of MS medium. The flasks were closed with 10-mm two-hole rigid polypropylene lids covered by 0.45-µm-diameter pore hydrophobic fluoropore polytetrafluoroethylene membranes (MilliSeal® AVS-045 Air Vent, Millipore, Tokyo, Japan), allowing CO_2 exchange at 25 µL L^{-1} s^{-1} (Batista et al. 2017) (Supplementary Fig. 1).

The plants were kept in a growth room with controlled irradiance of 60 µmol m^{-2} s^{-1} with two LED lamps (SMD 100, 18 W, Vilux®, Vitória, ES, Brazil) and temperature of

25 ± 1 °C. To test the influence of photoperiod, plants were cultivated for 40 days under the following photoperiods: 4, 8, 16, and 24 h. The experimental design was completely randomized, consisting of a 2 × 4 factorial (two accessions × four photoperiods) with seven replications each; the experimental unit was three plants per flask.

Plant growth parameters

At the end of the experiment, plants were collected and measured for shoot and root length (cm). To determine leaf area, the leaves were fixed on white paper, photographed with a digital camera, images were adjusted, and ImageJ software (Schneider et al. 2012) was used to calculate the leaf area attribute (cm^2). Leaves, stems, and roots were dried separately at 50 °C until constant weight to obtain the dry weight parameter (g).

Histological characterization

For anatomical studies, samples of the median stem region and the third fully expanded apical leaf were fixed in Karnovsky solution (Karnovsky 1965). After fixation, the samples were dehydrated in ethanolic series and included in acrylic resin (Historesin, Leica Instruments, Wetzlar, Germany). Transverse and longitudinal sections of 5 µm thickness were obtained on a rotary automatic microtome (RM2155, Leica Microsystems Inc., Buffalo Grove, IL, USA) and stained with toluidine blue (pH 3.2) for structural characterization (O'Brien and McCully 1981). The sections were then mounted on glass slides with synthetic Permount® SP15-500 resin (Fisher Chemicals, Thermo Fisher Scientific, Waltham, MA, USA). Images were captured on a light microscope (AX70 TRF; Olympus Optical, Tokyo, Japan) with a U-photo system, coupled to a digital color camera (Spot Insight 3.2.0; Diagnostic Instruments Inc., Sterling Heights, MI, USA) and microcomputer with the Spot Basic image capture program.

Photosynthetic rate and chlorophyll fluorescence parameters

Quantification of the in vitro photosynthetic rate was performed as described previously (Castro et al. 2019). CO_2 gas exchange was calculated based on the leaves' dry weight per plant (g), and the photosynthetic rate was expressed in µmol $CO_2\ s^{-1}\ plant^{-1}$.

Chlorophyll fluorescence parameters were measured using leaves previously acclimatized for 30 min in the dark, and then analyzed by a portable fluorometer (Mini-Pam; Heinz Walz GmbH, Effeltrich, Germany). Minimum fluorescence (F_0), maximum fluorescence (Fm), and variable fluorescence (Fv) were measured and maximum quantum productivity of photosystem II (Fv/Fm) was calculated as $Fv/Fm = [(Fm - F_0)/Fm)]$. The photochemical quenching (qP) was calculated as $qP = (Fm' - Fs)/(Fm' - F_{0'})$, and non-photochemical quenching (NQP) was calculated as $NPQ = (Fm - Fm')/Fm$ (Baker 2008). The apparent electron transport rate (ETR) was calculated as $ETR = \Delta F/Fm' \times PPFD \times 0.84 \times 0.5$, where PPFD is the photon flux (µmol $m^{-2}\ s^{-1}$) incident on the leaf; 0.5 is the value corresponding to the fraction of excitation energy distributed to PSII; and 0.84 corresponds to the fraction of incident light absorbed by the photosynthetic tissues of the leaves (Maxwell and Johnson 2000).

Determination of chlorophylls and carotenoids

For the extraction of chlorophyll and carotenoids, 500 µL of 80% acetone (Sigma-Aldrich, St. Louis, MO, USA) were added to 10 mg of freeze-dried and macerated leaf samples. Samples were vortexed, centrifuged at 12,000 rpm for 10 min, and added to microplate and supernatant absorbance (A) was assessed in a spectrophotometer at wavelengths of 470, 646, and 663 nm. Subsequently, calculations were performed according to the following equations (Wellburn 1994):

$$Chlorophyll\ a = \left(12.21 \times A_{663}\right) - (2.81 \times A_{646})$$

$$Chlorophyll\ b = (20.13 \times A_{646}) - (5.03 \times A_{663})$$

$$Carotenoids = \frac{(1000 \times A_{470}) - (3.27 \times Chlorophyll\ a) - (104 \times Chlorophyll\ b)}{198}$$

Determination of anthocyanin content

Relative anthocyanin content was measured as proposed previously (Neff and Chory 1998). Briefly, 300 µL of acidified methanol (1% HCl w/v) were added to 10 mg of freeze-dried and macerated leaf samples and incubated overnight under shaking at room temperature. Subsequently, 200 µL of water and 500 µL of chloroform were added to separate the chlorophyll phase from anthocyanins. The solution was then vortexed and centrifuged at 14,000×*g* for 5 min; 200 µL of the upper portion of the solution were mounted on a microplate and readings were taken at 530 and 657 nm. The relative anthocyanin content was expressed as ($A_{657} - A_{530}$) in relation to dry weight (DW).

Quantification of carbohydrates and amino acid levels

Extraction was performed as proposed previously (Lisec et al. 2006). Briefly, 700 µL of 100% methanol were added to 20 mg of freeze-dried and macerated leaf samples. The solution was vortexed for 10 s, stirred for 15 min at 950 rpm and 70 °C (Thermomixer comfort; Eppendorf, Hamburg, Germany), and then centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant (500 µL) was transferred to a new tube, to which 350 µL of chloroform and 750 µL of water were added. The solution was then vortexed, centrifuged at 4000 rpm for 15 min and 4 °C, and 800 µL of the upper portion were collected for subsequent amino acid (Cross et al. 2006) and soluble sugar (Fernie et al. 2001) quantification.

For starch quantification (Fernie et al. 2001), the first extraction pellet was washed in 100% ethanol and centrifuged twice at 14,000 rpm for 15 min at 4 °C. After hydrolysis and reaction preparation, absorbance was measured at 340 nm in a microplate reader. Starch content was then calculated, and results expressed in µmol glucose g^{-1} DW.

Antioxidant activity

The enzymes catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), superoxide dismutase (SOD, EC 1.15.1.1), and peroxidase oxidoreductase (POD, EC1.11.1.7) were quantified. Briefly, 50 mg of macerated leaf samples were collected and extracted in 1 mL extraction medium containing 0.1 M potassium phosphate buffer, pH 6.8; 0.1 mM ethylenediaminetetraacetic acid; 1 mM phenylmethylsulfonyl fluoride and 1% (w/v) polyvinylpolypyrrolidone. Then, the sample was centrifuged at 16,000×*g* for 15 min at 4 °C, and the supernatant was removed and set aside on ice for enzyme assays plus protein determination (Bradford 1976).

CAT, APX, and POD activities were determined as proposed previously (Chance and Maehly 1955; Nakano and Asada 1981; Havir and McHale 1987) and expressed as μmol^{-1} min^{-1} g^{-1} protein. SOD activity was measured as described earlier (Giannopolitis and Ries 1977) and expressed as U min^{-1} g^{-1}protein, with 1 U being equivalent to the concentration of SOD required to inhibit 50% of nitro blue tetrazolium photoreduction.

Determination of lipid peroxidation

Lipid peroxidation was determined based on the quantification of malondialdehyde (MDA), as proposed by Heath and Packer (1968) with some modifications. Extraction was performed by adding 1 mL of 1% trichloroacetic acid (TCA) to 100 mg of fresh and macerated leaf samples. The solution was vortexed and centrifuged at 12,000×*g* for 15 min at 4 °C, after which 250 µL of the supernatant were transferred to new tubes, along with 750 µL of 0.5% 2-thiobarbituric acid (w/v) in 20% TCA (w/v). The reaction proceeded for 30 min with shaking at 95 °C, and was blocked by incubation in an ice bath. The supernatants were transferred to new tubes, centrifuged at 10,000×*g* for 10 min at 4 °C, and read on a spectrophotometer at 532 and 600 nm. The concentration of MDA was calculated as $(A_{600} - A_{532})$ based on and extinction coefficient of 155 mM cm^{-1}.

Quantification of 20-E

20-E was measured as proposed previously (Kamada et al. 2009). Extraction was obtained by adding 10 mL of methanol to 100 mg DW of *P. glomerata* shoots and roots, and incubating with daily agitation for 7 days in the dark. Subsequently, the extracts were centrifuged twice at 3000×*g* for 15 min, and the extracts were collected for analysis. 20-E levels were quantified by Shimadzu Proeminence liquid chromatography system (Kyoto, Japan) equipped with RP column (150 mm × 4.6 mm i.d., 5 µm particle size; C18 stationary phase) from Phenomenex (Torrance, CA, USA) and a Shimadzu SPD-M20A photodiode array detector (monitoring 246 nm). The mobile phase of 1:1 (v/v) methanol:water, flow rate of 1.0 mL min^{-1}, injection of 20 µL of extract, and with reading at 245 nm for 15 min. The calibration curve was obtained by preparing standard 20-E solutions (Sigma-Aldrich, St. Louis, MO, USA), and the 20-E expressed by the content (mg g^{-1} DW) and 20-E accumulation (g).

RNA extraction, cDNA synthesis, and quantitative PCR analysis

Total RNA was extracted of shoot samples using the TRI Reagent® (Sigma-Aldrich Co.) as recommended by the manufacturer. To remove contamination with genomic DNA, RNA was treated with DNase I (NanoDrop™ Technologies, Wilmington, DE, USA). Then, cDNA was synthesized with the Super Script™ III kit (Invitrogen, Carlsbad, CA, USA). We evaluated the expression of two genes of the P450 family, *Phantom* (CYP76C) and *Spook* (CYP83A) (Batista et al. 2018), by quantitative real-time PCR using the Step One Plus™ system (Applied Biosystems®, Foster City, CA, USA). The *glyceraldehyde-3-phosphate dehydrogenase* gene was used for normalization (Batista et al. 2019). Transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), with three biological replicates with at least two technical replicates each.

Statistical analysis

Statistical analysis was performed using Genes software version Windows/2004.2.1 (Cruz 2016). Experiments were repeated at least once. Data were submitted to one-away analysis of variance and means were compared by Dunnett's (gene expression) or Tukey's test (other analyzes) at 5% significance.

Results

Longer photoperiods increase growth and induce anatomical changes

Longer photoperiods enhanced growth parameters, regardless of the evaluated accession (Fig. 1). The lowest average length of Ac22 (20% shorter) was observed with a photoperiod of 4 h, but did not differ significantly to the other conditions tested. In contrast, Ac43 plants showed an increase in length as the photoperiod increased. A similar increase in shoot, leaf, and root dry weight, as well as leaf area in relation to longer photoperiods, was observed for both accessions (Fig. 2).

Anatomical analysis revealed that the photoperiod affected leaf and stem structure of *P. glomerata* (Fig. 3). Stem development was similar between accessions, with less thickening observed during the shortest photoperiod. Instead, leaf structure differed between accessions. Except for the 8-h light treatment, in which the leaves presented reduced thickness and greater cell disorganization, Ac22 exhibited no structural changes to the leaf during the other photoperiods. In comparison, the leaves of Ac43 grown under a 24-h light photoperiod displayed less thickening and less vascular development, with more intercellular spaces and reduced organization of mesophyll cells.

Photoperiod affects pigment production and photosynthetic performance

In general, *P. glomerata* accessions presented similar chlorophyll content, which was lowest in plants cultivated

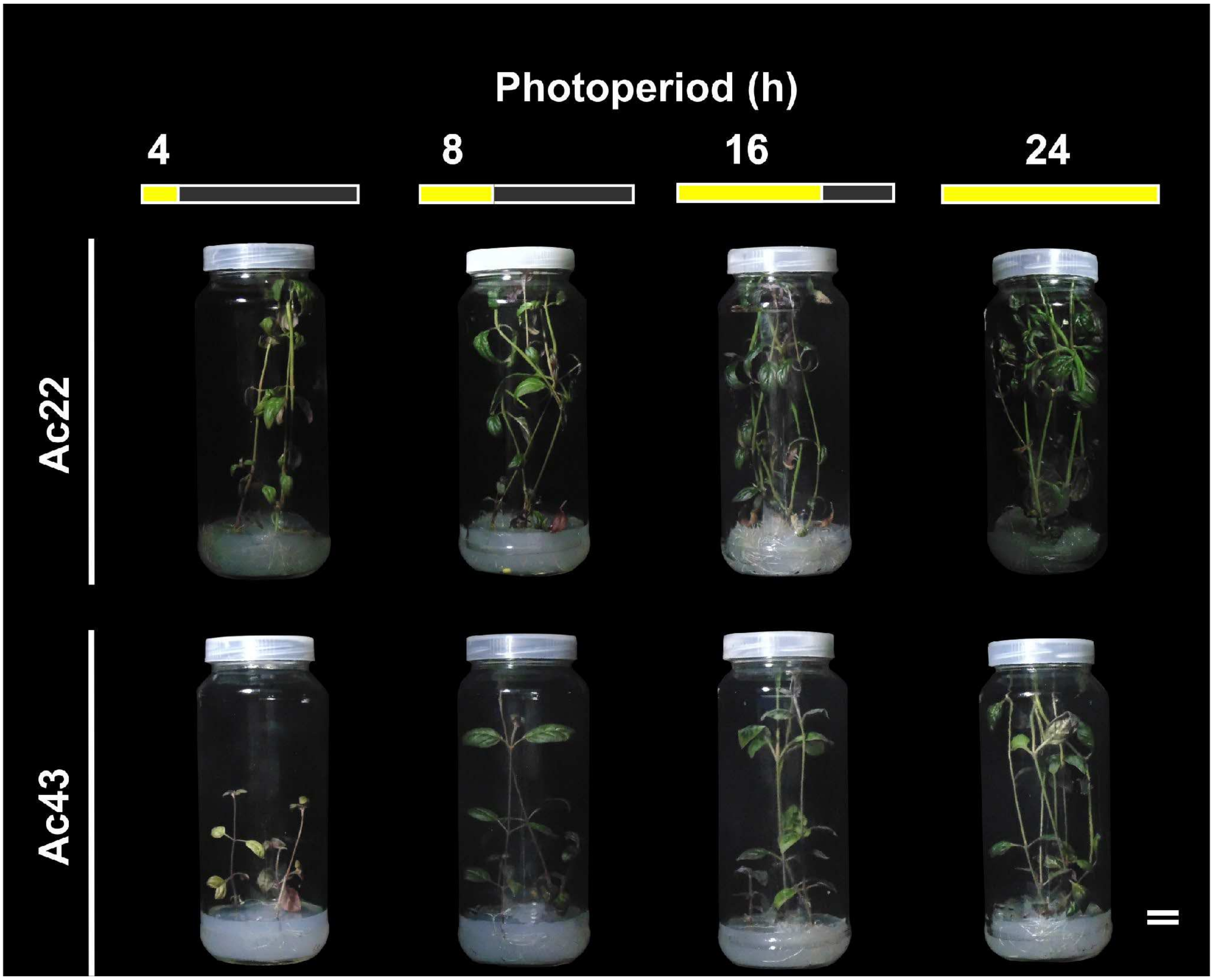

Fig. 1 Plants from the accessions 22 and 43 of *Pfaffia glomerata* after 40 days of in vitro culture under different daily photoperiod: 4, 8, 16, and 24 h. Bar = 2 cm

under the shorter photoperiod (Fig. 4). The production of carotenoids did not respond to the different photoperiods tested. Regarding anthocyanin content, *P. glomerata* accessions showed contrasting responses. On the one hand, Ac22 responded with a low anthocyanin content at 4 h, but a significantly higher one with longer photoperiods. On the other hand, for Ac43, the highest amount of anthocyanin was recorded with a short photoperiod, followed by its drop as the photoperiod lengthened.

Accession and photoperiod conditions tested affected all chlorophyll fluorescence parameters evaluated (Fig. 5). The highest average values of NPQ were observed in Ac43 cultivated over the shortest photoperiod. Instead, ETR was highest in Ac22 grown for 16 h.

The photosynthetic rate differed significantly between accessions, with the highest values observed for Ac22 under all conditions. Regarding the photoperiod, plants cultivated over 4 h of light presented the lowest rates of CO_2 assimilation. An increase in daily photoperiod resulted in a significant increase in plant photosynthetic rate (30–70%), although not so much at longer photoperiods (Fig. 5d).

Photoperiod changes modulate primary metabolism

Compounds related to primary metabolism, such as soluble carbohydrates, starch, and amino acids changed as a function of variations in photoperiod and accessions tested (Fig. 6). Longer photoperiods led to a significant increase in glucose and fructose production, but lower sucrose. No significant differences between accessions were observed for glucose and Ac22 exhibited the highest fructose means.

Longer photoperiods reduced total amino acid production (Fig. 6e). Under 16 and 24 h photoperiods, averages were more than twice lower than those observed for the 4 h photoperiod, which coincided with the highest values. Only at 4 h, there was no significant difference between accessions; under longer photoperiods, Ac22 exhibited higher averages.

Photoperiod affects lipid peroxidation and antioxidant enzyme activity

The level of lipid peroxidation/MDA formation was strongly affected by the photoperiod conditions tested (Fig. 7a). The lowest MDA values were observed under 4 h of light for both accessions. Ac43 plants displayed a constant increase in MDA formation with longer photoperiods, with the highest values observed after 24 h (29% greater than after 4 h). In contrast, no significant difference in lipid peroxidation was observed with photoperiods longer than 8 h. MDA averages were higher for Ac43 (25%) in the 4- and 24-h photoperiods but did not differ from those of Ac22 in the other treatments tested.

Antioxidant enzyme activity differed according to the treatments tested (Fig. 7b–e). The photoperiod did not affect CAT activity and, in terms of accession, the highest average CAT activity was observed for Ac43. Regarding APX activity, the lowest values were observed for Ac22 following a 24 h photoperiod, and for Ac43 following 8 h of light. There was a minor yet significant difference in SOD activity under a 24 h photoperiod, with average values being highest for Ac22. Finally, a photoperiod of 4 h resulted in greater POD activity for both accessions.

Photoperiod regulates 20-E production but does not alter their biosynthetic pathway- genes expression

20-E content in the shoots (Fig. 8a) was not influenced by the photoperiods tested. The lowest levels of 20-E in the roots were observed in Ac22 grown over shorter photoperiods (Fig. 8b). In comparison, total 20-E content of shoots and roots was strongly affected by longer photoperiods in both accessions, with the highest averages observed over the longer photoperiods (Fig. 8c and d).

Although directly related to the 20-E biosynthesis pathway, relative quantification of the *Phantom* and *Spook* genes revealed no significant differences among the tested photoperiods (Fig. 8e and f).

Discussion

Plants can sense seasonal variations in daily photoperiods, and modulate their life cycle and development to achieve greater fitness (Dodd 2005; de Wit et al. 2016; Serrano-Bueno et al. 2017). In this work, it was possible to observe the direct influence of photoperiod on the primary and secondary metabolism of two *P. glomerata* accessions. To our knowledge, this is the first study to demonstrate the effect of longer photoperiods on 20-E accumulation in plants, as well as an accession-dependent regulation of light conditions on the production of anthocyanins in *P. glomerata*.

We observed that, regardless of the accession, there was a significant increase in *P. glomerata* growth in response to an increase in the photoperiod. The higher growth was manifested as improved length, dry weight of shoots and roots, and leaf area. In line with these changes, *P. glomerata* photosynthetic rate was also significantly higher, indicating that the faster growth was a result of carbon assimilation due to higher energy availability under longer photoperiods.

Plants can modulate photosynthesis according to the photon flux in which they are exposed in order to optimize light capture or its dissipation (Dietz 2015). With increase

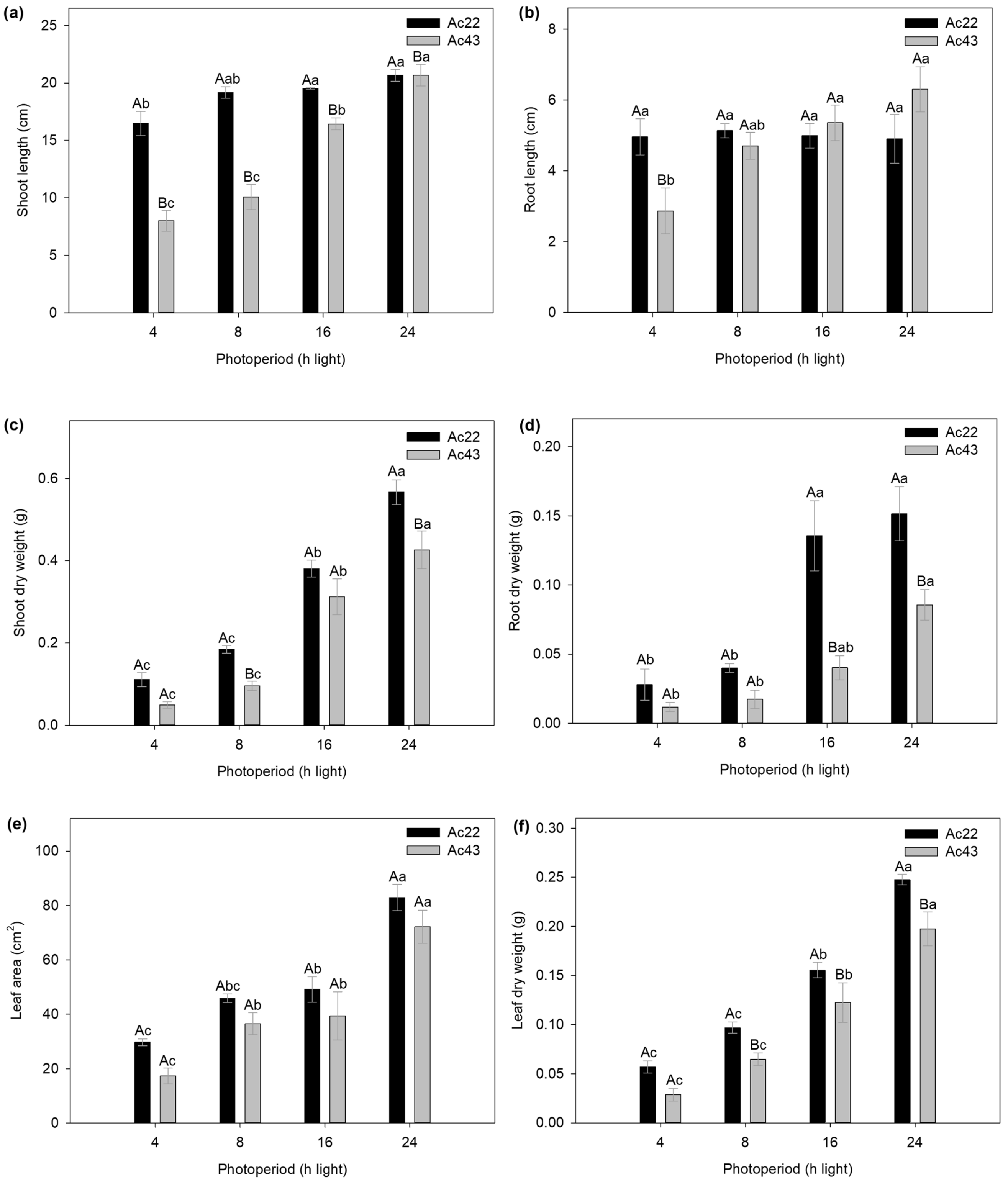

Fig. 2 Growth parameters in two *Pfaffia glomerata* accessions (22 and 43) grown in vitro under different daily photoperiod: 4, 8, 16, and 24 h. **a** Shoot length; **b** root length; **c** shoot dry weight; **d** root dry weight; **e** leaf area; **f** leaf dry weight. Uppercase letters represent comparisons among the two accessions under the same photoperiod; lowercase letters represent comparisons between the same accession under different photoperiod conditions. Identical uppercase or lowercase letters indicate no difference at a 5% significance level according to Tukey's test; values represent means (n = 4) ± standard error

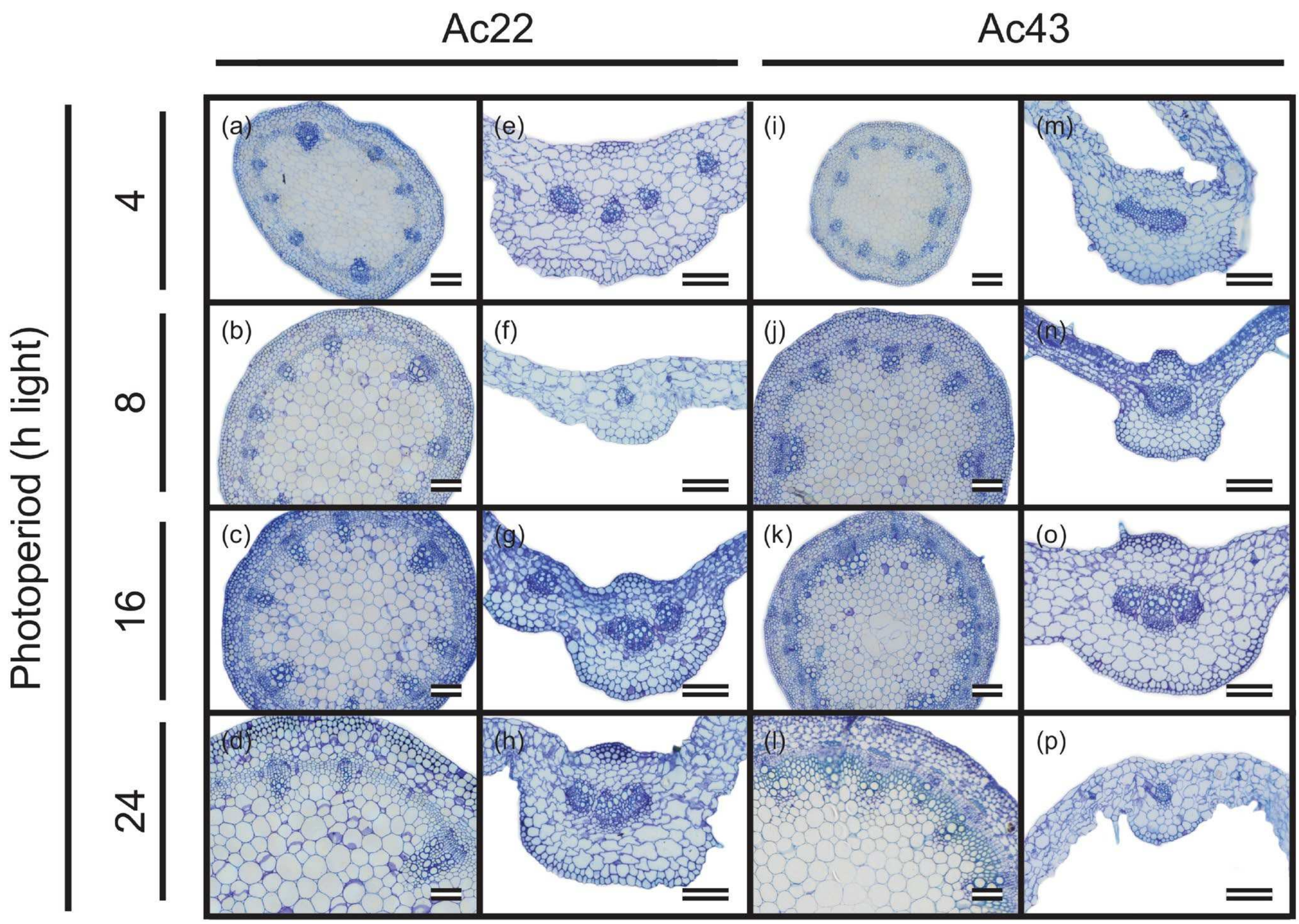

Fig. 3 Anatomic characterization of two *Pfaffia glomerata* accessions (22 and 43) grown in vitro under different daily photoperiod: 4, 8, 16, and 24 h. Cross-sections of stems (**a**–**d**) and leaf blades (**e**–**h**) of accession 22. Cross-sections of stems (**i**–**l**) and leaf blades (**m**–**p**) of accession 43. Bar = 30 µm

in the photon flux, photosynthetic performance can increase due to higher transfer of energy between the photosystems (Feng et al. 2019), in order to occur a greater consumption of ATP and NADPH by carbon assimilation reactions of the Calvin cycle (Bukhov 2004). Thus, photoperiod can modulate plant growth by regulating carbon fixation processes during photosynthesis, as well as sugar mobilization and starch degradation at night (Smith and Stitt 2007; Mengin et al. 2017). This influence can also occur indirectly, affecting the leaf area of plants, either by changing the number of cells or their size. Thus, longer photoperiods are generally associated with higher leaf area, chlorophyll content, photosynthetic and carbon fixation rates, and ultimately lead to better growth (Adams and Langton 2005), as observed in our work with *P. glomerata*.

Plants grown under excess light may suffer from photoinhibition, which lowers the photosynthetic rate and causes damage to the photosynthetic machinery (Powles and Thorne 1981). However, here, we did not observe these effects in plants grown over long photoperiods, and no change in the maximum quantum productivity of photosystem II (*F*v/*F*m) was observed. Indeed, the average *F*v/*F*m was close to 0.8, which is typical of healthy leaves (Maxwell and Johnson 2000; Vankoughnett et al. 2016). The lowest chlorophyll content was found in the shortest photoperiod tested (4 h), which may reflect the short light period these plants were exposed to. As a result, these plants exhibited also higher NPQ, which is probably related to photosystem saturation and release of excess energy to avoid damage to the photosynthetic apparatus (Adams 2018; Malnoë 2018). Plants grown under low light conditions are less energy-efficient and have higher NPQ because they are more susceptible to damage by excess light, possibly due to a breakdown of the antenna complexes (Ware et al. 2015).

Variations in photoperiod strongly affect primary metabolism and, as observed here for *P. glomerata*, shorter photoperiods lead to a reduction in soluble sugar content and an increase in amino acid and starch levels. As demonstrated

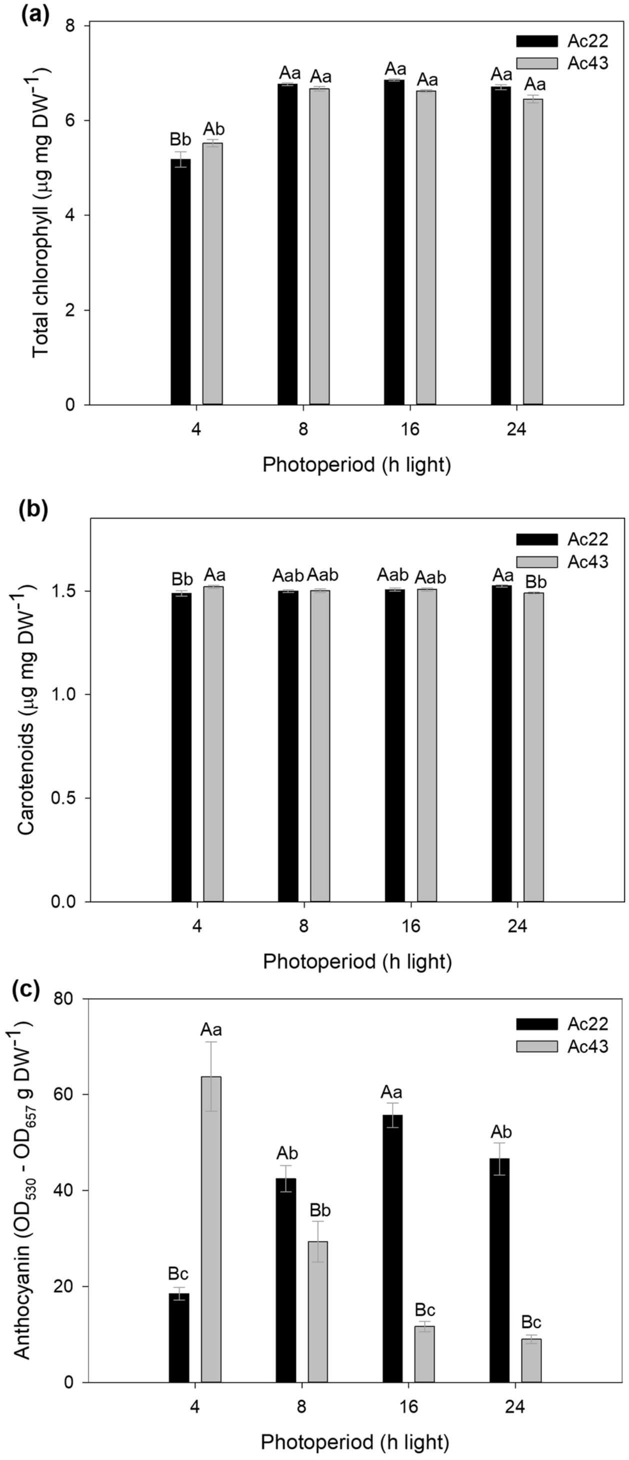

Fig. 4 Pigments contents in two *Pfaffia glomerata* accessions (Ac22 and Ac43) grown in vitro under different daily photoperiod: 4, 8, 16, and 24 h. **a** Total chlorophyll; **b** carotenoids; (**c**) anthocyanins. Uppercase letter represents comparisons between accessions during the same photoperiod; lowercase letters represent comparisons between the same accession under different photoperiods. The same uppercase or lowercase letters indicate no significant difference at a 5% level according to Tukey's test; values represent means (n = 5) ± SE

in our work, photosynthetic efficiency is affected by photoperiod conditions, thus altering the availability of carbon for the different metabolic processes. With shorter photoperiods, plants adjust their growth to synthesize more starch during the light period and degrade less of it during the night, resulting in a lower availability of hexoses (Gibon et al. 2009). Nitrogen uptake and amino acid catabolism are similarly affected. The conversion of amino acids into other structural molecules such as proteins is complex and requires substantial energy (Gibon et al. 2009). Therefore, lower carbon availability is expected to lead to higher amino acid levels, providing an additional energy source for substrate respiration (Hildebrandt et al. 2015).

Plants have developed numerous strategies for surviving under excess light conditions. Anthocyanin production is an important mechanism, as this pigment can act as a filter to protect against excess light or to attenuate oxidative stress (Gould et al. 2018). Here, we observed a significant increase in the biosynthesis of anthocyanin in accession 22 of *P. glomerata* during longer photoperiods. In contrast, accession 43 employed a different strategy, whereby the highest anthocyanin levels were observed during the shorter photoperiods, reflecting earlier studies on *Euphorbia pulcherrima* (Kannangara and Hansson 1998; Gu et al. 2018) and *Begonia semperflorens* (Zhang et al. 2016). Plants grown over short photoperiods may show an increase in carbohydrate production, such as sucrose and starch, and hormones such as abscisic acid, which may act to signal anthocyanin biosynthesis (Zhang et al. 2016). Anthocyanin production can result in increased energy efficiency, as it allows plants to capture photons in the visible region, where chlorophylls and carotenoids do not absorb (Merzlyak and Chivkunova 2000).

Short photoperiods may lead to plants producing more reactive oxygen species (ROS) due to changes in electron flow in the photosystem transport chain (Michelet and Krieger-Liszkay 2012). ROS accumulation induces cell damage that can result in death, and plants respond by producing antioxidant enzymes such as peroxidases (Cui et al. 2016). Here, *P. glomerata* exhibited an increase in POD activity to counteract ROS-induced damage and enable survival during short photoperiod conditions. Long photoperiods resulted in higher MDA content in leaves of *P. glomerata*. Increases in MDA content may lead to higher levels of membrane lipid peroxidation, signaling photooxidative damage (Martins et al. 2014). Transient MDA production may also be associated with photoprotection mechanisms, through signaling that promotes expression of genes involved in stress defenses and subsequent acclimatization (Weber et al. 2004; Morales and Munné-Bosch 2019). Based on the *Fv/Fm* results, no apparent oxidative damage was observed in the photosynthetic machinery. Hence, the accumulation of MDA in *P.*

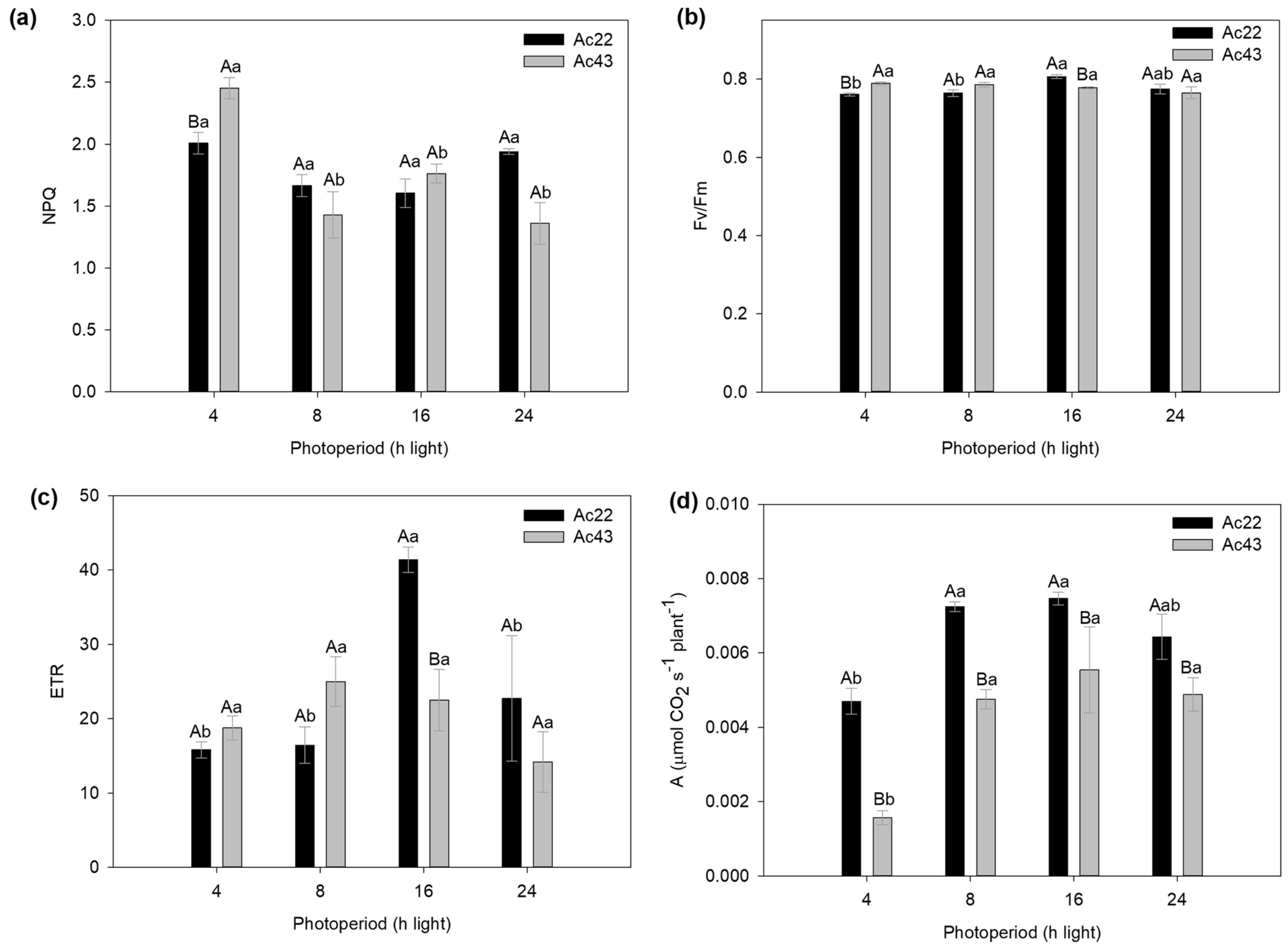

Fig. 5 Photosynthetic parameters in two *Pfaffia glomerata* accessions (22 and 43) grown in vitro under different daily photoperiod: 4, 8, 16, and 24 h. **a** Non-photochemical quenching (NQP); **b** maximum quantum productivity of photosystem II (*F*v/*F*m); **c** apparent electron transport rate (ETR); **d** photosynthetic rate. Uppercase letters represent comparisons between accessions under the same photoperiod; lowercase letters represent comparisons between the same accession under different photoperiods. The same uppercase or lowercase letters indicate no significant difference at a 5% level according to Tukey's test; values represent means (n = 4) ± SE

glomerata may be related to excess energy tolerance under longer photoperiods.

Longer photoperiods promoted the accumulation of 20E in *P. glomerata* on photosynthetic performance- and biomass-dependent manner. This increase was likely independent of transcriptional regulation, as no difference was observed in the relative expression of *Spook* and *Phantom* genes associated with its biosynthesis. Instead, the increase in 20-E accumulation was probably due to higher photosynthetic performance and the consequent increase in carbon fixation (Felipe et al. 2019b). Under beneficial conditions for carbon assimilation, plants can increase biomass accumulation and, consequently, attain better growth. Increased carbohydrate levels may alter hormonal modulation and thus stimulate the production of secondary metabolites (Huang et al. 2017).

In conclusion, our work shows that *P. glomerata* presents high physiological plasticity to photoperiod changes. During long photoperiods, photosynthetic performance is stimulated, promoting carbon availability. This, in turn, benefits secondary metabolism, as observed by the higher accumulation of 20-E in both shoots and roots. Accession-dependent changes in anthocyanin biosynthesis were observed in response to the different photoperiod tested. Thus, our results contribute to a better understanding of plant tolerance strategies to varying photoperiods. Furthermore, they demonstrate that longer photoperiods may be a beneficial strategy to increase the production of 20-E in in vitro cultures of *P. glomerata*.

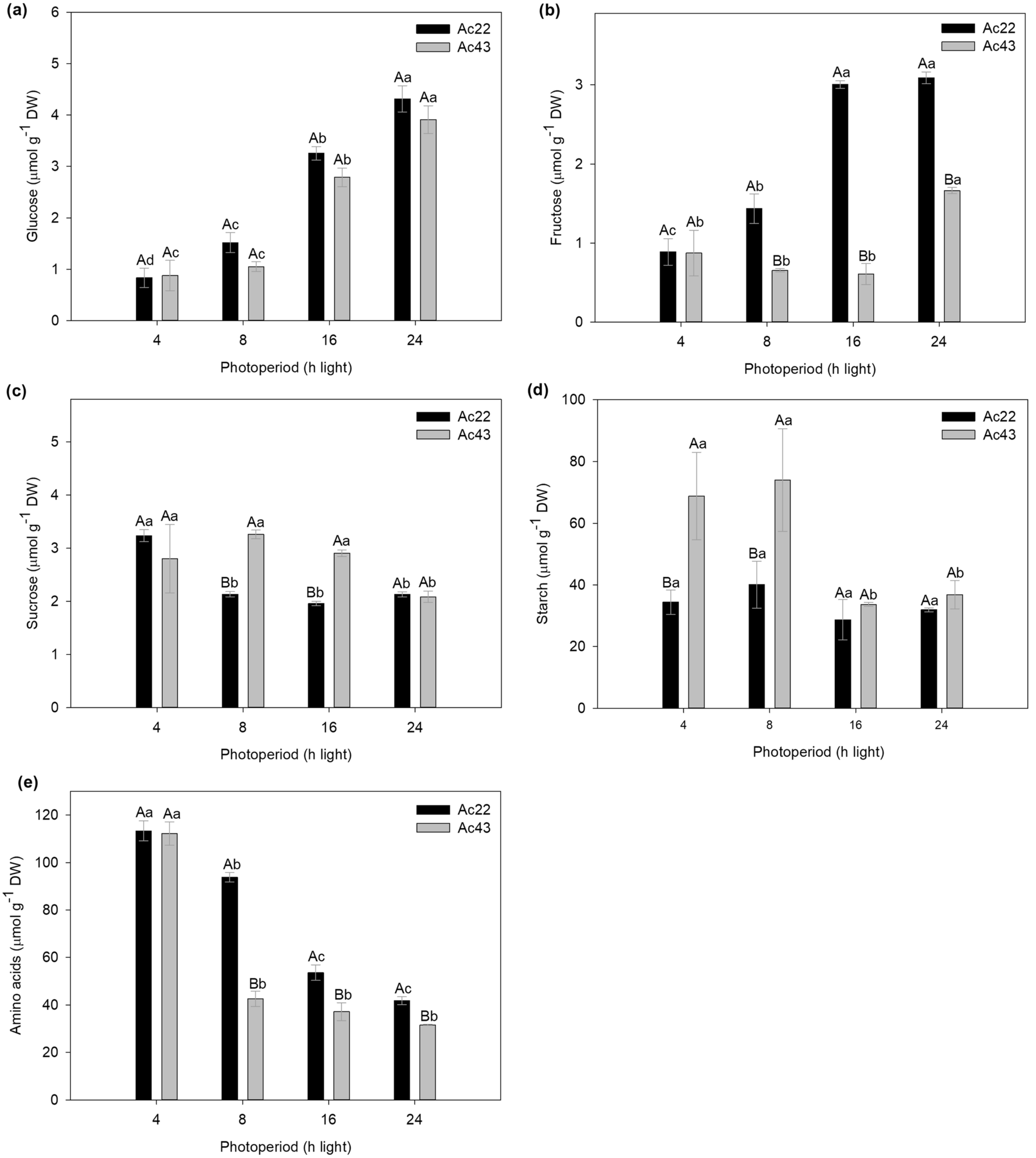

Fig. 6 Metabolites contents in two *Pfaffia glomerata* accessions (Ac22 and Ac43) grown in vitro under different daily photoperiod: 4, 8, 16, and 24 h. **a** Glucose; **b** fructose; **c** sucrose; **d** starch; **e** amino acids. Uppercase letters represent comparisons between accessions under the same photoperiod; lowercase letters represent comparisons between the same accession under different photoperiods. The same uppercase or lowercase letters indicate no significant difference at a 5% level according to Tukey's test; values represent means (n = 4) ± SE

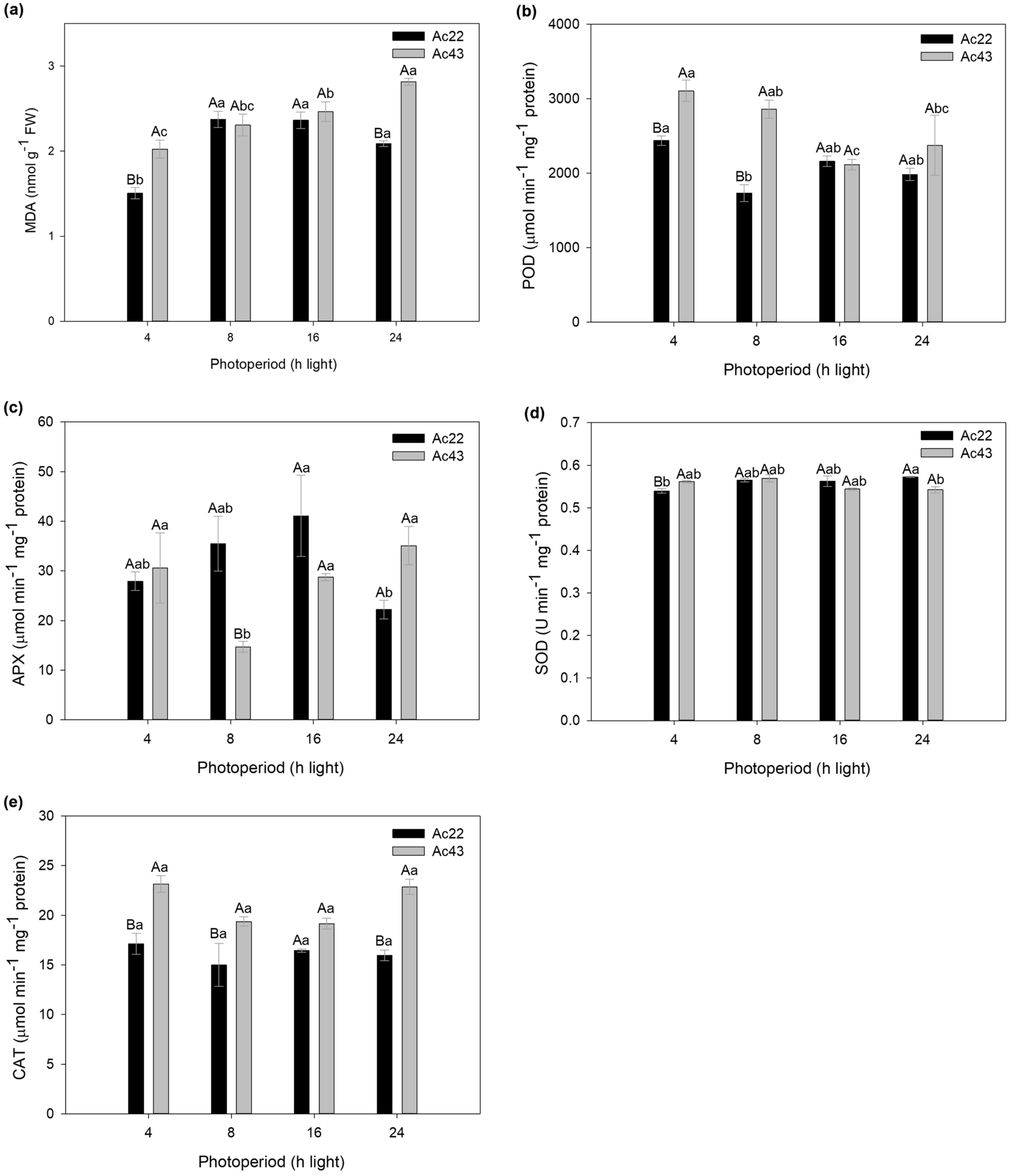

Fig. 7 Lipid peroxidation (MDA content) and antioxidant enzymatic activity in two *Pfaffia glomerata* accessions (Ac22 and Ac43) grown in vitro under different daily photoperiod: 4, 8, 16, and 24 h. **a** Malondialdehyde content (MDA); **b** peroxidase oxidoreductase (POD); **c** ascorbate peroxidase (APX); **d** superoxide dismutase (SOD); **e** catalase (CAT). Uppercase letters represent comparisons between accessions under the same photoperiod; lowercase letters represent comparisons between the same accession under different photoperiods. The same uppercase or lowercase letters indicate no significant difference at a 5% level according to Tukey's test; values represent means (n = 3) ± SE

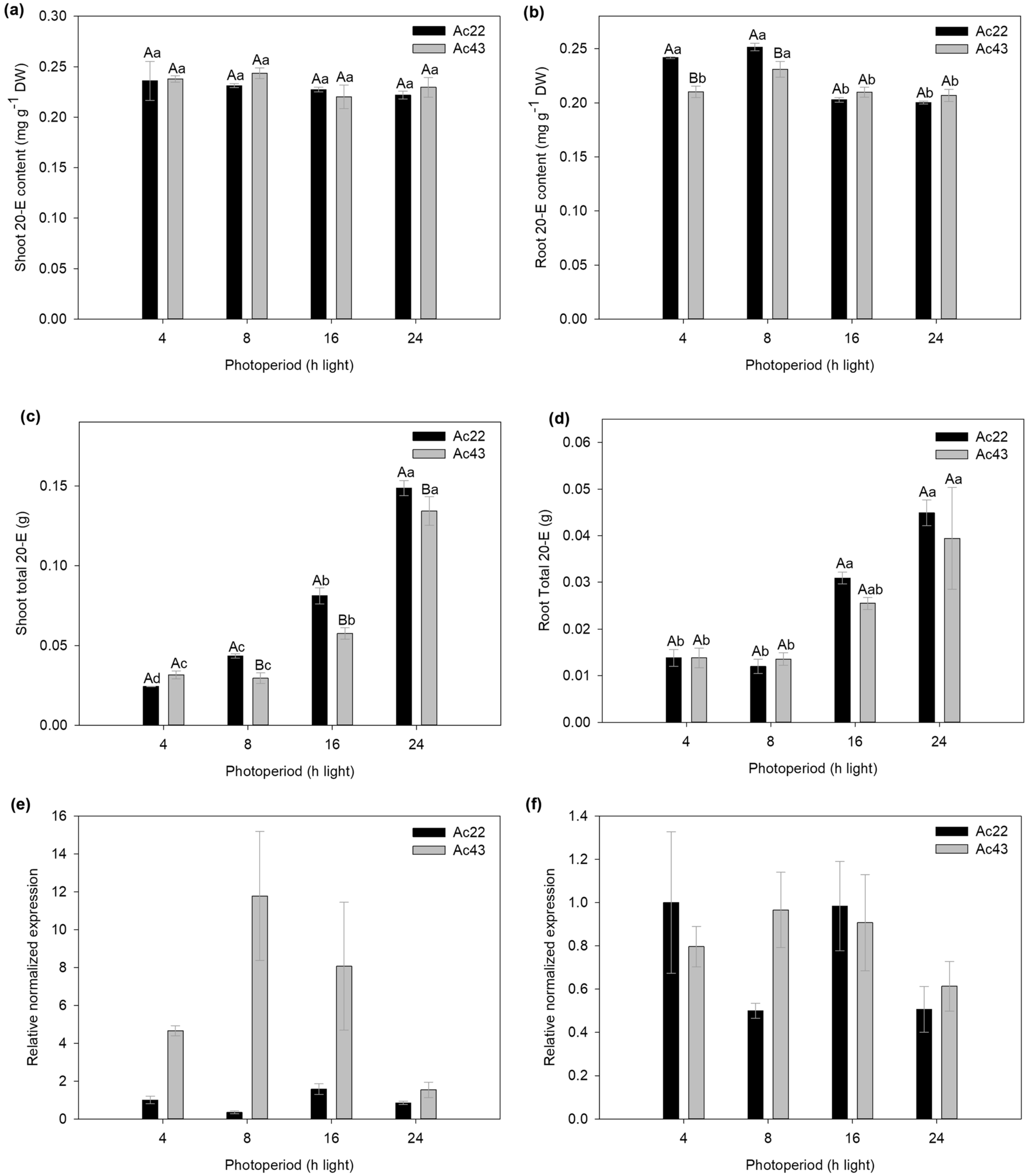

Fig. 8 20-E content and relative normalized gene expression in two *Pfaffia glomerata* accessions (22 and 43) grown in vitro under different daily photoperiod: 4, 8, 16, and 24 h. 20-E content (mg g^{-1} DW) in **a** shoots and **b** roots. Total 20-E content in **c** shoots and **d** roots. Uppercase letters represent comparisons between accessions under the same photoperiod; lowercase letters represent comparisons between the same accession under different photoperiods. The same uppercase or lowercase letters indicate no significant difference at a 5% level according to Tukey's test; values represent means (n = 4) ± SE. Relative normalized expression of **e** *Phantom* and **f** *Spook* genes in two *Pfaffia glomerata* accessions (22 and 43) grown in vitro under different daily photoperiod: 4, 8, 16, and 24 h. Gene expression is relative to the control gene *glyceraldehyde-3-phosphate dehydrogenase*. Values represent means (n = 3) ± SE and are compared by Dunnett's test at 5% probability

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Author contributions EAF, DSB, and WCO designed the study; EAF performed most of the experiments; EAF, KMC, TDS, SHSF, LNFC, KC, LMF, and JPVL performed physiological and biochemical analyses; EAF, SHSF, KC, KMC, and TDS evaluated photosynthetic performance; EAF, DSB, and LNFC performed molecular analyses; EAF and DSB analyzed the data; EAF and WCO wrote the article with input from all other authors. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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CHAPTER 2

The effect of salt stress and 5-azacytidine on the morpho-physiology, proteomic profile, and biosynthesis of 20-hydroxyecdysone in *Pfaffia glomerata*

Abstract

Plants adjust complex molecular, biochemical, and metabolic processes to overcome salt stress. Here, we investigate the proteomic and the epigenetic alterations involved in the morphophysiological responses shown by *Pfaffia glomerata*, a medicinal plant species, when cultivated under salt stress and treated with the demethylating agent 5-azacitidine (5-azaC), as well as how these changes affect the biosynthesis of 20-hydroxyecdysone (20-E), a secondary metabolite of great medicinal and pharmacological interest. To this, plants were cultivated in vitro for 40 days in Murashige and Skoog (MS) medium (control) and in MS supplemented with NaCl (50 mM), 5-azaC (25 μM), and NaCl+5-azaC. Compared to the control, the treatments reduced growth, photosynthetic rates, and photosynthetic pigment content. The primary metabolism was also affected by the treatments, with an increase of sucrose, total amino acids, and proline, and a reduction of starch and protein. Comparative proteomic analyses revealed 268 common differential accumulated proteins (DAP). We identified DAPs involved in 87 metabolic pathways, most of them related to the metabolism of amino acids, carbohydrates, and secondary metabolites, which may have played a key role in the salt stress tolerance responses observed. Plants treated with 5-azaC and NaCl+5-azaC reduced the 20-E levels, suggesting that the biosynthesis pathway can be regulated by DNA methylation. Moreover, the down-accumulation of a key protein of jasmonate biosynthesis, indicates the fundamental role of this hormone in the biosynthesis of 20-E. Taken together, our results highlight the candidate regulatory proteins relate to salt stress tolerance responses and 20-E biosynthesis in *P. glomerata*. Also, it was demonstrated a possible epigenetic control in the biosynthesis of 20-E, paving the way for works elucidating the mechanisms involved in this regulation.

Keywords: Proteomics, DNA methylation, phytoecdysteroids, medicinal plant, abiotic stress.

Introduction

During their life cycle, plants are exposed to several abiotic stresses that directly affect their development. Among these adversities, salinity stands out as one of the main problems affecting arable soils around the world, which imposes significant losses in plant productivity (Munns and Gilliham 2015; Van Zelm et al. 2020). Moreover, climate change scenarios forecast that by 2050 half of the arable areas will be impacted by salinization (Bates et al. 2008). Therefore, understanding the mechanisms of tolerance of plants to salt stress can be a key strategy to increase the productivity of crops to face this scenario (Wu et al. 2018), and a useful tool for the study and conservation of plant species.

Salinity impacts the development of plants in multiple ways. Initially, it limits soil water absorption, inducing osmotic stress and, consequently, water deficit (Arif et al. 2020). Then, the excessive accumulation of some ions in the cells, mainly sodium (Na^+) and chloride (Cl^-), induces cytotoxicity and reduces the absorption capacity of other ions that are essential for cellular processes, such as calcium (Ca^{2+}) and potassium (K^+), altering the ionic homeostasis (Wu et al. 2018; Isayenkov and Maathuis 2019). There is also an increase in the production of reactive oxygen species (ROS), leading to oxidative stress (Yang and Guo 2018a; Arif et al. 2020). As a consequence, there may be a reduction in seed germination, and plant growth, and productivity, as well as the induction of senescence processes and reduction of survival rate (Munns and Tester 2008).

Plants can adjust molecular, biochemical, and metabolic processes in salt stress conditions, but the magnitude of responses varies among species, time of exposure and the severity of stress (Munns and Tester 2008; Gupta and Huang 2014; Van Zelm et al. 2020; Shahid et al. 2020). The perception of stress occurs through cellular receptors, which readily induce a signaling network to activate osmotic adjustment mechanisms and ionic homeostasis

(Yang and Guo 2018a, b; Isayenkov and Maathuis 2019). These mechanisms are involved in the stomata closure, the activation of enzymatic and non-enzymatic systems for ROS detoxification, and the production of osmoregulating molecules, such as soluble sugars and proteins (Munns and Gilliham 2015; Saddhe et al. 2019).

Plants under salt stress present a significant increase in the expression of genes related to stress tolerance mechanisms, with a consequent change in the proteomic profile. As products of gene expression, proteins play a key role in regulating the physiological and morphological changes of plants in response to salinity (Sun et al. 2017; Kosová et al. 2018), as well as being directly involved in the maintenance of cell homeostasis (Liu et al. 2019). In this perspective, proteomic analyses become a fundamental tool for understanding the complex biological processes involved in the mechanisms of tolerance to salt stress.

Alterations in the metabolism and redox state of plants can induce epigenetic changes, which, in turn, regulate the expression of genes and key enzymes of metabolism (Shen et al. 2016; Samo et al. 2021). Epigenetic modifications involve processes such as DNA methylation and post-translational modifications of the histones, and play a crucial role in regulating responses to abiotic stresses, since they reversibly affect gene expression without altering the sequences of the genome, and can be transmitted hereditarily (Agarwal et al. 2020). Many studies correlate changes in DNA methylation pattern with salt stress tolerance responses, but the mechanisms involved in this regulation are still poorly described (Sun et al. 2019; Han et al. 2020). The use of chemical compounds, such as 5-azacytidine (5-azaC), can be a valuable approach to understand the dynamics of epigenetic changes on plant development (Santos et al. 2017).

5-azaC is a potent hypomethylating agent since it inhibits the activity of DNA methyltransferases, reducing the DNA methylation and altering the distribution pattern of methylated DNA (Solís et al. 2015), which may result in increased expression of genes related to saline stress tolerance, as observed in *Oryza sativa* (Zhu et al. 2015) and *Hibiscus cannabinus* (Li et al. 2021). Soybean plant under saline stress showed an increase in expression of *MYB84*, a transcription factor associated with salinity tolerance, and the treatment with 5-azaC reversed this effect, indicating that salt stress tolerance responses can be reversibly regulated by DNA methylation dynamics (Zhang et al. 2020).

Moreover, changes in the pattern of DNA methylation can regulate the secondary metabolism of plants (Yang et al. 2018). Treatments with 5-azaC induced the hypomethylation of DNA and affected the production of carotenoids (Faria et al. 2020), phenolic compounds (Yang et al. 2018), terpenes (Zeng et al. 2020), and alkaloids (Parashar et al. 2020). Thus, 5-azaC is a compound with a great potential to be used in the investigation of the biosynthesis pathways of secondary metabolites, with medicinal and pharmacological applications.

Pfaffia glomerata (Amaranthaceae) is a plant native to South America, commonly used in folk medicine, and with proven therapeutic properties as an anti-inflammatory, anti-depressant, analgesic, aphrodisiac, and in the treatment of muscle atrophy (Neto et al. 2005; Tóth et al. 2008; Dias et al. 2019; Franco et al. 2021). *P. glomerata* stands out for being one of the main producing plants of 20-hydroxyecdysone (20-E), a compound of great medicinal and biotechnological potential, which can be related to some of the medicinal properties presented by the species (Festucci-Buselli et al. 2008; Franco et al. 2021). A previous study showed that *P. glomerata*, when cultivated under mild salt stress, presented a complex mechanism of osmotic and ionic adjustment and up-regulation of the 20-E biosynthesis, to achieve physiological homeostasis in the sub-optimal conditions of the environment (Felipe et al. 2019). However, the way of regulating these mechanisms of salt stress response in proteomic and epigenetic levels still unclear in *P. glomerata*. Thus, the objective of this work was to investigate how changes in the proteome and global DNA methylation dynamics affect the morphophysiological responses and 20-E biosynthesis in *P. glomerata* treated with NaCl and 5-azaC.

Materials and methods

Plant material and experimental design

P. glomerata plants (Accession 22) used in this work come from a germplasm bank of the Plant Tissue Culture Laboratory (LCT-II, BIOAGRO, Universidade Federal de Viçosa). Voucher material was deposited at the Leopoldo Krieger Herbarium (UFJF, Juiz de Fora, MG, Brazil) under code number CESJ 63317.

The plants were kept under monthly subcultures from nodal explants in MS medium, supplemented with a vitamin complex (0.01 g L^{-1} of thiamine.HCl, 0.05 g L^{-1} of pyridoxine.HCl, 0.05 g L^{-1} of nicotinic acid, and 0.2 g L^{-1} of glycine), myo-inositol (100 mg L^{-1}), 3% (w/v) of sucrose and solidified with 5.5 g L^{-1} agar (PhytoTecnology Laboratories®, Kansas, USA).

Nodal segments (3 explants, with a mean length of 1.5 cm) were transferred to glass flasks (62 mm diameter × 15.5 mm height; AZ 200; Embalagens Rio, Nova Friburgo, Brazil) containing 100 mL of semi-solid MS in the same conditions mentioned above. For the conduction of stress treatments, 4 different supplements were performed in the MS medium: **1-** 50 mM of sodium chloride (NaCl, ACS reagent, ≥ 99%, Sigma-Aldrich, St. Louis, MO); **2-** Sterilized aqueous solution of 5-Azacydidine (5-azaC, Sigma-Aldrich, St. Louis, MO), in the concentration of 25 μM; **3-** Interaction between NaCl and 5-azaC (NaCl + 5-azaC), in the identical concentrations of the previous treatments; **4-** Control (without supplementation of NaCl and 5-azaC in MS medium). The concentrations of NaCl and 5-azaC were determined according to a previous test. The flasks were closed with rigid polypropylene lids with two 10 mm holes covered by two 0.45 μm-pore hydrophobic fluoropore polytetrafluoroethylene membranes (MilliSeal AVS-045 Air Vent, Tokyo, Japan), with the CO_2 exchange rate of 25 μL L^{-1} s^{-1} (Batista et al. 2017). The plants were kept for 40 days in a growth room with controlled irradiance of 60 µmol m^{-2} s^{-1}, by using two LED lamps (SMD 100, 18W, Vilux®, Vitória, Brazil) and an average temperature of 25 °C.

Plant growth parameters

After 40 days of in vitro culture, plants were collected and separated in shoot and root for length measurement (cm). Leaves, stems, and roots were dried separately at 50°C until constant weight to obtain the dry weight parameter (g).

Determination of photosynthetic rate and pigments content

The photosynthetic performance was determined according to Silva et al. (2020), through an S151 Infrared CO_2 analyzer (Qubit Systems, Kingston, ON, Canada) and a LoggerLite 1.8.1 software (Vernier Software & Technology, Beaverton, OR, USA) for measurements and data collection, respectively. CO_2 gas exchange was calculated based on the dry weight of leaves per plant (g), and the photosynthetic rate was expressed in µmol CO_2 s^{-1} $plant^{-1}$.

For the quantification of chlorophylls and carotenoids, 600 µL of MeOH 100% was added in 10 mg of freeze-dried and macerated leaf sample. The solution was vigorously vortexed (30 s) and incubated at 4 ºC under agitation in the dark for 10 min. Later, the samples were centrifuged at 12,000*g* (4 ºC, 10 min), and the supernatant was collected. The pellet was extracted once again according to the procedures described above, and the supernatant was collected and added together with that of the first extraction. After extractions, 200 µL of the supernatant was collected and added to a microplate for the absorbance readings (A) at wavelengths of 470, 646, and 663 nm. The contents of total chlorophylls (Chl *a* + Chl *b*) and carotenoids were determined according to the following equations (Wellburn 1994), and the concentrations were expressed by µg mL^{-1}.

$$\mathrm{Chl}\ a\ = (15.65 \times \mathrm{A}_{666}) - 7.34 \times \mathrm{A}_{653})$$

$$\text{Chl } b = (27.05 \times \text{A}_{653}) - (11.21 \times \text{A}_{666})$$

$$\text{Carotenoids} = \frac{(1000 \times \text{A}_{470}) - (2.86 \times \text{Chl } a) - (129.2 \times \text{Chl } b)}{221}$$

Quantification of anthocyanins was performed as described previously (Neff and Chory 1998). After measuring the extracts by spectrophotometer, the relative anthocyanin content was expressed as (A657 − A530) g^{-1} dry weight (DW).

Quantification of carbohydrates, proteins, total amino acids, and proline levels

The samples (leaves) were collected, freeze-dried, and macerated after 40 days of in vitro culture. For the quantification of carbohydrates, proteins, and total amino acids, approximately 20 mg of samples were extracted in methanol, as previously described (Lisec et al. 2006). The supernatant from the extraction was used to determine the soluble sugars (glucose, fructose, sucrose) as described by Fernie et al. (2001) and the total amino acid (Cross et al. 2006). From the pellets of extraction, the contents of starch (Fernie et al. 2001) and proteins (Cross et al. 2006) were determined. For the quantification of the proline content, approximately 10 mg of samples (leaves and roots) were freeze-dried and macerated. The extraction was performed according to the methodology proposed by Bates et al. (1973), with some modifications described previously by Felipe et al. (2019). The readings were taken at 520 nm wavelength, and a calibration curve was prepared using a proline standard solution (Sigma-Aldrich, St. Louis, MO). The content of proline was expressed by mmol mg^{-1} DW.

Quantification of 20-E

The 20-E quantification was performed according to the methodology described by Corrêa et al. (2015). Briefly, a methanolic extraction of macerated and freeze-dried samples of leaves and roots was performed. The determination of 20-E was done using a Shimadzu Proeminence liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with RP column (150 mm x 4,6 mm i.d., 5 µm particle size; C18 stationary phase) from Phenomenex (Torrance, CA) and a Shimadzu SPD –M20A photodiode array detector (monitoring 246 nm). The mobile phase of 1:1 (*v/v*) methanol:water, the flow rate of 1.0 mL min^{-1}, injection of 20 µL of extract, and with reading at 245 nm for 15 min. A 20-E standard (Sigma-Aldrich, St. Louis, MO, USA) solution was prepared to quantify a calibration curve (0–120 mg L$^{−1}$). 20-E content was expressed by mg g^{-1} DW.

Protein extraction and digestion

Proteomic analyses were performed using freeze-dried and macerated leaf samples (three biological replicates, 30 mg DW each sample). The extraction was performed by the methodology described by Damerval et al. (1986), by suspending the samples in a buffer solution (10% (w/v) Trichloroacetic acid/acetone (SigmaAldrich) together with 20 mM of Dithiothreitol (DTT, GE Healthcare, Piscataway, NJ, USA), with subsequent vortex mixing (5 min, 8 °C). The solution was kept for 60 min at -20 ° C and then centrifuged (16,000 *g*, 30 min, 4 °C). The pellets from the previous step were washed 3 times in acetone solution with DTT (20 mM) and centrifuged for 5 min each wash. Subsequently, the pellets were dried under vacuum, solubilized in a buffer composed of 7 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), 2% Triton X-100 (GE Healthcare), 1% DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), and 5 µM pepstatin (Sigma-Aldrich), and incubated for 30 min on ice. The samples were vortexed (16,000 *g*, 30 min, 8 °C), the supernatants were collected, and the protein concentration was measured using a 2-D Quant Kit (GE Healthcare).

Before the trypsin digestion step, protein samples (100 µg from each biological replicate) were precipitated using the methanol/chloroform, and the samples were resuspended in a solution buffer (urea 7 M/thiourea 2 M). Protein digestion was performed by filter-aided sample preparation (FASP) methodology according to Reis et al. (2021). Then, the peptides and proteins were quantified through a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA), at a wavelength of 205 nm.

Mass spectrometry analysis

Mass spectrometry was performed using a nanoAcquity UPLC connected to a Q-TOF SYNAPT G2-Si instrument (Waters, Manchester, UK) according to Passamani et al. (2020). Briefly, runs consisted of three biological replicates of 1 µg of digested peptides. During separation, samples were loaded onto the nanoAcquity UPLC M-Class Symmetry C18 5 µm trap column (180 µm × 20 mm) at 5 µl min^{-1} for 3 min and then onto the nanoAcquity M-Class HSS T3 1.8 µm analytical reversed-phase column (75 µm × 150 mm) at 400 nl min^{-1}, with a column temperature of 45°C. For peptide elution, a binary gradient was used, with mobile phase A consisting of water (Tedia; Fairfield, Ohio, USA) and 0.1% formic acid (Sigma-Aldrich) and mobile phase B consisting of acetonitrile (Sigma-Aldrich) and 0.1% formic acid. The gradient elution started at 7% B, then ramped from 7 B to 40% B until 91.12 min, then ramped again from 40 B to 99.9% B until 92.72 min, then remained at 99.9% until 106.00 min, then decreased to 7% B until 106.1 min, and finally remained at 7% B until the end of run at 120 min. Mass spectrometry was performed in positive and resolution mode (V mode), at 35,000 FWHM, with ion mobility, and in data-independent acquisition mode (HDMS^{E}). Human [Glu1]-fibrinopeptide B at 100 fmol µl^{-1} was used as an external calibrant, and lock mass acquisition was performed every 30 s. Mass spectra were acquired by MassLynx v 4.0 software.

Proteomic data analysis

Spectra processing and comparative analysis were performed according to Passamani et al (2020). Spectra processing and database searching were performed using ProteinLynx Global Server (PLGS) software v. 3.0.2 (Waters) and comparative label-free quantification was performed using ISOQuant software v. 1.7 (Distler et al. 2014). Briefly, for ISOQuant the following parameters were used to identify proteins: a 1% FDR, a peptide score greater than six, a minimum peptide length of six amino acids, and at least two peptides per protein were required for label-free quantitation using the TOP3 approach, followed by the multidimensional normalized process within ISOQuant. The proteomics data were processed against the *P. glomerata* database (http://www.ncbi.nlm.nih.gov/biosample/8103044; Batista et al. 2019).

For comparative proteomic analysis, only proteins present in all three biological replications, or absent in all replicates (for unique proteins) were considered. Data were analyzed using Student's t-test ($P \leq 0.05$). Also, the criterion of Log_2 fold change (FC) was considered for the calculation of the differential protein abundance, being considered up-accumulated when Log_2 FC > 0.6 and down-accumulated when Log_2 FC < -0.6. Description and functional annotation were performed for the differentially abundant proteins using OmicsBox software (https://www.biobam.com/omicsbox).

Statistical analyses

The experiment was designed in a completely randomized design. Data were submitted to one-way analysis of variance and means were compared by Student's t-test (proteomic analyses) or Tukey's test (other analyses) ($P \leq 0.05$). A principal component analysis (PCA) was performed to determine the statistical similarity among the treatments based on the differential abundance of proteins. Besides, a HeatMap was generated through the HemI software (Deng et al. 2014), presenting the pattern of protein variation related to the main metabolic pathways, identified by KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway annotation. Statistical analyses were conducted using the software R v3.4.0 (www.r-project.org) and RStudio v1.1.456 (http://www.rstudio.org/).

Results

Saline stress and 5-azaC affected growth parameters

After 40 days of in vitro culture, *P. glomerata* treated with 5-azaC and under salt stress showed a significant reduction in development and phenotypic changes, such as lower branching and number of leaves, and symptoms of leaf chlorosis (Fig. 1). Compared to the control, the plant length was significantly decreased in the treatments with NaCl (34%), 5-azaC (31%), and NaCl+5-azaC (42%). In addition, the treatments also led to a significant reduction in plant biomass, as seen in the dry mass of leaves (31-52%), stems (42-50%), and roots (30-59%). However, there was no significant difference in root length among the treatments.

Salt stress and 5-azaC *decreased pigment content and photosynthesis*

Compared to the control, the chlorophyll content was significantly reduced in plants treated with NaCl (23%), 5-azaC (24%), but mainly in NaCl+5-azaC (45%) (Fig. 2a). Besides having the lowest chlorophyll content, plants treated with NaCl+5-azaC also showed a significant reduction in carotenoids content (22%) (Fig. 2b). Regarding anthocyanins, only plants treated with NaCl differed in the contents of this pigment, with a reduction of 32% when compared to the control (Fig. 2c).

The photosynthetic performance was significantly decreased in plants treated with NaCl (59%), 5-azaC (44%), and NaCl+5-azaC (75%), compared to the control (Fig. 2d).

Saline stress and 5-azaC affected the primary metabolism

The content of hexoses was antagonistically affected by the treatments applied (Fig. 3a-b). Compared to the control, plants treated with NaCl showed a significant increase in glucose (39%) and fructose (62%) content. On the other hand, plants treated with 5-azaC and

NaCl+5-azaC significantly reduced their glucose content (29% and 40%, respectively) and fructose (38% and 40%, respectively).

Compared to the control plants, there was a significant increase in sucrose (Fig. 3c) and amino acid (Fig. 3e) content in all treatments. Conversely, the starch (Fig. 3d) and protein (Fig. 3f) contents were significantly reduced.

The treatments with 5-azaC and NaCl also affected the accumulation of proline in *P. glomerata*, but differently depending on the organ evaluated (Fig. 3g-h). Compared to the control, we observed a significant increase in the content of proline in leaves of plants treated with NaCl (72%) and NaCl+5-azaC (33%), but without a difference in those treated with 5-azaC only. However, we observed that all treatments significantly increased (155-240%) the proline content in the roots.

Effects of treatments of NaCl and 5-azaC on 20-E content

Compared to the control, there was a significant reduction in the 20-E content in the leaves and roots of plants treated with 5-azaC and NaCl+5-azaC. On the other hand, plants treated with NaCl did not differ significantly in metabolite production (Fig. 4).

Effects of treatments with 5-azaC and saline stress on the proteomic profile

Proteomic analyzes of plants treated with the hypomethylating agent 5-azaC and under salt stress revealed significant changes in the abundance of proteins in *P. glomerata*. In total, 1274 proteins were identified, of which 1243 (97%) were found in plants from all treatments (Supplementary Figure 1; Supplementary Table 1). We observed 268 common differentially accumulated proteins (DAP), and the NaCl/Control, 5-azaC/Control and 5-azaC+NaCl/Control comparisons shared 14 protein up-accumulated and 5 down-accumulated (Figure 5a-b). Compared to the control, 185 common proteins were up-accumulated and 81 down-accumulated in plants grown in at least in one of the comparisons with NaCl, 5-azaC and NaCl+5-azaC.

A PCA was applied to compare proteomic profiles among the different treatments to which *P. glomerata* plants were submitted (Figure 5c). Taken together, PC1, PC2, and PC3 explained 73.9% of the total variability. The PCA showed greater similarity between the proteome of plants treated with NaCl and control plants. On the other hand, the least similarity was observed between the control treatment and the plants treated with 5-azaC. As expected, plants treated with NaCl+5-azaC showed an intermediate proteomic profile between treatments with NaCl and those with 5-azaC, according to the cluster generated by the PCA.

GO analysis of DAP was performed using the Blast2GO software (Fig. 6). Proteins were identified and categorized into Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). The analysis revealed that most accumulated proteins by 5-azaC and salt stress are related to cellular and metabolic processes, responses to stress, photosynthesis, and methylation. For nearly all these shared GO terms, plants treated with NaCl had more up- or down-accumulated DAP, when compared to plants from the other treatments.

KEGG analyzes were performed and identified DAPs on 87 different metabolic pathways (Supplementary Table 2). The largest groups observed were “Thiamine and purine metabolism” (25 proteins), “Glycolysis/gluconeogenesis” (19 proteins), “Starch and sucrose metabolism” (18 proteins), and “Cysteine and methionine metabolism” (14 proteins). The abundance of DAPs involved in the main pathways is shown in Fig. 7.

Discussion

Salinity is a major problem in croplands around the world and imposes severe limitations on plant growth. In contrast, plants can adjust molecular, biochemical, and metabolic processes to resist these unfavorable conditions (Shahid et al. 2020; Van Zelm et al. 2020). Here, we cultivated in vitro the medicinal plant *P. glomerata* under saline stress and treated with the hypomethylating agent 5-azaC. Our findings suggest that these treatments altered the proteomic levels and the global methylation pattern of the DNA, which may have regulated the accumulation of biomass and physiological characteristics exhibited by the plants, as well as the biosynthesis of 20-E.

We observed that *P. glomerata* treated with NaCl and 5-azaC decreased the rate of CO_2 assimilation, with a consequent reduction in growth and biomass. Similar results were found by Felipe et al. (2019), in which *P. glomerata* plants submitted to severe salt stress showed signs of photoinhibition and photooxidative damage. Salinity limits water absorption by plants, inducing stomata closure and photorespiration, also triggering non-stomata responses, such as changes in the rate of electron transport and ultrastructural modifications in cellular components (Acosta-Motos et al. 2017; Pan et al. 2020). Plants treated with NaCl and 5-azaC showed inhibition in the accumulation of cytosolic glyceraldehyde-3-phosphate dehydrogenase, indicating a reduction in the efficiency of the Calvin cycle (Lu et al. 2020), which may explain the reduction in the assimilation rates of CO_2. On the other hand, we observed a significant increase in photosynthesis-related enzymes such as chloroplastic fructose-bisphosphate aldolase and NADP-dependent malic enzyme-like. These proteins may be involved in stress tolerance responses (Lv et al. 2017; Chen et al. 2019), mitigating the formation of ROS and producing osmoregulatory compounds (Chen et al. 2019).

Lower photosynthetic rates in plants under salt stress may also be associated with a reduction in the content of photosynthetic pigments. Here, we observe a reduction in the chlorophylls and carotenoids content in plants treated with NaCl, as well as in plants treated with 5-azaC, indicating that the biosynthesis or degradation of these pigments can be regulated by the level of methylation of the DNA (Xu et al. 2017; Yuan et al. 2020; Zhong et al. 2020). The reduction in the content of these pigments may be related to the down-accumulation of oxygen-dependent coproporphyrinogen-III oxidase, geranylgeranyl diphosphate reductase, and zeta-carotene desaturase (Goto et al. 2010; Wang et al. 2014a; Flores-Ortiz et al. 2020). Only NaCl treatments affected the accumulation of anthocyanins, leading to a significant reduction in their levels, as also observed in other works with plants cultivated under high saline stress (Li et al. 2013; Trivellini et al. 2014). However, *P. glomerata* treated with NaCl+5-azaC showed an increase in the accumulation of anthocyanins when compared to those treated only with NaCl, indicating that DNA methylation can regulate genes involved with anthocyanins biosynthesis (Zhu et al. 2020).

Plants grown under abiotic stress may alter carbohydrate metabolism in order to respond to unfavorable growth conditions (Zhang et al. 2017; Diniz et al. 2020). *P. glomerata* treated with NaCl showed a reduction in starch content and an increase in hexoses content, corroborating with the results found by Felipe et al. (2019). On the other hand, treatments with 5-azaC and NaCl+5-azaC resulted in a reduction in hexoses content, indicating the carbohydrate metabolism can be controlled by the genome methylation pattern (Li et al. 2015; Genitoni et al. 2020), but how these processes regulation work still unclear. Epigenetic regulation of primary metabolism involves the control of gene expression and key metabolism enzymes, which in turn control epigenetic changes through the availability of metabolites and chromatin status modification (Shen et al. 2016; Samo et al. 2021). In this work, we identified 14 pathways of carbohydrate metabolism with DAPs, the most abundant being those related to glycolysis/gluconeogenesis, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, and fructose and mannose metabolism (Supplementary Table 2). Interestingly, the highest abundance of DAPs involved in these metabolic pathways was found in plants treated with NaCl compared to plants in control. The increase in the accumulation of some proteins, such as fructokinase-4, pyruvate decarboxylase 2, pyruvate dehydrogenase, aldose 1-epimerase-like, and UDP-glucose 6-dehydrogenase 5, may indicate a rise in carbohydrate production and energy supplementation to resist saline stress and keep cellular processes active (Damaris et al. 2016). Besides the fundamental role of energy source, the increase of sugar content in stressed plants can help in stress tolerance mechanisms, acting as osmoregulation, antioxidant, and ionic balance molecules (Bolouri-Moghaddam et al. 2010; Yuan et al. 2016).

Plants treated with NaCl and 5-azaC showed an increase in the content of proline and total amino acids and a significant reduction in protein content. This demonstrates that amino acid metabolism is crucial in stress response mechanisms in plants (Zhang et al. 2017; Hildebrandt 2018). We also identified a differential accumulation of proteins related to 19 different metabolic pathways of the amino acid metabolism class; the largest amount of DAPs being also found in plants treated with NaCl compared to control (Supplementary Table 2). Cysteine and methionine metabolism was the route with the highest number of DAPs, and the increase in these proteins may be associated with antioxidant responses and control of ROS formation (Liao et al. 2019). The increase in the metabolism of some aromatic amino acids (phenylalanine and tyrosine) and branched chains (leucine, isoleucine, and valine) can be related to the increase in the degradation of proteins in response to osmotic stress, through the activity of proteases regulated by abscisic acid (Huang and Jander 2017). Proline accumulation is a well-preserved mechanism in plants exposed to abiotic stresses, working mainly as osmoregulation compounds, in ion adjustment, as ROS scavengers, and in membrane protection (Per et al. 2017; Arif et al. 2020). Here, we observe a greater accumulation of polyamine oxidase and Δ-1-pyrroline-5-carboxylate dehydrogenase. The accumulation of these two proteins can directly regulate the metabolism of polyamines and proline, and affect the tolerance of plants to salt stress, through signaling for the production of solutes and the mitigation of oxidative stress (Zarza et al. 2017; Guan et al. 2020).

P. glomerata treated with 5-azaC and NaCl+5-azaC showed down- accumulation of probable leucine-rich repeat receptor-like protein kinase (LRR-RLK) compared to plants from control, whilst plants treated only with NaCl showed no significant difference in the accumulation of this protein comparing to control plants. The expression of LRR-RLK is directly associated with tolerance responses to biotic and abiotic stresses by regulating jasmonate biosynthesis (Van der Does et al. 2017; Ye et al. 2020). Several works have demonstrated that the pattern of DNA methylation is related to hormonal regulation and jasmonate production by plants (López Sánchez et al. 2016; Latzel et al. 2020). Thus, our results suggest that the hypomethylation of DNA promoted by the action of 5-azaC can lead to the reduction of the accumulation of LRR-RLK, and, as a consequence, reduce the biosynthesis of jasmonate. This negative regulation of jasmonate biosynthesis may be associated with the suppression of stress tolerance responses, such as the lower abundance of glucan-endo-1,3-beta-glucosidase-like (Balasubramanian et al. 2012; Mageroy et al. 2020), and 60 kDa jasmonate-induced protein-like (Chen et al. 2017; Przydacz et al. 2020) (as also observed here). Furthermore, several works have shown that jasmonate is related to the signaling of the 20-E biosynthesis pathway in plants (Wang et al. 2014b; John et al. 2018; Erst et al. 2019). In this sense, 20-E biosynthesis in *P. glomerata* can be regulated by epigenetic mechanisms. The reduction in the content of 20-E observed in plants treated with 5-azaC can be related to the lower signaling of jasmonates, due to DNA hypomethylation.

In conclusion, we verify that saline stress and 5-azaC treatment strongly affected the photosynthetic process in *P. glomerata*, resulting in a significant reduction in biomass accumulation, and an adjustment in primary metabolism. At the proteomic level, 268 common DAPs were identified, mainly related to the metabolism of amino acids, carbohydrates, and energy, which may have played a fundamental role in the tolerance stress responses observed. Some of these responses were contrasting between plants treated with NaCl and those treated with 5-azaC, suggesting that the methylation dynamics affect the plant ability to respond to salinity. Finally, our observations support the idea that jasmonate plays a key role in 20-E biosynthesis pathway in plants, and that DNA hypomethylation negatively affects hormone production, with consequent reduction of 20-E production. Our findings shed light on the role of DNA methylation in controlling the biosynthesis of this 20-E in *P. glomerata* and paves the way for studies that seek to elucidate the epigenetic mechanisms involved in the regulation of secondary metabolism pathways in plants.

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Figures

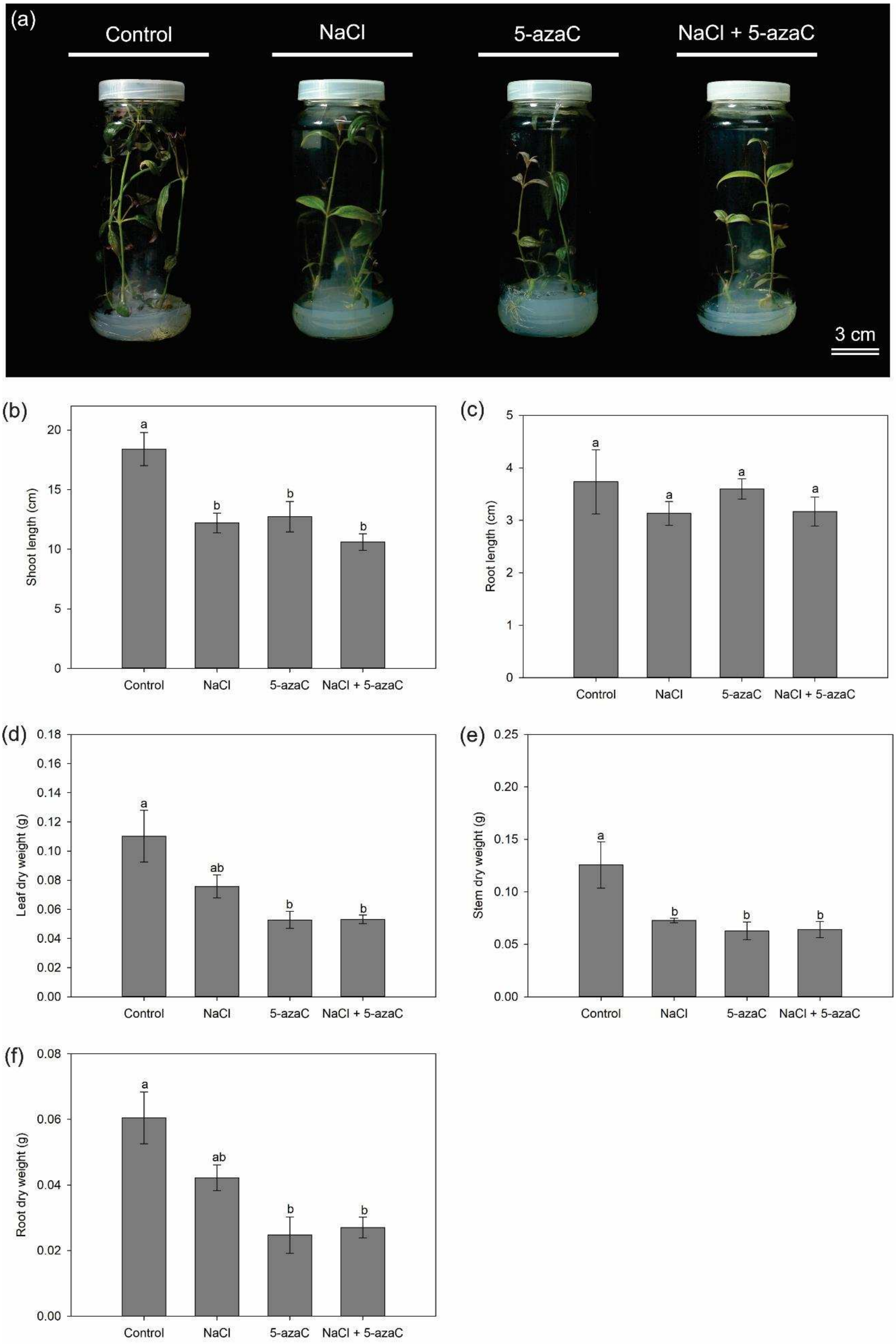

Figure 1. Experimental set-up and growth analysis. **(a)** Plants of *Pfaffia glomerata* treated with

5-azacytidine (5-azaC) and under salt stress (NaCl) after 40 days of in vitro culture; **(b–e)** Growth parameters of *P. glomerata*. Equal letters do not differ at 5% level by Tukey's test; values represent means (n = 5) ± standard error (SE).

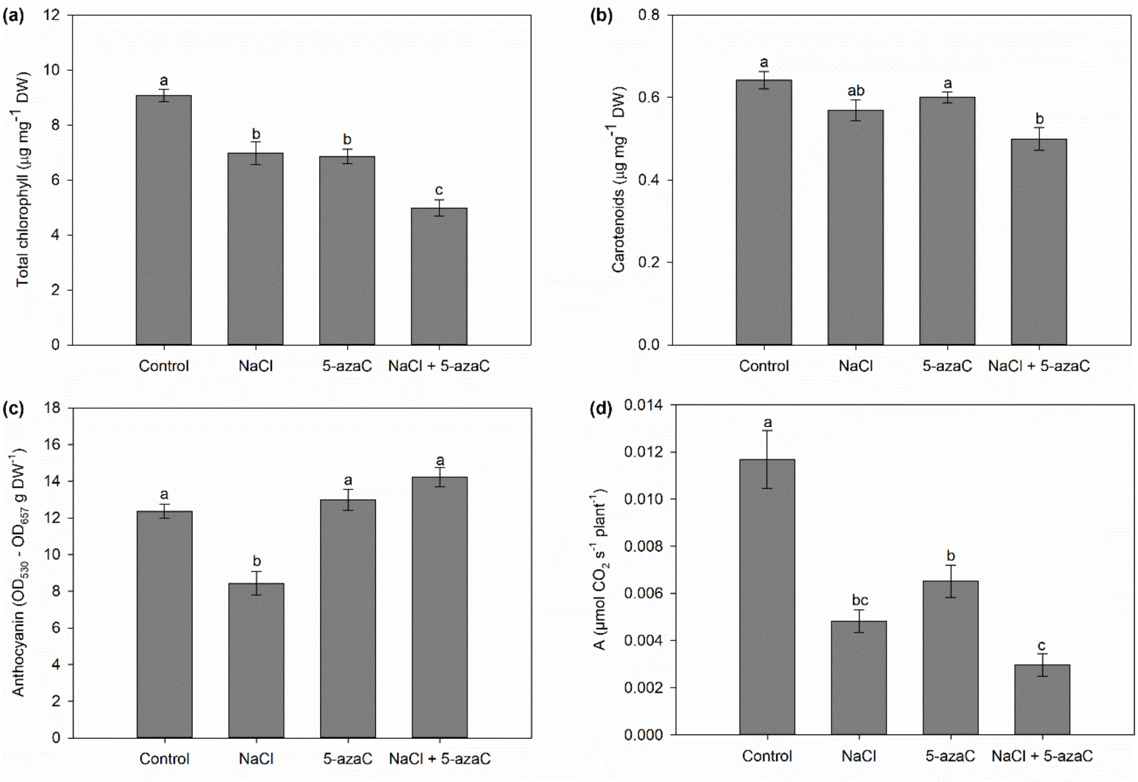

Figure 2. Pigments contents and photosynthetic performance in *P. glomerata* plants treated with 5-azacytidine (5-azaC) and under salt stress (NaCl) after 40 days of in vitro culture. **(a)** Total chlorophyll; **(b)** Carotenoids; **(c)** Anthocyanins; **(d)** Photosynthetic rate (A). Equal letters do not differ at 5% level by Tukey's test; values represent means (n = 5) ± (SE).

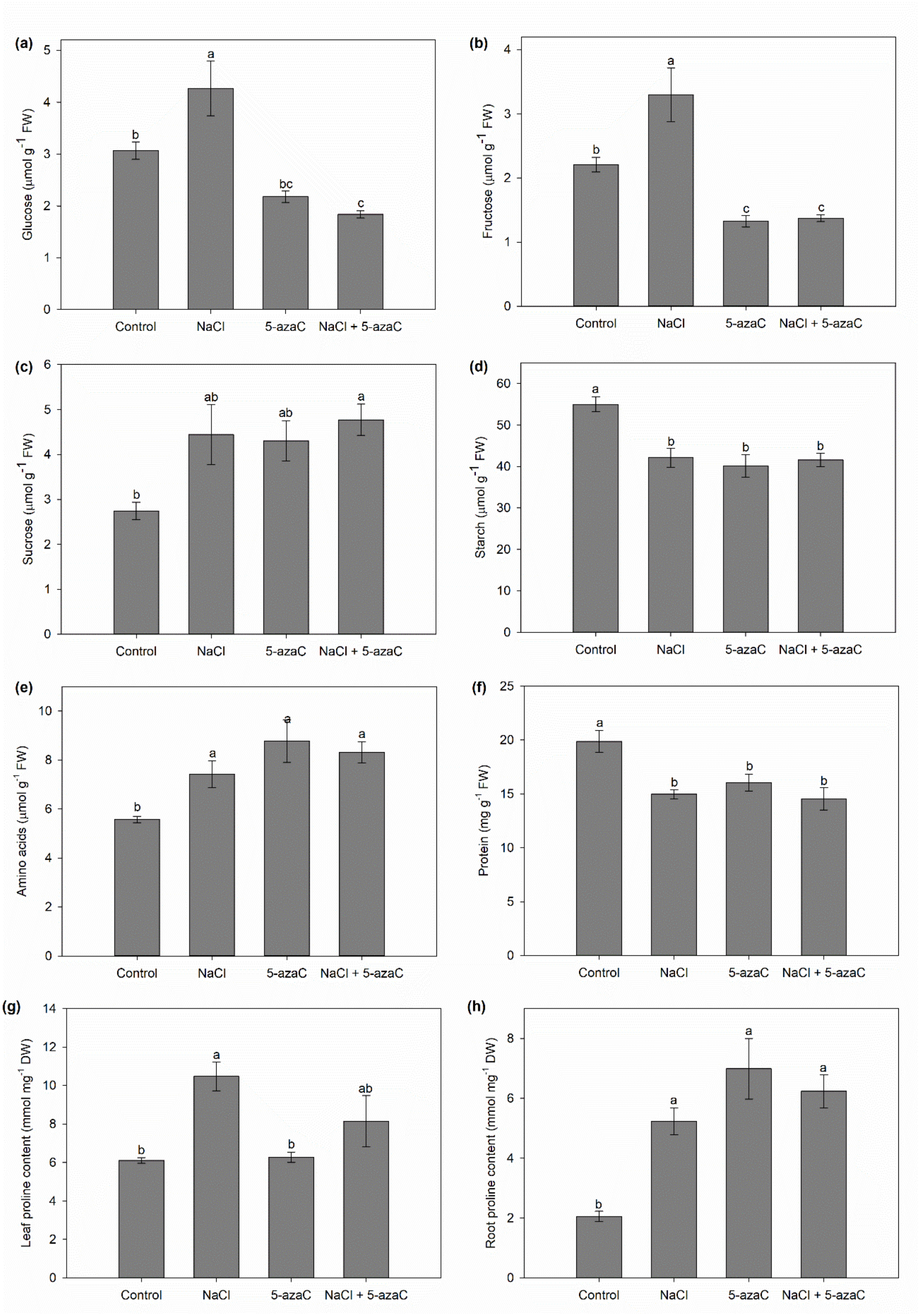

Figure 3. Metabolites contents in *P. glomerata* plants treated with 5-azacytidine (5-azaC) and under salt stress (NaCl) after 40 days of in vitro culture. (**a**) Glucose; (**b**) Fructose; (**c**) Sucrose; (**d**) Starch; (**e**) Amino acids; **(f)** Protein; **(g)** Leaf proline; **(h)** Root proline. Equal letters do not

differ at 5% level by Tukey's test; values represent means (n = 5) ± (SE).

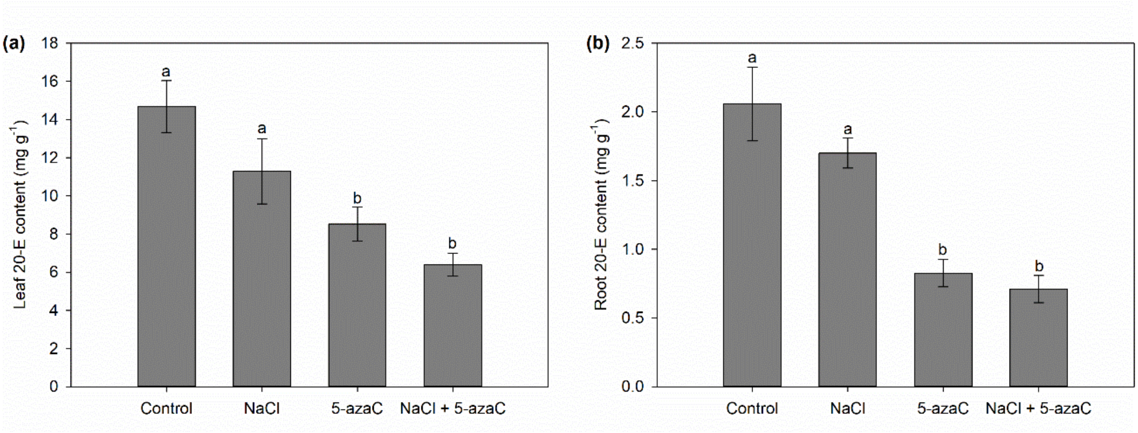

Figure 4. 20-E content in (**a**) leaves and (**b**) roots of *P. glomerata* plants treated with 5-azacytidine (5-azaC) and under salt stress (NaCl) after 40 days of in vitro culture. Same letters do not differ at 5% level by Tukey's test; values represent means (n = 4) ± (SE).

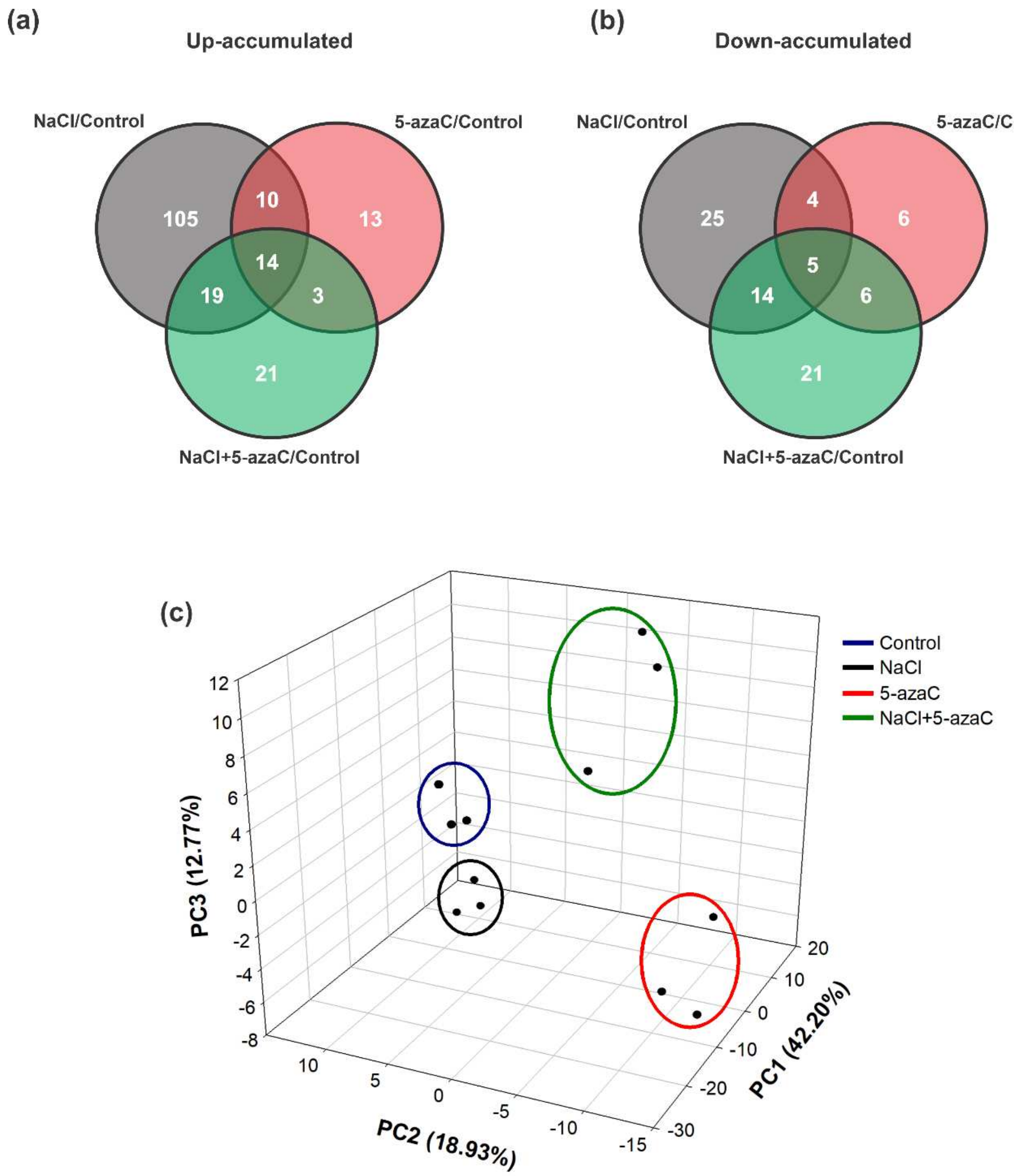

Fig. 5. Differentially accumulated proteins (DAP) in *P. glometara* plants treated with 5-azacytidine (5-azaC) and under salt stress (NaCl). Venn diagrams showing common DAP proteins up- **(a)** and down-accumulated **(b)** in plants of *P. glomerata* in the comparisons NaCl/Control, 5-azaC/Control and 5-azaC+NaCl/Control. **(c)** PCA results show the similarity of the treatments in which the plants were submitted, based on the differential abundance of proteins identified by the proteomic analysis.

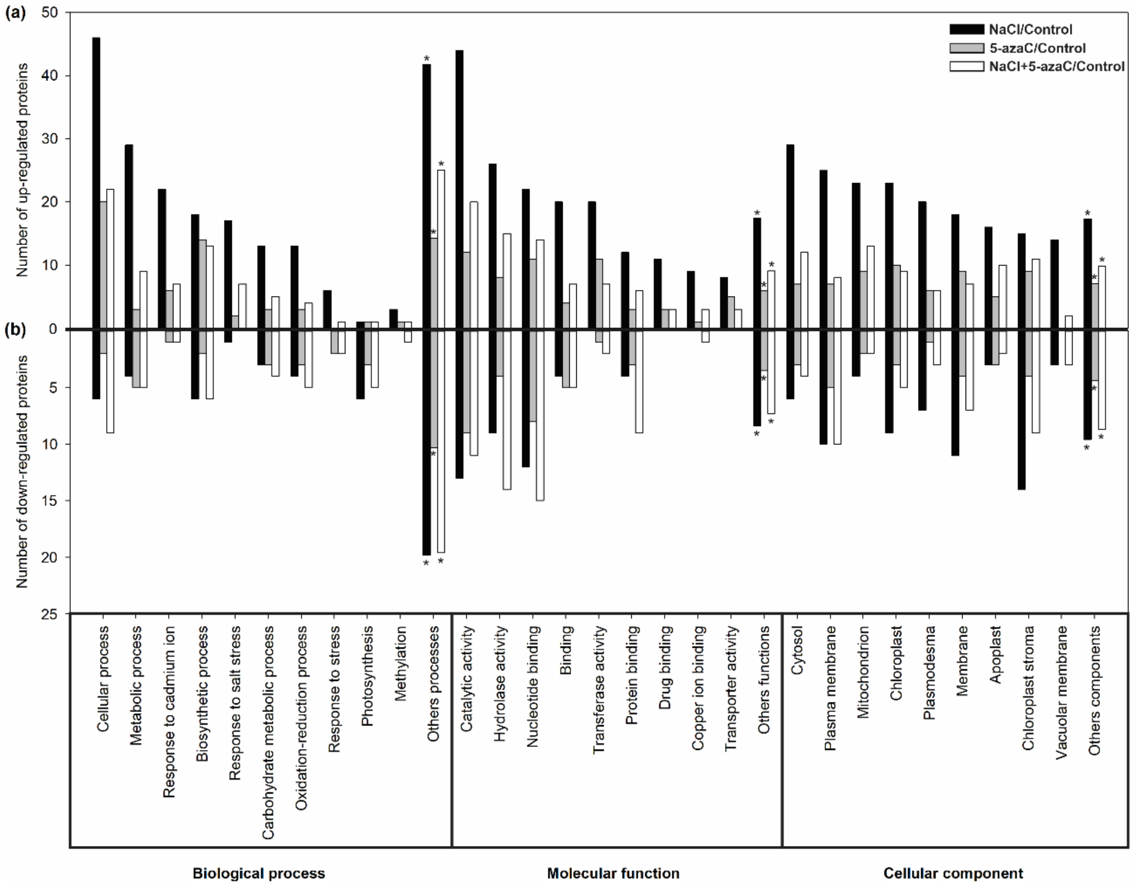

Fig. 6. Functional classification based on the gene ontology (GO) of **(a)** up- and **(b)** down-accumulated proteins of *P. glomerata* plants under salt stress (NaCl) and treated with 5-azacytidine (5-azaC). Processes, functions, and components identified as Others (highlighted with *) correspond to all the other GO terms added together, and the total number of proteins in these terms was divided by 10 for graphical representation.

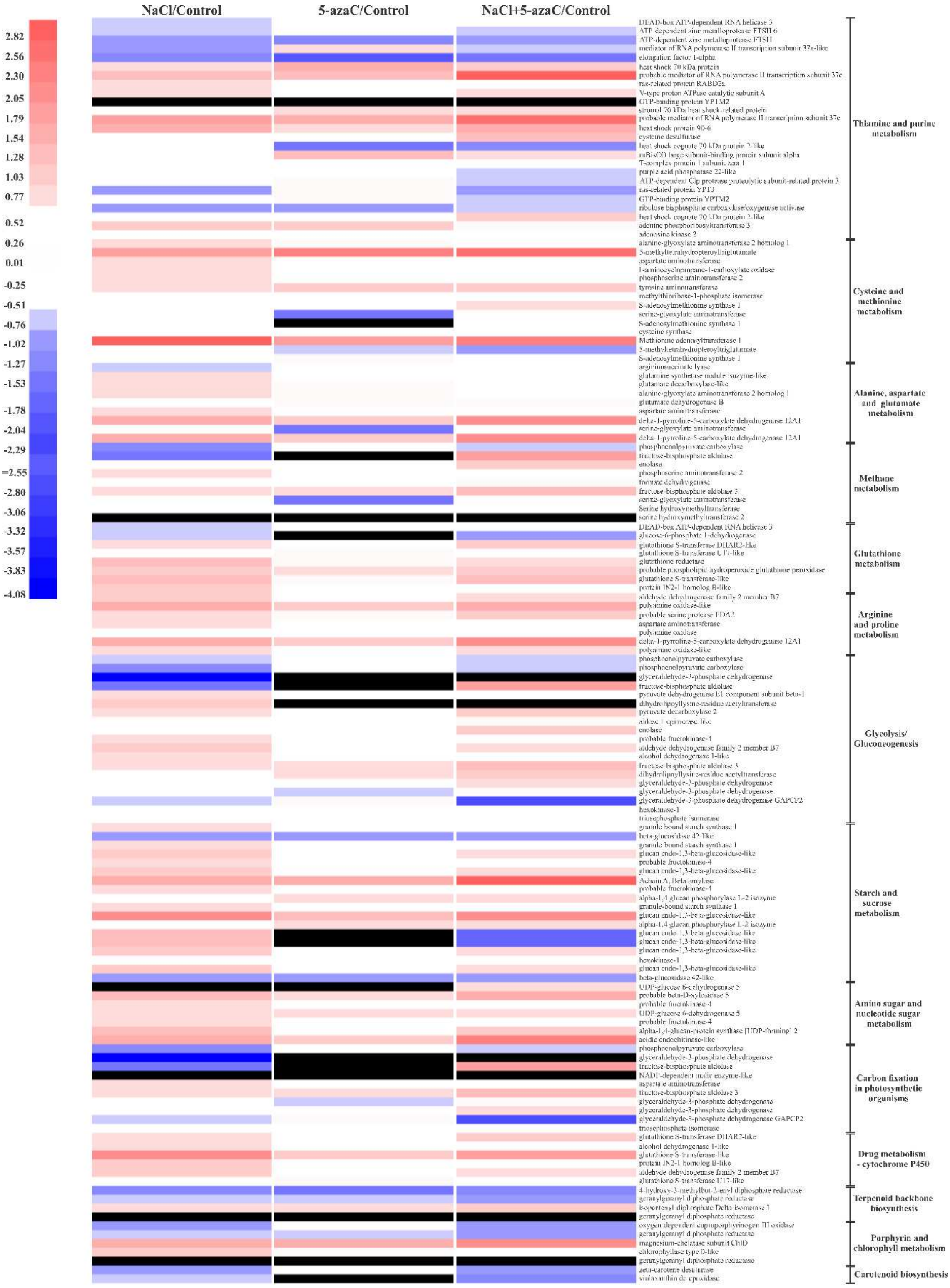

Fig. 7. Heatmap of DAPs in *P. glomerata* plants under salt stress (NaCl) and treated with 5-azacytidine (5-azaC), after 40 days of in vitro culture. Expression profile of DAPs involved in 14 of the main pathways revealed by KEGG analyzes (Kyoto Encyclopedia of Genes and Genomes). For the construction of the heatmap, the Log2 Fold change (FC) criterion was used to calculate the differential protein abundance, being considered up-accumulated when Log2

FC > 0.6 and down-accumulated when Log2 FC < -0.6. Red, up- accumulated. Blue, downregulated. White, unchanged. Black, missing.

Supplementary material

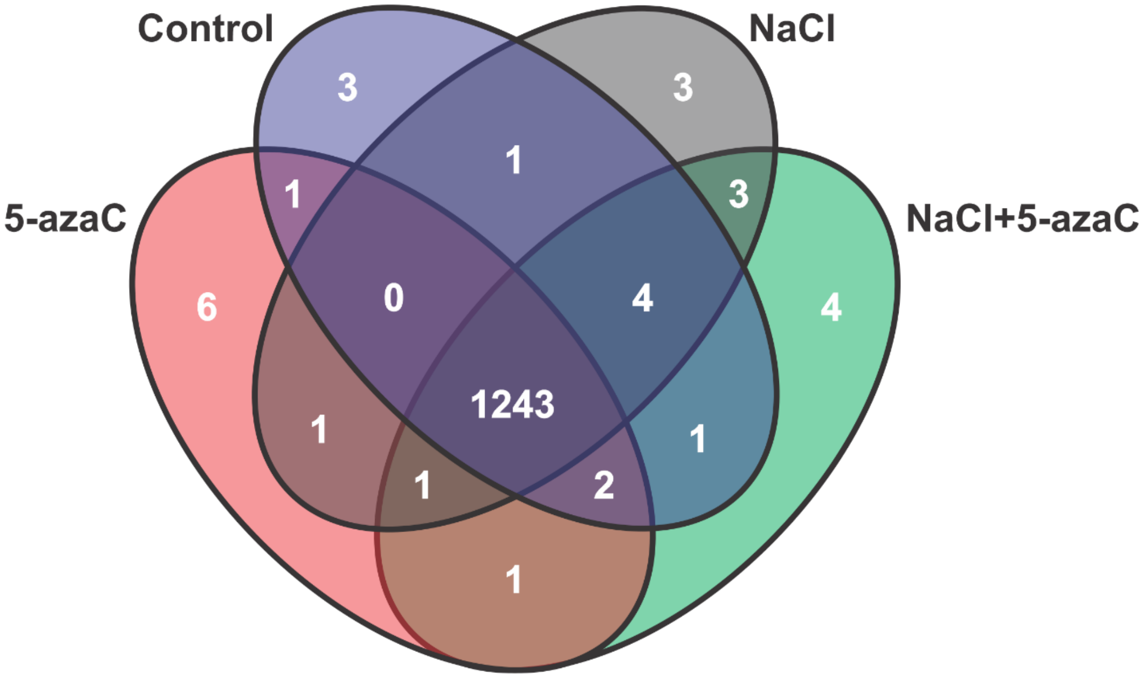

Figure S1. Venn diagram illustrating the numbers of proteins identified in plants of *P. glomerata* under salt stress (NaCl) and treated with 5-azacytidine (5-azaC). The diagram shows common and unique proteins at each treatment.

Table S1. Complete list of identified proteins, functional protein annotations, and configuration parameters.

https://1drv.ms/x/s!AhsdNhfp1NBbhJ8GWPYqpQVIoxRFCA?e=yAQqkj

Table S2. List of metabolic pathways identified in the KEGG (Kyoto Encyclopedia of Genes and Genomes), created from the analysis of differential accumulated proteins.

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CHAPTER 3

Elicitation with methyl jasmonate and methyl salicylate leads to changes in morphophysiology and biosynthesis of 20-hydroxyecdysone of *Pfaffia glomerata* [(Spreng.) Pedersen]

Abstract

Pfaffia glomerata is an important medicinal species and a major producer of the secondary metabolite 20-hydroxyecdysone (20-E), a compound of biotechnological, agrochemical, and pharmacological interest. Here, we aimed to evaluate the role of ploidy level and elicitation in the regulation of biosynthesis and expression of genes from the 20-E pathway, as well as the morphophysiological responses exhibited by *P. glomerata*. For this, we cultivated in vitro for 40 days plants of two different ploidies (A22, diploid; P28, an A22-derived tetraploid), which were exposed in the first 20 days to elicitation with methyl jasmonate (methyl-JA) and methyl salicylate (methyl-SA). Both elicitors reduced photosynthesis and photosynthetic pigment content, which reduced growth and changed the profile of primary metabolites. Elicitation also promoted evident modifications in the anatomical structure of the leaves. We found that elicitors potentiated the oxidative burst, with a consequent increase in the activity of antioxidant enzymes, and these changes were more significant in diploid plants. Besides, tetraploid plants exhibited considerable increment in 20-E content when compared to diploids. 20-E content was also affected by the elicitors: methyl-JA induced a significant increment of 20-E in leaves; on the other hand, methyl-SA downregulated *Phantom* expression, with a consequent reduction in the 20-E production. In conclusion, our data suggest that elicitation with methyl-JA and polyploidy induction may be efficient strategies for increasing 20-E production in *P. glomerata* plants.

Keywords: Brazilian ginseng, jasmonate, medicinal plant, phytoecdysteroids, polyploidy, salicylate

Introduction

As sessile organisms, plants are continuously exposed to harmful conditions, which directly impact their development, resulting in the phenomenon of "growth-defense trade-off" (Huot et al. 2014). Plants can recognize stress signals through internal and external receptors and activate complex defense mechanisms (Rojas et al. 2014; Gust et al. 2017), among which is the biosynthesis of secondary metabolites. The signaling process involves the perception of environmental stimuli and their transmission from cell to cell or between organs, through signaling molecules such as hormones and peptides (Takahashi and Shinozaki 2019).

Jasmonate and salicylate are two key hormones in triggering defense responses in plants (Huot et al. 2014). The signaling mediated by jasmonate and its derivatives (e.g. methyl jasmonate, jasmonate iso-leucine conjugate) is involved in the tolerance to abiotic stresses (Wang et al. 2020; Raza et al. 2020), as well as in the activation of immunity induced by insects attack and necrotrophic pathogens (Wasternack and Song 2017). Salicylates, such as salicylic acid and methyl salicylate, are central players in the activation of systemic acquired resistance, and mediate tolerance responses to biotic (mainly biotrophic pathogens) and abiotic stresses (Klessig et al. 2018; Ye et al. 2020; Santisree et al. 2020). The hormonal signaling network is complex, and it has been shown that the mechanisms related to stress defense are not signaled only by a specific pathway, but rather by the combined action and interactions among several hormones (Klessig et al. 2018). Usually, it is attributed to salicylate an antagonistic role regarding jasmonate-induced signaling, but both can act synergistically. The mechanisms involved in these interplay are still poorly recognized (Klessig et al. 2018; Li et al. 2019). The elicitation with hormonal elicitors can contribute to understand the complex mechanisms of hormone-induced stress responses and how they affect the physiological and biochemical characteristics of plants (Arif et al. 2020), and to develop relevant biotechnological tools for the increment of secondary metabolites (Nabi et al. 2021).

Furthermore, the plasticity of plants under stress can be related to aspects of their genome, such as ploidy level (Moura et al. 2021). Polyploidy events are widely dispersed among plants, consisting of whole-genome duplication (WGD) processes, resulting in organisms with multiple sets of chromosomes (Van De Peer et al. 2017). Polyploids can exhibit greater genetic diversity due to the complexity of their genome, as well as modify molecular, biochemical, and epigenetic mechanisms, resulting in a potential increase in tolerance to adverse conditions (Fox et al. 2020; Scarrow et al. 2020). The metabolic and morpho-physiological adjustment exhibited by polyploids can generate traits of resistance and enhanced fitness in plants under stresses such as salt (Zhu et al. 2018), drought (Rao et al. 2020), and herbivory (Scholes and Paige 2014; Shimizu-Inatsugi et al. 2020). WGD can also affect biosynthetic pathways related to plant secondary metabolism, and as a consequence modifying quantity, chemical structure, and the profile of the metabolites produced (Gaynor et al. 2020; Kumar 2021). Despite the ecological and economic importance of secondary metabolites, little is known about how they are affected by polyploidy events. Thus, more studies are needed to unravel this regulation (Gaynor et al. 2020).

Secondary metabolites play an important role in the regulation of plant growth and defense mechanisms against biotic and abiotic stresses (Erb and Kliebenstein 2020). According to the metabolic pathway of origin and the chemical structure, they can be classified into phenolic compounds, alkaloids, and terpenes (Yang et al. 2018). Among the terpenes, an important group of metabolites are the ecdysteroids. They can be found in a wide diversity of organisms, performing essential physiological functions for development (Dinan et al. 2009; Hunyadi et al. 2016), such as the regulation of oogenesis and in the signaling of ecdysis stages in arthropods (Tsukagoshi et al. 2016; Swevers 2019). In plants, the main ecdysteroid synthesized is the 20-hydroxyecdysone (20-E), which acts mainly in defense against attack by herbivorous insects and parasitic nematodes (Schmelz et al. 1999; Marion-Poll and Descoins

2002; Soriano et al. 2004), however, the physiological and molecular mechanisms controlling 20-E biosynthesis in plants are little known compared to other organisms, such as insects (Tsukagoshi et al. 2016).

The enzymes of the P450 family are central players in the regulation of 20-E biosynthesis (Festucci-Buselli et al. 2008a), and two genes related to this enzyme family, *Phantom* and *Spook*, have been described in the medicinal plant *Pfaffia glomerata* (Batista et al. 2019a). Moreover, characteristics of the culture environment and stress conditions can induce alteration in the expression of these genes, which may explain the differences in 20-E biosynthesis exhibited by *P. glomerata* (Felipe et al. 2019a, b; Batista et al. 2019a). Thus, *P. glomerata* can be used as a model for the study of the function and the biosynthesis of 20-E in plants, since this species stands out as one of the main 20-E producing plant (Festucci-Buselli et al. 2008b), is widely used in folk medicine, and the effectiveness of many of its pharmacological and phytotherapeutic properties have been confirmed (Tóth et al. 2008; Dias et al. 2019; Franco et al. 2021).

A previous study described an efficient protocol for polyploid induction in *P. glomerata* plants (Gomes et al. 2014), and from this, it was possible to find a relationship between ploidy level and 20-E production (Gomes et al. 2014; Corrêa et al. 2016). However, the molecular, physiological, and hormonal mechanisms involved in the biosynthesis of this compound as a function of different ploidy levels are still unclear. Here, we aimed to provide new insights into the role of polyploidy and elicitation with hormones (methyl jasmonate and methyl salicylate) on the control of biosynthesis and expression of 20-E pathway genes, as well as on the morphophysiological responses in *P. glomerata*.

Materials and methods

Plant material

Plants of *Pfaffia glomerata* were obtained from the Germplasm Bank of the Plant Tissue Culture Laboratory (LCT II, BIOAGRO, Federal University of Viçosa, Brazil). Diploid plants from accession 22 (A22) and tetraploids from accession 28 (P28) were used. Voucher material of A22 (code number CESJ 63,317) was deposited at the Leopoldo Krieger Herbarium (UFJF, Juiz de Fora, MG, Brazil); P28 voucher material was deposited at the VIC Herbarium (UFV, Viçosa, MG, Brazil) under the code number VIC 53,563.

A22 and P28 explants with a nodal segment (approximately 2 cm) were cultured in glass flasks (62 mm diameter × 15.5 mm height; AZ 200; Embalagens Rio, Nova Friburgo, Brazil) containing 100 mL of MS medium (Murashige and Skoog 1962), supplemented with MS vitamins, myo-inositol (100 mg L^{-1}), 3% (*w/v*) sucrose, 5.5 g L^{-1} agar (PhytoTechnology Laboratories®, Overland Park, USA), pH adjusted to 5.7 ± 0.1, and autoclaved at 121°C and 108 kPa for 20 min. The plants were kept in a growth room with controlled irradiance of 60 $\mu mol\ m^{-2}\ s^{-1}$ with two white LED lamps (SMD 100, 18W, Vilux®, Vitória, ES, Brazil) and temperature of 25 ± 1°C.

Hormone treatments

To test the influence of hormones, the plants (A22 and P28) were subdivided into three treatments: Control, Methyl-Jasmonate (Methyl-JA, Sigma-Aldrich, St. Louis, MO), and Methyl-Salicylate (Methyl-SA, Sigma-Aldrich, St. Louis, MO). The methyl-JA and methyl-SA solutions were prepared by diluting the hormones in distilled water at a final concentration of 10 mM, with subsequent filter-sterilization.

To conduct the elicitation, microtubes containing 1 mL of water (control) or hormone aqueous solution were introduced into culture flasks closed with rigid lids of polypropylene without membranes, since the first day of the experiment. The elicitation occurred until the 20th day of the experiment, consisting of the evaporation of the hormones to the flask headspace (Figure 1). From the 20th day on, the elicitation treatments were interrupted, and the plants were cultivated for more 20 days in flasks closed with 10-mm two-hole rigid polypropylene lids covered by 0.45-μm-diameter pore hydrophobic fluoropore polytetrafluoroethylene membranes (MilliSeal® AVS-045 Air Vent, Millipore, Tokyo, Japan), allowing a CO_2 exchange rate of 25 $\mu L\ L^{-1}\ s^{-1}$ (Batista et al. 2017).

Plant growth parameters

After 40 days of in vitro culture, plants were collected and measured for the shoot and root length (cm). Shoots and roots were dried separately at 50°C until constant weight to obtain the dry weight (g).

Anatomical analysis

For anatomical characterization, the second fully expanded apical leaf of three plants per treatment was sampled. The leaf blades were sectioned in the central region, and the sections were fixed in Karnovsky's solution (Karnovsky 1965). Subsequently, the samples were dehydrated in ethanolic solutions (10-95%) and included in acrylic resin (Historesin; Leica Instruments, Wetzlar, Germany). Then, the samples were transversely sectioned on an autotuning rotary microtome (RM2155; Leica Microsystems Inc., Buffalo Grove, IL), and 5 μm thick sections were obtained. For structural characterization, the samples were stained in toluidine blue (pH 3.2) (O'Brien and McCully 1981). Image capture was performed using a light microscope (AX70 TRF; Olympus Optical, Tokyo, Japan) with a U-photo system, coupled to a digital camera (Spot Insightcolour 3.2.0, Diagnostic Instruments Inc., Sterling Heights, MI).

Quantification of photosynthetic pigments and photosynthetic rate

For the quantification of chlorophyll and carotenoids content, a methanolic extraction of freeze-dried and macerated leaf samples (10 mg) was performed. Aliquots of 200 µL of the samples were taken from the extraction supernatant and added to a microplate. The absorbances were measured in a spectrophotometer at wavelengths of 470, 653, and 666 nm. Chlorophyll and carotenoid contents were determined according to Wellburn (1994) and were expressed by µg mL^{-1}.

The quantification of the photosynthetic performance was conducted as previously described by Silva et al. (2020). Measurement of CO_2 gas exchange was carried out with an IRGA (model S151; Qubit Systems, ON, Canada), and data collection was performed with LoggerLite software (LoggerLite, 1.8.1, Vernier Software & Technology Caliper, Beaverton). CO_2 gas exchange was calculated according to the leaf dry weight per plant (g), and the photosynthesis was expressed in µmol CO_2 s^{-1} $plant^{-1}$.

Quantification of soluble sugars, starch, proteins, and amino acids

Freeze-dried and macerated leaf samples (~ 20 mg) were extracted with methanol as described by Lisec et al. (2006). After the extraction, aliquots of sample were taken from the supernatant, which were used in the reactions for the quantification of soluble sugars (glucose, fructose and sucrose) (Fernie et al. 2001) and amino acids (Cross et al. 2006). From the insoluble pellet the contents of protein (Cross et al. 2006), and starch (Fernie et al. 2001) were determined.

Enzyme assay

Leaf samples (approximately 50 mg fresh weight (FW)) were macerated and extracted in 1 mL of the extraction solution (0.1 M potassium phosphate buffer pH 6.8; 0.1 mM ethylenediaminetetraacetic acid; 1 mM phenylmethylsulfonyl fluoride and 1% *w*/*v* polyvinylpolypyrrolidone). Then, the extract was centrifuged at 12,000 rpm (15 min, 4 °C), and the supernatant was kept on ice for protein quantification (Bradford 1976) and to perform the enzyme assay. Subsequently, the activities of catalase (CAT, EC 1.11.1.6) (Havir and McHale 1987), ascorbate peroxidase (APX, EC 1.11.1.11) (Nakano and Asada 1981), peroxidase oxidoreductase (POD, EC1.11.1.7) (Chance and Maehly 1955), and superoxide dismutase (SOD, EC 1.15.1.1) (Giannopolitis and Ries 1977) were quantified. The activity of CAT, APX, and POD were expressed by μmol^{-1} min^{-1} g^{-1} protein. SOD was expressed by U min^{-1} g^{-1} protein, with 1 U being equivalent to the concentration of SOD required to inhibit 50% of nitro blue tetrazolium photoreduction.

Determination of lipid peroxidation

Lipid peroxidation was determined as a function of malondialdehyde (MDA) production, according to the methodology described by Heath and Packer (1968). Leaf samples (approximately 100 mg FW) were macerated and then extracted in trichloroacetic acid (TCA; 1% *w*/*v*). Then 250 µL of the supernatant was added to 750 µL of the solution of 2-thiobarbituric acid (TBA; 0.5% w/v) in TCA (20% w/v), and the reaction was kept under agitation for 30 min at 95 °C. Subsequently, the samples were added to a microplate, and absorbance readings were carried out in a spectrophotometer at wavelengths of 532 and 600 nm. The MDA content was expressed by nmol g^{-1} FW.

Quantification of hydrogen peroxide

H_2O_2 content was determined as proposed by Velikova et al. (2000). Briefly, macerated leaf samples (approximately 50 mg FW) were extracted 500 µL of TCA (0.1 % *w*/*v*). The reaction was performed by adding 20 µL supernatant, 80 µL potassium phosphate buffer (10 mM, pH 7.0), and 100 µL potassium iodide (1 M) to a microplate and keeping it in the dark for 45 min. The absorbance was read in a spectrophotometer at a wavelength of 390 nm. The calibration curve was prepared, and the H_2O_2 content was expressed by µmol g^{-1} FW.

Determination of 20-E

For the quantification of 20-E, the macerated and freeze-dried samples (leaves and roots) were extracted in MeOH, as described by Corrêa et al. (2015). For the determination of the 20-E contents, a Shimadzu Prominence liquid chromatography system (Kyoto, Japan) equipped with RP column (150 mm x 4,6 mm i.d., 5 µm particle size; C18 stationary phase) from Phenomenex (Torrance, CA, USA) and a Shimadzu SPD –M20A photodiode array detector (monitoring 246 nm). The mobile phase was composed of methanol:water solution (1:1 *v*/*v*). It was injected 20 µL of the extracts, with a flow rate of 1.0 mL per min. The readings were taken after 15 min of injection, at a wavelength of 245 nm. The calibration curve was prepared from a 20-E standard (Sigma-Aldrich, St. Louis, MO), and the 20-E content was expressed by mg g^{-1} DW.

RNA extraction, cDNA synthesis, and quantitative PCR analysis

Approximately 100 mg of fresh leaf samples were macerated and then extracted in TRI Reagent® (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol. Then, RNA was treated with DNAse to eliminate the contamination with genomic DNA. From the purified RNA, first-strand cDNA was synthesized using the Super Script™ III Kit (Invitrogen®, Carlsbad, CA, USA), diluted to a concentration of 10 ng L^{-1}. The genes of the P450 family, *Phantom* (*CYP76C*) and *Spook* (*CYP83A*), were obtained from a *P. glomerata* transcriptome

(Batista et al. 2019a), and the gene *glyceraldehyde-3-phosphate dehydrogenase* was used as internal reference (Batista et al. 2019b). The gene expression was evaluated by quantitative real-time PCR using the Step One Plus™ system (Applied Biosystems®, Foster City, CA, USA). Transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), with three biological replicates and with two technical replicates each.

Statistical analysis

The experimental design was completely randomized, in a 2 × 3 factorial scheme (two ploidy levels: A22 (diploid) and P28 (tetraploid) × three elicitation treatments: control, methyl-JA, and methyl-SA), with ten replications, being the experimental unit consisted by three plants per flask. Experiments were repeated twice. The statistical analyses of all experiments were performed using the software Genes version Windows/2004.2.1 (Cruz 2016). Data were submitted to analysis of variance and the means were compared by Student's t-test (gene expression) or Tukey's test (other analyses) ($p \leq 0.05$).

Results

Methyl-JA and Methyl-SA inhibited growth and promoted anatomical changes

Plants treated with methyl-JA and methyl-SA exhibited a reduced development and visible physiological changes, such as lower branching, yellowing, and necrotic lesions on the leaves (Fig. 2a). Compared to the control, there was a significant reduction in all growth parameters evaluated (Fig. 2b-e). However, the length and biomass of the shoot and root did not differ significantly between the plants treated with methyl-JA and methyl-SA. The ploidy level did not influence the development of *P. glomerata*, except for root biomass, where a significant reduction was observed in tetraploid plants treated with methyl JA (43%) and methyl-SA (24%). The interaction between ploidy level and elicitors was significant for the shoot and root length.

The anatomical analysis revealed that the leaves of *P. glomerata* presented the same histological organization, independently of the ploidy level of the plants (Fig. 3). It consists of a large central vascular bundle with parenchyma distributed along the midrib, a layer of uniseriate epidermis, and subepidermal collenchyma on both surfaces. However, methyl-JA and mainly methyl-SA treatments affected the histological differentiation of leaves. On the one hand, methyl-JA treated plants exhibited slightly thicker leaves with prominent midrib. On the other hand, plants treated with methyl-SA showed thinner leaves, with a less thick and disorganized vascular cylinder, more juxtaposed parenchymal tissue, and with smaller cells.

Elicitation with methyl-JA and methyl-SA, as well as ploidy levels, affected pigment content and photosynthetic performance

Chlorophyll content was significantly reduced in diploid and tetraploid plants treated with methyl-JA (respectively 48% and 24%) and methyl-SA (respectively 49% and 14%) when compared to the control (Fig. 4a). Regarding ploidy levels, tetraploid plants showed increased chlorophyll content in control (23%) and methyl-SA treated (43%). Also, a significant reduction in carotenoid content was observed in methyl-JA treated plants, regardless of ploidy level (Fig. 4b).

The photosynthetic rate was significantly reduced in diploid and tetraploid plants treated with methyl-JA (40% and 53%, respectively) and methyl-SA (60% and 37%, respectively) (Fig. 4c). In methyl-SA treatments, tetraploid plants showed a photosynthetic rate 70% higher than diploids.

Elicitation with methyl-JA and methyl-SA affects primary metabolite content

The highest contents of glucose, fructose, and sucrose were found in methyl-SA treated plants, however with no significant difference to control and methyl-JA treated plants (Fig. 5a-c). In addition, tetraploid plants treated with methyl-SA showed a 25% increase in glucose content and a 28% increase in sucrose content when compared to diploid plants.

It was observed a significant interaction between the ploidy level and the treatments with phytohormones for the concentrations of starch, amino acids, and proteins (Fig. 5d-f). Control diploid plants exhibited the lowest starch contents when compared to tetraploids, as well as with the other elicitation treatments. Methyl-JA-treated plants showed a significant reduction in amino acid concentration, regardless of ploidy level. Regarding protein content, a significant increase was observed in tetraploid plants treated with methyl-JA (53%) and methyl-SA (51%), when compared to diploids. Besides, tetraploid plants treated with methyl-JA and methyl-SA showed a 40% increase in protein content compared to the control treatment.

Ploidy level and treatments with methyl-JA and methyl-SA affected the antioxidant enzyme activity

The antioxidant enzyme activity was strongly influenced by elicitation and the ploidy level of the plants (Fig. 6a-d), and there was a significant interaction between these factors for CAT and SOD. The highest CAT activities were found in tetraploid plants treated with methyl-JA and diploid plants treated with methyl-SA. Elicitation with methyl-JA also stimulated SOD activity, as observed by the 55% increase in diploid and 32% increase in tetraploid plants, when compared to the control. The ploidy level was a determining factor for APX activity, with diploid plants showing higher enzyme activity compared to tetraploids in the control (38%) and methyl-SA (46%) treatments. Similarly, diploid plants showed a significant increase in POD activity when treated with methyl-JA (150%) and methyl-SA (36%). Furthermore, plants treated with methyl-SA showed the highest POD activity when compared to the diploid (180%) and tetraploid (176%) control plants.

H_2O_2 contents and MDA formation were affected by elicitation and ploidy level of the plants, with no significant interaction between these factors (Fig. 6e-f). Compared to the control, plants treated with methyl-JA and methyl-SA showed a significant increase in H_2O_2 and MDA production, for both ploidy levels. In addition, diploid plants showed higher H_2O_2 content than tetraploid plants in the control (187%), and in elicitation with methyl-JA (34%) and with methyl-SA (49%).

Elicitation and ploidy level affect 20-E biosynthesis

Elicitation affected the expression of genes related to 20-E biosynthesis. Methyl-SA down-regulated *Phantom* expression, whereas there were no significant differences in plants treated with methyl-JA (Fig. 7a). Regarding *Spook*, there was a down-regulation in tetraploid plants treated with methyl-JA, with no difference in expression for the other treatments (Fig. 7b).

The 20-E content in methyl-SA treated plants followed the same response pattern as the expression of *Phantom*. Elicitation with methyl-SA induced a drastic reduction in the 20-E content of leaves (Fig. 7c) and roots (Fig. 7d) in both diploid (45% and 68%, respectively) and tetraploid (39% and 62%, respectively) plants. On the other hand, a significant increase in 20-E production occurred in leaves of diploid (52%) and tetraploid (31%) plants treated with methyl-JA compared to the control. In roots, there was a significant (62%) reduction in 20-E content in diploid plants treated with methyl-JA, with no difference in tetraploid plants. Besides, tetraploid plants exhibited higher 20-E content than diploid plants, both in leaves and roots, and regardless of the treatment.

Discussion

This is the first work to investigate the role of methyl-JA and methyl-SA elicitation on the production of 20-E in *P. glomerata* plants with different ploidy levels. Our findings indicate that 20-E biosynthesis is tightly regulated by jasmonate and salicylate, which act antagonistically. The biosynthesis of 20-E is also regulated by the level of ploidy, with a significant increase in tetraploid plants when compared to its parental diploid.

Plants often exhibit morphological changes when exposed to stressful conditions, and changes in the anatomy of leaf tissues may demonstrate traits of sensitivity or mechanisms of stress tolerance (Paula et al. 2019; Silva et al. 2020). These modifications can be mediated by hormones, and here we observed that salicylate and jasmonate contrastingly affect the anatomical structure and histological differentiation of leaves of *P. glomerata*. Elicitation with methyl-SA led to reduced leaf thickening, disarray in the midrib and parenchyma, as well as a reduction in cell size. The salicylate-induced changes in leaf anatomy may be related to mechanisms of maintaining water status by reducing transpiration, as well as may result in reduced photosynthesis due to the disarrangement of photosynthetic tissues (Uzunova and Popova 2000). Furthermore, hormone-induced metabolic changes may be involved in regulating cell wall architecture and composition (Napoleão et al. 2017), which may explain the changes observed in *P. glomerata*.

P. glomerata treated with methyl-JA and methyl-SA exhibited reduced chlorophyll contents and photosynthetic performance, as well as changes in carbohydrate, amino acid, and protein contents. As a result, there was a significant reduction in plant growth and biomass accumulation. Jasmonate and salicylate mediate the signaling for a complex regulatory mechanism of chlorophyll degradation, and the accumulation of these hormones can trigger senescence processes (Zhu et al. 2017; Ullah et al. 2019). The lower content of photosynthetic pigments may have contributed to the reduction in photosynthetic rates, due to the reduced capacity to capture and absorb photon energy (Jung 2004). Moreover, a mechanism often employed by stressed plants is the increase of some hormones (eg. jasmonate, salicylate) and reduction of the expression of genes involved in the process of photosynthesis, to increase stress tolerance (Bilgin et al. 2010; Nabity et al. 2013; Coppola et al. 2013; Foucher et al. 2020). As a result, a reallocation of energy and resources from processes involved with photosynthesis and primary metabolism to the biosynthesis of plant defense-related compounds may occur (Bilgin et al. 2010; Guo et al. 2018).

Photochemical limitations and dysfunctions in the photosynthetic apparatus can induce the formation of reactive oxygen species (ROS) in plants under stress (Felipe et al. 2019b). Here, plants treated with methyl-JA and methyl-SA had a significant increase in lipid peroxidation (MDA formation) and H_2O_2 content, indicating that elicitation with the phytohormones potentiated the oxidative burst. As a consequence, there was also an increase in the activity of antioxidant enzymes, to mitigate the toxic effects of ROS. The interaction between ROS production and some hormones, such as jasmonate and salicylate, enables the triggering of a complex signaling cascade, leading to molecular, biochemical, and physiological adjustment as stress tolerance responses (Devireddy et al. 2020; Poór 2020). The ROS-hormone interaction may also be crucial in the regulation of the biosynthesis pathway of several secondary metabolites in plants (Lv et al. 2019; Ho et al. 2020).

Polyploidization events occur spontaneously in nature, and confer to plants potential evolutionary advantages to face of biotic and abiotic stresses (Van de Peer et al. 2020). These events can also be induced artificially, as a tool for plant breeding, improving cultivars, and increasing tolerance to stresses (Renny-Byfield and Wendel 2014). Here, we observed that tetraploid plants of *P. glomerata* reduced H_2O_2 content when compared to diploid plants. The ploidy level can induce molecular and physiological changes in order to achieve oxidative homeostasis under stress conditions (Del Pozo and Ramirez-Parra 2015). The lower ROS production in polyploid plants may also be linked to the higher content of chlorophylls, indicating that the antenna complexes of the photosystems were more preserved, then reducing photoinhibition (López-Jurado et al. 2020).

Moreover, artificial polyploidization can be used as a biotechnological tool of high potential in the induction of secondary metabolism of interest in plants (Gantait and Mukherjee 2021). Here, we found that tetraploid plants of *P. glomerata* increased significantly the contents of 20-E, like was related before by Corrêa et al. (2015). However, the increase in 20-E production was not related to the expression of *Spook* and *Phantom* genes. The mechanisms involved in the regulation of secondary metabolism are not still clear (Kumar 2021), but may be related to genome reorganization and epigenetic changes promoted by polyploidization (Del Pozo and Ramirez-Parra 2015).

Additionally, our results indicate that 20-E biosynthesis is contrastingly regulated by elicitation with methyl-JA and methyl-SA. Jasmonate induced a significant increase in 20-E content in leaves of *P. glomerata* plants, as also observed in other species (Chamnipa et al. 2012; Wang et al. 2013; John et al. 2018; Erst et al. 2019; Gorelick et al. 2020). It is well known that jasmonate is a key hormone in signaling for tolerance responses to biotic stresses (herbivorous insects and necrotrophic pathogens) (Wu and Ye 2020), and here we demonstrate that it leads to the enhance of 20-E, a secondary metabolite associated with defense against insect herbivory and nematode parasitism (Schmelz et al. 1999; Marion-Poll and Descoins 2002; Soriano et al. 2004). On the other hand, methyl-SA down-regulated *Phantom* gene expression, with a consequent significant reduction in 20 E contents in leaves and roots of *P. glomerata*. Salicylate is a key hormone in the activation of systemic acquired resistance (SAR) against biotrophic pathogens (Klessig et al. 2018; Ye et al. 2020), and the responses mediated by this hormone may suppress jasmonate-activated signaling pathways (Li et al. 2019), which could resulted in the decrease of 20E biosynthesis observed here.

In conclusion, our results indicate that *P. glomerata* plants exhibit great morpho-physiological plasticity, both between different ploidies and in response to elicitation by methyl JA and methyl SA. Jasmonate and salicylate induce a complex signaling network that results in an adjustment of photosynthetic performance, primary metabolism, and redox activity, with consequent modulation of secondary metabolism. Finally, this study makes a valuable contribution to the understanding of the role played by the hormones jasmonate and salicylate in the regulation of 20-E biosynthesis. Also, the elicitation with methyl-JA and polyploidy induction may be efficient strategies in inducing 20-E production in *P. glomerata* plants.

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Figures

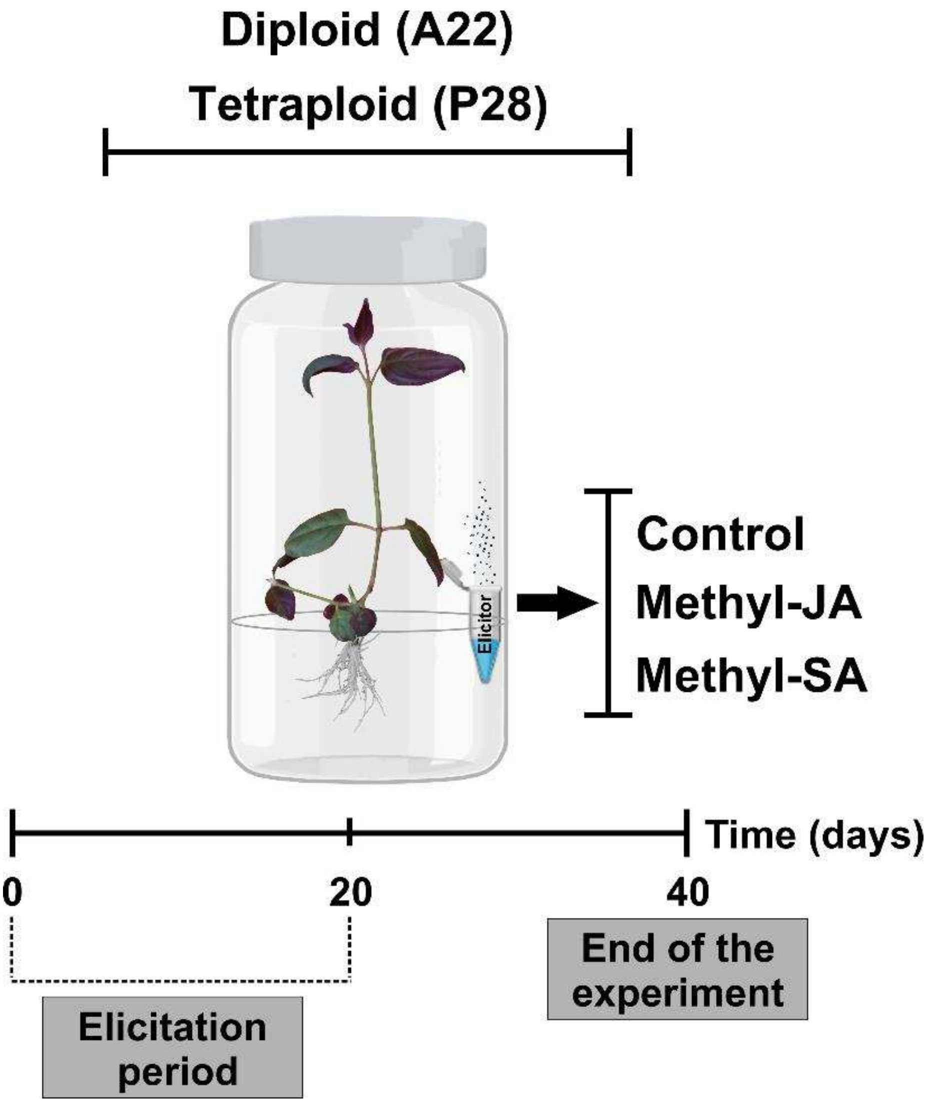

Figure 1. Hormone treatment. Microtubes with 1 mL of water (control), methyl-JA, or methyl-SA (10 mM) were added into the flasks on the 1st day of in vitro culture of diploid (A22) and tetraploid (P28) *Pfaffia glomerata* plants and kept until the 20th day. Then, the plants were cultivated for more 20 days without the hormones.

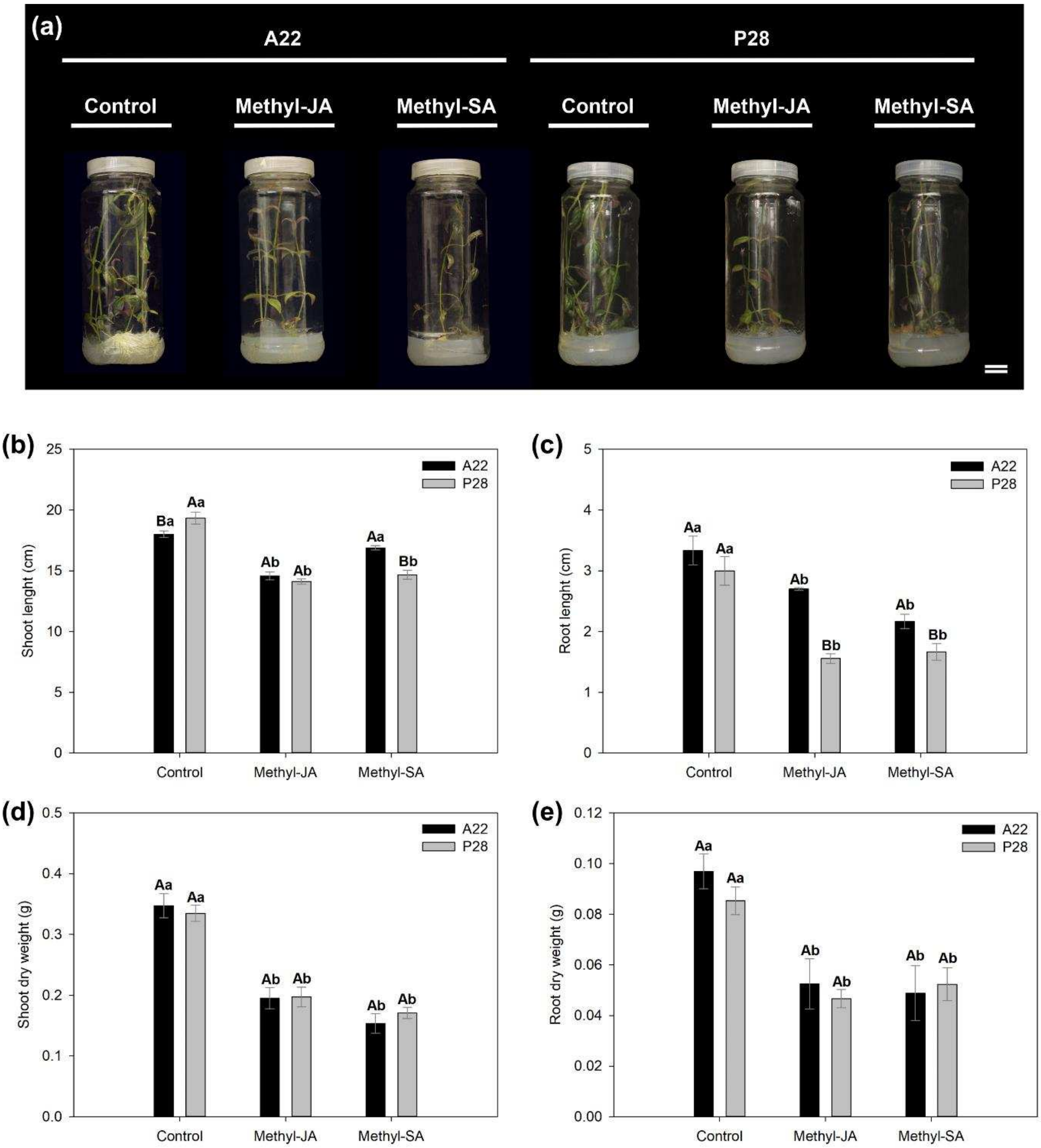

Figure 2. Experimental set-up and growth analysis. **(a)** Diploid (A22) and tetraploid (P28) plants of *Pfaffia glomerata* after 40 days of in vitro culture under chemical elicitation by methyl-jasmonate (Methyl-JA) and methyl-salicylate (Methyl-SA). Bar = 2 cm. **(b–e)** Growth parameters of *P. glomerata*. Uppercase letters compare between different ploidy levels under the same elicitation treatment; lowercase letters compare among different elicitation treatments under the same ploidy level according to Tukey's test ($P \leq 0.05$); values represent means (n = 4) ± standard error.

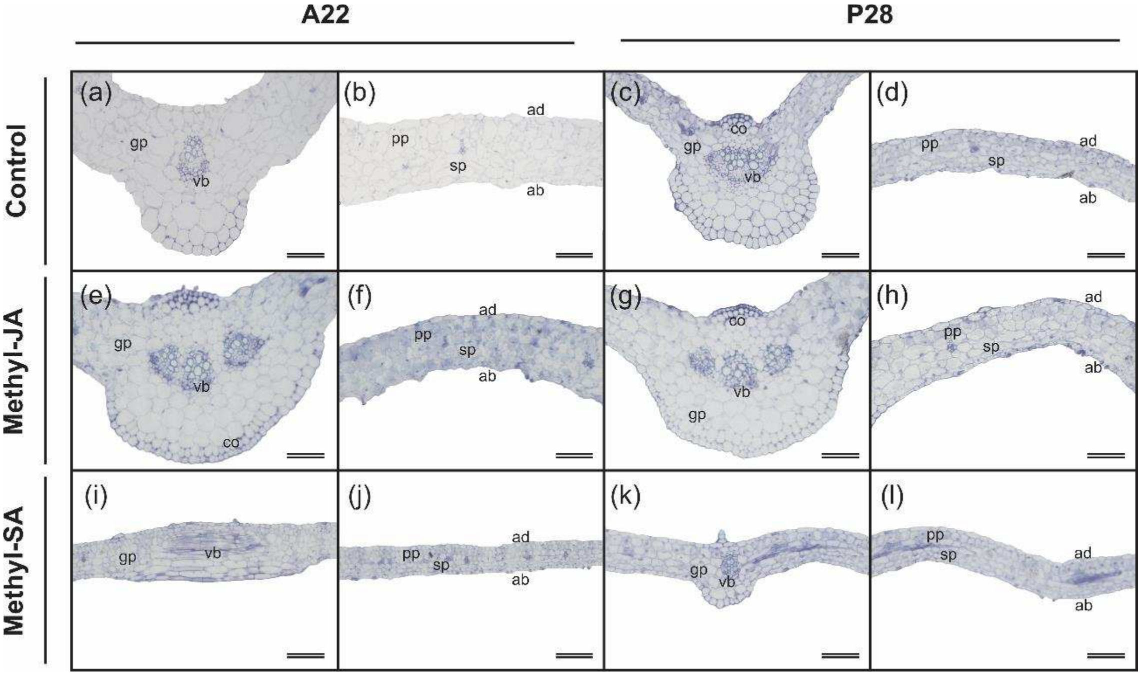

Figure 3. Leaf cross-sections of diploid (A22) and tetraploid (P28) plants of *Pfaffia glomerata* stained with toluidine blue after 40 days of in vitro culture under chemical elicitation by methyl-jasmonate (Methyl-JA) or methyl-salicylate (Methyl-SA). Co, collenchyma; gp, ground parenchyma; vb, vascular bundle; ad, adaxial surface; ab, abaxial surface; pp, palisade parenchyma; sp, palisade parenchyma. Bars = 300 µm.

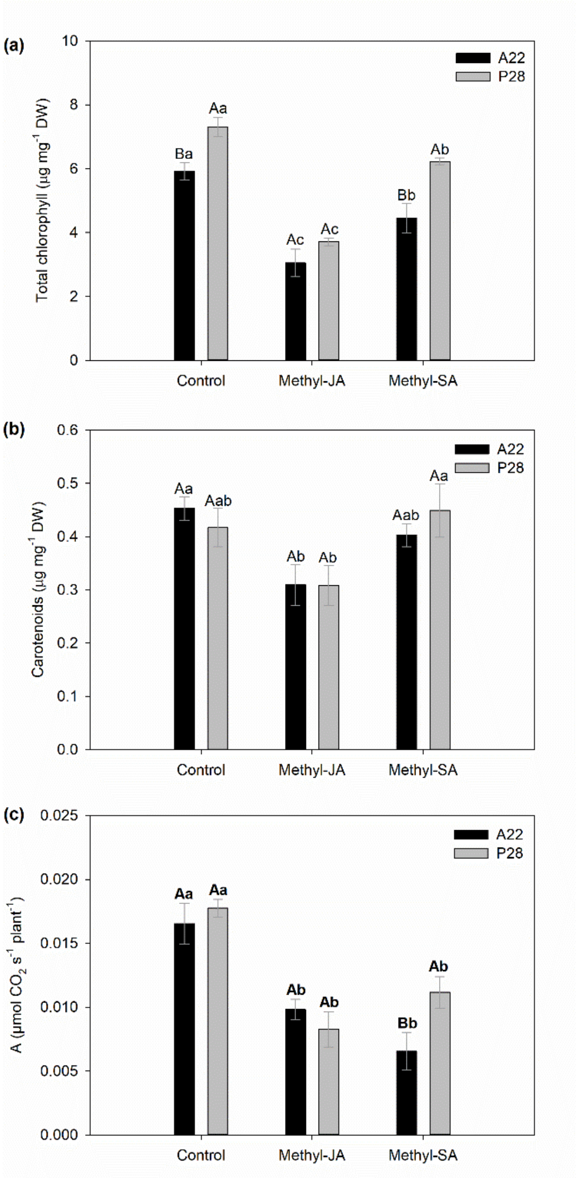

Figure 4. Pigments contents and photosynthetic performance in diploid (A22) and tetraploid (P28) plants of *Pfaffia glomerata* after 40 days of in vitro culture under chemical elicitation by methyl-jasmonate (Methyl-JA) and or methyl-salicylate (Methyl-SA). **(a)** Total chlorophyll; **(b)** Carotenoids; **(c)** Photosynthetic rate. Uppercase letters compare between different ploidy levels under the same elicitation treatment; lowercase letters compare among different elicitation treatments under the same ploidy level, according to Tukey’s test ($P \leq 0.05$); values represent means (n = 4) ± standard error.

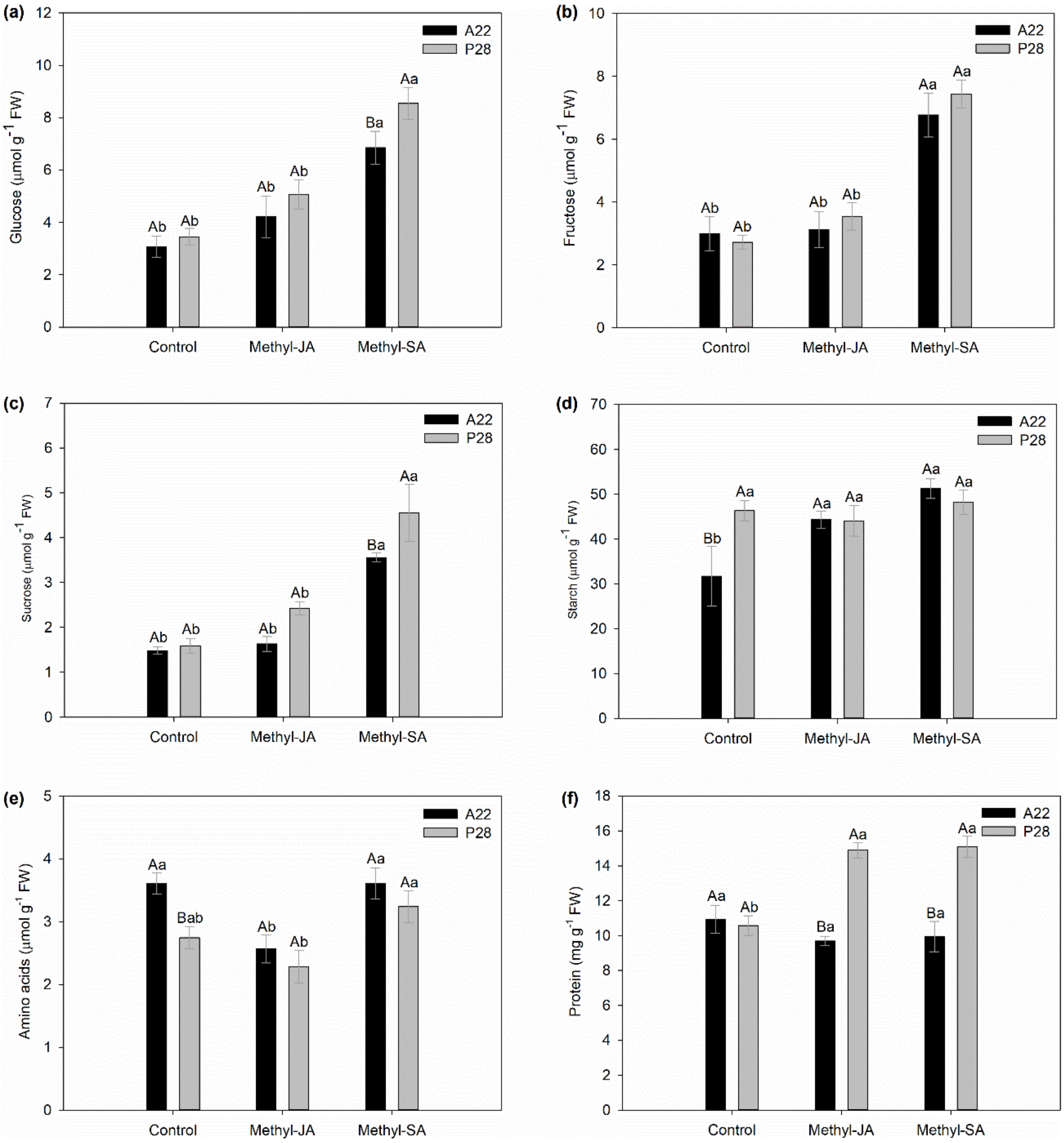

Figure 5. Metabolites contents in diploid (A22) and tetraploid (P28) plants of *Pfaffia glomerata* after 40 days of in vitro culture under chemical elicitation by methyl-jasmonate (Methyl-JA) or methyl-salicylate (Methyl-SA). (**a**) Glucose; (**b**) Fructose; (**c**) Sucrose; (**d**) Starch; (**e**) Amino acids; **(f)** Protein. Uppercase letters compare between different ploidy levels under the same elicitation treatment; lowercase letters compare among different elicitation treatments under the same ploidy level, according to Tukey's test ($P \leq 0.05$); values represent means (n = 4) ± standard error.

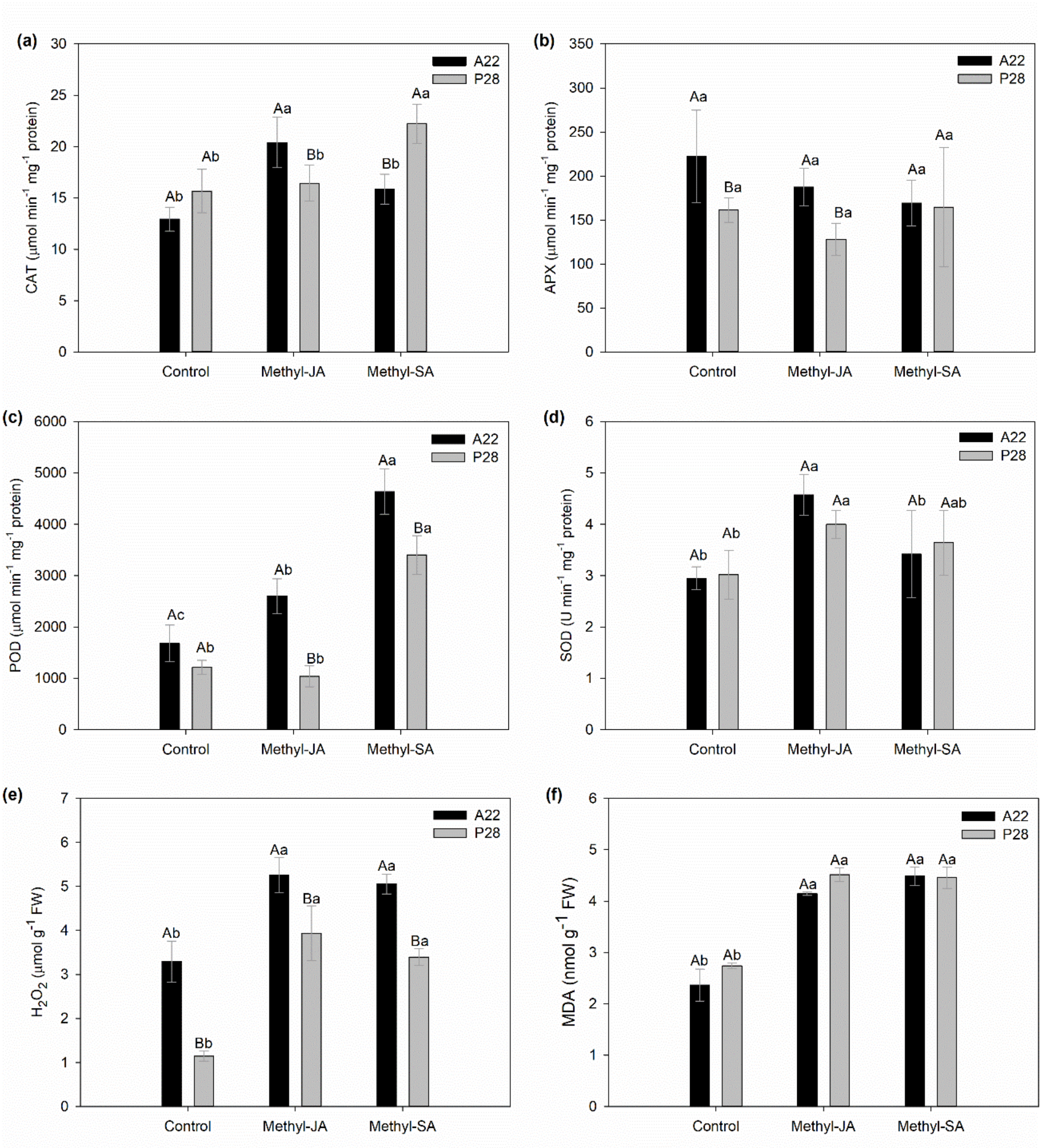

Figure 6. Antioxidant enzymatic activity and peroxidation production in diploid (A22) and tetraploid (P28) plants of *Pfaffia glomerata* after 40 days of in vitro culture under chemical elicitation by methyl-jasmonate (Methyl-JA) and methyl-salicylate (Methyl-SA). (**a**) Peroxidase oxidoreductase (POD); (**b**) Ascorbate peroxidase (APX); (**c**) Superoxide dismutase (SOD); (**d**) Catalase (CAT); **(e)** Free peroxide (H_2O_2) (**f**) Malondialdehyde content (MDA); Uppercase letters compare between different ploidy levels under the same elicitation treatment; lowercase letters compare among different elicitation treatments under the same ploidy level, according to Tukey's test ($P \leq 0.05$); values represent means (n = 4) ± standard error.

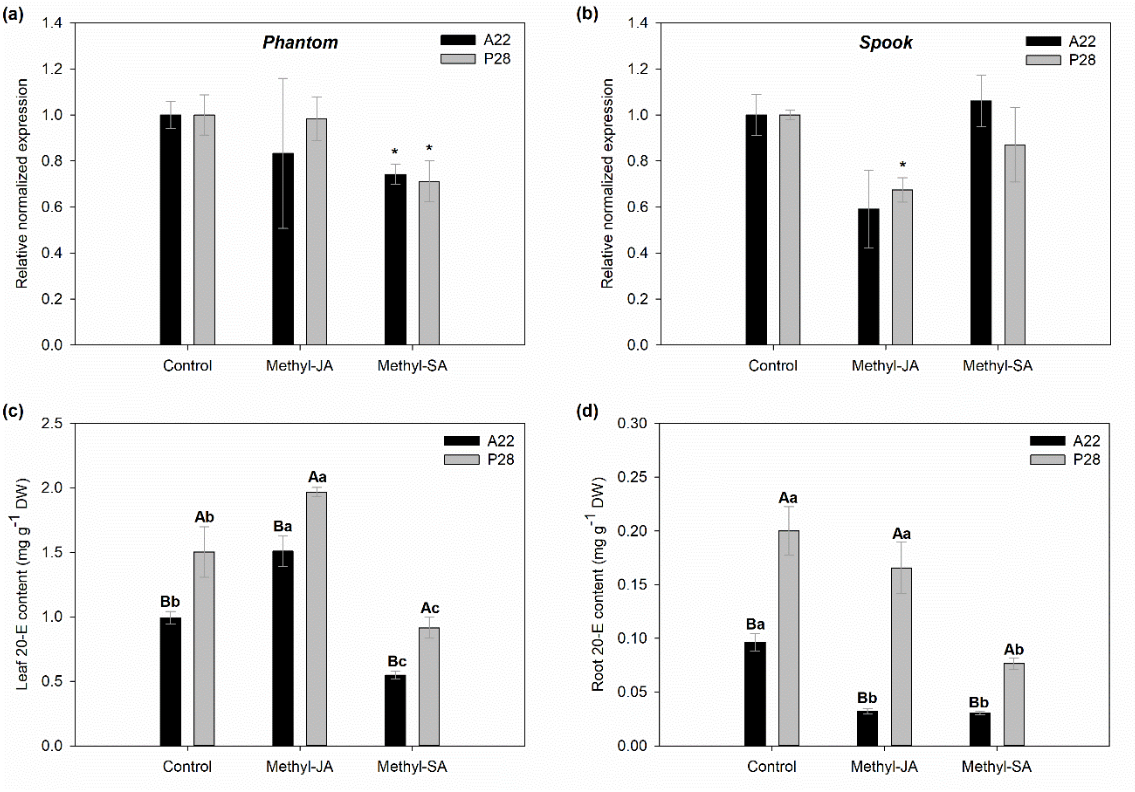

Figure 7. Relative normalized gene expression and 20-E content in diploid (A22) and tetraploid (P28) plants of *Pfaffia glomerata* after 40 days of in vitro culture under chemical elicitation by methyl-jasmonate (Methyl-JA) or methyl-salicylate (Methyl-SA). Relative normalized expression of **(a)** *Phantom* and **(b)** *Spook* genes. Gene expression is relative to the control gene *glyceraldeyde-3-phosphate* dehydrogenase. Values represent means (n = 3) ± SE and are compared by Dunnett's test at 5% probability. 20-E content (mg g^{-1} DW) in **(c)** leaves and **(d)** roots. Uppercase letters compare between different ploidy levels under the same elicitation treatment; lowercase letters compare among different elicitation treatments under the same ploidy level, according to Tukey's test ($P \leq 0.05$); values represent means (n = 4) ± standard error.

CONCLUDING REMARKS

From our results, under the experimental conditions, the following conclusions can be drawn:

- *P. glomerata* show high plasticity to changes in photoperiod, and the tolerance responses exhibited by the plants are accession-dependent. Longer photoperiods induce higher photosynthetic rates in plants, which results in higher carbon availability and greater partitioning to secondary metabolism;
- Salt stress and 5-azaC treatments affect the proteomic profile of *P. glomerata*, and here we highlight differentially accumulated proteins involved in the regulation of photosynthesis, carbohydrates, and amino acids metabolism. Our results also show that the accumulation of jasmonate biosynthesis-related protein can be affected by DNA methylation, and suggest that this hormone plays a key role in the regulation of 20-E biosynthesis;
- Elicitation by methyl-JA results in a significant increase in 20-E production, confirming our hypothesis that jasmonate is involved in the signaling biosynthesis of this compound. On the other hand, elicitation by methyl-SA down-regulates 20-E biosynthesis, suggesting that salicylate has an antagonistic role to jasmonate-induced signaling;
- Tetraploid plants show higher 20-E content than diploid plants, demonstrating that the level of ploidy is involved in the regulation of secondary metabolism in *P. glomerata*.

Collectively, our results provide new insights into the morpho-physiological adjustments involved in the tolerance responses of *P. glomerata* to environmental conditions. Also, abiotic, molecular, and hormonal factors are directly involved in the regulation of 20-E biosynthesis in plants. Considering the medicinal importance of *P. glomerata*, these results provide efficient biotechnological strategies for increasing the 20-E production in plants grown in vitro.