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# Unraveling the protective and multifaceted roles of exogenous melatonin in Pfaffia glomerata (Spreng.) Pedersen plants under aluminum stress

Michelle Maylla Viana de Almeida
*Doctor Scientiae*

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**MICHELLE MAYLLA VIANA DE ALMEIDA**

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Thesis submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Wagner Campos Otoni

Co-adviser: Tatiane Dulcineia Silva

**VIÇOSA - MINAS GERAIS**
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*À minha amada filha Yasmin.*
*Para ti e por ti. Tudo. Sempre.*
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## ABSTRACT

ALMEIDA, Michelle Maylla Viana de, D.Sc., Universidade Federal de Viçosa, August, 2024. **Unraveling the protective and multifaceted roles of exogenous melatonin in Pfaffia glomerata (Spreng.) Pedersen plants under aluminum stress**. Adviser: Wagner Campos Otoni. Co-adviser: Tatiane Dulcineia Silva.

Aluminum (Al) toxicity is the main factor limiting agricultural production in acidic soils worldwide. Excess Al inhibits the growth and function of roots and consequently damages crop development. In this sense, scientists are trying to develop technologies and products to generate plants that are more resistant to metal stress. Plant growth regulators, such as melatonin (MET), have been considered a promising approach to improving crop tolerance to abiotic stress conditions, including stress caused by heavy metals. Among the species of phytotherapeutic interest is *Pfaffia glomerata* (Spreng.), a plant native to Brazil and popularly known as Brazilian-ginseng. Its medicinal properties are mainly due to the production of the secondary metabolite 20-hydroxyecdysone (20E), a phytoecdysteroid with proven therapeutic and nutraceutical activities in mammals. Studies by our research group have revealed that the species has adaptive plasticity in the face of various abiotic stresses, raising the hypothesis that this adaptation is mainly due to the modulation of secondary metabolism. In this sense, the imposition of aluminum (Al) stress would shed light on the adaptations of *P. glomerata* to yet another type of abiotic stress and the role of melatonin (MET), a protective and multifaceted molecule that acts as an important free radical scavenger, relieving stress-induced oxidative damage and mediating various physiological responses, including rooting, photosynthesis and senescence. Therefore, we evaluated the effect of high Al toxicity on morphophysiological and molecular aspects and the effect of melatonin application. To this end, two independent in vitro experiments were carried out. Both experiments used seedlings derived from clonal micropropagation from the in vitro germplasm bank. In experiment I, we analyzed the influence of 4 doses of Al: 0, 200, 400, 600 and 800 µM of Al. After 40 days of cultivation, our data showed that concentrations above 600 negatively affected photosynthesis, biomass accumulation, leaf area, volume and total length of roots, photosynthetic pigments and the rate of net photosynthesis. On the other hand, Al toxicity positively altered the content of carbohydrates, starch, carotenoids and total phenols, malate and fumarate, as well as raising proline levels at all the Al concentrations tested. We also observed an increase in some oxidative stress enzymes, especially in the roots (CAT, POD and SOD), as well as an increase in MDA

levels in response to the increase in the concentration of Al ions in solution. Al promoted fluctuations in the relative expression of genes in the 20E metabolite and lignin biosynthesis pathway. In experiment II, the physiological and molecular responses of plants exposed to Al and application of MET were analyzed: (I) control; (II) Al 500 μM; (III) MET 100 μM (IIII) Al+MET. Our data showed that MET alleviates the impacts of Al toxicity by maintaining growth parameters, biomass accumulation, chlorophyll and photosynthetic rate. MET also significantly decreases Al accumulation in roots and maintenance of total amino acid and protein content. MET reduced overall levels of DNA endoreduplication. Proteomic profiling revealed 174 differentially accumulated proteins (DPAs), of which 11 were positively regulated by Al, 23 by MET and 24 by the Al+MET interaction. 24, 22 and 26 proteins were negatively accumulated in Al, MET and Al+MET, respectively. Gene ontology and cluster analysis revealed an increase in the relative abundance of proteins involved in transmembrane transport, energy-intensive metabolic transport, energy production in plants such as photosynthesis, ascorbate metabolism, oxidative phosphorylation, carbon metabolism, pentose phosphate and other metabolic pathways associated with the biosynthesis of amino acids (e.g. phenylalanine and tryptophan) and secondary metabolites. DAPs were predominantly present in vacuoles, the cell wall, chloroplasts, and apoplasts. Our results suggest that modification of molecular structure is one of the main causes of cell damage and that vacuolar sequestration of Al ions is an important mechanism of Al resistance in plants. Overall, our results indicate a possible role for MET in protecting *P. glomerata* against Al toxicity, paving the way for future studies.

Keywords: aluminum ; abiotic stress ; brazilian-ginseng ; 20-hydroxyecdysone ; proline; in vitro culture

# RESUMO

ALMEIDA, Michelle Maylla Viana de, D.Sc., Universidade Federal de Viçosa, agosto de 2024. **Aspectos morfofisiológicos, bioquímicos e moleculares em Pfaffia glomerata (Spreng.) Pedersen em resposta ao estresse de alumínio e à aplicação de melatonina**. Orientador: Wagner Campos Otoni. Coorientadora: Tatiane Dulcineia Silva.

A toxicidade do alumínio (Al) é o principal fator que limita a produção agrícola em solos ácidos em todo o mundo. O excesso de Al inibe o crescimento e a função das raízes e, consequentemente, prejudica o desenvolvimento das culturas. Nesse sentido cientistas estão a tentar desenvolver tecnologias e produtos para gerar plantas que sejam mais resistentes ao stress do metal. Os reguladores de crescimento das plantas, como a melatonina (MET), têm sido considerados uma abordagem promissora para melhorar a tolerância das culturas a condições de stress abiótico, incluindo o stress causado por metais pesados. Entre as espécies de interesse fitoterápico, destaca-se a *Pfaffia glomerata* (Spreng.), planta nativa do Brasil e popularmente conhecida como ginseng-brasileiro. As suas propriedades medicinais devem-se majoritariamente à produção do metabólito secundário 20-hidroxiecdisona (20E), um fitoecdisteroide com comprovadas atividades terapêutica e nutracêutica em mamíferos. Estudos do nosso grupo de pesquisa têm revelado que a espécie tem uma plasticidade adaptativa frente a diversos estresse abióticos, levantando a hipótese que essa adaptação se deve principalmente pela modulação do metabolismo secundário. Nesse sentido, a imposição ao estresse por alumínio (Al) lançaria luz de quais seriam as adaptações em *P. glomerata* frente a mais um tipo de estresse abiótico e qual seria o papel da melatonina (MET), uma molécula protetora e multifacetada, que atua como importante eliminador de radicais livres, promovendo o alívio dos danos oxidativos induzidos pelo stress e mediando várias respostas fisiológicas, incluindo enraizamento, fotossíntese, senescência. Portanto, avaliamos o efeito da toxicidade do a Al elevada nos aspectos morfofisiológicos e moleculares, bem como o efeito da aplicação de melatonina. Para tal, foram realizados dois experimentos independentes in vitro. Para ambos os experimentos, foram utilizadas plântulas derivadas da micropropagação clonal a partir do banco de germoplasma in vitro. No experimento I, foram analisados a influência de 4 doses de Al: 0, 200, 400, 600 e 800 µM de Al. Após 40 dias de cultivo, os nossos dados mostraram que concentrações a partir de 600 µM de Al afetou negativamente a fotossíntese, o acúmulo de biomassa, área foliar, volume e comprimento total de raízes, os pigmentos fotossintéticos e a taxa de fotossíntese liquida. Por outro lado, a toxicidade do Al alterou positivamente o

conteúdo de carboidratos, amido, carotenóides e fenóis totais, malato e fumarato, além de elevar os níveis de prolina em todas as concentrações de Al testadas. Observamos também aumento de algumas enzimas do estresse oxidativo especialmente em raízes (CAT, POD e SOD), além de aumento nos níveis de MDA em resposta ao aumento da concentração de íons Al em solução. O Al promoveu flutuações na expressão relativa de genes da rota de biossíntese do metabólito 20E e da lignina. No experimento II, foram analisadas as respostas fisiológicas e moleculares de plantas expostas ao Al e aplicação de MET: (I) control; (II) Al 500 µM; (III) MET 100 µM (IIII) Al+MET. Os nossos dados demonstraram que a MET alivia os impactos da toxicidade de Al através da manutenção dos parâmetros de crescimento, acúmulo de biomassa, clorofila e taxa fotossintética. A MET também diminui significativamente o acúmulo de Al em raízes e manutenção do teor de aminoácidos e proteínas totais. O MET reduziu os níveis globais de endoreduplicação do DNA. O perfil proteômico revelou um total de 174 proteínas diferencialmente acumuladas (DPAs), das quais 11 foram reguladas positivamente pelo Al, 23 pelo MET e 24 pela interação Al+MET. 24, 22 e 26 proteínas foram acumuladas negativamente em Al, MET e Al+MET, respetivamente. A ontologia genética e a análise de clusters revelaram um aumento da abundância relativa de proteínas envolvidas no transporte transmembranar, no transporte metabólico intensivo de energia, na produção de energia nas plantas, como a fotossíntese, no metabolismo do ascorbato, na fosforilação oxidativa, no metabolismo do carbono, no fosfato de pentoses e noutras vias metabólicas associadas à biossíntese de aminoácidos (por exemplo, fenilalanina e triptofano) e de metabolitos secundários. Os DAPs estavam predominantemente presentes nos vacúolos, na parede celular, nos cloroplastos e apoplastos. Os nossos resultados sugerem que a modificação da estrutura molecular é uma das principais causas de danos celulares e que o sequestro vacuolar de íons Al é um mecanismo importante de resistência ao Al nas plantas. Em geral, os nossos resultados indicam um possível papel da MET na proteção da *P. glomerata* contra a toxicidade do Al, abrindo caminho para estudos futuros.

Palavras-chave: alumínio ; estresse abiótico; ginseng-brasileiro ; 20-hidroxiecdisona ; prolina ; cultura in vitro

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# GENERAL INTRODUCTION

## Aluminum phytotoxicity in acidic environments

The alarming increase in soil acidity is a global problem, posing a significant threat to plant sustainable growth and development worldwide. Soil acidity generally results from the loss of basic cations from soils through certain agricultural practices and acid rain (Chauhan *et al*., 2021). It has been estimated that approximately 30% of the world's arable land and 50% of the underlying agricultural land suffer from soil acidity (FAO, 2015).

The toxicity of aluminum (Al) is the main factor limiting crop production in acidic soils worldwide (Singh *et al*., 2017; Rahman *et al*., 2024). At soil pH values equal to or less than 5.5 solubilization reactions lead to the production of the $Al^{3+}$ ion. This ion is mobile and highly reactive and becomes dangerous after interacting with the cell walls and membranes and simplasma of the root apex cells (Chauhan *et al*., 2021; Rahman and Upadhyaya, 2021), inhibiting root growth and function and, consequently, negatively impacting crop development (Kennedy, 1986; Frankowski, 2015; Singh *et al*., 2017; Rahman *et al*., 2024).

The main symptoms of Al toxicity include inhibition of root growth (Singh *et al*., 2017; Li *et al*., 2023), followed by inhibition of cell division and root elongation, formation of root hairs, which in turn makes them inefficient in absorbing nutrients and water (Bojórquez-Quintal *et al.,* 2017; Singh *et al.*, 2017; Kar *et al*., 2020). Al toxicity also promotes water stress, leading to nutritional deficiencies in plants due to impaired absorption of water and mineral nutrients (Singh *et al*., 2017; Rahman *et al*., 2021), changes in photosynthetic activities through disturbed $CO_2$ assimilation, and changes in stomatal conductance and leaf transpiration rate in plants (Sade *et al*., 2016; Cárcamo *et al*., 2019; Phukunkamkaew *et al*., 2021). In addition, it has been described that Al toxicity induces other toxic effects on different cell organelles, such as Al-induced disruption of free cytosolic $Ca^{2+}$, deposition of callose in plasmodesmata, inhibition of mitochondrial respiration, overproduction of reactive oxygen species (ROS) and DNA damage (Rengel, 2004; Sivaguru *et al*., 2000; Yamamoto *et al*., 2002; Phukunkamkaew *et al*., 2021).

Surprisingly, exposure to micromolar concentrations in a short space of time (Ownby and Popham, 1990; Matsumoto *et al.*, 2012) is sufficient to induce several symptoms of irreversible toxicity, such as the rapid and transient overgeneration of ROS, produced mainly in the mitochondria, chloroplasts or peroxisome, resulting in a cascade of oxidative reactions and

lipid peroxidation leading to protein denaturation and ultimately programmed cell death (Chauhan *et al*., 2021; Phukunkamkaew *et al*., 2021).

In addition, Al disrupts the homeostasis of biomolecules and alters the expression of various stress-responsive and regulatory genes. For example, the interaction of Al ions with the cell's plasma membrane stimulates the composition of callose, disrupting the redox balance (Sun *et al*., 2020). This disruption can result from the formation of an electrostatic bond between Al and oxygen-donating compounds, such as phosphate $(PO_4)^{3-}$ or carboxylate (COOH) groups, leading to lipid peroxidation, disruption in cell rigidity and deposition of cell wall compounds, through cross-linking of pectin and/or proteins (Ren *et al*., 2022), resulting in a decrease in the flexibility and hydraulic gradient of the cell (Manquián-Cerda *et al*., 2018) and alters the integrity, coupling and polymerization of microtubules and the cytoskeleton (Wu *et al*., 2018).

In most plant species, there is considerable genotypic variation in the ability to resist Al toxicity, given that plants employ different mechanisms to overcome Al stress. Initially, the exclusion of $Al^{3+}$ as a tolerance strategy involves the process of chelation and detoxification, where organic acids - OAs (such as citrate, oxalate and malate) are secreted, bind to and chelate Al ions external to the cell, inhibiting their absorption into the cytoplasm of root cells (Nunes-Nesi *et al*., 2014; Kochian *et al*., 2015; Ranjan *et al*., 2021). In addition, an increase in rhizosphere pH can protect the plant against the harmful effects of Al, since the bioavailability of Al is inhibited at alkaline pH. Some Al manages to cross the cell wall and plasma membrane, at which point there is an increase in the biosynthesis of organic acids that form stable complexes in the cytoplasm and sequester Al in vacuoles (Singh *et al*., 2017; Chauhan *et al*., 2021).

In addition, plants have also developed antioxidant defense mechanisms to regulate and adapt to Al-induced oxidative stress. The antioxidant defense mechanism involves several enzymatic components (superoxide dismutase; SOD, glutathione peroxidase; GPXs, catalase; CAT, peroxidase; PRXs) and non-enzymatic components (polyphenol, vitamin E, glutathione; vitamin C and carotenoids) to modulate ROS triggered by Al (Singh *et al*., 2017; Chauhan *et al*., 2021). In addition, recent advances in increasing plant tolerance to Al stress focus on increasing OA exudation to prevent Al from entering the plant and developing Al-tolerant varieties. Families of gene transporters, such as ATP-Binding Cassette (ABC), Aluminum-activated Malate Transporter (ALMT), Natural resistance-associated macrophage protein (Nramp), Multidrug and Toxic compounds Extrusion (MATE) and aquaporin, play a crucial role in regulating Al toxicity (Rahman *et al*., 2024).

Thus, it is imperative to understand the molecular reasons behind Al toxicity because this knowledge can lead to discoveries and mechanisms for designing Al-tolerant crop plants. In addition to understanding the patterns of Al bioavailability, translocation and accumulation, improving our understanding of the metabolic and molecular responses employed by plants is crucial in regulating Al toxicity.

**Melatonin: The stress protector**

The use of emerging plant growth regulators such as melatonin (MET) has been considered a promising approach to improve crop tolerance to abiotic stress conditions, which is why it has attracted the attention of researchers around the world (Dawood, 2022). Melatonin - MET (N-acetyl-5-methoxytryptamine) is a low molecular weight indole amine derived from tryptophan, which is a ubiquitous molecule in animals, plants, fungi and bacteria (Dawood, 2022; Hassan *et al*., 2022; Altaf *et al*., 2024). MET was first isolated from bovine pineal glands in 1958 (Lerner *et al*., 1958; Reiter, 1991). A MET recebeu seu nome em 1957, quando foi relatado seu papel no clareamento da pele de sapos e envolvimento no controle dos ritmos circadianos em diversos vertebrados (Lerner *et al*., 1958; Tan *et al*., 2019). Os níveis máximos de MET durante a noite indicam sua importância na sinalização noturna (Reiter, 1991). Em plantas, a presença de MET foi descoberta em várias famílias de monocotiledôneas e dicotiledôneas (Reiter *et al*., 2009; Nawaz *et al*., 2016).

Since its discovery in *Pharbitis nil* in 1993, several studies have demonstrated the beneficial effects and biological functions of MET in plants (Lei *et al.*, 2023). In plants, MET is reported to be a biostimulator of plant growth, especially under conditions of environmental stress, constituting a multifunctional signaling molecule (Zhang *et al*., 2019; Back *et al*., 2021; Huang *et al*., 2024). Its presence in various parts of the plant (root, stem, leaves, fruit, flowers and seeds) indicates its importance in plant growth and development throughout the plant kingdom (Nawaz *et al*., 2016; Wei *et al*., 2015) and many studies have been carried out on MET, reinforcing its positive role in plants (Antoniou *et al.,* 2017; Zhang *et al*., 2018; Zhao *et al*., 2021; Rajora *et al*., 2022; Park *et al*., 2024).

Evidence shows that MET is of great importance as a free radical scavenger, promoting relief from stress-induced oxidative damage and mediating various physiological responses, including rooting, seed germination, photosynthesis, flowering, aging and fruit ripening (Shi *et al*., 2016; Arnao and Hernández-Ruiz, 2020; Kołodziejczyk and Kaźmierczak *et al*., 2021),

representing a new and effective strategy for crop improvement (Zhang *et al.*, 2014; Khan *et al.*, 2020).

In addition, MET modulates plant tolerance or resistance to abiotic stresses, including salinity, drought, heat, cold, water deficit, toxic metals, nutritional deficiency, osmotic and ionic stress, ultraviolet radiation and cold injury (Sharma end Zheng, 2019; Bose and Howlader, 2020; Moustafa-Farag *et al,* 2020; Arnao and Hernández-Ruiz *et al*., 2020; Park *et al*., 2024; Saqib *et al*., 2024). MET increases tolerance to toxic metal stress in plants mainly by activating the redox network, membrane integrity, osmoregulation and modulating polyamine metabolism and phytochelatin biosynthesis (Hasan *et al*., 2015; Ke *et al*., 2018; Jannatizadeh, 2019; Xing *et al*., 2023). Thus, MET plays an essential role in improving the growth and development of plants subjected to abiotic stresses.

In addition, studies indicate that MET is closely related to a feedback mechanism in plants that comprises different regulatory elements of the redox network, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), mainly through the action of H2O2 (Arnao and Hernández-Ruiz, 2019). However, the mechanism by which MET affects tolerance to abiotic stress, including Al, mainly includes two important aspects: protecting root apices and activating the antioxidant system. Other functions include regulating the expression of related genes, participating in related metabolic pathways and protecting plant growth. In this sense, the role of MET in regulating plant tolerance to Al stress needs to be clarified.

***Pfaffia glomerata*: Adaptive plasticity to abiotic stresses**

The *Pfaffia glomerata* (Amaranthaceae), popularly known as fáfia or Brazilian ginseng, due to the morphological similarity of its roots to those of Korean ginseng (*Panax ginseng* C.A. Meyer.) (Neves *et al*., 2016). It is an herbaceous species native to South America, widely distributed in Brazil, and is found in almost all regions, occurring mainly in places with moist, sandy soil, making up riparian vegetation and river flood fields (Marchioretto, 2015; Dias *et al*., 2019; Franco *et al*., 2021; Ribeiro *et al*., 2024).

The species is widely used in traditional medicine because of its medicinal properties such as immunostimulant, sedative, hypocholesterolemic, analgesic, anabolic, anti-inflammatory and other functions, which have already been described in the literature (Nascimento *et al*., 2007; Neto *et al*., 2005; Neves *et al*., 2016; Dias *et al*., 2019; Franco *et al*., 2021). These medicinal properties are mainly due to 20-hydroxyecdysone (20E), pterosterone, polypodin B and ginsenosides. Among these compounds, 20E is the majority in *P. glomerata*

and its active principles are obtained from its roots by extraction (Kamada *et al*., 2009; Neves *et al*., 2016; Dinan *et al*., 2021). Due to its pharmacological properties, 20E has been widely studied and it has been found that different environmental changes modulate the production of 20E in *P. glomerata*, mainly due to the species' remarkable plasticity and adaptability to changes in abiotic conditions. For example, *P. glomerata* proved to be adjustable to different levels of irradiance, spectral quality and photoperiod conditions, showing changes in its morphophysiological profile and secondary metabolism (20E) (Silva *et al*., 2020; Felipe *et al*., 2019a; Silva *et al*., 2021; Fortini *et al*., 2020). On the other hand, salt stress induced the expression of the *Spook* and *Phantom* genes, which participate in the 20E biosynthesis pathway, by up to five times (Felipe *et al*. 2019b).

*P. glomerata* has been shown to exhibit moderate tolerance to excess zinc, mainly by modulating the enzymatic and non-enzymatic systems (anthocyanins and 20E) (Bernardy *et al.*, 2020). The same tolerance profile was also observed under mercury, cadmium, lead and arsenic toxicity (Calgaroto *et al*., 2010; Gomes *et al*., 2012; Gupta *et al*., 2013; Pereira *et al*., 2018). The only work available in the literature on the effects of Al on the growth of *P. glomerata* shows that the metal negatively affects the growth of the plant, especially the roots; this effect varied between the two accessions investigated (BRA and JB/UFSM), the latter proving to be more sensitive (Maldaner *et al*., 2015). However, little is known about the physiological, biochemical and molecular mechanisms employed by *P. glomerata* when exposed to Al, the impacts of Al toxicity on 20E production, and the effect of MET application on these responses.

## Melatonin-induced protection against Al toxicity in *P. glomerata*

Al toxicity represents a major limitation for global crop production (Singh *et al*., 2017; Rahman *et al*., 2020). In this sense, the search for molecules that can protect plant roots by preventing this highly reactive ion entry into the root apex cell symplasm has become a very important topic of research worldwide.

MET, an emerging molecule that is a vital regulator of plant growth under stress conditions, is an excellent tool for protecting against Al damage, especially in root apices (Hassan *et al.,* 2022). This molecule, an antioxidant with appreciable potential for eliminating ROS and improving stress tolerance, has been the subject of several studies (Debnath *et al*., 2019; Khan *et al*., 2023; Jan *et al*., 2023).

Transcriptomic studies in model plants, such as *Arabidopsis thaliana* and *Oryza sativa*, revealed numerous important transcriptional changes occurring in Al-stressed plants. This data, coupled with other multi-omics approaches, generates a high-throughput dataset vital in revealing several key genes, proteins and metabolic pathways underlying numerous tolerance traits in response to different stresses. However, this approach is especially challenging in plants due to the length of genomes, multiple organelles and diverse secondary metabolites (Singh *et al*., 2017).

Al stress appears to be multifaceted, including genes linked to signal transduction, membrane transporters, transcription factors, energy and oxidative stress pathways, cytoskeleton dynamics, protein damage, DNA, and metabolism, and several candidate genes (such as heavy metal transporters) or pathways (such as the glutathione and diterpenoid pathway) are involved in regulating Al tolerance (Singh *et al*., 2017; Liu *et al.,* 2021). The main genes involved in Al tolerance are the transporter family ALMT, MATE, ABC, Nramp and aquaporins, which are regulated by several transcription factors (Ma *et al*., 2014; Arenhart *et al*., 2014). However, enzymes, proteins and transcription factors that act downstream of these genes and pathways also play crucial roles in biological processes in higher plants.

In *P. glomerata*, responses to Al toxicity appear to be a complex phenomenon involving multiple genes and physiological mechanisms that have not yet been characterized, and due to the lack, to date, of studies involving Al toxicity and MET application in an in vitro environment, this became a pioneering approach involving physiological, biochemical and molecular aspects in this non-model medicinal species. Thus, these combined approaches may improve the understanding of the mechanisms involved in regulating Al tolerance and the underlying mechanisms by which MET could alleviate the inhibitory effects of this stress on the early development of *P. glomerata*.

Previous studies have shown the roles of MET in reducing Al concentration and increasing antioxidant capacity in *Brassica napus*, and also modulating cadmium tolerance via chelators and critical heavy metal transporters in *Raphanus sativus* (Sami *et al.*, 2020; Xu *et al*., 2020). However, the metabolic processes associated with Al-induced growth inhibition and a possible MET-induced mitigation of this damage in *P. glomerata* seedlings lack detailed studies, although its multiple actions also point to it being a key regulator of redox homeostasis in plants. The findings here will support the understanding of Al toxicity in *P. glomerata* and the tolerance mechanisms displayed by the species, as well as favoring the optimization of 20E production and the effect of MET application on responses to Al toxicity in the species.

Here, we hypothesize that: (1) MET could mitigate the inhibitory effect of Al stress on metabolism and as a result, could increase Al tolerance in *P. glomerata* plants, growth and photosynthetic parameters, enzyme activities and 20E accumulation; (2) MET reduces Al accumulation in roots and leaves of *P. glomerata*; (3) MET reduces Al accumulation in roots and leaves of *P. glomerata*; (3) the application of MET induce the production of 20E and the expression of key genes of this metabolite in *P. glomerata* under Al stress, in addition to acting in the differentiated deposition of cell wall compounds, especially in roots; (4) the combination of Al and MET promotes protection against the entry of Al into root apices and, as a result, protects DNA damage.

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## Chapter 1 – Morphophysiological, biochemical and molecular responses of *Pfaffia glomerata* under Aluminum stress

### Abstract

Al toxicity, especially in acidic soils, significantly inhibits plant growth and development worldwide. Understanding the mechanisms involved in the timing of to Al toxicity remains poorly explored in *Pfaffia glomerata*, a medicinal species. *P. glomerata* plantlets were exposed in vitro for 40 days to five concentrations of Al (0, 200, 400, 600 and 800 μM) to analyze morphophysiological effects, redox metabolism and molecular aspects in response to Al toxicity. Plant growth was negatively affected by Al concentrations above 600 μM. The seedlings showed a significant accumulation of Al in root and leaf tissues, demonstrating the translocation of this ion through the plant body. We observed a high accumulation of glucose and fructose and, to a lesser extent, sucrose and starch at concentrations of 600μM of Al. Starch, proline malate and fumarate content increased in response to Al toxicity. However, the seedlings suffered oxidative stress, as suggested by the significant increase in POD and SOD antioxidant activity and malondialdehyde levels in roots. The *PgSpook* and *PgPhanton* genes were negatively regulated, while *PgCCR*, *PgCAD*, *PgC4H* were positively regulated in leaves and negatively regulated in roots. Therefore, taken together our data reinforce the hypothesis that *P. glomerata* plants show moderate tolerance to Al stress through modulation of primary metabolism and redox metabolism and fluctuation in the expression of genes related to cell wall composition, mainly in roots, and 20-E biosynthesis (non-enzymatic metabolism), together with structural changes in the morphology of *P. glomerata* grown *in vitro*.

**Key words:** Aluminum, Brazilian-ginseng, proline, 20-hydroxyecdysone

**INTRODUCION**

The increase in soil acidity caused by extreme natural events and anthropogenic activities represents a severe problem for the growth and sustainable development of plants worldwide (Chauhan *et al*., 2021; Zhang *et al*., 2021; Farooq *et al*., 2022; Rahman *et al*., 2024). One of the main components of acidity in soils with a pH below 5 is the release of trivalent aluminum ($Al^{3+}$) (Chamier *et al*., 2015). Excess aluminum (Al) in the soil solution disrupts the physiological and metabolic functioning of plants by inhibiting cell division and root elongation, decreasing root hair formation, which in turn makes them inefficient at absorbing water and essential nutrients, leading to a decrease in overall biomass (Nunes-Nesi *et al*., 2014; Rahman *et al*., 2021). Excess Al also triggers the overproduction of reactive oxygen species (ROS), promoting oxidative burst and peroxidation of membrane lipids (Singh *et al*., 2017; Chauhan *et al*., 2021).

Al toxicity promotes changes in photosynthetic activities through disturbing $CO_2$ assimilation, stomatal conductance and leaf transpiration rate in plants (Sade *et al*., 2016; Cárcamo *et al*., 2019; Phukunkamkaew *et al*., 2021; Horst *et al*., 2010; Bojórquez-Quintal *et al*., 2017; Singh *et al*., 2017; Kar *et al*., 2020). Finally, Al can bind to the phosphodiester skeleton of genomic DNA and induce damage to the composition and replication of the genetic material (Grupa *et al*., 2013; Singh *et al*., 2017; Phukunkamkaew *et al*., 2021). Plants employ different strategies to deal with Al toxicity, including the sequestration of Al in vacuoles, the exudation of organic acids (OAs) such as citrate, oxalate and malate from root apex cells, forming Al-OAs complexes and activating the antioxidant machinery, as well as promoting the overexpression of genes responsive to Al stress (Rahman *et al*., 2024).

Excess aluminum in plants can also coordinate changes in the secondary metabolism of plants, such as in the phytoecdysteroid pathway. Phytoecdysteroids are polyhydroxylated, a class of biologically active chemicals synthesized by plants for defense against phytophagous insects (Das *et al*., 2021), but their physiological functions in plants are not fully elucidated. 20-hydroxyecdysteroid (20-E) has been considered the major phytoecdysteroid in *P. glomerata*, which is the most economically important ginseng species in Brazil (Nascimento *et al*., 2007; Ribeiro *et al*., 2024), because it has medicinal properties, and its active ingredient (20-E) is extracted mainly from plant roots in an extractive manner (Silva *et al*., 2021), it is a perennial plant with moderate tolerance to heavy metals such as cadmium (Gomes *et al*., 2013), lead

(Gupta *et al*., 2011), zinc (Calgaroto *et al*., 2011), arsenic (Gupta *et al*., 2013) and aluminum (Maldaner *et al*., 2015).

In addition, Kamada et al. (2009) reported high divergence between individuals of *P. glomerata* belonging to different populations concerning the content of specific phytoecdysteroids in different plant tissues. Similarly, several researchers suggest that abiotic factors can modulate the secondary metabolism of *P. glomerata* and modulate phytoecdysteroid production, for example, ultraviolet-B radiation (Felipe *et al*., 2019), moderate salt stress (Felipe *et al*., 2019b), long photoperiods (Fortini *et al*., 2020), spectral quality (red and blue LEDs) (Silva *et al*., 2020) and higher irradiances (Silva *et al*., 2020b). Therefore, it is assumed that the plasticity of secondary metabolism observed in different accessions of *P. glomerata* may be involved in greater tolerance to extreme environments.

It is worth noting that *P. glomerata* plants can be found in soils rich in aluminum, as exemplified in the Cerrado Biome (Senna, 2024). However, the possible mechanisms used by these medicinally important species to deal with this metal in the soil are unknown, just as little is known about the levels of aluminum accumulated in their tissues. In this sense, there is a lack of studies on the effects of Al on redox metabolism and the expression of genes involved in the production of phytoecdysteroids and cell wall compounds since Al binds mainly to these compounds in root apices and other secondary compounds, such as 20-E, as well as the translocation pattern of this metal in order to control and reduce the concentration of Al in plants and, ultimately, in the human body, in the case of a medicinal plant.

Here we hypothesize that *P. glomerata* plants show moderate tolerance to Al stress through the modulation of redox metabolism and fluctuation in the expression of genes related to cell wall composition, mainly in roots, and in the production of 20-E (non-enzymatic metabolism), together with structural changes in the morphology of *P. glomerata* grown in vitro.

## MATERIALS AND METHODS

### Plant material and in vitro cultivation

The work was carried out at the Plant Tissue Culture Laboratory (LCT) of the Institute of Biotechnology Applied to Agriculture (BIOAGRO) of the Federal University of Viçosa (UFV). Access to genetic material (permission number AA2A367) was granted by the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under existing Brazilian biodiversity legislation.

The plants (Alto do Caparaó accession) used in the experiments came from the LCT-BIOAGRO in vitro germplasm bank and were grown monthly in MS culture medium (Murashige and Skoog, 1962) supplemented with myo-inositol (100 mg $L^{-1}$) and 3% sucrose (w/v) (Sigma-Aldrich Co, St Louis, MO, USA), solidified with 5.5 g $L^{-1}$ of agar (PhytoTechnology Laboratories®, Kansas, United States).

Three nodal segments (accession Caparaó), about 2 cm long, were inoculated into glass flasks each containing 100 mL-aliquots of liquid MS medium. The nodal explants were placed in adapted polypropylene supports. The flasks were sealed with rigid polypropylene lids with two 10 mm holes covered with 0.45-µm-pore size hydrophobic fluoropore membranes (MilliSeal® AVS-045 Air Vent, Tokyo, Japan), which allow a gas exchange rate with the external medium of 25 µL $L^{-1}$ $s^{-1}$ of $CO_2$ (Batista *et al.*, 2017). The cultures were maintained at 25 ± 2 °C, under an irradiance of 60 µmol $m^{-2}$ $s^{-1}$ and a 16-h photoperiod for 40 days.

To induce Al stress, five increasing concentrations of Al (0, 200, 400, 600, 800 µM) (Sigma-Aldrich Co, St Louis, MO, USA) were used, sterilized by filtration under a laminar flow chamber (Millipore® filters – Sigma-Aldrich) and added to the previously autoclaved medium (1.5 atm, 120 °C, 20 min). The pH was adjusted to 4.5 in all the conditions tested. The plants were kept in a growth room with a 16-hour photoperiod, a temperature of 25 ± 2 °C and an irradiance of 60 µmol $m^{-2}$ $s^{-1}$ provided by two LED lamps (SMD 100, 18W, Vilux®, Vitória, ES, Brazil).

The experimental design was entirely randomized with 5 growing conditions (0, 200, 400, 600, 800 µM of Al). Each treatment had 20 replicates and the experimental unit consisted of a flask with 3 explants. The plants were grown for 40 days.

### Growth parameters

The following growth variables were assessed: root, stem and leaf dry mass (g), total shoot length (cm), root length (cm), leaf area and number of leaves. In addition, the roots were placed in distilled water for analysis in the WinRhizo Pro system (Régent Instr. Inc.) and the following root growth variables were obtained: total length (cm), surface area ($cm^2$), diameter (cm) and volume ($cm^3$).

**Photosynthetic rate *in vitro***

Gas exchange and in vitro photosynthetic rate quantification were performed as proposed by Costa *et al*. (2014) with some modifications (Silva *et al*., 2020). Measurements and data collection were performed using an AQ-S151 Infrared $CO_2$ analyzer (Qubit Systems, Kingston, ON, Canada) and LoggerLite 1.8.1 software (Vernier Software e Technology, Beaverton, OR, USA), respectively. Reference $CO_2$ was calculated by entering air into an empty flask pumped from the external environment at a constant airflow of 300 mL $min^{-1}$ inside a lighted chamber (blue and red LED lamps). LEDs were connected in a direct current circuit under a voltage of 12 V, formed by two parallel sets of four LEDs connected in series (600 µmol $m^{-2}$ $s^{-1}$). Plants were kept in darkness for 8 h before analysis. Soon after the reference $CO_2$ measurement, flasks containing the plants were coupled to the system and the $CO_2$ was calculated at the stabilization point. Gas exchange was measured by calculating the difference between the reference $CO_2$ and the $CO_2$ of plants exposed to atmospheric air. A Spec sensor measured air temperature and humidity in the balloon (Thermo Recorder RS-11; Takai Spec Corp., Aichi, Japan). In vitro photosynthetic rate (A) was calculated by the following formula:

$$A\ (\mu\text{mol m}^{-2}\ \text{s}^{-1}) = \frac{\Delta\ \text{CO}_2}{\text{Mol Flow}}$$

where,

$$\Delta\ \text{CO}_2(\text{ppm}) = \text{Reference CO}_2 - \text{Analysis CO}_2$$

$$\text{Mol Flow} = \frac{Air\ flow\ rate\ (L\ min^{-1})}{\left[\dfrac{\dfrac{Constant\ for\ perfect\ gases\ (22.4)\ x\ Temperature\ (K)}{60000}}{Leaf\ DW\ per\ plant\ (g)}\right]}$$

**Anatomical characterization**

After 40 days of cultivation in an induction medium, the third pair of fully expanded leaves and the root apices were collected and fixed in 4% glutaraldehyde (Karnovsky, 1965). The fixed samples were dehydrated in ethanolic series, embedded in methacrylate resin (Historesin, Leica®) and placed in plastic histomolds (Leica®). Five-micrometer-thick cross-sections were obtained on an automatic feed rotary microtome (RM2155, Leica Microsystems Inc., USA) and stained with toluidine blue (pH 3.2) for 3 min (O'Brien and McCully, 1981). The specimens were mounted in Permount on glass slides. The images were captured using 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 a microcomputer with Spot Basic image capture software.

**Aluminum quantification**

The Al concentration was determined using 0.5 g of leaves and roots dehydrated at 50 °C for 72 h, which were crushed using a knife mill to a size smaller than 1 mm. The extracts were obtained by digestion in nitroperchloric solution (Malavolta *et al*., 1989) and the Al content was determined using an inductively coupled plasma atomic emission spectrometer (ICP-OES, Optima 7300 DV PerkinElmer, Inc. Shelton, CT, USA). The values were expressed in mg $kg^{-1}$ of dry matter.

**Determination of primary metabolites**

The leaf samples were collected and subjected to methanolic extraction (Lisec *et al*., 2006). Approximately 20 mg of plant material, frozen in liquid nitrogen, crushed and freeze-dried, was sampled in microtubes. Photosynthetic pigments were determined as described by Wellburn (1994). Carbohydrates (starch, sucrose, glucose and fructose) were assessed as described by Fernie *et al*. (2001). Soluble total protein was assessed as described by Bradford (1976), and total amino acid contents were analyzed according to Yemn and Cocking (1995). Malate content was determined as described by Nunes-Nesi *et al.* (2007). Proline content followed the methodology proposed by Carillo and Gibon (2011).

**Determination of antioxidant system enzyme activity**

Activities of oxidative stress enzymes, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) were assessed. Briefly, 50 mg of frozen fresh material (aerial part) were homogenized in 1 mL extraction medium containing 0.1 M potassium phosphate buffer, pH 6.8; 0.1 mM ethylenediaminetetraacetic acid; 1 mM phenylmethylsulfonyl fuoride and 1% (w/v) polyvinylpolypyrrolidone. The samples were vortexed for 10 s, centrifuged at 12.000 rpm for 15 min at 4$^{o}$C, and the supernatant was removed and set aside on ice for enzyme assays plus protein determination (Bradford, 1976). CAT, and POD activities were determined as proposed previously (Chance and Maehly, 1955; Nakano and Asada, 1981; Havir and McHale, 1987) and expressed as $\mu M^{-1}$ $min^{-1}$ $g^{-1}$ 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 levels**

Lipid peroxidation was determined by quantifying the malonodialdehyde (MDA) levels, using the methodology proposed by Heath e Packer (1968), with modifications. 1 mL of 1% trichloroacetic acid (TCA) was added to 100 mg of fresh, macerated plant material. The solution was stirred in a vortex and centrifuged at 12,000 *g* for 15 min at 4 ºC. Then 250 µL of the supernatant was transferred to new tubes and 750 µL of 0.5% 2-thiobarbituric acid (TBA w/v) in 20% (w/v) TCA. The reaction was incubated by shaking at 95 °C and stopped in an ice bath after 30 min. The supernatants were transferred to new tubes and centrifuged (10000 *g*, 10 min, 4 °C), and read on a spectrophotometer at 532 ($A_{532}$) and 600 ($A_{600}$) nm. The MDA concentration was obtained by subtracting $A_{600}$ from $A_{532}$, and calculated according to the extinction coefficient of 155 mM $cm^{-1}$.

**Expression analysis using qRT-PCR**

The expression of the genes of the 20E biosynthesis pathway was determined: ecdysteroid 25-hydroxylase (*Phantom* - Cyp306a1) and cytochrome P450 family subfamily A (*Spook* - Cyp307a1), as well as the lignin biosynthesis pathway genes associated with root cell wall stiffening: *cinnamate 4-hydroxylase* (*PgC4H*), *caffeoylCoA O-methyltransferase* (*PgCCoAOMT*), *cinnamoyl-CoA reductase* (*PgCCR*) and *cinnamyl alcohol dehydrogenase* (*PgCAD*). The leaves or roots (100 mg) were collected in liquid nitrogen, macerated and subjected to total RNA extraction using TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA).

The material was treated with DNAse I (Thermo Scientific NanoDrop Technology, Wilmington, DE, USA) to remove possible contamination with genomic DNA, and around 500 ng of the extracted total RNA was converted to complementary DNA (cDNA) using the SuperScript™ III First-Strand Synthesis System kit (Invitrogen®, Carlsbad, CA, USA). The qRT-PCR analyses were carried out on a CFX96 TouchTM detection device (BIO-RAD), using specific primers from the *P. glomerata* transcriptome (Batista *et al*., 2019). The *glyceraldehyde-3-phosphate dehydrogenase* gene from *P. glomerata* (*PgGAPDH*) was used as an internal reference (Batista *et al*., 2019).

The reactions were performed with three biological replicates in duplicate technique each, in a reaction volume of 10 µL (4 µL of SYBR-Green, 1 µL (4 µM) of each primer, 3 µL of diethylpyrocarbonate-treated water and 1 µL (40 ng) of cDNA. The amplification conditions were performed in the following steps: 2 min at 50 ºC and 10 min at 95 ºC, followed by 40 cycles of 95 ºC for 16 s and 60 ºC for 60 s, and the dissociation curve from 60 to 95 ºC at 0.1 ºC $s^{-1}$. 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. The primer sequences used are shown in Table S1. The melting curves were checked for non-specific amplification and primer dimerization.

**Statistical analysis**

All experiments were designed in a completely randomized distribution with four biological replicates of each treatment. In addition, the experiments were repeated twice. Data were statistically tested for normality and subsequently examined using ANOVA ($P < 0.05$). Differences in means ($P < 0.05$) presented in the figures were examined using the Tukey test. Gene expression data were compared using Dunnett's test. All statistical analyses were carried out using R statistical software (www.r-project.org).

## RESULTS

### Aluminum alters biomass accumulation in *P. glomerata*

Al acted differently on the growth of the roots and the aerial part of the plants compared to the control plants (Figs. 1 and 2). We observed increased stem and root length when subjected to concentrations equal to or less than 400 μM of Al. On the other hand, concentrations above 600 μM of Al promoted drastic reductions in root length (50%) and stem length (20%) compared to the control (Fig. 2a-b). The same pattern was observed for stem dry mass, where concentrations of 600 and 800 μM of Al caused a reduction of 30 and 45%, respectively (Fig. 2c). In the roots, Al caused reductions in dry mass accumulation at all levels analyzed (200, 400, 600 and 800 μM), causing reductions of 33, 16, 34 and 50%, respectively. Leaf area was also negatively affected (27 and 17%) by the 600 and 800 μM Al concentrations, respectively (Fig. 2e). Concerning average root diameter, a significant decrease (18%) was only observed for the 400 μM concentration (Fig. 2g). In addition, the 200, 400, 600 and 800 μM doses of Al reduced the total length of *P. glomerata* roots by 35, 47, 41 and 55%. The total volume of the roots was negatively affected by 35% only at the highest Al concentration tested (800 μM) (Fig. 2i).

### Al accumulates in roots and some of this metal is translocated to the leaves of *P. glomerata*

After exposure to Al, *P. glomerata* plants accumulated Al in their leaf tissues (Fig. 3). The concentrations of 200, 400, 600 and 800 μM of Al promoted an accumulation of this metal in the roots of 2.4, 5, 20 and 12 times to the control treatment (Fig. 3a). In leaves, we also observed Al accumulation of 46, 64, 74 and 81%, respectively, when compared to plants not treated with the metal (Fig. 3b).

### Al induced morphoanatomical changes in *P. glomerata*

Morphological changes were observed in the leaves of plants under Al toxicity (Figs. 4). The leaves have a uniseriate epidermis, a large central vascular bundle with parenchyma distributed along the main vein, and collenchyma subdivided into palisade and spongy parenchyma (Fig. 4a). However, the plants subjected to Al showed different characteristics: there was a visual increase in the size of the parenchyma cells and a disturbance in the arrangement of the vascular bundles, in the palisade parenchyma there were changes in cell shape, and in the spongy parenchyma there were no intercellular spaces as the Al concentration

increased (Fig. 4h, k and n). In the roots, we found that Al altered the size of the cortex (Figs. 4 f, i, and o). The development of the xylem was visually more pronounced with increasing Al concentration compared to the control plants (Fig. 4 i, l and o.).

**Al promotes changes in pigments and photosynthetic rate**

Al toxicity caused significant reductions in chlorophyll (Chl*a*) and total Chl *a* contents from the 600 µM concentration onwards (Fig. 5). Plants grown at concentrations of 600 and 800 µM Al reduced Chl *a* content by 22 and 32% and total Chls by 18 and 26%, respectively (Fig. 5a). There were no significant changes in Chl *b* content at any of the Al concentrations tested (Fig. 5b). For the Chl *a*/Chl *b* ratio, we did not observe a specific pattern of changes in content (Fig. 5d). In addition, Al promoted an increase in carotenoid content up to a concentration of 600 µM compared to the control (Fig. 5e). Meanwhile, there was a significant reduction in photosynthetic rate in plants grown in the presence of Al at concentrations of 600 and 800 µM compared to the control (Fig. 5f).

**Changes in metabolism occurred in response to Al toxicity**

The primary metabolism of *P. glomerata* plants was altered in response to Al toxicity (Fig. 6). A pronounced increase in glucose and fructose content was observed from the 600 µM Al concentration onwards, with increases of 120% and 160%, respectively (Fig 6a-b). As for the fructose content, this behavior was repeated, with an increase of 46 and 63%, respectively, for the 600 and 800 µM Al concentrations when compared to the control (Fig. 6b). We observed a tendency for the sucrose content to increase as the concentration of Al in the culture medium increased, with the 800 µM Al concentration promoting the greatest increase in sucrose content (38%) (Fig. 6c). The starch content followed the same upward trend, with concentrations of 600 and 800 µM of Al promoting increases of 60 and 28%, respectively (Fig. 6d).

In relation to total amino acids, we observed a significant increase (74%) only for the 600 µM Al concentration; the other concentrations tested did not differ from the control (Fig. 6e). We did not observe a specific pattern of significant changes in protein content (Fig 6f). In addition, an increase in total phenolics was observed at dosages above 600 µM Al, (16 and 8%, respectively) (Fig. 6g). At dosages above 200 µM of Al, the proline content increased significantly compared to the control, this increase was of the order of 90% in all the concentrations analyzed (200, 400, 600 and 800 µM of Al) (Fig. 6h). Similarly, malate levels

increased in response to the increase in Al concentration at all the concentrations tested, with the 600 µM Al concentration promoting the highest accumulations of this metabolite, expressing a 100% increase compared to the control (Fig. 6i). The presence of Al in the culture medium showed a tendency to increase the fumarate content. However, we only observed significant differences for the 600 µM Al concentration (56%) compared to the control (Fig. 6j).

**Antioxidant enzymes and MDA content are not affected by high Al**

The activities of the antioxidant enzymes (CAT, POD and SOD) were significantly influenced by the presence of Al in the culture medium (Fig. 7). Compared to the control, 200, 600 and 800µM of Al promoted a 32%, 17 and 20% increase in CAT activity, respectively. For POD, plants under to Al toxicity increased its activity in the leaves and roots at all the Al doses tested. This significant increase was more pronounced at the 600 µM Al dose than the control in both analyzed organs (Fig. 7c-d).

For SOD, the plants under to Al toxicity increased their activity mainly in the roots at all the Al doses tested. This increase was 34, 37, 29 and 46%, for the concentrations of 200, 400, 600 and 800 µM, respectively (Fig. 7f). As expected, we observed an increase in the levels of MDA in the roots of the plants for all the concentrations of Al tested, this increase was 15, 23, 43 and 27%, for the respective concentrations of 200, 400, 600 and 800 µM of Al, respectively) (Fig. 7h). We observed a significant decrease in malonaldehyde content formed in the leaves at all the Al concentrations tested.

**Aluminum toxicity modulated the expression of genes related to the ecdysteroid biosynthetic pathway**

In the ecdysteroid biosynthesis pathway, the expression of the *PgSpook* and *PgPhantom* genes related to the biosynthesis of 20-hydroxyecdysone was negatively influenced by to Al toxicity in the roots of *P. glomerata* at all the concentrations tested (Fig. 8b). We observed a 3-fold reduction in the expression of these genes in the roots in plants treated with 200 µM Al and this pattern of decrease was repeated in the other levels of to Al toxicity analyzed. On the other hand, in the leaves, we observed no significant difference in the expression of the *PgSpook* gene in Al-stressed plants compared to the control (Fig. 8a). Similarly, the *PgPhantom* gene was negatively regulated in leaves and roots at least once by to Al toxicity in *P. glomerata* plants in vitro (Fig. 8c -d).

**Al modulated the expression of genes related to the lignin biosynthetic pathway**

In the phenylpropanoid biosynthesis pathway, the expression of the *PgCCR* gene related to lignin production was positively influenced by 0.7 times only by the highest level of Al tested (800 µM) (Fig. 9a). Conversely, the *PgCCR* gene was negatively regulated (0.4 - 0.6 - 0.6 and 0.8 times) by to Al toxicity in the roots at all concentrations (200, 400, 600 and 800 µM of Al, respectively) (Fig. 9b). The expression of the *Cinnamyl alcohol dehydrogenase* (*PgCAD*) gene was positively regulated in *P. glomerata* leaves at all Al doses (Fig. 9c). On the other hand, this same gene showed no significant differences in its level of expression in roots, except for the highest concentration of Al (800 µM) showing a 0,7-fold reduction in its level of expression, differing significantly from the control (Fig. 9d).

With regard to the expression of *PgC4H* in leaves, at concentrations of 200, 400, 600 and 800 µM of Al, there was a 0.5 to 1.3-fold increase compared to the control (Fig. 9e). In contrast, the *PgC4H* gene had 0.4 and 0.7-fold reduction at concentrations of 600 and 800 µM of Al, respectively, compared to the control (Fig. 9f). The *PgCCoAOMT* gene did not differ significantly in its level of expression in leaves (Fig. 9g). In contrast, in roots, we observed a negative regulation (0.8 - 0.8 - 0.6 and 0.7-fold) for the concentrations of 200, 400, 600 and 800 µM of Al, respectively (Fig 9h).

**DISCUSSION**

This study investigated the effects of Al toxicity in morphophysiological and molecular responses of *P. glomerata* grown in vitro. The most common responses in the aerial part and leaves to Al toxicity were curling of young leaves, smaller leaves, purpling of stems and leaves, yellowing and death of leaf tips, chlorosis and leaf necrosis (Fig. 1). In the present study, Al promoted a reduction in root and shoot growth at concentrations above 600 Al, ultimately resulting in a significant reduction in total plant biomass (Fig. 2c-d). These statements agree with Maldaner et al. (2015), whose results indicated a reduction in root and shoot growth in two accessions of *P. glomerata* studied.

The inhibition of root growth caused by Al toxicity is the result of reduced cell division (Rahman *et al*., 2024). It points to a correlation between the inhibition of root elongation and the cessation of mitotic figures in the root apical meristem (RAM), interrupting normal cell division (Boniotti, 2022) and altering the integrity, coupling and polymerization of microtubules and the cytoskeleton. The degree of Al toxicity reported in the literature varies greatly depending on the plant species, growth conditions, Al concentrations and duration of exposure. Here, we observed that concentrations of Al equal to or greater than 600 caused more significant decreases in the growth of *P. glomerata* plants.

Interestingly, leaf area was maintained in plants treated with 200 and 400 μM of Al, while higher concentrations caused a drastic decrease in leaf area. On the other hand, no decrease in leaf number was observed in response to Al application, demonstrating that the photosynthetically active area is maintained to sustain gas exchange rates and the photosynthesis process, even under stress conditions. Some studies have reported a compensatory system in which the reduction in leaf area may be associated with an increase in cell division, which allows chloroplast density to be adjusted to modulate the photosynthetic rate (Ferjani *et al*., 2007; Jiang *et al*., 2012). Al toxicity drastically reduced total root length by at least 30% at all Al concentrations tested, the lower robustness of the root system of plants exposed to the Al stressor is associated with a decrease in the diameter of these roots, slow root elongation and deformed cells, resulting in poor development of root hairs and damage to root apices (Panda *et al*., 2009, Engel *et al*., 2021).

The Al concentration in the leaves of *P. glomerata* increased with increasing Al availability in the growing medium (Fig. 3), showing a linear response to increasing Al concentration (Fig. 3). We speculate that $Al^{3+}$ reached the conductive vessels in a mostly symplastic flow, crossing the membranes involving the expenditure of anergy and promoting

the influx of hydrogen ions in a diffusion process (Rahman *et al*., 2024). It is important to note that the influx of these ions ($H^+$) is a process that raises the pH of the rhizosphere (or growing medium) and consequently reduces the toxic effects of Al on the roots, thus constituting an adaptive strategy of plants to Al toxicity conditions.

In addition, accompanied by the entry of Al and interaction with similar targets, intracellular translocation is hampered by the formation of complexes with various organic acids (e.g. malate, oxalacetate and citrate), inactivating or compartmentalizing in vacuoles to maintain low levels of free $Al^{3+}$ in the cytosol of the plant, through various transporter proteins present in the membrane, maintaining the state of homeostasis and cell rigidity, which will favor the maintenance of growth and development of plants under stress (Sade *et al*., 2016; Gupta *et al*., 2013; Nunes-Nesi *et al*., 2014; Yang *et al*., 2019; Sami *et al*., 2020). Here, we suggest that the Al accumulated in roots and leaves was compartmentalized in vacuoles to minimize its harmful effects on different cellular compartments (chloroplasts, mitochondria, peroxisomes), correlating with the increase in the content of malate and fumarate, two important organic acids in this process, which were significantly higher in plants treated with 600 μM of Al.

Lower photosynthetic rates in plants under Al toxicity can also be associated with a decrease in the content of photosynthetic pigments. Here, we observed a decrease in chlorophyll content, followed by an increase in carotenoid content in plants treated with Al, indicating that the biosynthesis of these pigments may be under negative regulation by Al, since the increase in, for example, ROS in chloroplasts may damage the photosynthetic apparatus, compromising what we call optimal photosynthesis and proper leaf development, since they depend on the spatio-temporal regulation of Chl. metabolism (Nong *et al*., 2024). The impact of Al toxicity on photosynthesis is indirect. Due to Al toxicity, there is a disturbance in the architecture of the chloroplast. In addition, there is a decrease in photosynthesis due to reduced electron transport in photosystem II (PSII) (Panda et al., 2009).

Plant growth typically results in the accumulation of starch and soluble sugars (De la Mata *et al*., 2012), here we show that plants under Al toxicity showed a high accumulation of glucose and fructose and to a lesser extent sucrose from Al concentrations of 600, possibly to sustain their initial growth under to toxicity conditions. Proline is an amino acid involved in various physiological processes and plant development (Spormann *et al*., 2023). In addition to being a proteinogenic amino acid, proline contributes to stress mitigation as a compatible solute for osmotic adjustment, a ROS scavenger, and a molecular chaperone stabilizing proteins and membranes. In addition, changes in proline metabolism can affect the cellular redox state, requiring metabolic adjustments (Ghosh *et al*., 2021). We observed an increase in proline levels

at all levels of Al toxicity. Exposure to metal altered proline levels, suggesting involvement in defense against metal-induced damage

The increase in proline levels has been suggested as a plant defense strategy against various types of stress (Felipe *et al*., 2019; Sousa *et al*., 2020; Fortini *et al*., 2023). We speculate that the role of proline contributed to buffering cytosolic pH and may act as a source of energy, carbon and nitrogen to support plant growth after stress relief (Szabados *et al*., 2010; Kishor *et al*., 2014).

In addition to Al detoxification mediated by organic acids, stress response genes, and morphological adaptations, plants have also developed antioxidant defense mechanisms to regulate and adapt to Al-induced oxidative stress. Here, *P. glomerata* plants exhibited enhanced antioxidant defense mechanisms in response to Al toxicity, as indicated by increased levels of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) activities (Fig. 6), and membrane damage, suggested by increased MDA production mainly in the roots (Fig. 6g-h). Activation of the antioxidant machinery prevents excessive accumulation of EROS, thus minimizing oxidative damage induced by Al toxicity, preventing damage to membrane lipids, proteins and DNA, which could ultimately lead to programmed cell death (Sami *et al*., 2020). We observed increased lipid peroxidation levels in the roots for all the Al concentrations analyzed. Similarly, Bernardy et al. (2020) observed an elevated MDA content in *P. glomerata* roots in response to high doses of zinc.

The interaction of Al with cell wall biomolecules and symplastic components, especially in root apices, disturbs the homeostasis of biomolecules and leads to an increase in the production of reactive oxygen species (ROS), due to the high reactivity of the stressor, which promotes potential binding to epidermal and cortical root cells, the surface of the plasma membrane, the cell wall, the nucleus and the cytoskeleton (Panda *et al*., 2009; Manquián-Cerda *et al*., 2018). For example, the interaction of Al ions with the cell plasma membrane of roots stimulates the composition of callose, restricting cell-to-cell transport and disturbing the redox balance (Sun *et al*., 2020). This can be proven by the significant increase in POD and SOD activity, especially in roots. It is known that enzymatic metabolism can act as a detoxification agents and play an important role in reducing oxidative damage to cellular components (Kesawat *et al*., 2023). In addition, Al interfered with the proper functioning of the plasma membrane through interaction with lipids, causing lipid peroxidation (Kochian, 2015). In conclusion, Al-induced ROS production can serve as signaling molecules to provoke changes in the expression of enzymes responsible for scavenging ROS, including hydrogen peroxide (H

$_2O_2$), hydroxyl radical ($HO^-$), and singlet oxygen ($_1O_2$), produced mainly in mitochondria, chloroplasts or peroxisomes (Guo *et al*., 2023).

Al promoted differential responses in the expression of genes involved in modulating the biosynthesis of phytoecdysteroids. The de novo assembly of the *P. glomerata* transcriptome revealed several genes of the CYP P450 family related to secondary metabolism and stress, which confers a dynamic plasticity of these genes (Batista *et al*., 2019). Here we evaluated the relative expression level of the Spook and Phantom genes, members of this family and previously involved in the biosynthesis of the phytoecdysteroid 20-hydroxyecdysone (20E) in *P. glomerata* under abiotic stresses (Batista *et al*., 2019). Interestingly, we found that the *PgSpook* gene (CYP83A) was negatively regulated at all levels of Al exposure, mainly in roots (Fig. 8 a-b).

The same regulation pattern was observed for the *PgPhanton* gene (CYP76C), which was negatively regulated in roots (5 to 0.5 times) and leaf tissues (5 times) in response to Al toxicity (Fig. 8 b-c). Our results differ from those found by Felipe *et al*. (2019), who showed that salt stress and UV-B radiation up-regulated the *Spook* and *PgPhantom* genes of the CYT P450 gene family in *P. glomerata*, suggesting the involvement of these genes in relieving the imposed stress resulting in fluctuations in the expression levels of genes related to 20E production. Furthermore, in *Panax ginseng*, heavy metal interactions with the cell membrane increased the expression of several defense-related genes via the jasmonic acid pathway (Balusamy *et al*., 2013).

In relation to the genes involved in the phenylpropanoid metabolic network also belonging to the multigenic CYT family (CYP73, CYP98 and CYP84) (Renault *et al*., 2017; Hansen *et al*., 2021), we analyzed the expression of *PgCCR*, *PgCAD*, *PgC4H* and *PgCCoAOMT* involved in the construction of structural biopolymers such as lignin and its fractions, suberin and cutin (Fig. 9). Al promoted positive regulation of *PgCCR* only at the highest concentration of Al tested in leaves. On the other hand, due to its strong binding to cell walls, Al promoted a negative regulation of *PgCCR* in roots, possibly due to the saturation of active sites by the $Al^{3+}$ ion, which may be altering the integrity, coupling and polymerization of microtubules and the cytoskeleton via Al toxicity. We observed a significant increase in Al-mediated *PgC4H* gene expression in leaves and a negative increase in roots.

Al induced the negative regulation of the genes of the enzymes involved in lignin biosynthesis *PgCCoAOMT* in *P. glomerata* roots. In leaves, we did not observe a significant difference in the expression of this gene. Thus, *caffeoylCoAO-methyltransferases* (C*CoAOMTs*)

catalyze the transfer of a methyl group from S-adenosylmethionine to a hydroxyl portion of *caffeoyl-CoA* as part of the lignin biosynthetic pathway (Weng *et al*., 2010).

**CONCLUSION**

In general, our data show that in *P. glomerata*, Al induced damage to photosynthesis, decreasing Chl*a* content, compromising carbon assimilation and limiting growth and biomass gain. On the other hand, concentrations equal to or greater than 600 µM of Al promoted the accumulation of carbohydrates (glucose and fructose) and proline at all levels of Al tested. In stressed plants, we observed a negative regulation for the genes involved in the 20E biosynthesis pathway (*PgSpook* and *PgPhanton*), suggesting a decrease in the content of this metabolite in *P. glomerata* leaves and roots. For the genes of the phenylpropanoid biosynthesis pathway, a precursor mainly of lignin, we observed an up-regulation in three genes (*PgCCR, PgCAD*, *PgC4H*) in leaves and a down-regulation in roots. Our data taken together reinforce the hypothesis that *P. glomerata* plants show moderate tolerance to Al toxicity through modulation of primary metabolism and redox metabolism and fluctuation in the expression of genes related to cell wall composition mainly in roots and in 20-E biosynthesis (non-enzymatic metabolism), together with structural changes in the morphology of *P. glomerata* grown in vitro.

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**FIGURES**

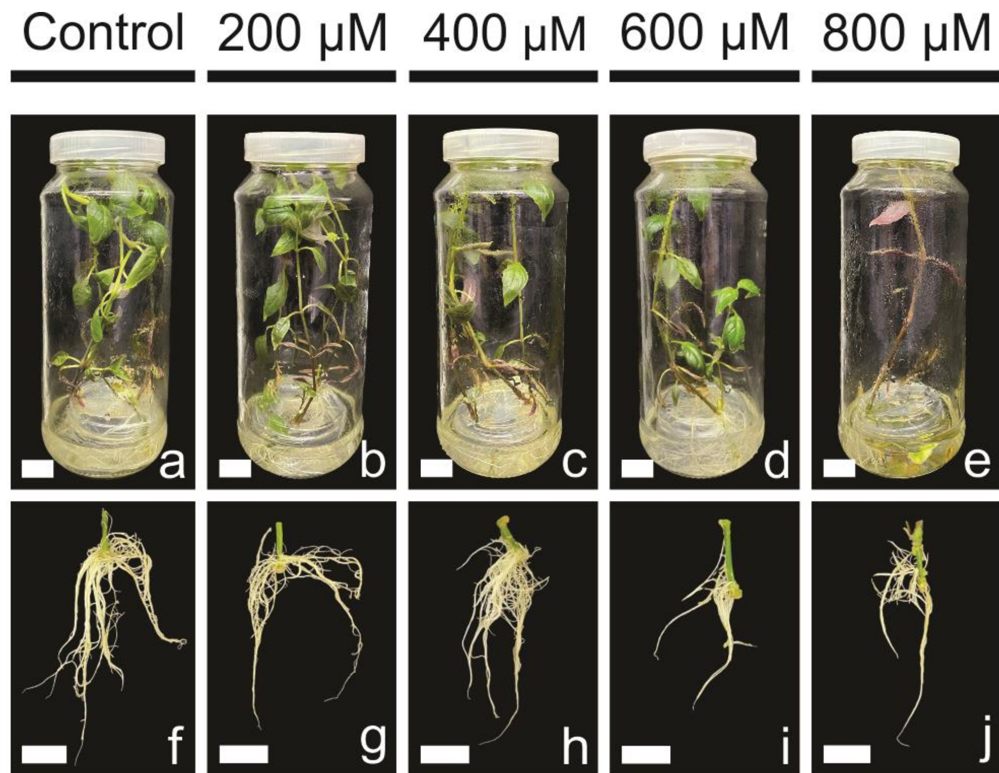



**Figure 1**. Plants of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: (**a-f**) Control; (**b-g**) 200 µM; (**c-h**); (**c-h**) 400 µM; (**d-i**); (**e-j**) 600 µM and (**d-i**) 800 µM of Al. Scale bars = 30 mm.

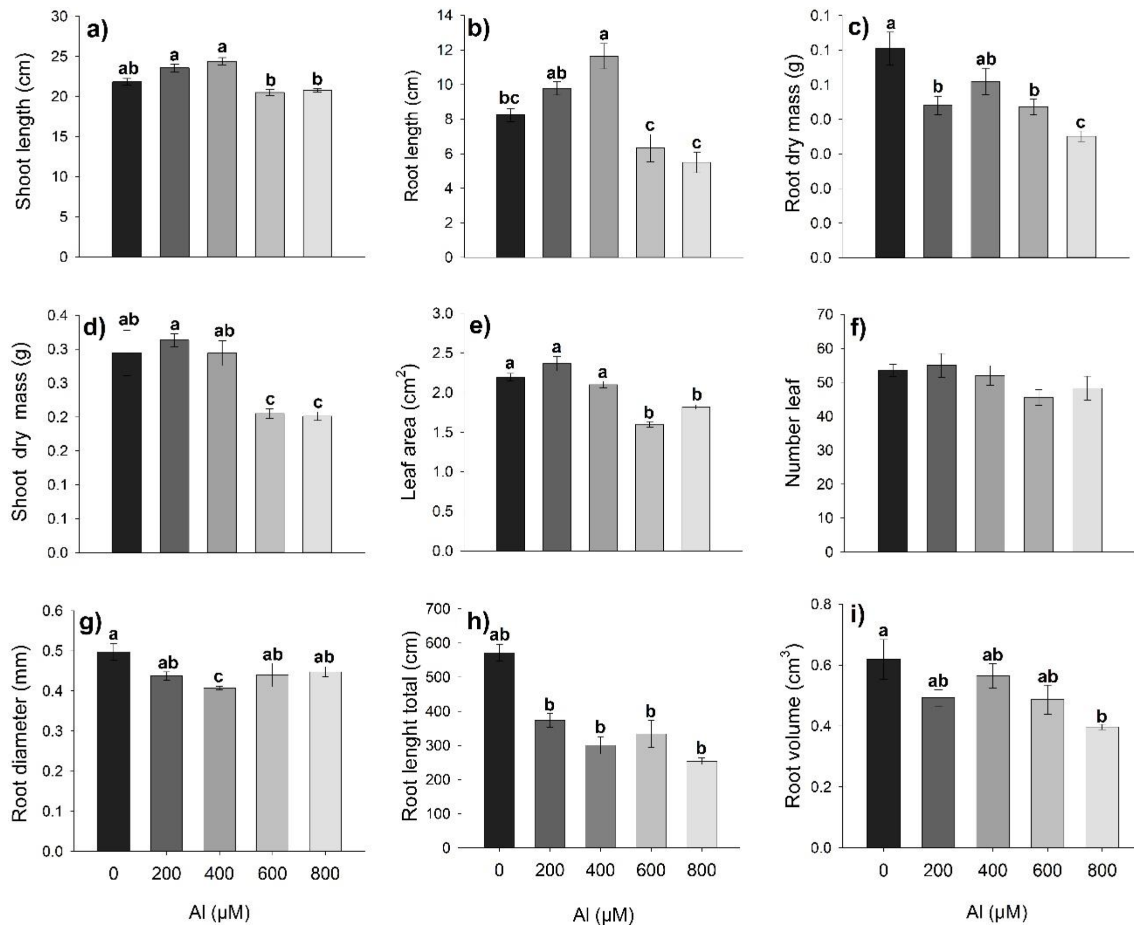



**Figure 2**. Growth parameters of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: Control; 200, 400, 600 and 800 µM of Al. (**a**) Shoot length; (**b**) Root length; (**c**) Root dry mass; (**d**) Shoot dry mass; (**e**) Leaf area (cm$^2$ plant$^{-1}$); (**f**) Number leaf; (**g**) Root diameter; (**h**) Root length total and (**i**) Root volume (cm$^{-3}$). Values are presented as means (n=4) ± standard error. Averages followed by equal letters did not differ according to the Tukey test ($P \leq 0.05$).

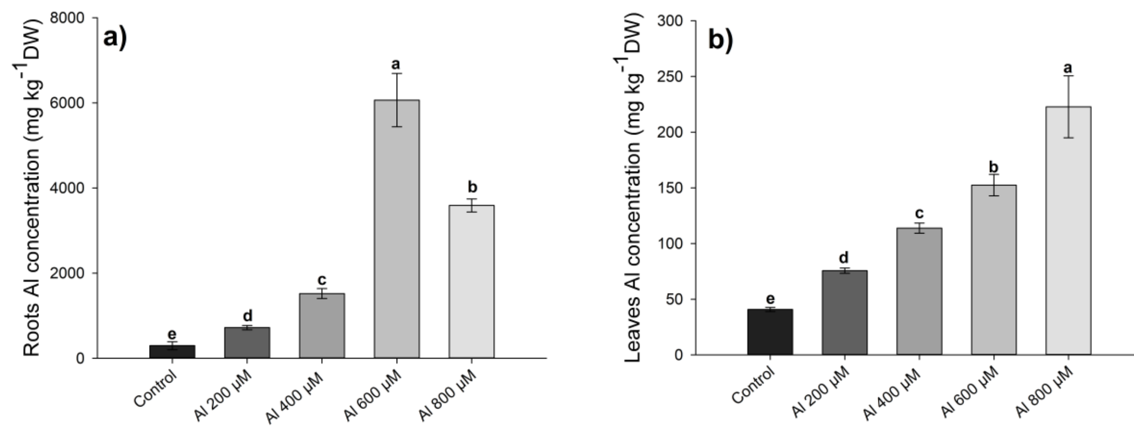



**Figure 3.** Quantification of Al in *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: Control; 200, 400, 600 and 800 µM of Al. (**a**) quantification Al in roots; and (**b**) quantification Al in leaves. Values are presented as means (n=4) ± standard error. Means followed by equal letters did not differ according to the Tukey test ($P \leq 0.05$).

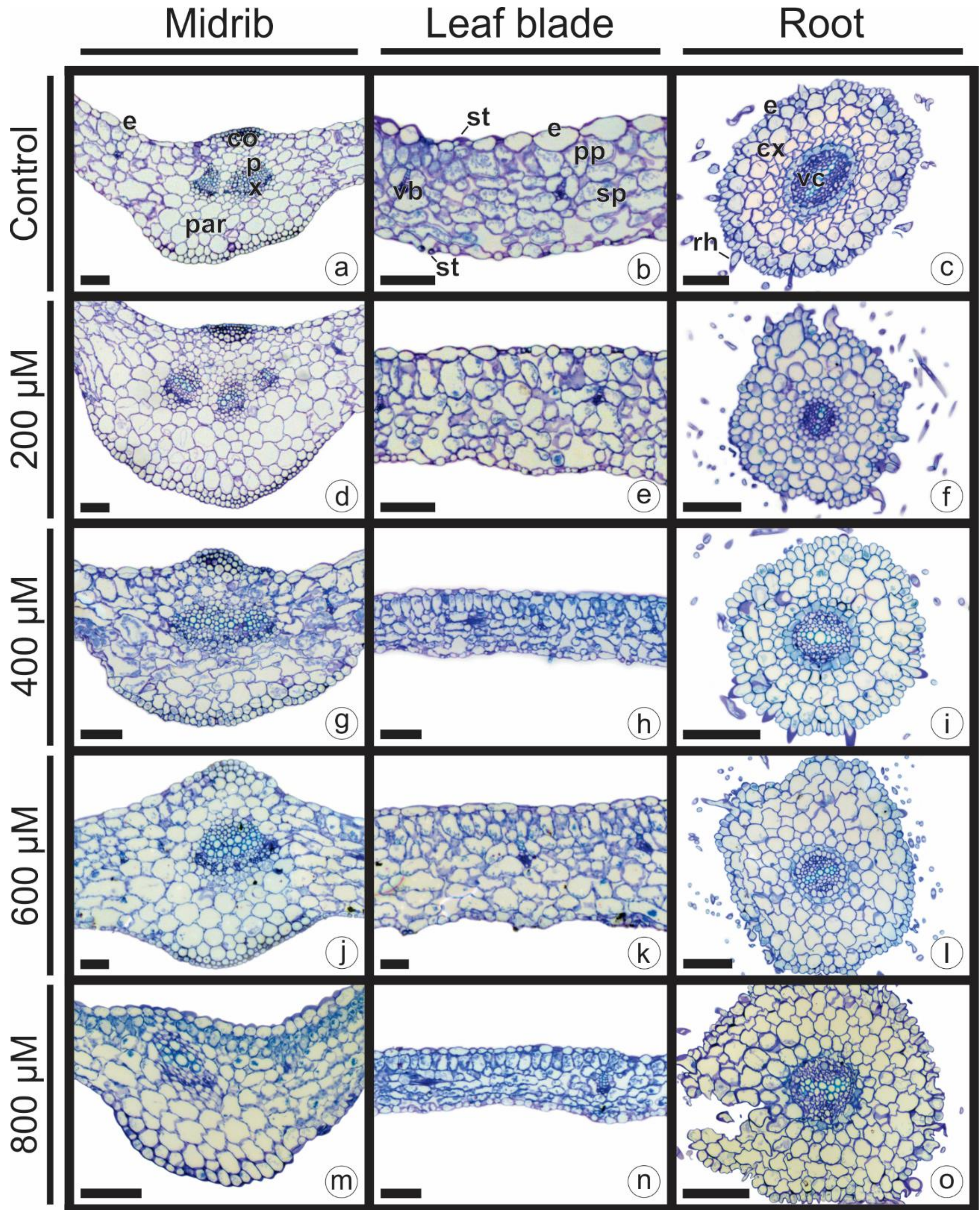

Midrib
Leaf blade
Root
Control
200 µM
400 µM
600 µM
800 µM
e
co
p
x
par
st
e
pp
vb
sp
st
e
cx
vc
rh
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o

**Figure 4.** Anatomical characterization of leaves (midrib and leaf blade) and roots in cross-section of *Pfaffia glomerata* plants after 40 days of in vitro cultivation under the following conditions: Control; 200, 400, 600 and 800 µM of Al. *Abbreviations: e* – epidermis; *co* – collenchyma; *fc* – fascicular cambium; *ic* – interfascicular cambium; *par* – parenchyma; *p* – phloem; *pp* – palisade parenchyma; *sp* – spongy parenchyma; *st* – stomata; *sx* – secondary xylem; *vb* – vascular bundle; *x* – xylem. Scale bars: *100 µm*.

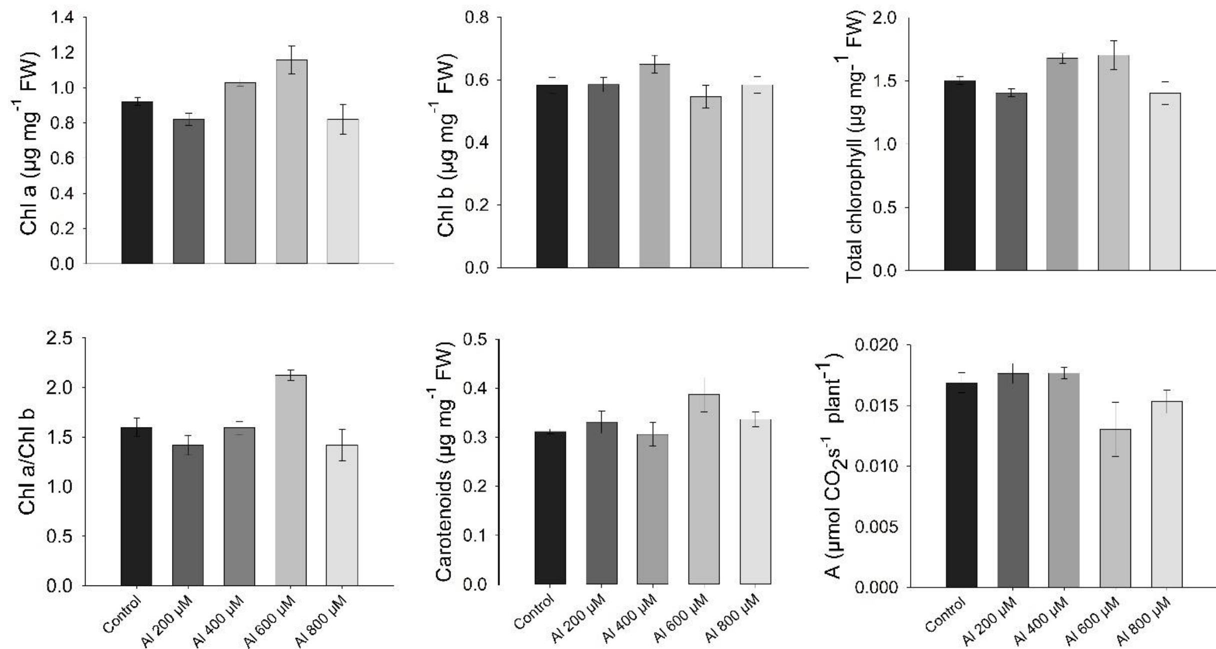



**Figure 5**. Leaf pigment content and photosynthetic rate of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: Control; 200, 400, 600 and 800 µM of Al. (**a**) chlorophyll *a*; (**b**) chlorophyll *b*; (**c**) total chlorophylls; (**d**); chlorophyll *a* and chlorophyll *b* ratio (Chl *a*/Chl *b*); (**e**) carotenoids; and (**f**) *in vitro* photosynthetic rate (µmol $CO_2$ $S^{-1}$ $plant^{-1}$). Values are presented as means (n=4) ± standard error. Values are presented as means (n=4) ± standard error. Means followed by equal letters did not differ according to Tukey's test ($P \leq 0.05$).

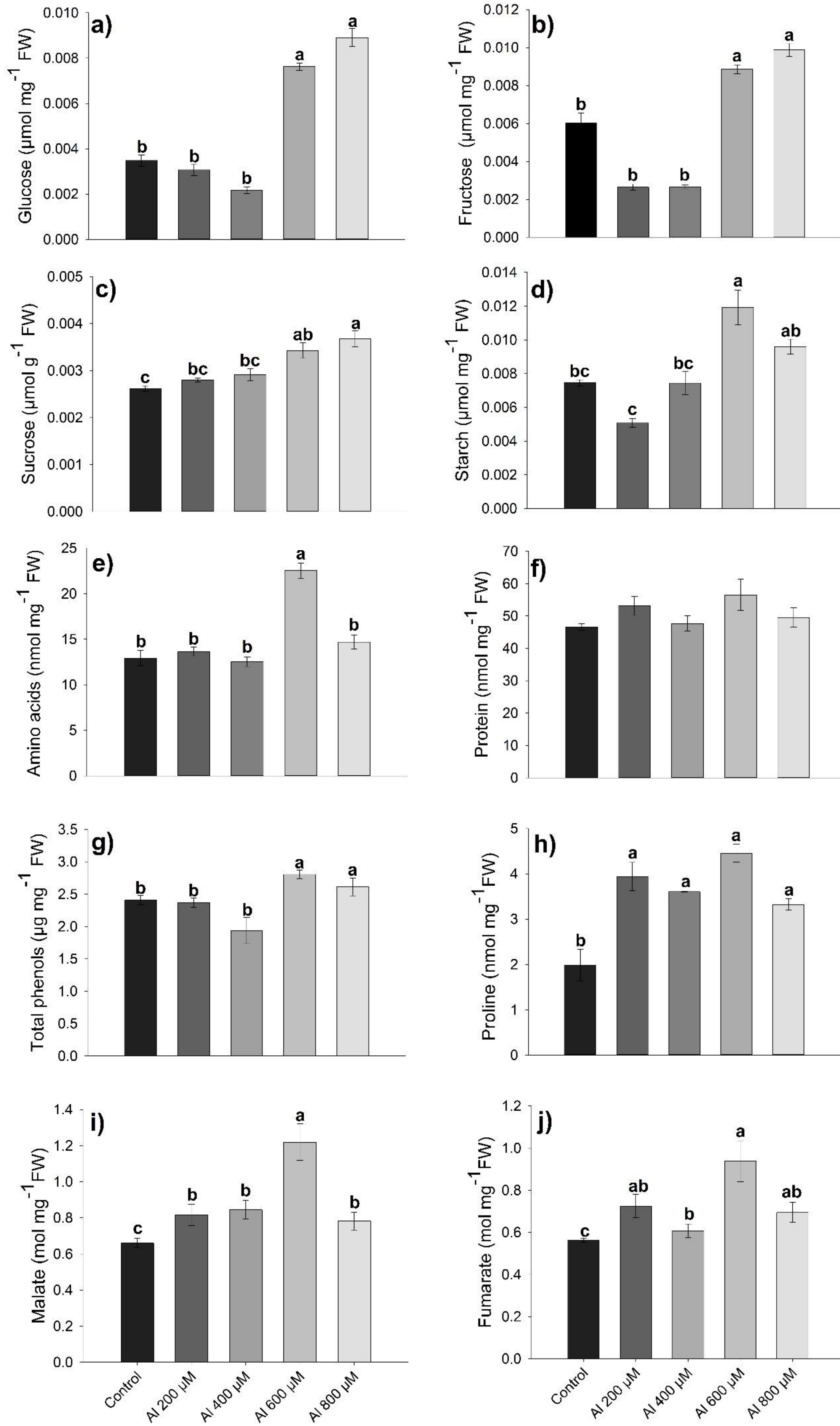
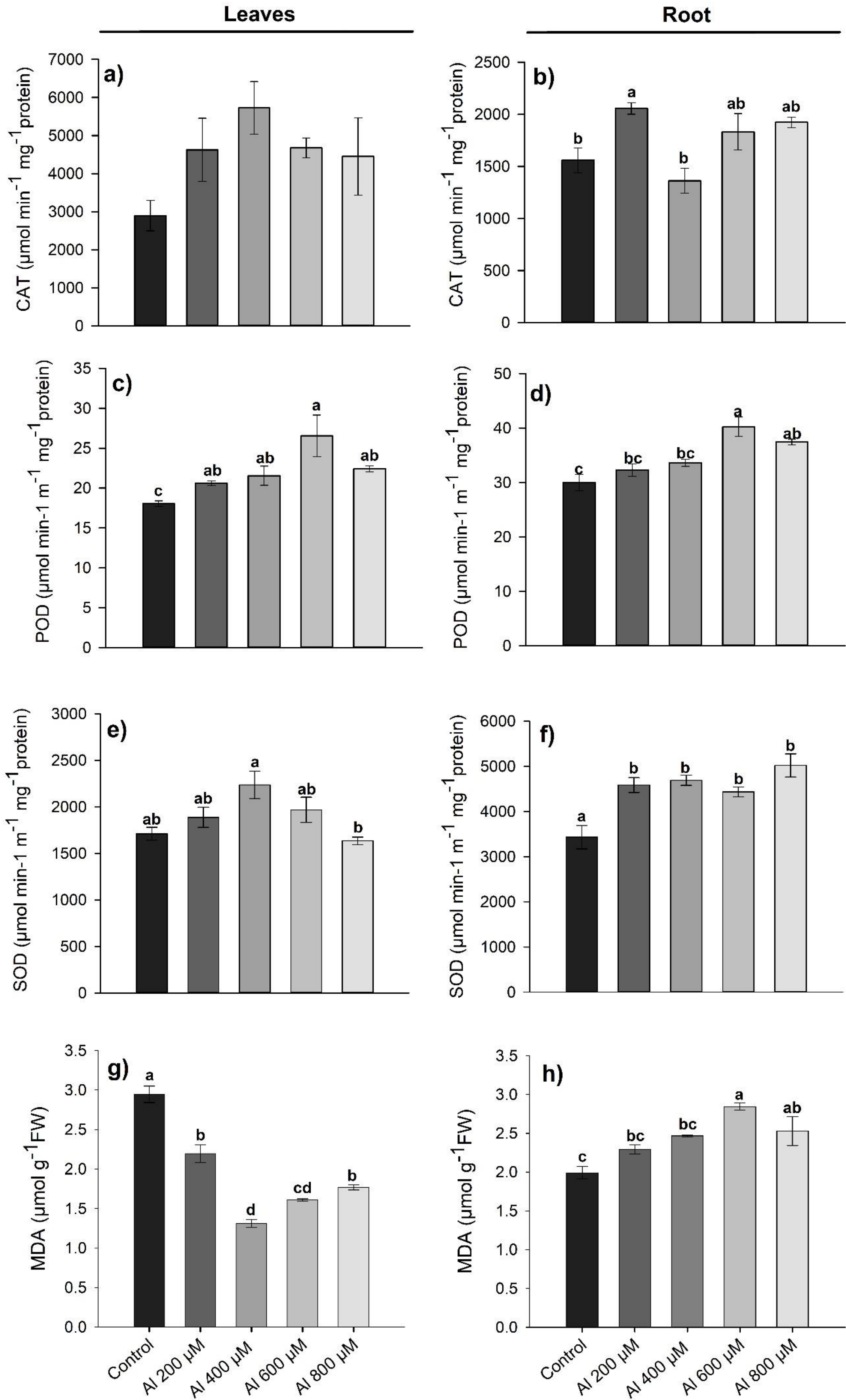

a)
Glucose (µmol mg-1 FW)
b)
Fructose (µmol mg-1 FW)
c)
Sucrose (µmol g-1 FW)
d)
Starch (µmol mg-1 FW)
e)
Amino acids (nmol mg-1 FW)
f)
Protein (nmol mg-1 FW)
g)
Total phenols (µg mg-1 FW)
h)
Proline (nmol mg-1 FW)
i)
Malate (mol mg-1 FW)
j)
Fumarate (mol mg-1 FW)
Control
Al 200 µM
Al 400 µM
Al 600 µM
Al 800 µM

**Figure 6.** Changes in the primary metabolism of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: Control; 200, 400, 600 and 800 μM of Al. (**a**) glucose; (**b**) fructose; (**c**) sucrose; (**d**) starch; (**e**) total proteins; (**f**) total amino acids; (**g**) total phenols and (**h**) proline. Values are presented as means (n = 4) ± standard error. Averages followed by equal letters did not differ according to Tukey's test ($P \leq 0.05$).



Leaves
Root
a)
b)
c)
d)
e)
f)
g)
h)
CAT (µmol min$^{-1}$ mg$^{-1}$protein)
POD (µmol min-1 m$^{-1}$ mg$^{-1}$protein)
SOD (µmol min-1 m$^{-1}$ mg$^{-1}$protein)
MDA (µmol g$^{-1}$FW)
Control
Al 200 µM
Al 400 µM
Al 600 µM
Al 800 µM

**Figure 7.** Antioxidant enzyme activity of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: control; 200, 400, 600 and 800 µM of Al. (**a**) Catalase - CAT (leaves); (**b**) Catalase - CAT (root); (**c**) Peroxidase - POD (leaves); (**d**) Peroxidase - POD (root); (**e**) Superoxide dismutase - SOD (leaves); (**f**) Superoxide dismutase - SOD (root); (**g**) malondialdehyde content – MDA (leaves) and (**h**) malondialdehyde content – MDA (roots). Values are presented as means (n=4) ± standard error. Means followed by equal letters did not differ according to the Tukey test ($P \leq 0.05$).

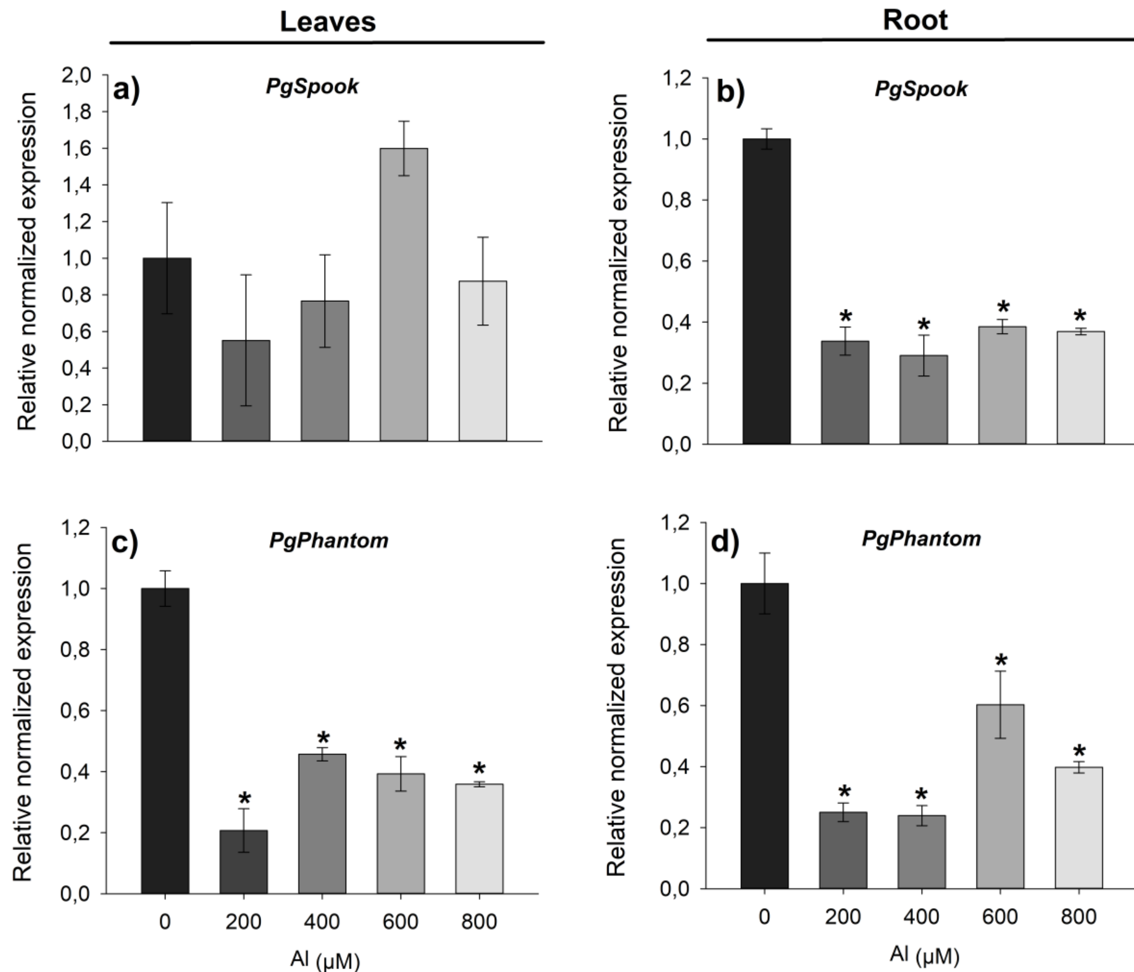
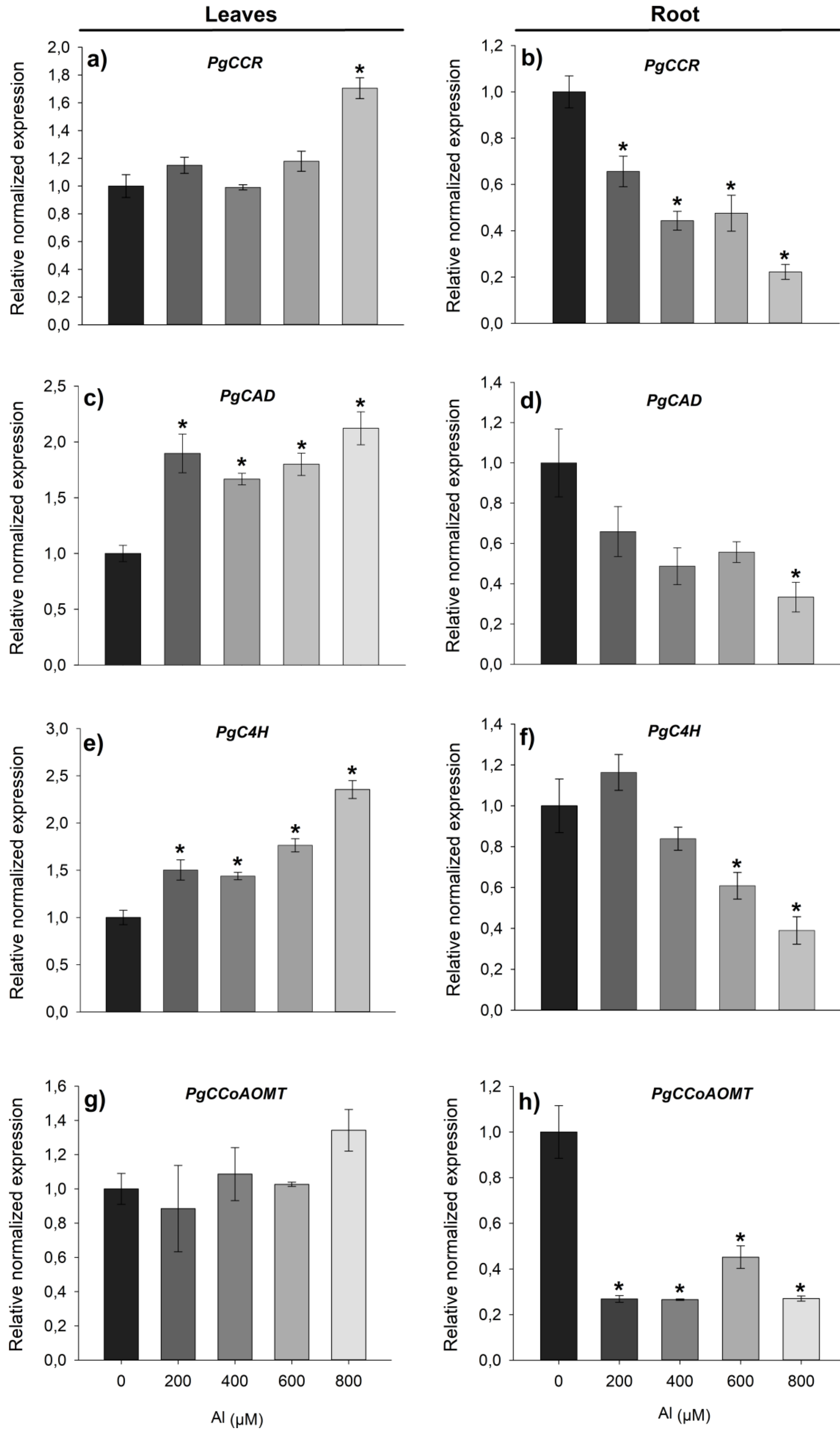



**Figure 8**. Relative expression of genes from the 20-hydroxyecdysone synthesis pathway in *P. glomerata* plants grown in vitro for 40 days under the following conditions: control; 200, 400, 600 and 800 µM of Al. (**a**) *PgSpook*; (**b**) *PgSpook*; (**c**) *PgPhantom*; (**d**) *PgPhantom*. Expression was normalized by the glyceraldehyde-3-phosphate dehydrogenase gene. Values are presented as means (n = 3) ± standard error. Asterisks indicate a significant difference by Dunnett's test ($P \leq 0.05$).



Leaves
Root
a)
PgCCR
b)
PgCCR
c)
PgCAD
d)
PgCAD
e)
PgC4H
f)
PgC4H
g)
PgCCoAOMT
h)
PgCCoAOMT
Relative normalized expression
0
200
400
600
800
Al (µM)

**Figure 9**. Relative expression of lignin synthesis pathway genes in *Pfaffia glomerata* plants grown in vitro for 40 days under the following conditions: control; 200, 400, 600 and 800 µM of Al. (**a**) *PgCCR*; (**b**) *PgCCR*; (**c**) *PgCAD*; (**d**) *PgCAD*; (**e**) *PgC4H*; (**f**) *PgC4H*; (**g**) *PgCCoAOMT*; (**h**) *PgCCoAOMT*. The glyceraldehyde-3-phosphate dehydrogenase gene normalized expression. Values are presented as means (n=3) ± standard error. Asterisks indicate a significant difference by Dunnett's test ($P \leq 0.05$).

## Chapter 2 - Positive modulation of melatonin application on physiological and molecular parameters under aluminum toxicity in *P. glomerata*

### Abstract

Aluminum (Al) toxicity is the main factor limiting agricultural production in acidic soils around the world. Excess Al inhibits the growth and function of roots and consequently damages crop development. That is why scientists worldwide are trying to develop technologies and products to generate plants that are more resistant to metal stress. Plant growth regulators, such as melatonin (MET), have been considered a promising approach to improving crop tolerance to abiotic stress conditions, including stress caused by heavy metals. Here, we investigated the changes in redox metabolism involved in the morphophysiological responses of *Pfaffia glomerata*, a medicinal plant, to Al toxicity and MET application. In addition, we investigated how these changes affected the biosynthesis of 20-hydroxyecdysone (20E), a metabolite of pharmacological importance. Compared to the control, the plants were grown in vitro for 40 days in Murashige and Skoog medium supplemented with Al, MET and Al + MET. We observed reduced growth, pigment content and photosynthetic rate under Al toxicity. In addition, there was a reduction in the content of total amino acids and proteins and an increase in the content of total phenols. On the other hand, all these parameters were improved in plants supplemented with MET under Al toxicity. MET reduced global DNA endoreduplication levels and induced 120 differentially accumulated proteins (DPAs), of which 11 were up-regulated by Al, 23 by MET and 24 for the Al+MET interaction. 24, 22 and 26 proteins were down-accumulated in Al, MET and Al+MET, respectively. Gene ontology and cluster analysis revealed an increase in the relative abundance of proteins involved in transmembrane transport, energy-intensive metabolic transport, energy production in plants such as photosynthesis, ascorbate metabolism, oxidative phosphorylation, carbon metabolism, pentose phosphate and other metabolic pathways associated with the biosynthesis of amino acids (e.g. phenylalanine and tryptophan) and secondary metabolites. DAPs were predominantly present in vacuoles, the cell wall, chloroplasts, and apoplasts. Our results suggest that modification of molecular structure is one of the leading causes of cell damage, and vacuolar sequestration of Al ions is an important mechanism of Al resistance in plants. Overall, our results indicate a possible role for MET in protecting *P. glomerata* against Al toxicity, paving the way for future studies.

**Keywords**: brazilian-ginseng, endorreduplication DNA, flow cytometry; lignin biosynthesis, melatonin, 20-hydroxyecdysone; proteomics.

**INTRODUCTION**

Aluminum (Al) is the most abundant metal in the earth's crust and limits crop production in acidic soils worldwide (Singh *et al*., 2017; Rahman *et al*., 2021). Al is solubilized in the liquid phase as a trivalent aluminum ion ($Al^{3+}$) in acidic soil with a pH below 5, disrupting the physiological and metabolic functioning of plants. Excess Al inhibits cell division and, root elongation, root hair formation, which in turn makes them inefficient at absorbing water and nutrients (Yang and Horst *et al*., 2015; Bojórquez-Quintal *et al*., 2017; Singh *et al*., 2017; Kar *et al*., 2020). In addition to causing a decrease in photosynthetic rate, stomatal conductance and leaf transpiration rate in plants (Sade *et al*., 2016; Cárcamo *et al*., 2019; Phukunkamkaew *et al*., 2021), leading to the overproduction of reactive oxygen species (ROS), lipid peroxidation and accumulation of various osmolytes, causing significant yield losses (Singh *et al*., 2017; Chauhan *et al*., 2021). In addition, Al induces genotoxic stress, causing damage to DNA nuclear composition and replication (Grupa *et al*., 2013; Singh *et al*., 2017).

However, some plants have developed resistance mechanisms to Al stress conditions, including modifications in metabolism and Al-induced exudation of organic acid (OA) anions, including citrate, malate and oxalate from roots, cell wall alterations, vacuolar compartmentalization of Al (Nunes-Nesi *et al*, 2014; Kochian *et al*., 2015; Singh *et al*., 2017; Phukunkamkaew *et al*., 2021), as well as DNA repair (Gupta *et al*., 2013 Eekhout *et al*., 2017).

The most accepted mechanism for increasing Al resistance in plants concerns the neutralization of metals with OA, neutralizing them and the formation of OA-metal complexes in the rhizosphere (Nunes-Nesi *et al*., 2014; Kochian *et al*., 2015). On the other hand, an increase in OA production does not always mean greater tolerance to Al, since Al-sensitive genotypes produce and exude large amounts of Al (Kochian *et al.,* 2005). In addition, in recent years, the most recently discussed mechanism for improving Al tolerance has been based on genetic manipulations of DNA checkpoints, which allows cell cycle progression in the presence of Al (Eekhout *et al*., 2017).

The use of emerging plant growth regulators such as melatonin (N-acetyl-5-methoxytryptamine - MET) has been considered a promising approach to improve crop tolerance to abiotic stress conditions such as drought, salt and heavy metals, which is why it has attracted the attention of researchers around the world (Dawood, 2022; Hassan *et al*., 2022). In plants, MET is reported as a biostimulator of plant growth, especially under conditions of environmental stress, constituting a multifunctional signaling molecule (Debnath *et al*., 2019; Zhang *et al*., 2019; Back *et al*., 2020) and many studies have been carried out on MET,

reinforcing its positive role in plants (Antoniou *et al*., 2017; Zhang *et al*., 2019; Zeng *et al*., 2022; Hussain *et al*., 2024).

Evidence shows that MET improves chlorophyll content, photosynthetic efficiency, protein accumulation and RuBisCO activities, in addition to activating the antioxidant defense system, presenting great importance as a free radical scavenger, promoting relief from stress-induced oxidative damage and mediating various physiological responses, including rooting, seed germination, flowering, aging, fruit ripening, and senescence (Shi *et al*., 2015; Arnao and Hernández-Ruiz 2020; Kłodziejczyk *et al*., 2021; Hassan *et al*., 2022), representing a new and effective strategy for crop improvement (Zhang *et al*., 201; Khan *et al*., 2020).

Previous studies have shown that exogenous application of MET triggered an antioxidant defense system under stress conditions, favoring the elimination of ROS and acting as a stress-protective molecule (Khan *et al*., 2020). MET improves carbon and nitrogen metabolism in maize leaves under Al stress (Ren *et al*., 2022). In addition, MET reduced Al toxicity, attenuated EROS accumulation and increased antioxidant activity in pea (*Pisum sativum*) (Ahmad *et al*., 2023), walnut (*Carya cathayensis*) (Sharma *et al*., 2020) and tomato (*Solanun lycopersicum*) plants (Ghorbani *et al*., 2023). MET also modulated the antioxidant defense system and secondary metabolites and reduced oxidative stress in rice (Jan *et al*., 2023). However, studies demonstrating that MET alleviates abiotic stress focus mainly on model plants, and the molecular mechanisms underlying MET remain elusive, especially in medicinal plants.

*Pfaffia glomerata*, popularly known as fáfia or brazilian-ginseng, is widely used in traditional medicine because of its medicinal properties such as immunostimulant, sedative, hypocholesterolemic, analgesic, anabolic, anti-inflammatory and other functions, which have already been described in the literature (Nascimento *et al*., 2007; Neves *et al*., 2016; Dias *et al*., 2019; Franco *et al*., 2021; Ribeiro *et al*, 2024). These medicinal properties are mainly due to the compounds 20-hydroxyecdysone (20E), pterosterone, polypodin B, and and triterpens (Shiobara *et al*., 1993). Among these compounds, 20E is the majority in *P. glomerata* and its active principles are obtained from its roots by extraction (Kamada *et al*., 2009; Neves *et al*., 2016; Dinan *et al*., 2021). Due to its pharmacological properties, 20E has been widely studied, and it has been found that different environmental changes modulate the production of 20E in *P. glomerata*.

This study explores the physiological and biochemical role of MET under to Al toxicity and its effects on *P. glomerata*. We also discuss the modulation of protein profile and 20E

production and the expression of key genes of this metabolite. The knowledge generated here will contribute to future research to provide insights into MET-mediated Al stress tolerance in medicinal plants.

## MATERIALS AND METHODS

### Obtaining plant material and in vitro cultivation

### Plant material and in vitro cultivation

The work was carried out at the Plant Tissue Culture Laboratory (LCT) of the Institute of Biotechnology Applied to Agriculture (BIOAGRO) of the Federal University of Viçosa (UFV). Access to genetic material (permission number AA2A367) was granted by the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under existing Brazilian biodiversity legislation.

The plants (Alto do Caparaó accession) used in the experiments came from the LCT-BIOAGRO in vitro germplasm bank and were grown monthly in MS culture medium (Murashige and Skoog, 1962) supplemented with myo-inositol (100 mg $L^{-1}$) and 3% sucrose (w/v) (Sigma-Aldrich Co, St Louis, MO, USA), solidified with 5.5 g $L^{-1}$ of agar (PhytoTechnology Laboratories®, Kansas, United States).

Three nodal segments (accession Caparaó), about 2 cm long, were inoculated into glass flasks each containing 100 mL-aliquots of liquid MS medium. The nodal explants were placed in adapted polypropylene supports. The flasks were sealed with rigid polypropylene lids with two 10 mm holes covered with 0.45-µm-pore size hydrophobic fluoropore membranes (MilliSeal® AVS-045 Air Vent, Tokyo, Japan)., which allow a gas exchange rate with the external medium of 25 µL $L^{-1}$ $s^{-1}$ of $CO_2$ (Batista *et al.*, 2017) The cultures were maintained at 25 ± 2 °C, under an irradiance of 60 µmol $m^{-2}$ $s^{-1}$ and a 16-h photoperiod for 40 days.

The treatments consisted of (I) control; (II) Al; (III) MET and (IV) Al + MET. To induce Al stress, 500 µM of Al was used and the concentration of melatonin applied was 100 µM (Sigma-Aldrich Co, St Louis, MO, USA). The pH was adjusted to 4.5 in all the conditions tested. The plants were kept in a growth room with a 16-h photoperiod, a temperature of 25 ± 2 °C and an irradiance of 60 µmol $m^{-2}$ $s^{-1}$ provided by two LED lamps (SMD 100, 18W, Vilux®, Vitória, ES, Brazil).

The experimental design used was entirely randomized with four growing conditions (: (I) control; (II) Al, stress; (III) MET, with the presence of; and (IV) Al and stress + MET). Each treatment had 20 replicates, with the experimental unit consisting of a flask containing three explants. The plants were grown for 40 days.

**Growth parameters**

The following growth variables were assessed: root, stem and leaf dry mass (g), total shoot length (cm), root length (cm), leaf area and number of leaves. In addition, the roots were placed in distilled water for analysis in the WinRhizo Pro system (Régent Instr. Inc.), and the following root growth variables were obtained: total length (cm), surface area ($cm^2$), diameter (cm) and volume ($cm^3$).

**Aluminum quantification**

The Al concentration was determined using 0.5 g of leaves and roots dehydrated at 50 °C for 72 h, which were crushed using a knife mill to a size smaller than 1 mm. The extracts were obtained by digestion in nitro perchloric solution (Malavolta *et al.*, 1989) and the Al content was determined using an inductively coupled plasma atomic emission spectrometer (ICP-OES, Optima 7300 DV PerkinElmer, Inc. Shelton, CT, USA).

**Histolocalization of aluminum in root apices**

The accumulation of Al in the root apices was analyzed by staining fresh tissues with ferric hematoxylin (Polle *et al.*, 1978). After exposing the plantlets to the treatments and control, the roots were dipped in a 0.2% (w/v) ferric hematoxylin solution in 0.02% (w/v) $NaIO_3$ for 10 min. The roots were transferred to containers containing deionized water to remove excess dye and washed for 30 min. Finally, the roots were removed and photographed under a stereomicroscope (Zeiss Stemi model DV4). The purplish color confirmed the positive reaction of hematoxylin with Al.

**Anatomical characterization**

After 40 days of cultivation in an induction medium, the explants were harvested and fixed in 4% Karnovsky's solution (Karnovsky, 1965), composed of: 2.5% glutaraldehyde, 4.0% paraformaldehyde, 5 mM CaCl2, in 0.1M cacodylate buffer, pH 6.8, and kept under refrigeration. The fixed samples were dehydrated in ethanolic series, embedded in methacrylate resin (Historesin, Leica®) and placed in plastic histomolds (Leica®). Five-micrometer-thick cross-sections were obtained on an automatic feed rotary microtome (RM2155, Leica

Microsystems Inc., USA) and stained with toluidine blue (pH 3.2) for 3 min (O'Brien and McCully, 1981). The specimens were mounted in Permount on glass slides. The images were captured using 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 a microcomputer with Spot Basic image capture software.

**Photosynthetic rate *in vitro***

Gas exchange and in vitro photosynthetic rate quantification were performed as proposed by Costa *et al*. (2014) with some modifications (Silva *et al*., 2020). Measurements and data collection were performed using an AQ-S151 Infrared $CO_2$ analyzer (Qubit Systems, Kingston, ON, Canada) and LoggerLite 1.8.1 software (Vernier Software e Technology, Beaverton, OR, USA), respectively. Reference $CO_2$ was calculated by entering air into an empty flask pumped from the external environment at a constant airflow of 300 mL min$^{-1}$ inside a lighted chamber (blue and red LED lamps). LEDs were connected in a direct current circuit under a voltage of 12 V, formed by two parallel sets of four LEDs connected in series (600 µmol m$^{-2}$ s$^{-1}$). Plants were kept in darkness for 8 h before analysis. Soon after the reference $CO_2$ measurement, flasks containing the plants were coupled to the system and the $CO_2$ was calculated at the stabilization point. Gas exchange was measured by calculating the difference between the reference $CO_2$ and the $CO_2$ of plants exposed to atmospheric air. A Spec sensor measured air temperature and humidity in the balloon (Thermo Recorder RS-11; Takai Spec Corp., Aichi, Japan). In vitro photosynthetic rate (A) was calculated by the following formula:

$$A\ (\mu mol\ m^{-2}\ s^{-1}) = \frac{\Delta CO_2}{Mol\ Flow}$$

where,

$$\Delta\ CO_2 (ppm) = Reference\ CO_2 - Analysis\ CO_2$$

$$Mol\ Flow = \frac{Air\ flow\ rate\ (L\ min^{-1})}{\left[\frac{\frac{Constant\ for\ perfect\ gases\ (22.4)\ x\ Temperature\ (K)}{60000}}{Leaf\ DW\ per\ plant\ (g)}\right]}$$

**Determination of primary metabolites**

The leaf samples were collected and subjected to methanolic extraction (Lisec *et al*., 2006). Approximately 20 mg of plant material, frozen in liquid nitrogen, crushed and freeze-dried, was sampled in microtubes. Photosynthetic pigments were determined as described by Wellburn (1994). Carbohydrates (starch, sucrose, glucose and fructose) were assessed as described by Fernie *et al*. (2001). Soluble total protein was assessed as described by Bradford (1976) and total amino acid contents were analyzed according to Yemn and Cocking (1995). Malate content was determined as previously described by Nunes-Nesi *et al.* (2007). Proline content followed the methodology proposed by Carillo and Gibson (2011).

**Determination of antioxidant system enzyme activity**

Activities of oxidative stress enzymes, superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) were assessed. Briefly, 50 mg of frozen fresh material (aerial part) were homogenized 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. The samples were vortexed for 10 s, centrifuged at 12.000 rpm for 15 min at 4 ºC, and the supernatant was removed and set aside on ice for enzyme assays plus protein determination (Bradford, 1976). CAT and POD activities were determined as proposed previously (Maehly and Chance, 1955; Nakano and Asada, 1981; Havir and McHale, 1987, respectively) and expressed as $\mu M^{-1}$ $min^{-1}$ $g^{-1}$ 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.

**Expression analysis using qRT-PCR**

The expression of the genes of the 20E biosynthesis pathway was determined: ecdysteroid 25-hydroxylase (*Phantom* – Cyp306a1) and cytochrome P450 family subfamily A (*Spook* – Cyp307a1), as well as the lignin biosynthesis pathway genes associated with root cell wall stiffening: *cinnamate 4-hydroxylase* (*PgC4H*), *caffeoylCoA O-methyltransferase* (*PgCCoAOMT*), *cinnamoyl-CoA reductase* (*PgCCR*) and *cinnamyl alcohol dehydrogenase* (*PgCAD*). The leaves or roots (100 mg) were collected in liquid nitrogen, macerated and subjected to total RNA extraction using TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA).

The material was treated with DNAse I (Thermo Scientific NanoDrop Technology, Wilmington, DE, USA) to remove possible contamination with genomic DNA, and around 500 ng of the extracted total RNA was converted to complementary DNA (cDNA) using the SuperScript™ III First-Strand Synthesis System kit (Invitrogen®, Carlsbad, CA, USA). The qRT-PCR analyses were carried out on a CFX96 TouchTM detection device (BIO-RAD), using specific primers from the *P. glomerata* transcriptome (Batista et al. 2019). The *glyceraldehyde-3-phosphate dehydrogenase* gene from *P. glomerata* (*PgGAPDH*) was used as an internal reference (Batista *et al*., 2019).

The reactions were performed with three biological replicates in duplicate technique each, in a reaction volume of 10 µL (4 µL of SYBR-Green, 1 µL (4 µM) of each primer, 3 µL of diethylpyrocarbonate-treated water and 1 µL (40 ng) of cDNA. The amplification conditions were performed in the following steps: 2 min at 50 ºC and 10 min at 95 ºC, followed by 40 cycles of 95 ºC for 16 s and 60 ºC for 60 s, and the dissociation curve from 60 to 95 ºC at 0.1 ºC s-1. 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 the primer sequences are shown in Table S1. The melting curves were checked for non-specific amplification and primer dimerization.

**Flow cytometry**

The genetic stability of the plants was assessed by quantifying the relative DNA content using a flow cytometer from leaf tissue and roots of plants grown in vitro. The nuclei suspensions were prepared by chopping young leaves and roots with a disposable steel blade (Galbraith *et al*., 1983) in WPB isolation buffer (Loureiro *et al*., 2007). The suspension generated was aspirated through two layers of gauze with a plastic pipette and filtered through a CellTrics® Nylon membrane with a mesh size of 30 µm to remove cell fragments and tissue debris, after which 25 µl (1mg $mL^{-1}$) of propidium iodide and 5 µl (1 mg $mL^{-1}$) of RNase were added to stain the DNA.

After 30 min in an environment protected from light, the samples were analyzed using a flow cytometer. Three repetitions were carried out and at least 10,000 nuclei were analyzed to quantify the fluorescence emission using the CytoFLEX flow cytometer (Beckman Coulter, CA, USA). The species *Glycine max* with 2C DNA content = 2.50 pg or *Pisum sativum* with 2C DNA content = 9.09 pg (Doležel *et al*., 1998) were used as internal standards. The

histograms were generated and analyzed using the CytExpert 2.0.1 program and the DNA content (pg) was calculated according to Doležel and Bartos (2005) and Doležel *et al.* (2007).

**Proteomic analysis**

Mass spectrometry was performed using a nano Acquity UPLC connected to a SINAPT Q-TOF Instrument G2-Si (Waters, Manchester, UK), 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 label-free comparative quantification was performed using ISOQuant software v. 1.7 (Distler et al. 2014) as previously described by (Passamani *et al*. 2020). In summary, for ISOQuant the following parameters were used to identify proteins: at 1% FDR, a peptide scores greater than six, a minimum peptide length of six amino acids, and at least two peptides per protein was required for label-free quantification using the TOP3 approach, followed by the multidimensional normalized process within ISOQuant. The proteomic 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 the three biological replicates or absent in all replicates (for single proteins) were considered. Data were analyzed using Student's t-test ($P \leq 0.05$). In addition, the $Log^2$ fold change (FC) criterion was considered for calculating 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 carried out for the differentially abundant proteins using OmicsBox software (https://www.biobam.com/omicsbox).

**Statistical analysis**

All experiments were designed in a completely randomized distribution with four biological replicates of each treatment. In addition, the experiments were repeated twice. Data were statistically tested for normality and subsequently examined using ANOVA ($P < 0.05$). Differences in means ($P < 0.05$) presented in the figures were examined using the Tukey test. Gene expression data were compared using Dunnett's test. All statistical analyses were carried out using R statistical software (www.r-project.org).

## RESULTS

### Aluminum stress leads to a decrease in growth and biomass accumulation in *P. glomerata* plants

After 40 days both in the roots and the aerial parts (Fig. 1) in 500 µM Al-treated plants of *P. glomerata* showed characteristic phenotypic changes, compared to non-exposed Al plants. The roots of plants treated with MET alone and in Al + MET combination showed a yellowish color (Fig. 1b-c). The growth of *P. glomerata* was significantly reduced under conditions of to Al toxicity (Fig. 2). Al caused a 23% reduction in root growth (Fig. 2a). However, there was a recovery in the growth of this organ when we associated Al + MET, which did not differ significantly from the control condition (Fig. 2a). At the same time, Al caused a 16.5% reduction in the aerial part compared to its counterpart (Fig. 2b).

We observed that plants subjected to Al toxicity showed a significant reduction in root length (23%), aerial part (16.5%), root dry mass (37.5%), leaf area (11%), number of leaves (35%), diameter (13%), total length (34%) and root volume (38%) (Fig. 2a-c, e-f, g-i). Furthermore, when we combined Al + MET, the plants maintained their growth, increasing 13% in root length and 14% in total leaf area, compared to plants grown in the presence of Al (Fig. 2 a-e, h). Our results showed that the interaction of Al + MET led to greater total root growth (34%) compared to plants treated with Al alone (Fig. 2a). Furthermore, under Al + MET combination, the plants showed a 13% increase in root length and a 14% increase in total leaf area compared to stressed plants (Fig. 2 a, e). Our results showed that the interaction of Al + MET led to greater root growth (20%) compared to plants treated with Al alone (Fig. 2a).

### Melatonin treatment reduced aluminum accumulation to avoid toxicity in *P. glomerata* roots

After exposure to Al, *P. glomerata* plants accumulated Al in their roots and leaves, but mainly in the roots (Fig. 3). In the treatment with Al, we observed a high accumulation of this metal, expressing values 14.8 times higher than the control treatment, while the roots maintained in the Al and MET interaction showed a reduction in Al accumulation of 18% compared to the roots maintained under Al toxicity (Fig. 3a).

In the treatment with Al and the Al + MET interaction, the plants accumulated 82 and 85% more Al in the leaves than in the control; both treatments did not differ (Fig. 3b). No significant difference was observed in the roots and leaves of MET-treated plants compared to the control plants (Fig. 3).

**Al toxicity caused anatomical changes in the roots**

The positive result of the ferric hematoxylin staining of the root apices in the presence of Al is evidenced by the purple staining. In the control treatment, the purple staining was absent, showing that there was no detectable presence of Al in these roots (Fig. 4a). In the case of the roots exposed to Al, it was possible to observe an accumulation of this metal, mainly in the coif cells (Fig. 4d). In these cells, we assume that the Al was mainly bound to the cell wall. We did not observe any detectable presence of Al in the plants supplemented with MET (Fig. 4g). In plants grown in the presence of Al and MET, we observed a slight detection of Al (Fig. 4j).

Toluidine blue staining showed that the roots treated with Al had structural changes. The cross-sections of the roots showed that Al caused changes in the epidermis, making it irregularly uniseriate, a cortex with rounded and juxtaposed tips, and a poorly developed vascular cambium, with a predominance of vessel elements (Fig. 4b). Plants treated with MET showed a well-developed vascular cambium, with an increase in the diameter of the metaxylem and a regular cortex, similar to the control condition (Fig 4h). For the Al and MET interaction, we observed a uniseriate epidermis with irregular cells and poorly developed vascular cambium (Fig. 4K).

We found notable structural anatomical changes in the leaves anatomy of the plants under Al toxicity (Fig. 5). The control plants had a large central vascular bundle with well-defined cells, subepidermal collenchyma on the adaxial surface and lamina subdivided into palisade and spongy parenchyma (Fig. 5 a-b). In the presence of Al, the leaves have a uniseriate epidermis, three large central vascular bundles with parenchyma distributed along the main vein, subepidermal collenchyma on the adaxial surface and lamina subdivided into palisade parenchyma that is poorly developed and has fewer intercellular spaces (Fig. 5 d-e), a similar pattern was observed for the plants supplemented with MET and for the Al and MET interaction. Concerning the stem, the plants exposed to Al showed more differentiated vascular tissue, greater vascularization and cambial activity, and a greater number of secondary xylem vessels (Fig. 5f), differing from the other treatments.

**Al promotes changes in pigments and photosynthetic parameters**

Al toxicity caused significant reductions in chlorophyll (Chl) content (Fig. 6). Plants grown under Al toxicity reduced the content of Chl *a* (16%), Chl *b* (15%) and total Chl (13%)

(Fig. 6 c). The content of carotenoids was also significantly affected by Al, with an 11% reduction compared to the control treatment (Fig. 6e). Regarding the Chl *a*/Chl *b* ratio, there was no significant difference between the treatments (Fig. 6d). At the same time, there was a significant reduction in photosynthetic rate in plants grown in the presence of Al (16%), and in the presence of MET (21%), both alone. Conversely, plants exposed to the Al + MET combination exhibited no significant difference in photosynthetic pigment content and photosynthetic rate compared to the control (Fig. 6). On the other hand, the interaction between MET + Al promoted an increase in photosynthetic rate (14%) compared to stressed plants (Fig. 6f).

**Changes in metabolism occurred in response to Al toxicity**

There was a decrease in fructose content (34%) for plants grown under Al toxicity, but no significant difference was observed in glucose and sucrose content compared to control plants (Fig. 7a - c). At the same time, plants treated with MET and in association with Al + MET showed a reduction in glucose levels of 53 and 41% and in fructose content of 64 and 48%, respectively (Fig. 7a - b). Concerning sucrose content, no significant pattern of change was observed between the treatments imposed (Fig. 7c).

Starch content increased by 20% in plants grown under to Al toxicity and MET application; in the other treatments, we did not observe any specific pattern of change in starch content compared to the control (Fig. 7d). Protein content was altered in plants grown in the presence of Al, with a decrease of (25%), in parallel with the content of total amino acids which also decreased (30%) (Fig. 7d-e). On the other hand, total amino acid levels were not altered in the other treatments and did not differ from the control condition.

Concerning total phenol levels, we observed a significant increase of 30% in plants grown under aluminum toxicity. In contrast, the other treatments did not differ from the control (Fig. 7g). Proline content was not significantly influenced by Al toxicity, melatonin application, or the interaction between these treatments (Fig. 7h).

**Exogenous MET alleviated Al toxicity in *P. glomerata***

The activities of the antioxidant enzymes (CAT, POD and SOD) were significantly influenced by the presence of Al and MET (Fig. 8). Al to toxicity promoted a 63% increase in CAT activity (Fig. 8a). Plants grown in the presence of MET and in the Al + MET interaction showed a significant reduction of 21% and 24% in CAT activity in the roots, compared to plants kept under Al toxicity. Similarly, Al caused a 76% increase in CAT activity in the leaves. Plants

treated only with MET showed a 73% increase and in the AL + MET interaction, plants showed a 78% increase, which did not differ from each other (Fig. 8b).

In the roots, no significant difference was observed between the treatments analyzed (Fig. 8c). In the leaves, there was a significant reduction in POD activity in the plants kept under Al toxicity (37%), which did not differ from the plants treated with AL + MET (Fig. 8d). As for SOD, an increase in its activity of 16% was observed only in plants supplemented with Al and MET in the leaves (Fig. 8f). At the same time, we observed an increase in SOD activity of 33% in the roots of plants exposed to Al compared to the control, and this increase was even more pronounced in plants grown in the presence of MET and Al + MET (Fig. 8e).

**Aluminum stress and MET application modulated the expression of genes related to the ecdysteroid biosynthetic pathway**

In the ecdysteroid biosynthesis pathway, the expression of the *PgSpook* and *PgPhantom* genes related to the biosynthesis of 20-hydroxyecdysone was influenced by to Al toxicity in *P. glomerata* roots (Fig. 9). Under Al toxicity, we observed a 0.6 - 0.4-fold reduction in the expression of these genes in roots (Fig. 9a, c). When Al toxicity was combined with MET application, we also observed a significant difference, expressing 0.5- (*PgSpook*) and 0.4-fold (*PgPhanton*) reductions, respectively, compared to the control (Fig. 9a-c)

On the other hand, in the leaves, no significant difference was observed in the expression of the *PgSpook* gene in any of the treatments imposed when compared to the control plants (Fig. 9b). On the other hand, *PgPhantom* was positively regulated (1.5-fold) in the roots by to Al toxicity in *P. glometara* the other treatments did not differ from the control (Fig. 9d).

**Genes related to the lignin biosynthetic pathway are differentially expressed in leaves and roots**

In the phenylpropanoid biosynthesis pathway, the expression of the *PgCCR* gene related to lignin production was negatively influenced by Al toxicity in roots and leaves (0.6 and 0.4 times, respectively) (Figs. 10a-b). The application of MET and the combination of Al + MET also negatively regulated the expression of *PgCCR* in leaves (Fig. 10b), which was not observed for roots (Fig. 10a).

On the other hand, the *PgCAD* genes showed no significant difference in their level of expression in *P. glomerata* roots and leaves (Fig. 10 c-d). The *PgC4H* gene was positively regulated in the roots (0.5 and 0.4 times) of plants treated only with MET and Al + MET, respectively, compared to the control (Fig. 10e). On the other hand, in the leaves, there was a

negative regulation in the expression of this gene in plants grown under Al and MET stress, compared to the control (Fig. 10f).

The *PgCCoAOMT* gene showed a significant difference in expression levels in the two organs analyzed (Fig. 10g-h). In the roots, we observed a negative regulation, which was more pronounced in plants treated with AL + MET (1.5 times) compared to the control (Fig. 10g). Conversely, in the leaves, this regulation was positive; this pattern was observed in all the treatments analyzed (Fig. 10h), and once again, we observed that this increase in *PgCCoAOMT* levels was more pronounced (0.6-fold) in the plants treated with Al + MET (Fig. 10h).

**Al promotes a reduction in the level of DNA ploidy in the roots and positive regulation of endoreduplication**

In order to assess ploidy levels by flow cytometry in *P. glomerata* plants in response to Al toxicity and the interaction between Al and MET, nuclei were isolated from leaves tissues and root apices (Fig. 11). Our results indicated a reduction in nuclear DNA content in root apex cells under to Al toxicity (Fig. 11a), while no changes were observed in leaves (Fig. 11b).

We then turned our attention to the root apices, which showed changes in ploidy levels, stimulating the appearance of polyploid cells (4 C, 8 C, 16 C) in the RAM (Fig. 11c), and a possible attempt to maintain MET-mediated repair of potential proliferative capacity under stress (Fig. 11c). Our results revealed a pronounced increase in the endoreduplication index in the presence of Al, and for the Al and MET interaction (Fig. 11 d).

**Al to toxicity causes changes in the protein profile**

Proteomic analyses of *P. glomerata* plants in response to Al toxicity and the Al and MET interaction revealed significant changes in protein abundance. In total, 1309 proteins were identified (Fig. 13), of which 1269 (97.7%) were found in the four comparisons analyzed: Al/Control; MET/Control; Al + MET/Control and Al/MET (Fig. 13a, Table S2). A total of 174 differentially accumulated proteins (DAPs) were found (Fig. 13a). Comparisons between Al/Control; MET/Control; Al + MET/Control and Al/MET, revealed 11, 23, 24 and 20 protein up-accumulated, respectively (Fig. 13a). Relatived and protein down-accumulated 24, 22, 26 and 24 protein, respectively (Fig. 13b).

Principal component analysis (PCA) revealed that the main differences in the proteome in relation to the treatments imposed were due to the presence of Al and Melatonin separately (Fig. 13c). Thus, the first principal component (PC1), responsible for 28.1% of the total variability, divided both treatments for the proteomic analysis. Determining PC2 (20%) for the

proteomic analysis showed that the treatments involving Al (individually or in combination with MET) clustered together, while the remaining treatments (Control and MET) were closer together. These groupings highlighted the toxicity of Al and its interference in the differential accumulation of proteins, since the Al and Al + MET samples showed more differences.

Gene ontology (GO) analyses of DAPs were carried out using OmicsBox software (Fig. 14). The proteins were identified and categorized into Biological Process and Cellular Component, Molecular Function and Metabolic Pathways (Fig. 14). The analysis revealed that most of the accumulated proteins are related to biological processes involving transmembrane transport, response to light stimuli, metabolic transport with energy expenditure, energy generation in plants, such as photosynthesis, ascorbate metabolism, oxidative phosphorylation, carbon metabolism, pentose phosphate and other metabolic pathways associated with the biosynthesis of amino acids (e.g. phenylalanine and tryptophan) and secondary metabolites (Fig. 14). The DAPs were predominantly present in the vacuoles, cell wall and chloroplasts and apoplasts (Fig. 14b).

**DISCUSSION**

Melatonin (MET) is a regulatory molecule that plays various biological roles in plant growth and development and in response to abiotic stresses, including the mitigation of aluminum (Al) stress (Hassan *et al*., 2022; Zeng *et al*., 2023). In the present work, we elucidated the alleviation of Al toxicity promoted by melatonin in *Pfaffia glomerata* plants grown in vitro. Our results indicate that exogenous MET can maintain the growth of *P. glomerata* and improve biomass increment characteristics under Al stress. MET also increases leaf area and leaf number, increasing photosynthetically active area and, ultimately, biomass production (Xu *et al*., 2020). Root growth variables revealed that plants under aluminum stress considerably reduce the length, diameter, total length and volume of the roots, which results in a smaller area of soil explored and, consequently, less absorption of water and essential nutrients. Al-induced inhibition of root elongation is known to be due to the active triggering of cell cycle arrest, terminal differentiation of the root tip and loss of the quiescent root center (QC), and all these processes are associated with loss of DNA integrity (Eekhout *et al*., 2017; Yang *et al*., 2019). We identified the accumulation of a putative histone H2A variant 1 - HTA8 ortholog in *Arabdopsis* (cds.comp18924_c0_seq1) (Fig. 15, Tables S2), a structural constituent and previously involved in chromatin compaction under Al stress, reinforcing our idea of impaired cell cycle progression.

We found that the supply of MET could mitigate the damage caused by aluminum in the root system of plants, promoting relief in the interruption of root growth under Al toxicity in *P. glomerata*. Thus, the present results show that melatonin can reduce the detrimental effects of Al toxicity on root growth, and these findings reinforce the need to identify processes and factors associated with MET's ability to be one of the multifactorial regulators of plants under Al toxicity.

Concerning Al accumulation, we observed that *P. glomerata* plants accumulated the metal in their tissues mainly in the root system, but that there was also translocation of the metal to the aerial part, possibly by increasing the expression or action of genes related to Al transport that are already known, for example, families of gene transporters such as ATP-binding cassette (ABC), aluminum-activated malate transporter (ALMTs) (Rahman *et al*., 2024). In this study, we showed the up-regulation of a protein member of the ABC transporter I family (ABCI17 - cds.comp52454_c0_seq1), involved in transmembrane transport and implicated in aluminum tolerance in Glycine soja (Wen *et al*., 2021).

Considering that, accompanied by the entry of Al and interaction with apoplastic and symplastic targets, plant cells generally allow the synthesis and efflux of various organic acids (e.g. malate, oxalacetate and citrate), forming complexes together with Al and promoting its sequestration in the vacuoles to maintain low levels of free Al in the cytosol of the plant, maintaining the state of homeostasis and cell rigidity, which will favor the maintenance of plant growth and development by reducing the cytotoxic impact of Al (Gupta *et al*, 2013; Nunes-Nesi *et al.*, 2014; Yang *et al*., 2019; Huang *et al*., 2021).

Interestingly, exogenous MET was not able to prevent the translocation of Al to the leaves, although it did promote less adherence of the metal to the roots. These results are corroborated by the low detection of Al in the fresh root tissues of plants under Al stress and supplied with MET (Fig. 4j). On the other hand, intense adhesion of Al molecules was observed in roots of plants treated with Al, represented by the intense purple coloration. Sun et al. (2020) confirmed that exogenous application of melatonin significantly decreases the amount of cell wall polysaccharides, thus increasing aluminum exclusion and promoting root growth in wheat. Suggesting that this mechanism of Al exclusion via cell wall modification may be optimized by melatonin application and governing the response of lower Al detection in *P. glomerata* roots supplemented with MET.

Al and MET inhibited the photosynthetic system in *P. glomerata* plants. However, the interaction between exogenous melatonin and Al may be able to specifically attenuate Al toxicity and restore photosynthetic rates, since it promoted the maintenance of photosynthetic activity in plants treated with Al + MET. Plants exposed to Al stress separately showed a reduction in chlorophylls *a*, *b* and total chlorophyll content. These data are corroborated by Fortini et al. (2023) who also observed reductions in chlorophyll content in *P. glomerata* subjected to salt stress in vitro.

When MET + Al were combined, there was a significant improvement in these parameters. This may be related to the accumulation of a chloroplast transketolase (TKL-1; cds.comp28452_c0_seq1), an essential enzyme of both the Calvin-Benson cycle and the pentose phosphate oxidative pathway, essential for the metabolism of carbohydrates (fructose, sucrose) in plants (Rocha *et al*., 2014). Thus, under Al toxicity, MET appears to exert significant effects on the enzyme's substrate saturation kinetics at specific physiological pH values, favoring carbon allocation and thus promoting the maintenance of photosynthetic activity (Hassan *et al*., 2022).

Therefore, our findings are in line with Hassan et al. (2015) and Wang et al. (2019), who showed that melatonin is effective in maintaining the photosynthesis process under abiotic stressors in different plant species. Thus, we suggest that melatonin functions as a potent regulator, improving plant photosynthesis in the presence of Al and strengthening plant tolerance to excess $Al^{3+}$ ions in the nutrient solution, sustaining the initial growth of *P. glomerata*.

In the metabolic evaluations, it was possible to observe a pattern of reduction in the concentration of non-structural carbohydrates (glucose and fructose) in response to stress in all the treatments analyzed, compared to the control plants, which explains, at least partially, reductions in the growth of the aerial part. Similarly, Qu et al. (2020) discussed that Al toxicity reduced overall plant biomass, hampering protein biosynthesis and reducing carbohydrate content in *Camellia oleifera* subjected to Al stress. MET alone promoted the lowest concentrations of glucose and fructose, indicating that glucose was consumed over time in these plants, which is in line with the decrease in photosynthetic rate observed in these plants.

In addition, Al toxicity significantly reduced protein and amino acid levels, suggesting that increased protein degradation an alternative respiratory substrate supply to the cell may occur under stress conditions (Araújo *et al*., 2011). We found a low accumulation of the chlorismate synthase 1, chloroplastic enzyme (EMB1144; cds.comp17932_c0_seq1) responsible for the biosynthesis of several aromatic amino acids, such as phenylalanine, tyrosine and tryptophan, which are precursors of several crucial plant compounds, such as lignin, anthocyanins, flavonoids and tocopherols (Bonawitz and Chapple, 2010; Vogt, 2010).

In contrast, exogenous MET increased protein and amino acid content during Al stress. Plants exposed to stress conditions are known to increase the synthesis of total amino acids, including proline. However, we did not observe a significant increase in the accumulation of this isolated osmoprotectant, indicating maintenance in the process of synthesis and degradation of this amino acid (Reis *et al*., 2012). Thus, the accumulation of amino acids and proteins in *P. glomerata* promoted by MET under stress may be related to the protection of the turnover of proteins that make up the photosynthetic apparatus, indicated by the maintenance of the photosynthetic rate and stabilization of *Chls* metabolism (Wang and Grimm, 2021).

The roots of plants treated with melatonin exhibited enhanced antioxidant defense mechanisms, as indicated by the increased levels of SOD, CAT and POD activities, thus minimizing the oxidative damage caused by Al toxicity. In addition, MET acted by reducing the toxicity caused by the entry of Al into plant cells, preserving cellular homeostasis,

maintaining redox balance and probably activating signaling transduction and the expression of genes linked to stress tolerance (Sharif *et al*., 2018; Xu *et al*., 2020; Tang *et al*., 2020). As MET is a free amphiphilic molecule that crosses cell membranes, it can directly eliminate ROS, improving antioxidant activities and conferring stress tolerance (Moustafa-Farag *et al*., 2020; Hassan *et al*., 2022). For example, the application of MET under Al stress conditions induced a significant increase in SOD, POD and CAT activity in the roots and leaves of *Zea mays* (Ren *et al*., 2022). Similarly, MET also alleviated arsenic toxicity in rice plants by modulating the antioxidant system (Rahmatullah *et al*., 2023).

Similarly, other authors have also reported that MET significantly improved the activities of CAT, POD, SOD and other antioxidant compounds under waterlogging, cold, cadmium, arsenic, drought, salinity, temperature and ultraviolet radiation (Hasan *et al.*, 2015; Li *et al.*, 2018; Campos *et al.,* 2019; Gu *et al*., 2021; Imran *et al*., 2021; Raza *et al*., 2022; Ren *et al*., 2022; Iqbal *et al*., 2024). Thus, all these findings endorsed that MET supplementation improves the antioxidant defense system to alleviate the effects of different abiotic stresses.

The cytochrome P450 (CYP) superfamily of enzymes plays important roles as flexible catalysts and is essential for the production of secondary metabolites, antioxidants, biopolymers for structural support and phytohormones in higher plants (Hansen *et al*., 2021; Chakraborty *et al*., 2023). These compounds provide a molecular basis for alleviating abiotic stress through different mechanisms and the active participation of CYPs in various biosynthetic and detoxification pathways. In this sense, it has been reported that some member genes of this family are involved in the biosynthesis of ecdysteroids in plants (Lei *et al*., 2018; Wang *et al*., 2018; Batista *et al*., 2019; Janeczko *et al*., 2021; Park *et al*., 2020). De novo assembly of the *P. glomerata* transcriptome revealed several CYP P450 family genes related to secondary metabolism and stress, which confers dynamic plasticity to these genes (Batista *et al*., 2019).

Here we evaluated the relative expression level of the *Spook* and *Phantom* genes, members of this family and previously involved in the biosynthesis of 20-hydroxyecdysone (20E) in *P. glomerata* under abiotic stresses (Batista *et al*., 2019). Interestingly, we demonstrated that the Spook gene (CYP83A) was negatively regulated by Al toxicity and that the supply of MET did not promote significant increases in its expression levels in leaves and roots. In addition, we observed the same regulation pattern for the *Phanton* gene (CYP76C), which was negatively regulated in roots in response to Al toxicity. On the other hand, *Phanton* increased its expression 1.4-fold in response to Al stress in leaf tissues. In contrast, Felipe et al. (2019) showed that salt stress and UV-B radiation up-regulated the *Spook* and *Phantom* genes

of the CYP P450 gene family in *P. glomerata*, suggesting the involvement of these genes in alleviating the imposed stress. Our results suggest that, although there was no increase in the expression of these genes in this study, cross-talk mechanisms with other pathways related to defense against abiotic stress may be at work.

With regard to genes involved in the phenylpropanoid metabolic network that also belong to the multigenic CYP family (CYP73, CYP98 and CYP84) (Renault *et al*., 2017; Hansen *et al*., 2021), we analyzed the expression of *PgCCR*, *PgCAD*, *PgC4H* and *PgCCoAOMT* involved in the construction of lignin biopolymers and their fractions (Hansen *et al*., 2021). We propose that due to the strong affinity of Al with cell walls, wall components were disrupted, and lower lignin synthesis was demonstrated by the low expression of *PgCCR* in roots. We identified a negative regulation of *caffeic acid 3-O-methyltransferase-like enzymes* (OMT1; cds.comp42612_c0_seq1) involved in the biosynthesis of phenylpropanoids, mainly lignin, reinforcing our idea of disturbance in the deposition of wall compounds, through impaired activity of *cinnamoyl-CoA reductases* via Al toxicity.

It is likely that the saturation of active sites in the roots by the $Al^{3+}$ ion may be altering the biosynthesis, integrity, coupling and polymerization of lignin microtubules via Al toxicity and reducing lignin biosynthesis in the roots, since cinnamoyl-coenzyme A (*CCRs*) is the first gene compromised in the lignin-specific pathway (Chao *et al*., 2017). Similarly, Van Acker *et al*. (2013) observed that a lower expression of the *CCR* gene is related to a reduction in lignin biosynthesis.

On the other hand, we observed a significant increase in melatonin-mediated PgC4H gene expression, the second step in the central phenylpropanoid pathway is known to be catalyzed by CYP73 (C4H) and is a hydroxylation of cinnamic acid (Yao *et al*., 2021), suggesting highly efficient cinnamic acid hydroxylase activity in melatonin-treated plants and for the Al + MET interaction sustaining lignin biosynthesis in leaves during Al stress in *P. glomerata*.

Al induced negative regulation of the genes of the enzymes involved in lignin biosynthesis *PgCCoAOMT* in the roots of *P. glomerata*, observed the opposite in leaves. This gene was up-regulated, thus increasing lignin biosynthesis, ensuring mechanical support, water retention and transport, but also serving as protective, antioxidant and defense compounds (Wang *et al*., 2022) and thus alleviating the damage caused by Al toxicity.

Upon entering the root, Al interacts with apoplastic targets, such as the cell wall and plasma membrane, and in the symplast, it binds mainly to the phosphodiester skeleton of DNA,

causing implications for the maintenance of QC integrity, especially of the root apical meristem (RAM) (Eekhout *et al*., 2017). Al toxicity can cause cell cycle progression interruption events and damage to DNA replication, two important cellular modifications that affect the maintenance of genome integrity, especially in the root apex (Gentric *et al*., 2021; Szurman-Zubrzycka *et al*., 2023). During Al toxicity, cell cycle arrest occurs, leading to damage to the differentiation of the root apical meristem (RAM), which progresses to loss of identity of the quiescent center and, ultimately, can alter DNA ploidy, compromising root elongation (Sjögrene *et al*., 2015)

Therefore, we isolated RAMs to assess DNA ploidy levels in response to Al stress and monitored cell divisions in root meristems using flow cytometry (Fig. 11). Our results revealed that roots dealing with Al toxicity showed a decrease in nuclear DNA content, but we did not observe the same in plants treated with MET alone. We also observed that Al stimulated the appearance of polyploid cells in the RAM. Generally, the roots of plants exposed to toxic concentrations of Al show the formation of a greater number of polyploid cells (Sjögren *et al*., 2015). In this sense, we believe that the tolerance of the species, corroborated by the low decrease in root growth rate under Al, may be associated with the positive regulation of DNA endoreduplication (Fig. 11d), suggesting that the root endopoliploidy pattern may be responsive to stresses (Bhosale *et al*., 2018).

Consequently, changes in cell volume directly impact the ultimate function of root cells, which are disturbed by the presence of Al, mainly by reductions in cell content promoted by Al. This was reversed by MET supply, indicating that MET signaling may be involved in activating the DNA damage response (DDR) pathway, inhibiting cell cycle progression and stimulating key DNA damage checkpoint genes, favoring the maintenance of genome replication (Fig. 14).

Cell cycle inhibitor genes include members of the SOG1 and SIAMESE RELATED (SMR) gene family that encode negative regulators of cyclin-dependent kinase complexes (CDKs) (Eekhout *et al*., 2017). After severe damage, DDR-induced cell cycle arrest is permanent, resulting in premature cell differentiation and growth arrest (Hu *et al*., 2016). Our idea of melatonin-induced protection against DNA damage in *P. glomerata* is endorsed by regulatory

Proteome analysis (Fig. 15 to Table S2) indicated low accumulation of proteins mainly involved in the biosynthesis of aromatic amino acids and secondary metabolites, such as chorismate synthase 1, chloroplastic (EMB1144; cds.comp17932_c0_seq1) like phenylalanine, tyrosine and tryptophan. In addition to low accumulation of delta-aminolevulinic acid

dehydratase, chloroplastic (HEMB1; cds.comp17367_c0_seq1) involved in the biosynthetic process of chlorophyll, as well as photosystem II 22 kDa protein, chloroplastic isoform X1 down-accumulated (PSBS; cds. comp66564_c0_seq1, compromising processes such as photosynthesis and light harvesting in photosystem II, corroborating the low content of these pigments and impaired photosynthesis in plants exposed to Al. It is important to note that in almost all of the pathways impaired by Al toxicity, the combination of Al and MET promoted more DAPs in response to Al toxicity separately, enriching the results.

Taken together, our results provide integrated information on the morphophysiology, redox balance and modulation of carbon metabolism under Al stress, as well as highlighting the protection induced by MET in maintaining photosynthetic rates, chlorophyll and carbon metabolism under stress, in addition to promoting alleviation of DNA endoreduplication and consequent protection of cell cycle progression.

## CONCLUSION

Our results showed that Al toxicity is probably attenuated by melatonin supplementation through the maintenance of physiological parameters. *P. glomerata* seems to attenuate the physiological responses of Al toxicity mainly by maintaining photosynthetic rates, sustaining its initial growth and providing protection to root apices from excess ions in the growth medium, thus damage to DNA replication seems to be attenuated. Comparative proteomic analysis revealed 1269 common DAPs involved in 22 metabolic pathways, mainly carbon metabolism, amino acid biosynthesis, and cofactor biosynthesis. Thus, we conclude that melatonin is a potential protective molecule against Al toxicity, promotes plant development, while regulating photosynthesis, the antioxidant defense system and oxidative stress. However, more research is needed to fully understand the molecular principles underlying the Al tolerance response mediated by MET.

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FIGURES

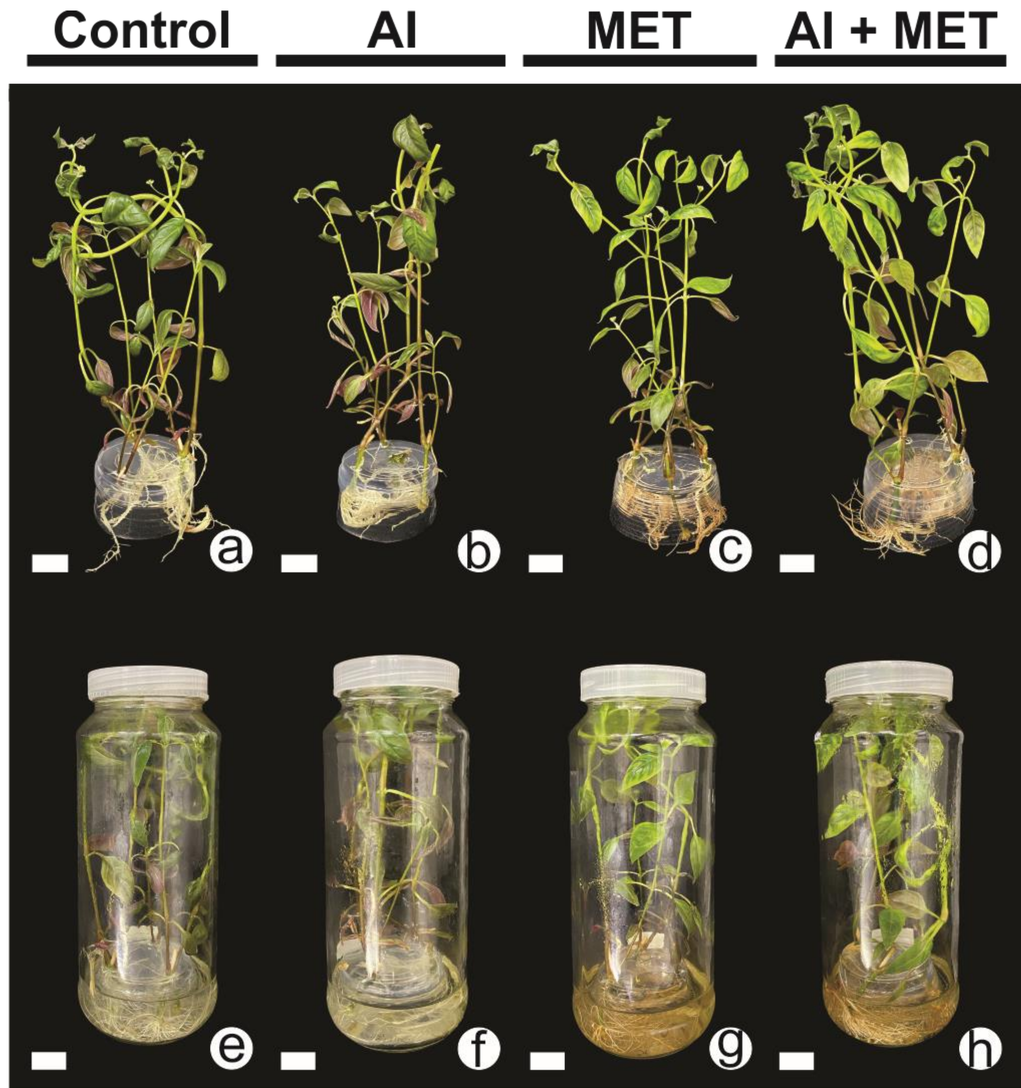


**Figure 1**. Plants of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: (**a-e**) control; (**b-f**) Al; (**c-g**) MET and (**d-h**) Al + MET. Scale bars = 2 cm.

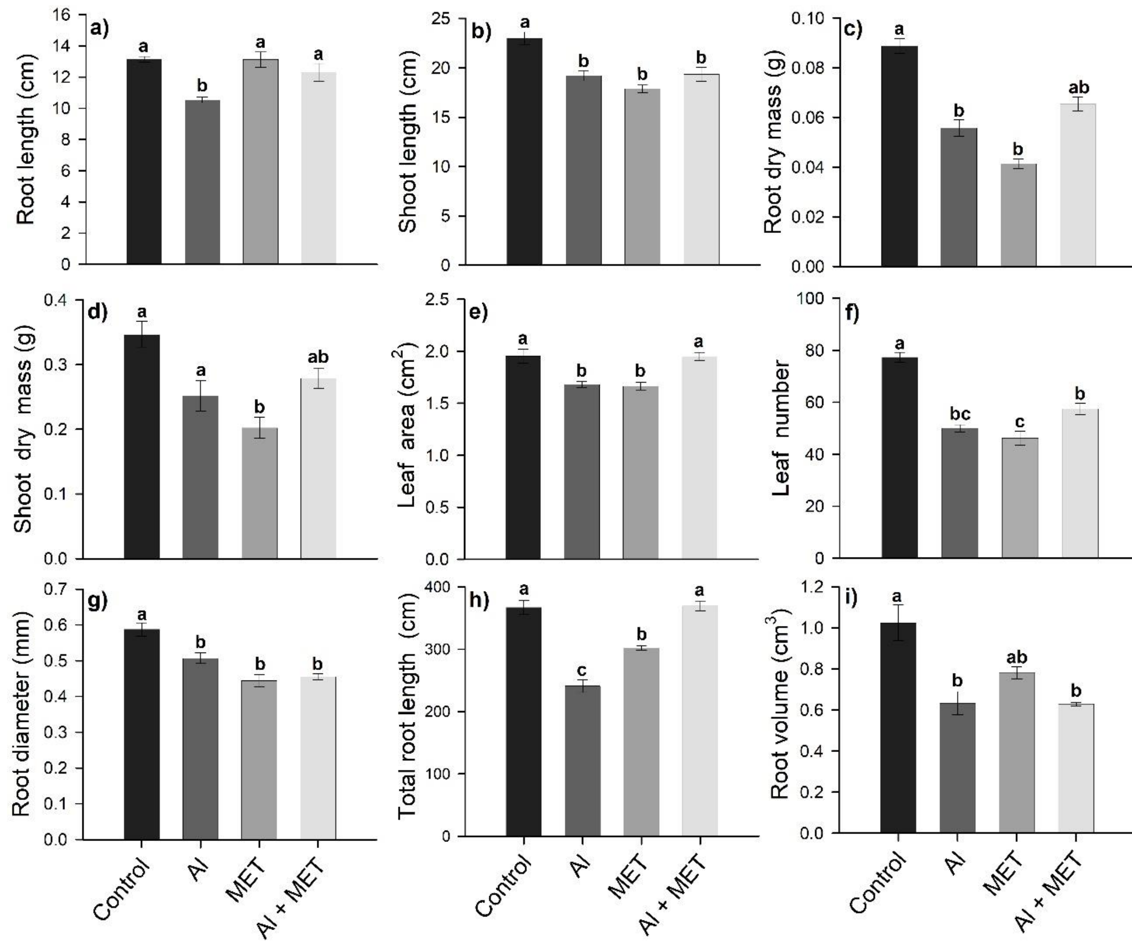


**Figure 2**. Growth parameters of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: Control; Al; MET and Al + MET. (**a**) root length; stem height; (**b**) stem length; (**c**) root dry mass; (**d**) Shoot dry mass; (**e**) Total leaf area); (**f**) Leaf number; (**g**) Root diameter; (**h**) Total root length and (**i**) Root volume ($cm^{-3}$). Values are presented as means (n=4) ± standard error. Averages followed by equal letters did not differ according to the Tukey test ($P \leq 0.05$).

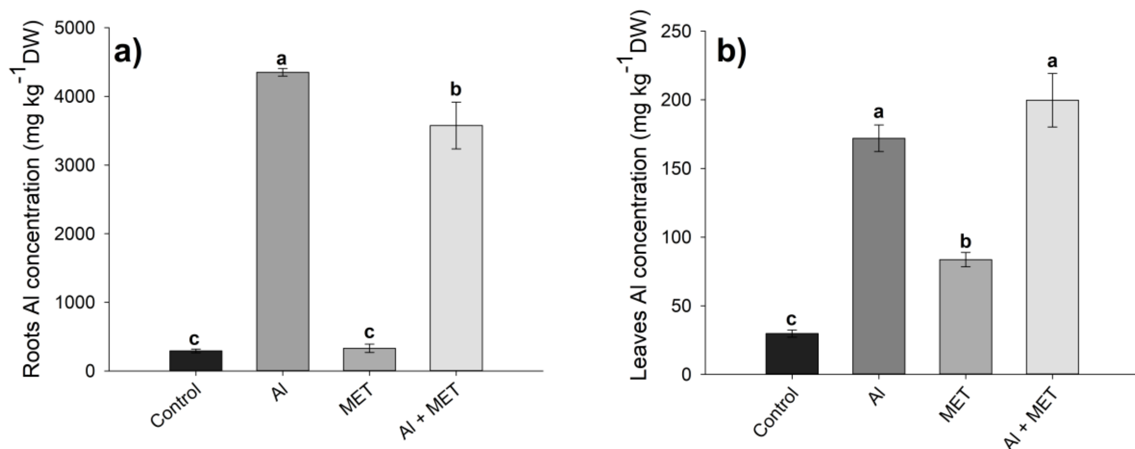

**Figure 3.** Quantification of Al in *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: Control; Al; MET and Al + MET. (**a**) quantification Al in roots; and (**b**) quantification Al in leaves. Values are presented as means (n=4) ± standard error. Means followed by equal letters did not differ according to the Tukey test ($P \leq 0.05$

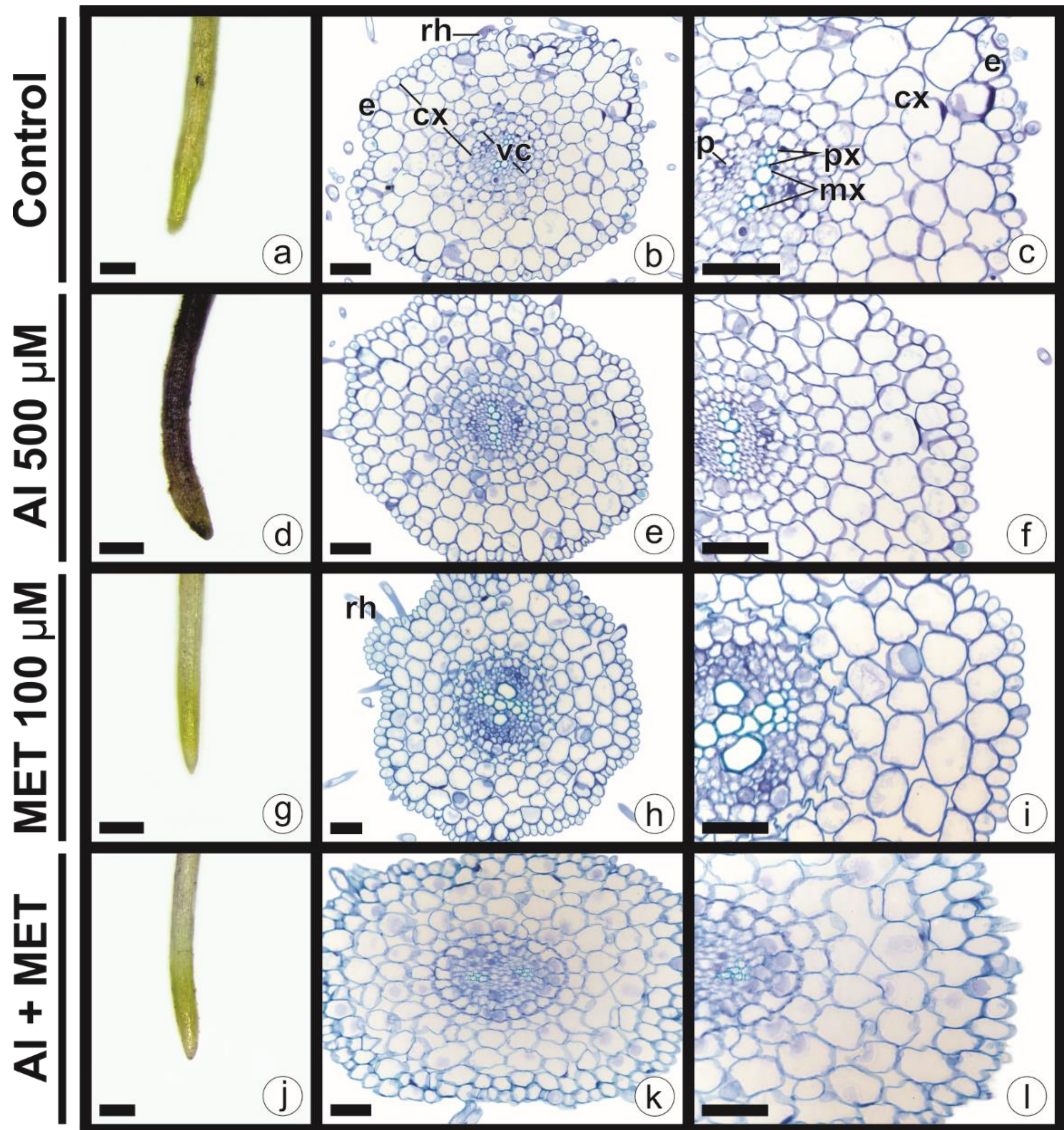


**Figure 4**. Histochemical analysis of the root apex and anatomical characterization of the roots in cross-section of *Pfaffia glomerata* plants after 40 days of in vitro cultivation under the following conditions: control; Al; MET and Al + MET and stained with the following reagents: Hematoxylin (**a**, **e**, **g**, **j**) and Toluidine blue hematoxylin (**b**, **c**, **e**, **f**, **h**, **i**, **k**, **l**). *Abbreviations*: *e* - epidermis; *cx* - cortex; *mx* - metaxylem; *p* - phloem; *px* - protoxylem; *rh* - root hairs. Scale bars: a, d, g, j = *5 mm*; b, c, e, f, h, i, k, l = *50 µm*.

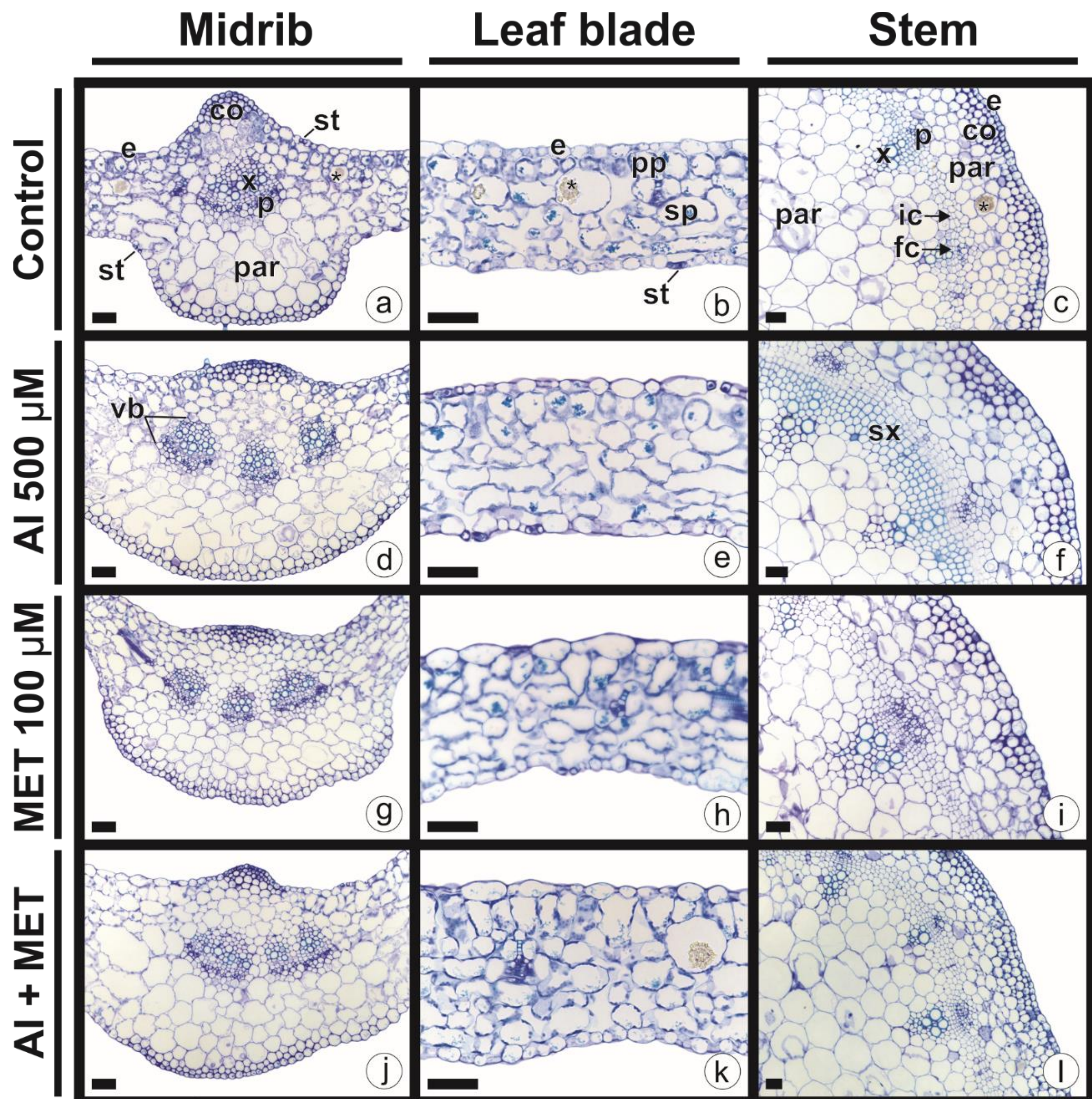


**Figure 5.** Anatomical characterization of leaves (midrib and leaf blade) and stems in cross-section of *Pfaffia glomerata* plants after 40 days of in vitro cultivation under the following conditions: Control; Al; MET and Al + MET. *Abbreviations: e* – epidermis; *co* – collenchyma; *fc* – fascicular cambium; *ic* – interfascicular cambium; *par* – parenchyma; *p* – phloem; *pp* – palisade parenchyma; *sp* – spongy parenchyma; *st* – stomata; *sx* – secondary xylem; *vb* – vascular bundle; *x* – xylem. * - druse crystal. Scale bars: *50 µm*.

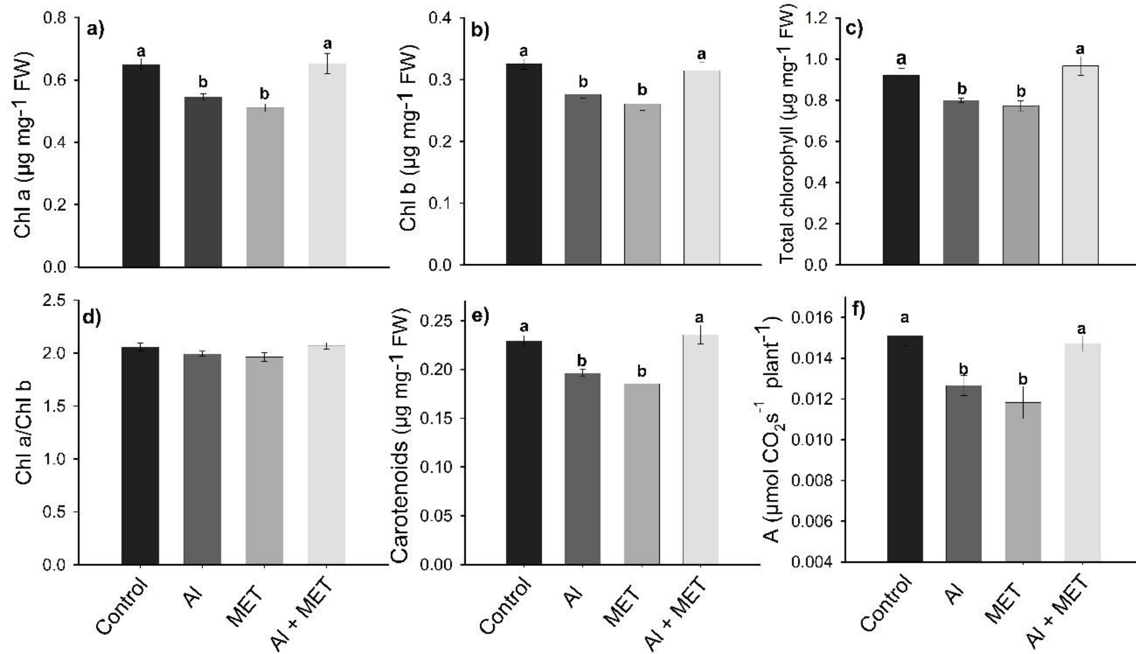


**Figure 6**. Leaf pigment content and photosynthetic rate of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: control; Al; MET and Al + MET. (**a**) chlorophyll *a*; (**b** chlorophyll *b*; (**c**) total chlorophylls; (**d**); chlorophyll *a* and chlorophyll *b* ratio (Chl *a*/Chl *b*); (**e**) carotenoids; and (**f**) *in vitro* photosynthetic rate (µmol $CO_2$ $S^{-1}$ $plant^{-1}$). Values are presented as means (n=4) ± standard error. Means followed by equal letters did not differ according to the Tukey test ($P \leq 0.05$).

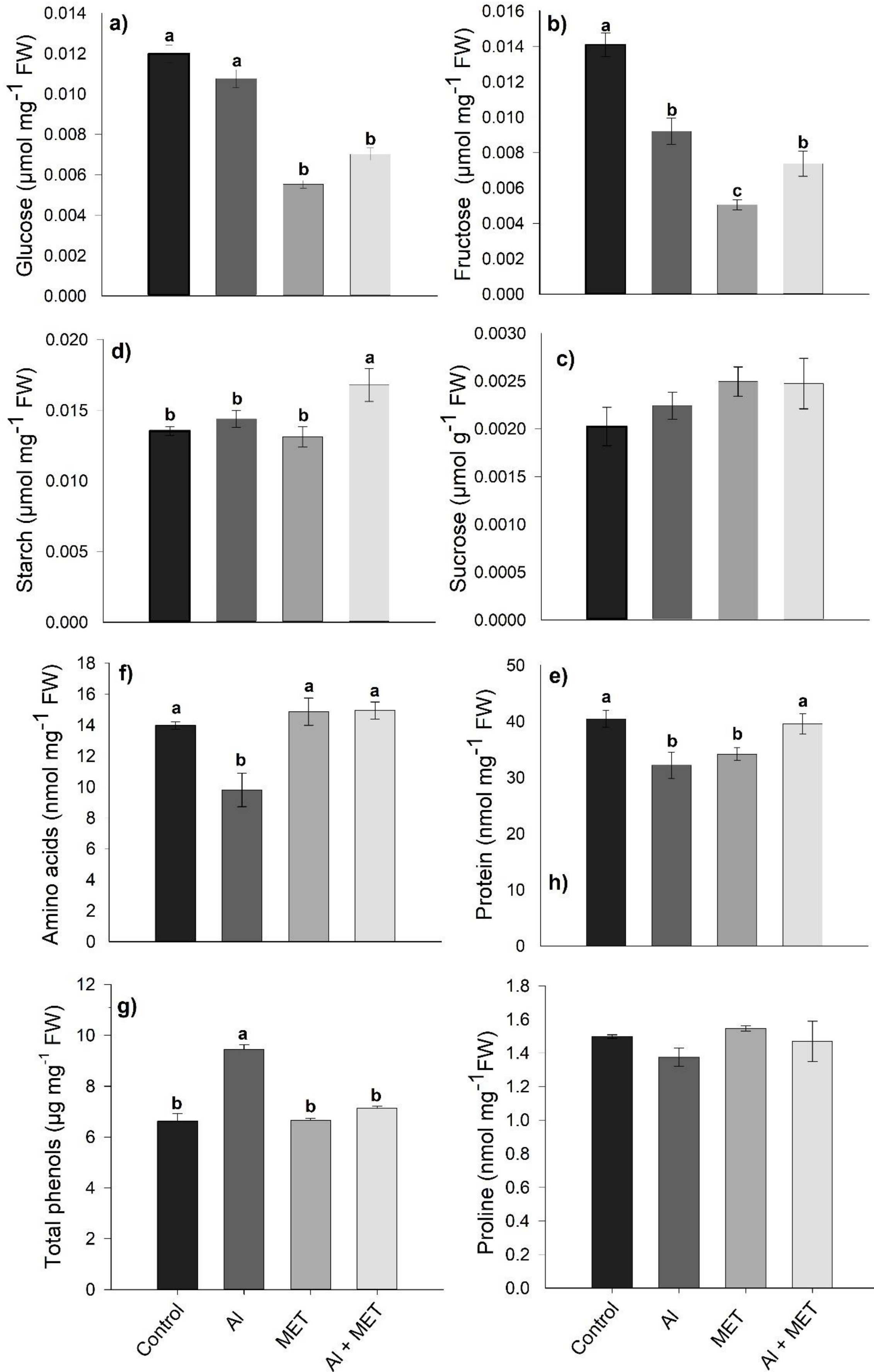
a)
Glucose (µmol mg$^{-1}$ FW)
b)
Fructose (µmol mg$^{-1}$ FW)
d)
Starch (µmol mg$^{-1}$ FW)
c)
Sucrose (µmol g$^{-1}$ FW)
f)
Amino acids (nmol mg$^{-1}$ FW)
e)
h)
Protein (nmol mg$^{-1}$ FW)
g)
Total phenols (µg mg$^{-1}$ FW)
Proline (nmol mg$^{-1}$FW)
Control
Al
MET
Al + MET

**Figure 7**. Changes in the primary metabolism of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: control; Al; MET and Al + MET. (**a**) glucose; (**b**) fructose; (**c**) sucrose; (**d**) starch; (**e**) total proteins; (**f**) total amino acids; (**g**) total phenols and (**h**) proline. Values are presented as means (n=4) ± standard error. Averages followed by equal letters did not differ according to Tukey's test (*$P \leq 0.05$*).

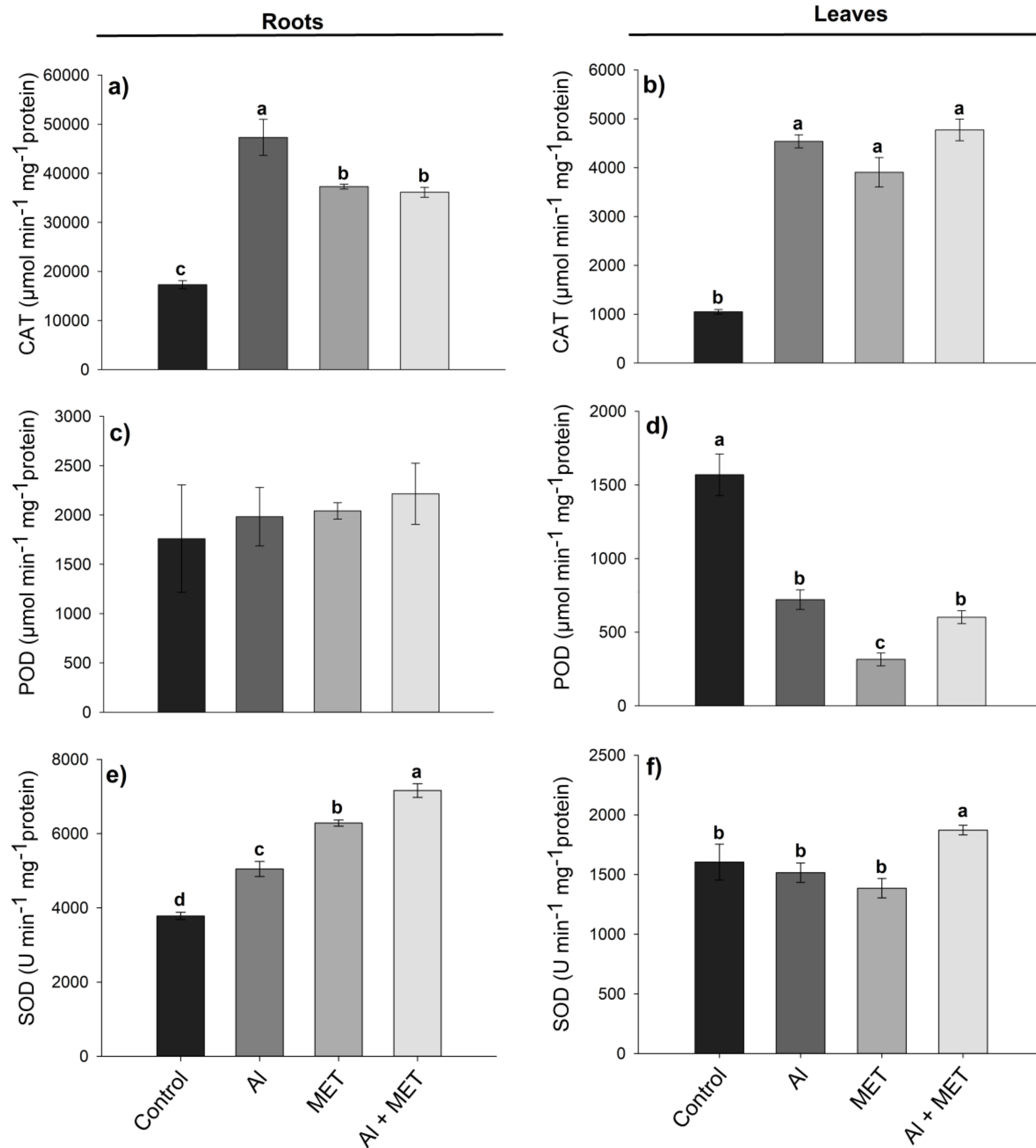

**Figure 8**. Antioxidant enzyme activities of *Pfaffia glomerata* after 40 days of in vitro cultivation under the following conditions: control; Al; MET and Al + MET. (**a**) Catalase - CAT (leaves); (**b**) Catalase - CAT (root); (**c**) Peroxidase - POD (leaves); (**d**) Peroxidase - POD (root); (**e**) Superoxide dismutase - SOD (leaves); (**f**) Superoxide dismutase - SOD (root). Values are presented as means (n=4) ± standard error. Means followed by equal letters did not differ according to the Tukey test ($P \leq 0.05$).

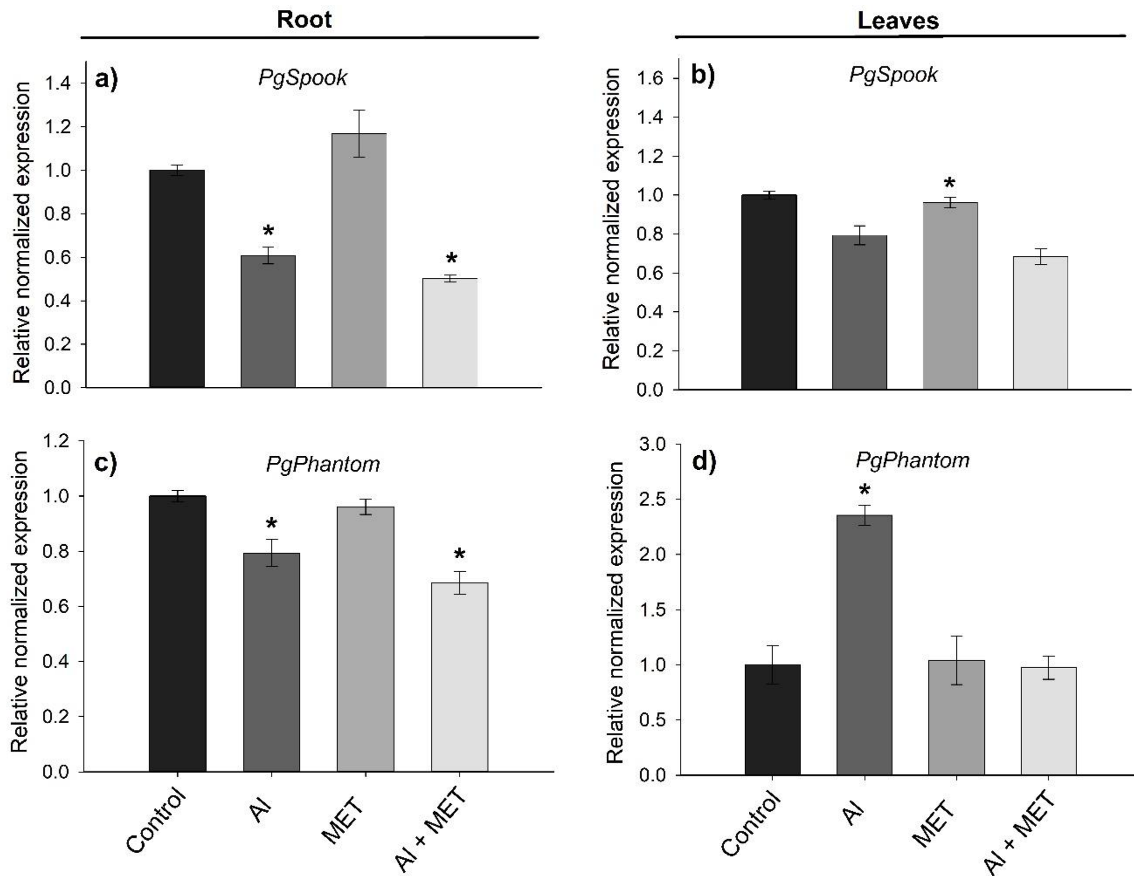


**Figure 9**. Relative expression of genes from the 20-hydroxyecdysone synthesis pathway in *P. glomerata* plants grown in vitro for 40 days under the following conditions: control; Al; MET and Al + MET. (**a**) *PgSpook*; (**b**) *PgSpook*; (**c**) *PgPhantom*; (**d**) *PgPhantom*. Expression was normalized by the *glyceraldehyde-3-phosphate dehydrogenase gene*. Values are presented as means (n=3) ± standard error. Asterisks indicate a significant difference by Dunnett's test ($P \leq 0.05$).

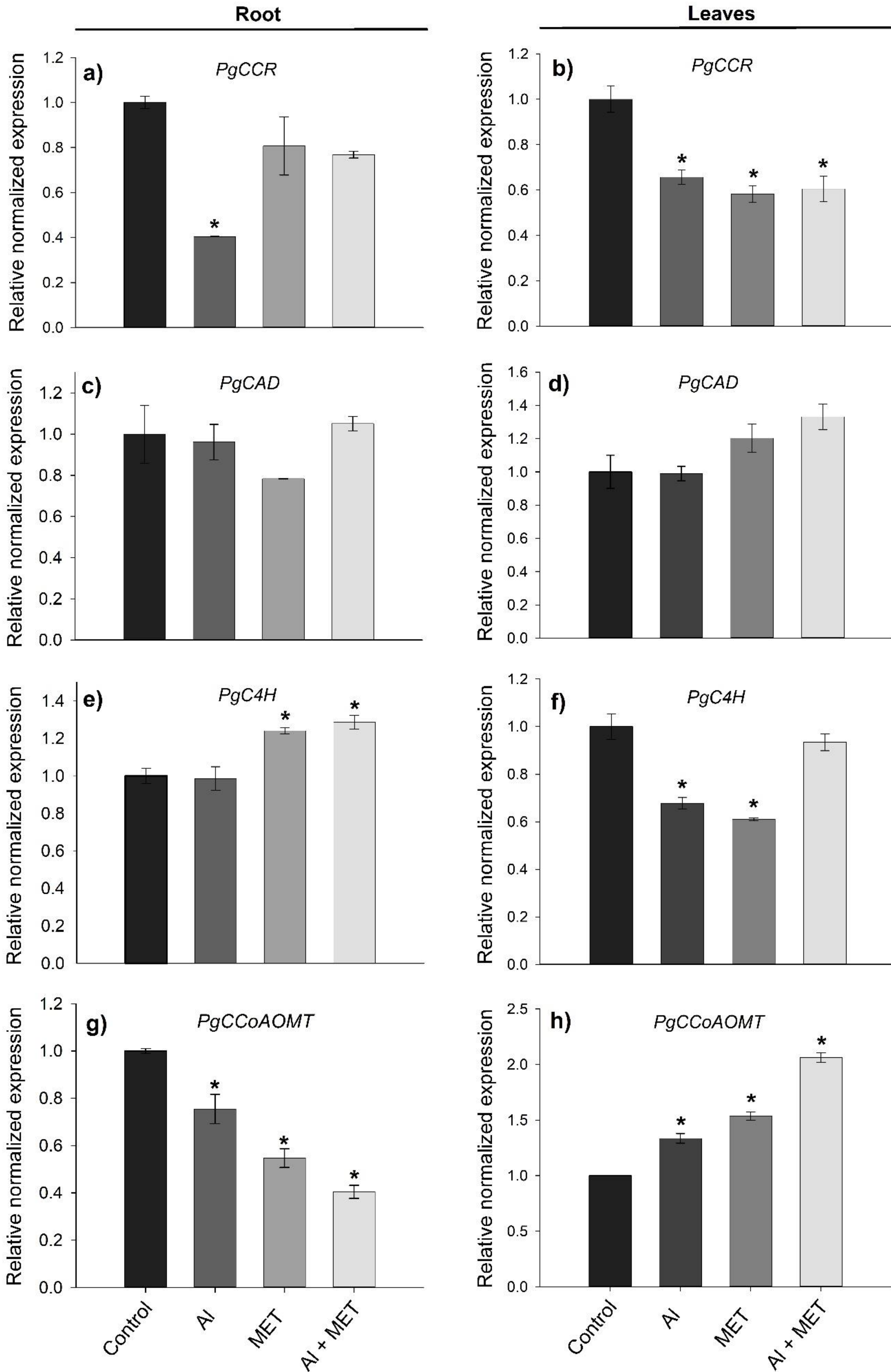
Root
Leaves
a)
PgCCR
b)
PgCCR
c)
PgCAD
d)
PgCAD
e)
PgC4H
f)
PgC4H
g)
PgCCoAOMT
h)
PgCCoAOMT
Relative normalized expression
Control
Al
MET
Al + MET

**Figure 10**. Relative expression of lignin synthesis pathway genes in *Pfaffia glomerata* plants grown in vitro for 40 days under the following conditions: control; Al; MET and Al + MET. (**a**) *PgCCR*; (**b**) *PgCCR*; (**c**) *PgCAD*; (**d**) *PgCAD*; (**e**) *PgC4H*; (**f**) *PgC4H*; (**g**) *PgCCoAOMT*; (**h**) *PgCCoAOMT*. The glyceraldehyde-3-phosphate dehydrogenase gene normalized expression. Values are presented as means (n=3) ± standard error. Asterisks indicate a significant difference by Dunnett's test ($P \leq 0.05$).

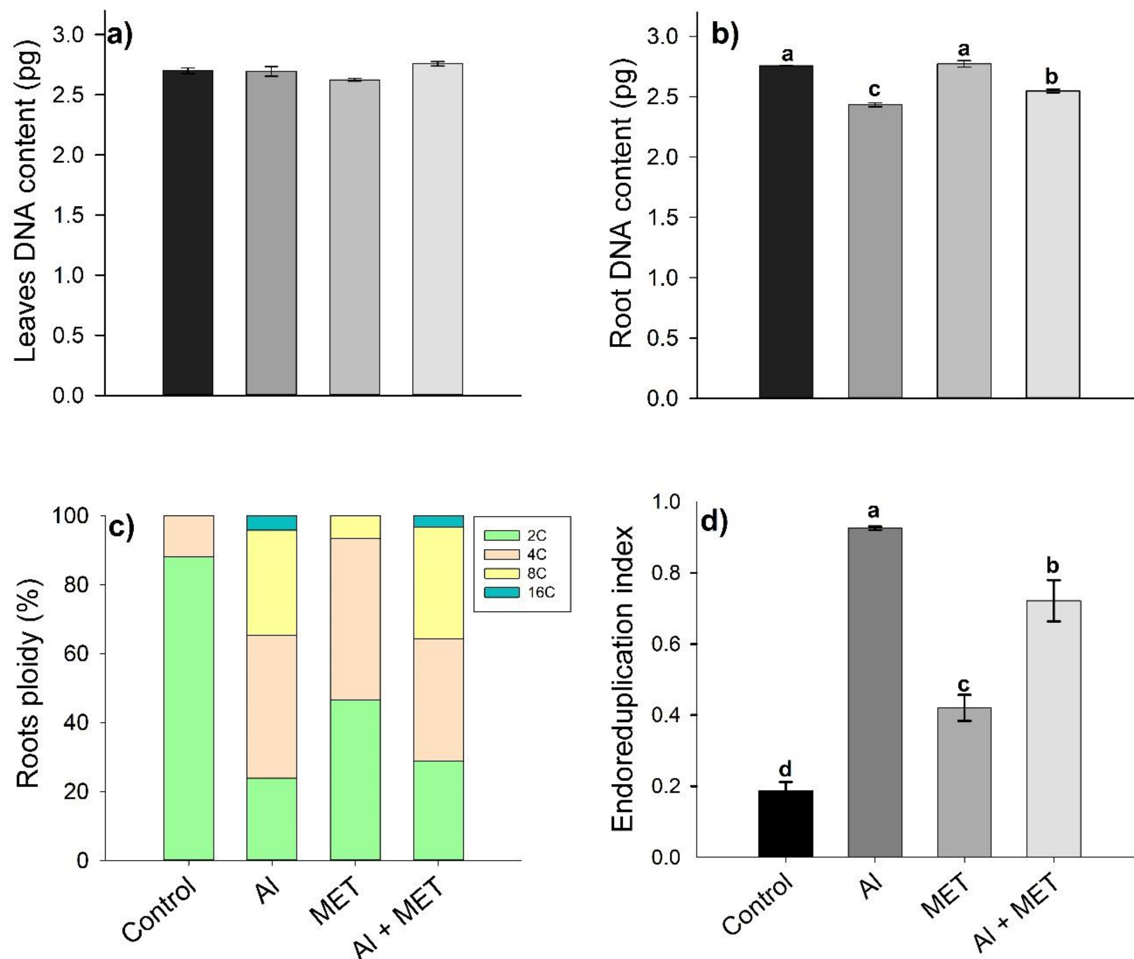


**Figure 11.** Differential DNA ploidy level and cell cycle regulation modulated by Al stress and melatonin application in *Pfaffia glomerata* plants grown in vitro for 40 days under the following conditions: control; Al; MET and Al + MET. **a**) DNA content (pg) of leaves, **b**) DNA content (pg) of roots, **c**) DNA ploidy level in root apical meristematic cells and **d**) Endoreduplication index. Values are presented as means (n=4) ± standard error. Means followed by equal letters did not differ by the Tukey test ($P \leq 0.05$).

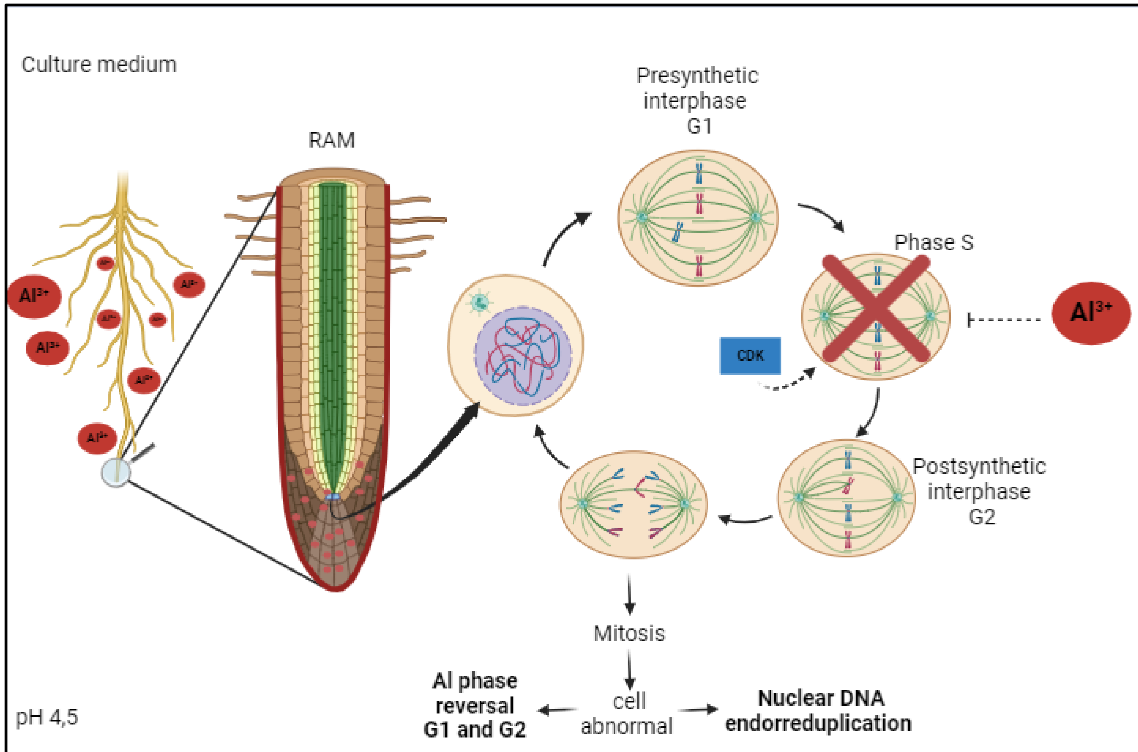


**Figure 12**. Hypothetical model of the involvement of Al in DNA endoreduplication events and the occurrence of abnormal cell divisions resulting from the inversion of cell cycle phases in *Pfaffia glomerata* monitored via flow cytometry.

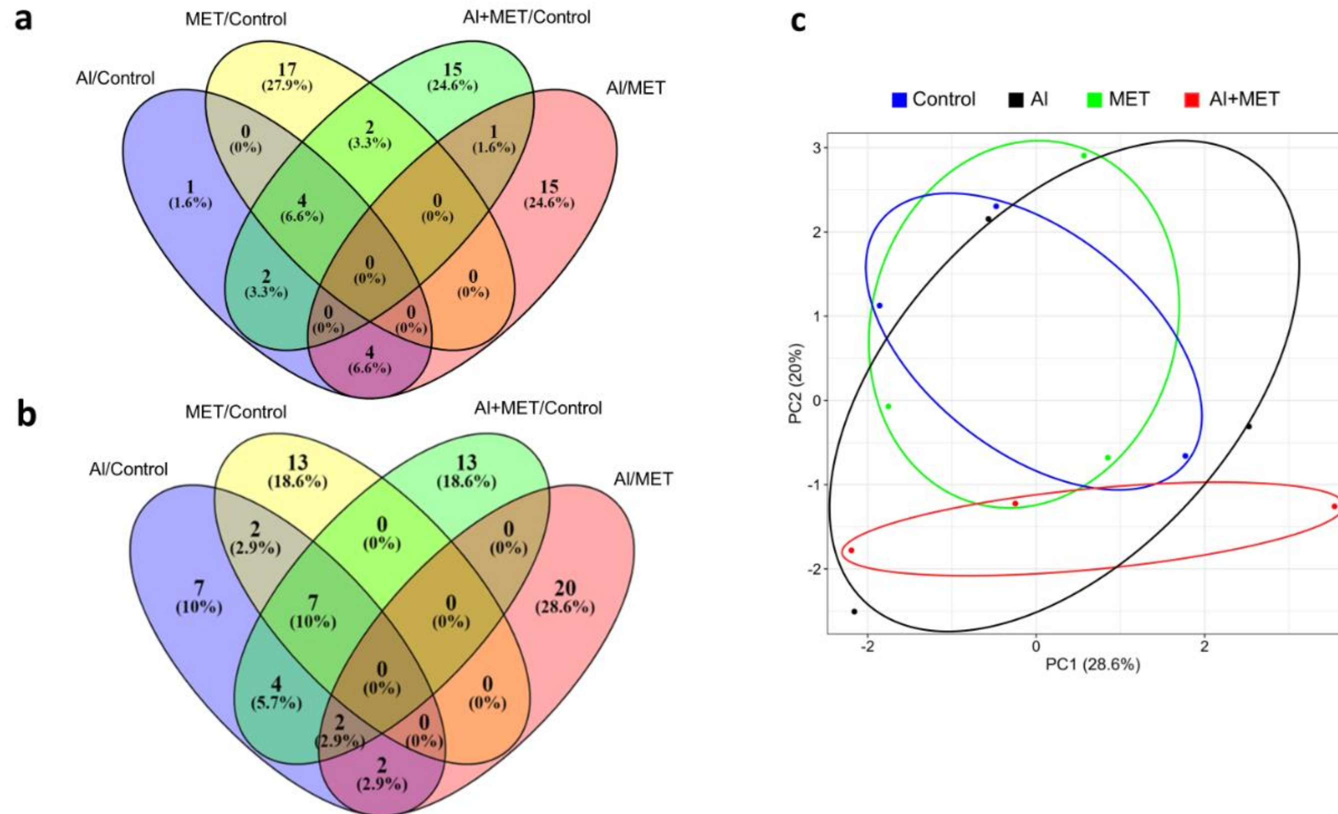


**Figure 13.** Analysis of differentially accumulated proteins (DAPs) in leaves of *P. glomerata* plants grown in vitro for 40 days under the following conditions: control; Al; MET and Al + MET. Venn diagram showing common DAPs proteins (**a**) up- and (**b**) down-accumulated and; (**c**) Principal component analysis (PCA) score plot of proteome profiles.

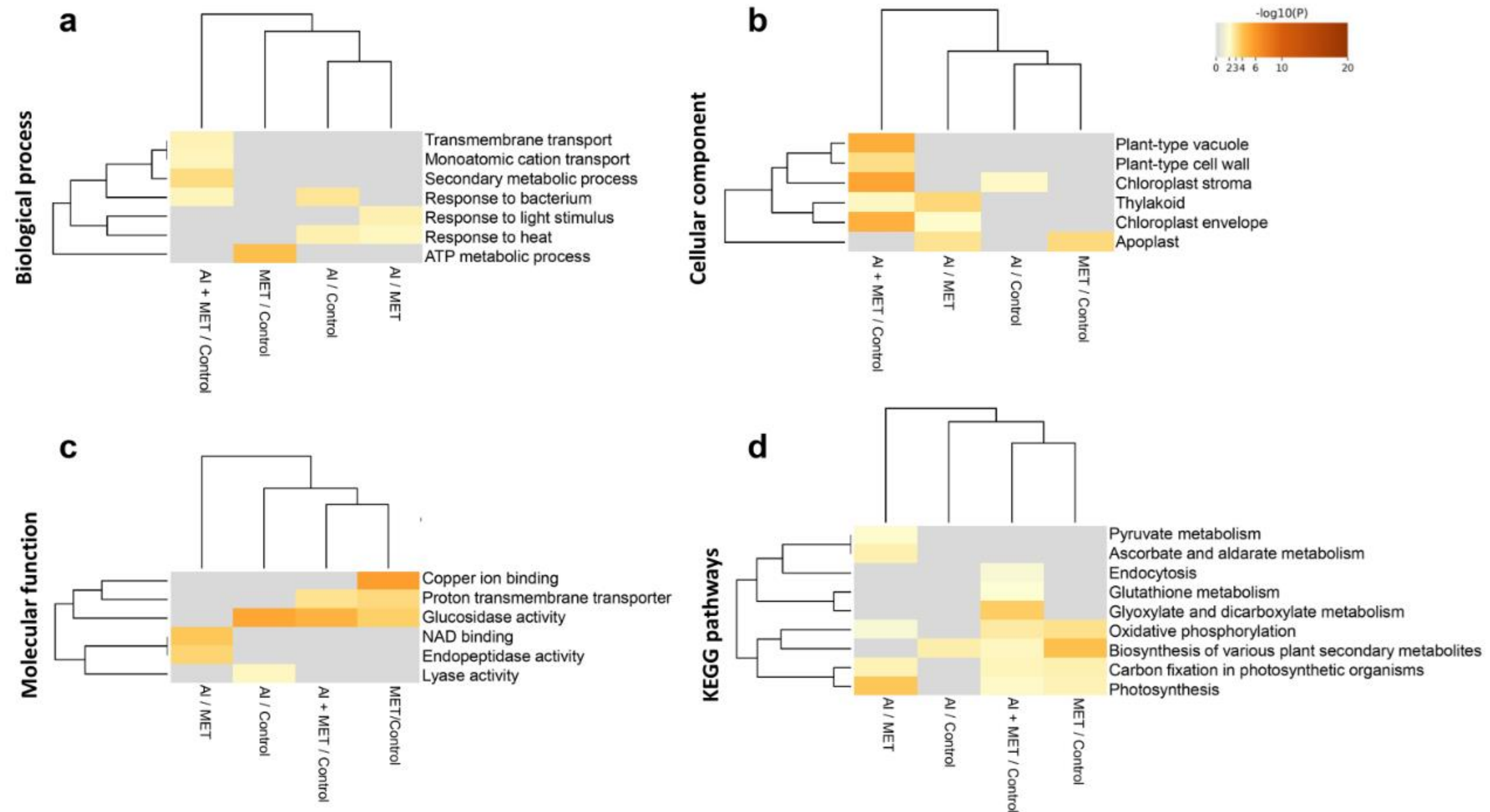


**Figure 14.** Functional classification based on the gene ontology (GO) of differentially accumulated proteins in leaves of *Pfaffia glomerata* plants grown in vitro for 40 days under the following conditions: control; Al; MET and Al + MET. (**A**) Biological process; and (**B**) cellular component. Processes and components identified as Others correspond to all the other GO terms added together. pathway enrichment analysis revealed by KEGG analyzes (Kyoto Encyclopedia of Genes and Genomes).

**SUPPLEMENTARY MATERIAL**

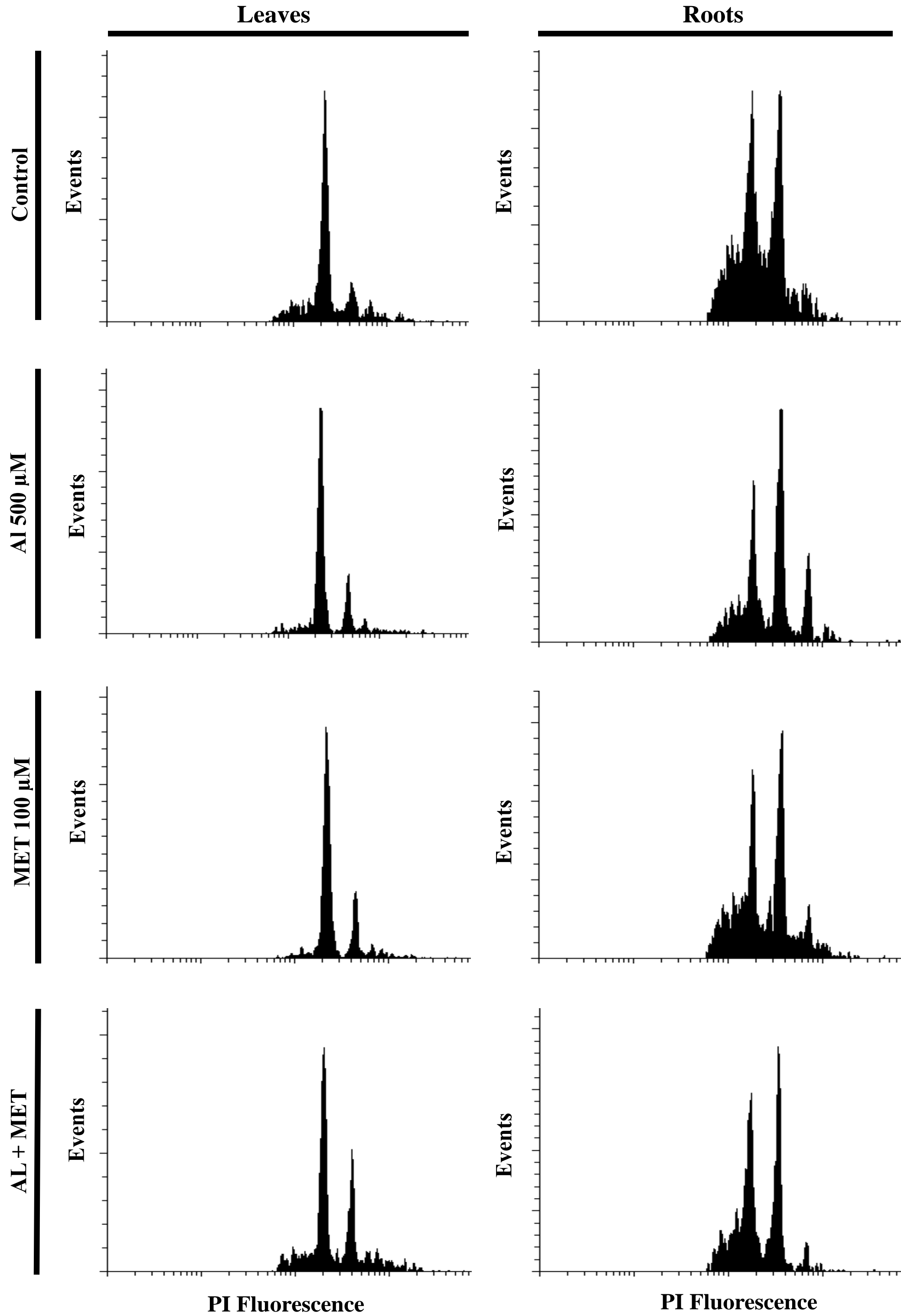


**Figure S1.** Histograms of flow cytometry in leaves and roots of *P. glomerata* plants grown in vitro under the following conditions: control; Al; MET and Al + MET.

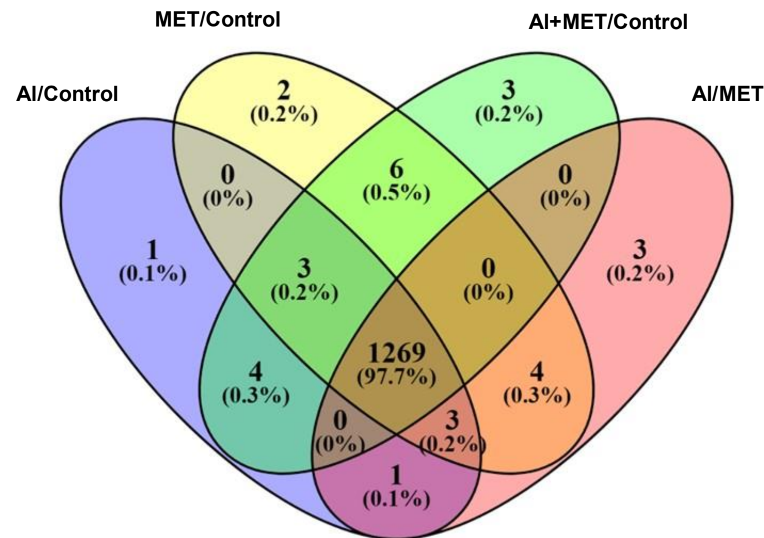


**Figure S2**. Venn diagram illustrating the numbers of proteins identified in plants of *P. glomerata* plants grown in vitro for 40 days under the following conditions: control; Al; MET and Al + MET. The diagram shows common and unique proteins at each treatment.

**Table S1**. Primer sequences used for real-time PCR.

| Primer | Name | Sequence 5'-3' |
|---|---|---|
| *PgSpook* | *ecdysteroid 25-hydroxylase (*Cyp307a1) | GATTCAACTGCGCCCCTCTT- **F**<br>ACCAAGCCCTCCTAAACCAC-**R** |
| *PgPhantom* | *ecdysteroid 25-hydroxylase* (Cyp306a1) | GACATTGTTACCAACTGCCGC-**F**<br>ACCCAAGAGATGCCGATAAGC-**R** |
| *PgCCR* | *cinnamoyl-CoA reductase* | ACTTCTCTCTCACCAGTCCGT- **F**<br>GCCTTCTTCATGGAGGCCTT- **R** |
| *PgCAD* | *Cinnamyl alcohol dehydrogenase* | GATTCAACTGCGCCCCTCTT- **F**<br>ACCAAGCCCTCCTAAACCAC-**R** |
| *PgC4H* | *cinnamate 4-hydroxylase* | TTCGCATTTGTTGAGGCTGA- **F**<br>AGGAAACGCGACAGAACCAA- **R** |
| *PgCCoAOMT* | *caffeoylCoA O-methyltransferase* | TTCGCATTTGTTGAGGCTGA- **F**<br>AGGAAACGCGACAGAACCAA- **R** |
| *PgGAPDH* (gene reference) | glyceraldehyde-3-phosphate dehydrogenase | ACTTCTCTCTCACCAGTCCGT- **F**<br>GCCTTCTTCATGGAGGCCTT- **R** |

**Table S2.** Complete list of identified proteins, functional protein annotations, and configuration parameters.

https://docs.google.com/spreadsheets/d/1f9e8wUOeUGmlPZ67d9T9F2pjuKYUcYzF/edit?usp=drive_link&ouid=112497179649771551195&rtpof=true&sd=true