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Melatonin Alleviates Aluminum Toxicity in *Pfaffia glomerata* by Modulating Tolerance Mechanisms

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Abstract
Aluminum (Al) toxicity is the main factor limiting agricultural production in acidic soils around the world. In this scenario, melatonin (MET) has emerged as a promising approach to alleviate Al toxicity. Thus, we investigated morphophysiological responses of *Pfaffia glomerata* (Spreng.) Pedersen, a medicinal plant, to Al toxicity and MET application. Plants were grown in vitro for 40 days in MS medium (control) supplemented with Al, MET, and Al+MET. Aluminum had genotoxic effects, inducing DNA damages and endoreduplication, which resulted in growth arrest, mainly in roots. Al also decreased the photosynthetic rate and the contents of pigments, carbohydrates, amino acids, and proteins. On the other hand, all these effects were improved in plants supplemented with MET. This is supported by the up-accumulation of proteins involved in organic acids exudation (AHA2), photosynthesis (transketolase, rubisco activase, and photosystem I P700 apoprotein A2), amino acid biosynthesis (chorismate synthase 1 and aspartokinase/homoserine dehydrogenase), and cell expansion and differentiation (rough sheath 2, beta tubulin b6, and tubulin beta chain-like). Moreover, MET enhanced CAT, SOD, and GABA, reinforcing its antioxidant role and reducing DNA damage and endoreduplication, which preserved QC identity and root elongation. Al toxicity impaired growth and carbon–nitrogen metabolism, whereas MET mitigated these effects by boosting photosynthesis, antioxidant defenses, and Al sequestration, decreasing Al adherence to roots. Thus, MET serves as a protective molecule against Al toxicity in *P. glomerata*.

Keywords Brazilian-ginseng · Endoreduplication DNA · Lignin biosynthesis · Metabolomics · 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 and Upadhyaya 2021). Al is solubilized in the liquid phase as a trivalent aluminum ion (Al^{3+}) in acidic soils with a pH below 5, disrupting the physiological and metabolic functioning of plants (Yang et al. 2019). Excess Al inhibits cell division, root elongation, and root hair formation, which in turn decreases water and nutrient uptake (Bojórquez-Quintal et al. 2017; Singh et al. 2017; Kar et al. 2021; Yang et al. 2021). In addition, Al stress decreases photosynthetic rate, stomatal conductance, and transpiration rate, also leading to the overproduction of reactive oxygen species (ROS) and lipid peroxidation, causing significant yield losses (Singh et al. 2017; Chauhan et al. 2021; Phukunkamkaew et al. 2021). In addition, Al induces genotoxic stress, causing DNA damage and disturbing replication (Gupta et al. 2013; Singh et al. 2017).

Due to its highly deleterious effects, some plants have developed tolerance mechanisms to Al stress conditions, including metabolism modulation, exudation of organic acids (OA), such as citrate, malate, and oxalate from roots, modifications on cell wall, vacuolar compartmentalization

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of Al, and DNA repair (Nunes-Nesi et al. 2014; Kochian et al. 2015; Eekhout et al. 2017; Singh et al. 2017; Phukunkamkaew et al. 2021). The role of OA exudation in Al tolerance is their capacity to form OA-metal complexes in the rhizosphere (Nunes-Nesi et al. 2014; Kochian et al. 2015). However, an increase in OA production does not always mean greater tolerance to Al, since Al-sensitive genotypes produce and exude large amounts of OA (Pineros et al. 2005). In turn, the interest in tolerance mechanisms related to DNA repair is increasing, with studies focusing on genetic manipulations of DNA checkpoints, which allow cell cycle progression even in the presence of Al (Eekhout et al. 2017; Hajiboland et al. 2023).

The use of emerging plant growth regulators, such as melatonin (N-acetyl-5-methoxytryptamine – MET), has been considered a promising strategy to enhance crop tolerance to a wide range of abiotic stresses, including drought, salinity, and heavy metal toxicity (Dawood 2022; Hassan et al. 2022). In plants, MET functions as a multifunctional signaling molecule and a potent biostimulator of growth, particularly under adverse environmental conditions (Debnath et al. 2019; Zhang et al. 2019; Back 2021). Recent studies have further reinforced its protective role, demonstrating that melatonin supplementation enhances pansy tolerance to salinity stress (Muhammad et al. 2024), alleviates salt-induced damage in tomato seedlings (Altaf et al. 2021), and improves heavy metal-stress tolerance in pepper by mitigating oxidative damage and reducing metal accumulation (Altaf et al. 2023). Collectively, these findings support the growing evidence of MET's broad and beneficial impact on plant stress resilience (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, carbon and nitrogen metabolism, protein accumulation and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activities, in addition to activating the antioxidant defense system. It functions as a free radical scavenger, relieving stress-induced oxidative damage and mediating various physiological responses, including rooting, seed germination, flowering, aging, fruit ripening, and senescence (Arnao and Hernández-Ruiz 2020; Hassan et al. 2022; Ren et al. 2022). Thus, exogenous MET has emerged as a promising and effective strategy for improving crop performance under stress (Zhang et al. 2019). Although MET has been shown to alleviate Al toxicity by scavenging ROS and enhancing antioxidant defenses in several crop species, including pea (Ahmad et al. 2023), walnut (Sharma et al. 2020), and tomato (Ghorbani et al. 2023), research on its effects in medicinal plants such as *Pfaffia glomerata* (Spreng.) Pedersen remains limited, and its molecular mechanisms are still not fully understood. Notably, aluminum toxicity is known to trigger alterations in the leaf proteome (Li et al. 2016), and integrated analyses of the transcriptome, metabolome, and physiological responses can elucidate the mechanisms underlying stress mitigation, including pH-mediated alleviation of Al toxicity (Wu et al. 2023).

P. 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 ginsenosides (Shiobara et al. 1993). Among these, 20E is the major compound in *P. glomerata*, mainly accumulated in its roots (Dinan 2001; Kamada et al. 2009; Neves et al. 2016). Due to its pharmacological properties, 20E has been widely studied, with environmental conditions and exogenous melatonin reported to modulate its production in *P. glomerata* (Fortini et al. 2023; Silva et al. 2025).

Considering the lack of information on the potential of melatonin to alleviate Al toxicity in medicinal plants, this study aims to investigate the physiological and biochemical roles of MET in P. glomerata exposed to Al stress. We also examine changes in protein and metabolic profiles, as well as the expression of key genes involved in 20E biosynthesis. We hypothesize that MET mitigates Al-induced damage by enhancing antioxidant defenses, modulating metabolic and molecular responses, and protecting root development. The knowledge generated here will support future research and provide insights into MET-mediated Al stress tolerance in medicinal plants.

Materials and Methods

Plant Material and In Vitro *Cultivation*

The experiment was conducted at the Plant Tissue Culture Laboratory (UFV, Brazil) with accession *Alto do Caparaó* maintained in the in vitro germplasm bank (Permission No. AA2A367, SISGEN). Stock cultures consisted of nodal segments (2 cm) placed onto MS basal medium with vitamins (Murashige and Skoog 1962; Powder mixture No. M519, Phytotech Labs, Lenexa, KS, USA), devoid of casein hydrolysate and plant growth regulators, and supplemented with 3.0% (w/v) sucrose. The medium was solidified with 0.55% (w/v) micropropagation grade agar from *Gelidium* (Agar Plant TC, A296, Gel strength 910 g cm^{-2}, Phytotech Labs). The pH was adjusted to 5.6 ± 0.1 prior to the addition of the gelling agent. Cultures were kept under controlled

conditions: 25 ± 2 °C, 16-h photoperiod, and irradiance of 60 µmol m^{-2} s^{-1} provided by white LED lighting (HO Bivolt 6500 K, 40 W, 240 cm, Avant, São Paulo, Brazil).

To set up the experiment, nodal segments (average 2 cm) were cultured on MS basal medium with vitamins (as previously described), however in a stationary liquid medium devoid of the gelling agent, with pH adjusted to 4.5. Treatments consisted of control, Al (500 µM, supplied as $AlCl_3 \cdot 6H_2O$, pH 4.5), MET (100 µM, ≥ 98% purity, HPLC grade; Sigma-Aldrich, St. Louis, MO, USA), and Al + MET (Zhang et al. 2013; Ahmad et al. 2024; Oliveira et al. 2025). MET was prepared from a water-based stock solution, as it is fully soluble under the experimental conditions.

The experiment followed a completely randomized design with four treatments and four replicates (each experimental unit was composed by a flask containing three explants), and plants were grown for 40 days. More details are available in the Online Resource 1 (Supplementary Methods).

Growth Parameters

The following variables were assessed: root, stem, and leaf dry mass (g), total shoot length (cm), root length (cm), leaf area, and number of leaves. Leaf area was determined from digital photographs analyzed using the ImageJ software. Root morphology was assessed using the WinRhizo Pro system (Régent Instr. Inc.), which provided measurements of total length (cm), surface area (cm^2), diameter (cm), and volume (cm^3).

Aluminum Quantification

Aluminum concentration was determined using 0.5 g of leaf and root tissues, which were dehydrated at 50 °C for 72 h and then ground in a knife mill to particles smaller than 1 mm. The extracts were obtained by digestion in a nitroperchloric solution (Malavolta et al. 1997), and the Al content was determined using an inductively coupled plasma atomic emission spectrometer (ICP-OES, Optima 7300 DV, PerkinElmer, Inc., Shelton, CT, USA). Values were expressed in mg kg^{-1} of dry matter.

Anatomical Characterization and Histolocalization of Al in Root Apices

After 40 days of cultivation, the third leaf pair and root apices were fixed in 4% glutaraldehyde (Karnovsky 1965), dehydrated in ethanol, embedded in methacrylate, sectioned (5 µm), and stained with toluidine blue (O'Brien and McCully 1981). Slides were mounted in Permount and imaged using an Olympus AX70 TRF microscope with a digital camera system. Al accumulation in root apices was assessed by ferric hematoxylin staining (Polle et al. 1978) and visualized under a stereomicroscope (Zeiss Stemi DV4). More details are available in Online Resource 1 (Supplementary Methods).

Carbohydrates, Amino Acids, and Protein Quantification

Leaf and root samples (~ 10 mg) were extracted with methanol following Lisec et al. (2006). Samples were frozen in liquid nitrogen, ground, and then lyophilized. They were subsequently incubated with methanol at 80 °C for 20 min. After centrifugation, supernatants were used to quantify photosynthetic pigments, sugars, and amino acids. Sugars were determined enzymatically using a 96-well microplate assay with sequential enzyme additions and absorbance readings at 340 nm. Amino acids were quantified using the ninhydrin reagent, with absorbance measured at 570 nm (Yemm et al. 1955). The remaining precipitate was used to determine starch and protein contents. Protein was quantified using the Bradford method (595 nm) (Bradford 1976), and starch was determined by enzymatic hydrolysis followed by spectrophotometric analysis at 340 nm. Calculations were based on standard curves and the Lambert–Beer equation. More details are available in Online Resource 1 (Supplementary Methods).

Photosynthetic Rate, Photosynthetic Pigments and Proline Content Determination

Gas exchange and in vitro photosynthetic rates were measured following Costa et al. (2014), with modifications by Silva et al. (2020), using an AQ-S151 infrared CO_2 analyzer (Qubit Systems). Reference CO_2 was obtained by channeling ambient air into an empty flask at 300 mL min^{-1} under red and blue LED lighting (600 µmol m^{-2} s^{-1}). Plants were dark-adapted for 8 h before analysis. CO_2 levels were then recorded with plant-containing flasks, and gas exchange was calculated as the difference from the reference value. Air temperature and humidity were monitored using a Spec sensor (Thermo Recorder RS-11). More details are available in Online Resource 1 (Supplementary Methods).

To quantify photosynthetic pigments, 25 µL of the previously prepared methanolic extract was combined with 175 µL of methanol in 96-well microplates. Absorbance readings were taken using a microplate reader (OptiMax Tunable Microplate Reader) at wavelengths of 470, 666, and 653 nm, corresponding to carotenoids, chlorophyll *a* (Chl *a*), and chlorophyll *b* (Chl *b*), respectively. Calculations were performed using the equations described by Wellburn (1994).

Proline content was determined according to the method described by Bates et al. (1973). In a microplate, 50 µL of the methanolic extract was mixed with 100 µL of an acidic ninhydrin solution (1% [w/v] ninhydrin in 60% [v/v] acetic acid and 20% [v/v] ethanol). The microplate was sealed with aluminum tape and incubated at 95 °C for 20 min. Absorbance was measured at 520 nm, and a calibration curve was prepared using a standard proline solution (Sigma-Aldrich, St. Louis, MO, USA).

Determination of Antioxidant System Enzyme Activity

Catalase, APX, SOD, and POD activities were measured from leaf and root extracts (50 mg tissue homogenized in phosphate buffer with EDTA, PMSF, and PVP) (Chance and Maehly 1955; Giannopolitis and Ries 1977; Nakano and Asada 1981; Havir and McHale 1987). After centrifugation, the supernatant was used for enzymatic assays in microplates. CAT, APX, and POD activities were monitored by changes in absorbance at 240, 290, and 470 nm, respectively, and expressed as µmol min^{-1} mg^{-1} protein. SOD activity was based on inhibition of nitro blue tetrazolium photoreduction under light and expressed as U min^{-1} mg^{-1} protein. More details are available in Online Resource 1 (Supplementary Methods).

Flow Cytometry

Genetic stability was assessed by flow cytometry of nuclei suspensions from in vitro leaf and root tissues, prepared in WPB buffer (Loureiro et al. 2007) following Galbraith et al. (1983). Samples were stained with propidium iodide and treated with RNase, then analyzed in a CytoFLEX cytometer (Beckman Coulter, USA) using *Glycine max* (2C = 2.50 pg) or *Pisum sativum* (2C = 9.09 pg) as internal standards (Doležel et al. 1998). At least 10,000 nuclei were measured per sample, and DNA content was calculated as described by Doležel and Bartoš (2005). More details are available in Online Resource 1 (Supplementary Methods).

Proteomic and Metabolomic Analyses

Proteomic analysis was performed on freeze-dried leaf samples (30 mg; three replicates) using nanoUPLC–Q-TOF mass spectrometry (Waters) as described by Passamani et al. (2020). Protein identification and label-free quantification (TOP3 approach) were conducted with PLGS v3.0.2 and ISOQuant v1.7 against the *P. glomerata* database (Batista et al. 2019). Differentially abundant proteins were identified using Student's t-test ($P \leq 0.05$) and Log2 fold change thresholds (> 0.6 or < -0.6) and functionally annotated with OmicsBox.

Metabolomic analyses were conducted on freeze-dried leaf samples (30 mg; three replicates), derivatized with MSFTA, and analyzed on a GC-EI-Q-MS platform using the splitless method. Metabolites were identified with Xcalibur® 2.1 software against proprietary and international databases. Intensities were corrected by blank values, and relative metabolite content was calculated by normalizing to ribitol and sample dry mass. More details are available in Online Resource 1 (Supplementary Methods).

Data Analyzes

All experiments followed a completely randomized design with three biological replicates for proteomics/metabolomics and four biological replicates for the other analyzes. In addition, the experiments were repeated twice. Data were statistically tested for normality and subsequently examined using ANOVA, with means compared by Tukey's test ($P \leq 0.05$). Gene expression data were compared using Dunnett's test ($P \leq 0.05$). For metabolomic data, the distance among treatments was identified by canonical discriminant analysis in a 3-D scatter plot and the treatments were grouped using Tocher optimization and the generalized squared interpoint distance of Mahalanobis (D^2). The relative contribution of each variable to discriminate treatments was quantified using the Singh (1981) criterion. All statistical analyses were carried out using R statistical software (www.r-project.org).

Results

Aluminum Stress Impacts Growth, Anatomy, and Photosynthetic Capacity in P. glomerata Plants

After 40 days, Al inhibited the overall growth of *P. glomerata*, as shown by the decreases in the root length, diameter, volume, and dry mass, as well as in the leaf area and number of leaves, compared to control plants (Fig. 1). When combined to Al, MET restored the Al-induced decreases in root length and leaf area, reaching values similar to the control (Fig. 1a, e, and h). However, MET alone also decreased some growth parameters compared to control, such as shoot length and dry mass, leaf area and leaf number, as well as root dry mass and diameter. In addition to the growth impairment, the roots of plants treated with MET alone and Al + MET showed a yellowish color (Fig. 1j).

Toluidine blue staining showed that the roots treated with Al and Al + MET had structural changes, with irregularly uniseriate epidermis and a poorly developed vascular

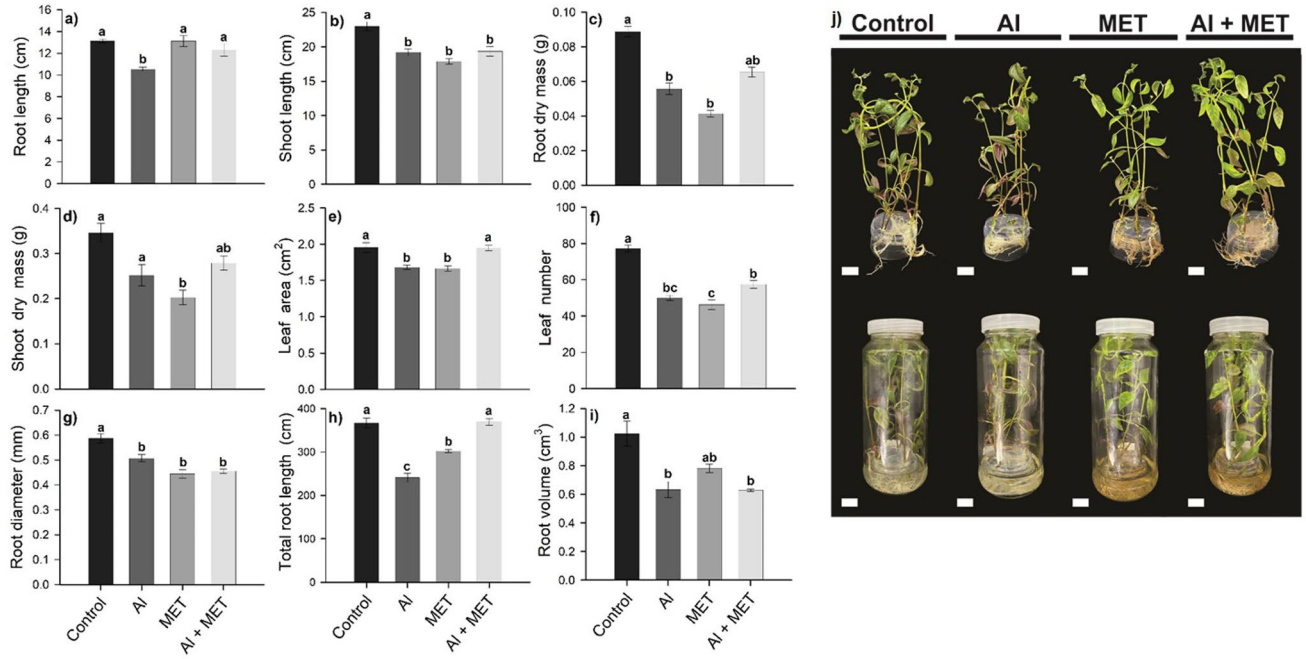


Fig. 1 Growth parameters of *Pfaffia glomerata* plants grown in vitro for 40 days on the effect of aluminum (Al), melatonin (MET), both combined (Al+MET) and neither of them (Control). (**a**) root length; stem height; (**b**) stem length; (**c**) root dry mass; (**d**) Shoot dry mass; (**e**) Total leaf area); (**f**) Leaf number; (**g**) Root diameter; (**h**) Total root length; (**i**) Root volume (cm^{-3}); and (**j**) pictures of representative plants. Values are presented as means ($n=4$) ± standard error. Averages followed by equal letters did not differ according to the Tukey test ($P \leq 0.05$). Scale bars = 2 cm

cambium, with a predominance of vessel elements (Online Resource 1, Supplementary Fig. 1). 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. In the presence of Al, the leaves had three large central vascular bundles with parenchyma distributed along the main vein, while only one was observed in the control (Online Resource 1, Supplementary Fig. 2). The lamina was also subdivided into a poorly developed palisade parenchyma with fewer intercellular spaces. A similar pattern was observed for plants supplemented with MET and Al+MET (Online Resource 1, Supplementary Fig. 2). Stems exposed to Al showed more differentiated vascular tissue, with greater vascularization and increased cambial activity, as well as a higher number of secondary xylem vessels, differing from the other treatments (Online Resource 1, Supplementary Fig. 2).

Al and MET alone also impacted the contents of photosynthetic pigments, decreasing the contents of Chl *a*, Chl *b*, total Chls, and carotenoids by more than 10% compared to the control, while there were no differences between the control and Al+MET (Fig. 2a-c). Moreover, no differences among treatments were found for Chl *a*/Chl *b* ratio (Fig. 2d). The photosynthetic rate (A), in turn, was decreased by 16% under Al, and by 21% under MET treatments, whereas the combination between Al+MET restored A to values similar to control levels (Fig. 2f).

Melatonin Decreases Aluminum Accumulation in Roots and Modulates Antioxidant Responses to Mitigate Toxicity in *P. glomerata*

After exposure to Al, *P. glomerata* plants accumulated Al in roots and leaves, with Al contents in roots and leaves being 14.8 and 3 times higher than in the control, respectively (Fig. 3a-b). The combination of Al+MET, in turn, decreased the Al accumulated in roots by 18% compared to Al treatment, while this effect was not observed in leaves (Fig. 3a). Interestingly, MET alone slightly increased the content of Al in leaves compared to the control (Fig. 3b). The decrease in Al accumulation in roots under Al+MET was further supported by anatomical analysis showing a lower Al detection through ferric hematoxylin staining in root apices in this treatment, while a strong staining occurred in Al alone (Online Resource 1, Supplementary Fig. 1). Moreover, no Al was detected in control and MET treatments.

Al and MET also modulated the activities of antioxidant enzymes, with Al increasing the activity of CAT in roots and leaves, and SOD in roots, compared to control (Online Resource 1, Supplementary Fig. S3). Compared to Al alone, Al+MET, slightly decreased CAT activity in roots, while

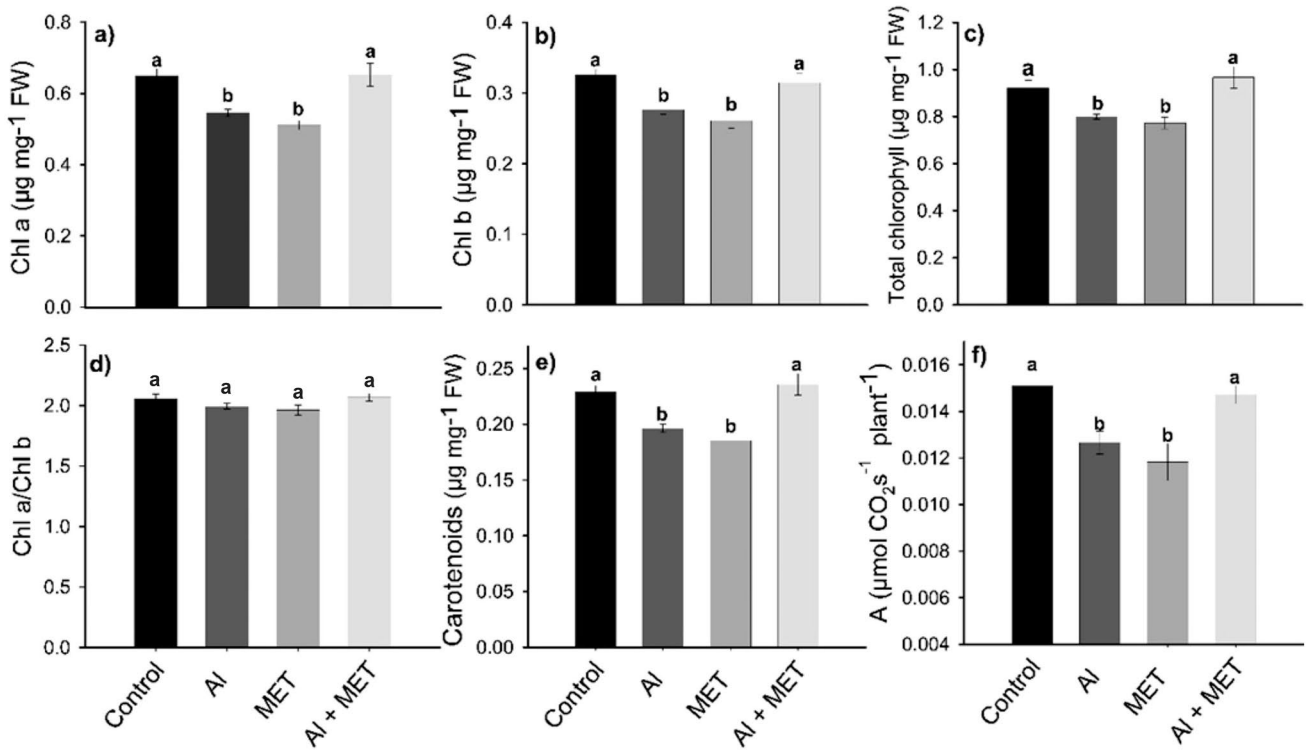


Fig. 2 Photosynthetic pigment contents and photosynthetic rate of *Pfaffia glomerata* plants grown in vitro for 40 days on the effect of aluminum (Al), melatonin (MET), both combined (Al+MET), and neither of them (Control). (**a**) chlorophyll *a*; (**b**) chlorophyll *b*; (**c**) total chlorophylls; (**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 ($P \leq 0.05$)

increasing SOD activity in both roots and leaves. Compared to the control, MET alone increased the activity of CAT in roots and leaves, while increasing SOD and decreasing POD activity in roots (Online Resource 1, Supplementary Fig. S3). Al stress also modulated the expression of genes related to the biosynthesis of 20-hydroxyecdysone (20E), down-regulating *PgSpook* and *PgPhantom* in roots and up-regulating *PgPhantom* in leaves, while MET was not able to reverse this effect (Online Resource 1, Supplementary Fig. S4).

Metabolic Responses of *P. glomerata* to Al and Melatonin Treatments

Starch content increased by 20% in plants grown under to Al+MET compared to all other treatments, with no differences among them (Fig. 3c). Al toxicity, in turn, decreased protein content by 25%, and total amino acids by 30% while increasing total phenols content by 30% (Fig. 3d-f). When combined to Al, MET (Al+MET) was able to restore total phenols, amino acids, and protein contents to values similar to those of control, while MET alone only decreased protein content, compared to control (3d-f). Sucrose and proline contents were not affected by any of the treatments.

The metabolic profile was modulated by Al and MET, with most metabolites showing lower relative metabolic content than the control (Fig. 4a). Compared to the control, plants exposed to Al had lower relative content of glutamine, pyroglutamic acid, and tyramine, and higher oxalic acid, while MET-treated plants had higher relative content of shikimic acid and succinic acid, and lower sucrose. Al, MET, and Al+MET resulted in lower relative contents of glycine, sorbitol, and urea (Fig. 4a). Interestingly, when combined, MET reversed the effect of Al on the relative content of ornithine, oxalic acid, pyroglutamic acid, and tyramine, remaining unchanged in relation to the control.

The canonical analysis of metabolic profile revealed that Al (red ellipse) and MET (green ellipse) single treatments formed individual groups, while control and Al+MET were grouped together (blue ellipse) (Fig. 4b). The variables that most contributed to this separation were threonic acid, GABA, glucose, glutamine, and sucrose, with contributions higher than 6% (Fig. 4c).

Regarding the protein profile, 1309 proteins were identified, of which 1269 (97.7%) were found in the four comparisons analyzed: Al/Control; MET/Control; Al+MET/Control and Al/MET (Fig. 5a, c, Table S2). A total of 174 differentially accumulated proteins (DAPs) were found

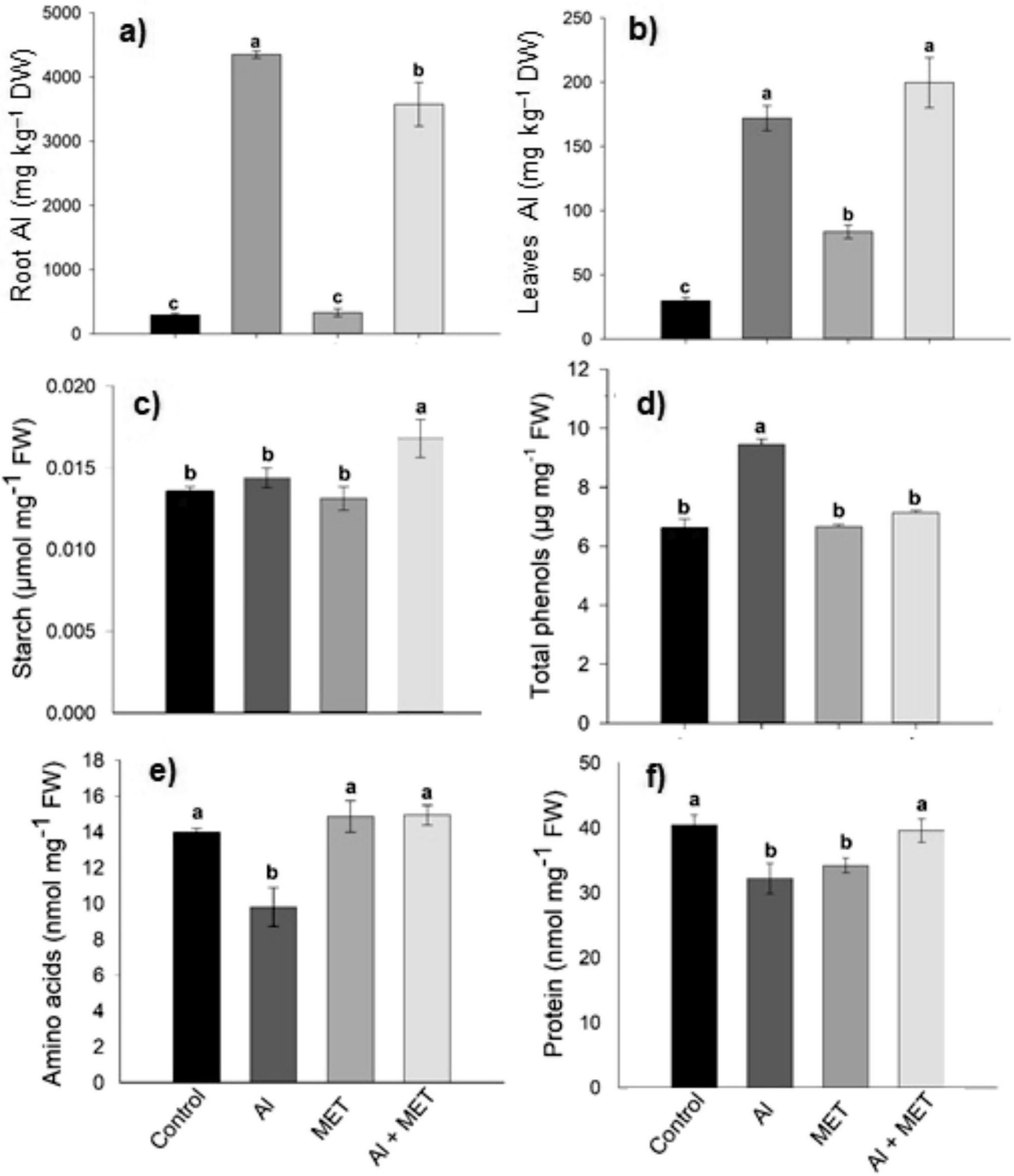


Fig. 3 Accumulation of Al and changes in the primary metabolism of *Pfaffia glomerata* plants grown in vitro for 40 days on the effect of aluminum (Al), melatonin (MET), both combined (Al + MET) and neither of them (Control). (**a**) Al contents in roots; (**b**) Al contents in shoots; (**c**) starch; (**d**) total phenols; (**e**) total amino acids; and (**f**) total proteins. Values are presented as means ($n = 4$) ± standard error. Averages followed by equal letters did not differ according to Tukey's test ($P \leq 0.05$)

a)

Metabolites	Al	MET	Al + MET
Lactic Acid	0.6	0.3	0.1
Glycolic Acid	**0.3**	**0.3**	**0.5**
Oxalic Acid	**4.2**	0.5	0.1
Malonic Acid	0.2	-0.1	-0.2
Urea	**-1**	**-1**	**-1**
Serine	0.1	0.1	0.1
Serine-O-acetyl	-0.4	-0.2	-0.3
Glycerol	0.1	0.1	0
Glycine	**-0.5**	**-0.5**	**-0.5**
Succinic Acid	0.2	**0.3**	0.2
Glyceric Acid	0.3	-0.2	-0.2
Fumaric Acid	0.1	0.1	0.6
Malic Acid	0.8	0.2	1.1
Glutaric Acid	0.8	0.6	1.3
Erythritol	0	0.1	0.1
Pyroglutamic Acid	**-0.3**	-0.1	-0.1
GABA	0.1	0	1.6
Threonic Acid	0.3	0.1	0.1
Glutamic Acid	0	0	-0.2
Phenylalanine	-0.5	-0.5	-0.6
Xylitol	-0.1	-0.1	-0.1
Arabitol	-0.1	-0.5	-0.5
Glycerol 3-phosphate	0	-0.1	-0.1
Glutamine	**-0.5**	-0.1	-0.3
Shikimic Acid	0.2	**0.4**	0.2
Ornithine	-0.6	0.2	0.2
Citric Acid	2.2	0.4	0.4
Fructose	-0.4	-0.4	-0.4
Glucose	-0.3	-0.2	-0.3
Lysine	-0.5	-0.2	-0.3
Sorbitol	**-0.4**	**-0.5**	**-0.5**
Allo-inositol	0.2	0.3	0
Galactonic Acid	0.7	0.1	0
Gluconic Acid	0.2	-0.4	-0.5
Tyramine	**-0.8**	-0.5	-0.3
Myo-inositol	0	-0.2	-0.2
Tryptophan	-0.2	0	-0.2
Sucrose	-0.3	**-0.4**	-0.4

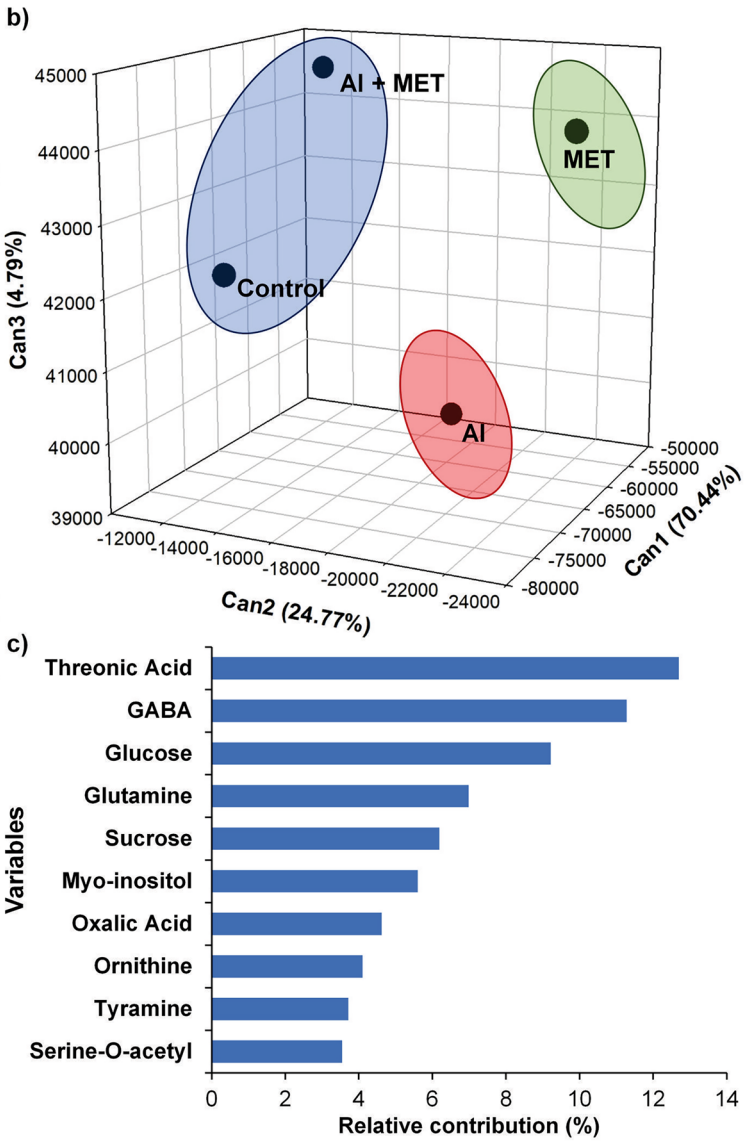


Fig. 4 Metabolic profile of *Pfaffia glomerata* under aluminum (Al), melatonin (MET), and their combination (Al+MET) or neither of them (Control). (**a**) Heat map representation of the relative changes in metabolite level in the leaves. Columns and rows represent treatments and metabolites, respectively. The heatmap was constructed by normalizing the relative level of the metabolites observed in the control. Given that all control ratios are equal to zero (0), and were omitted for simplicity. Values in blue and red indicate metabolites with increased and decreased levels, respectively. Bold numbers indicate values that are significantly different from the control by Dunnett's test ($P<0.05$). (**b**) Three-dimensional scatter plot of first three canonical variables (% total variance explained by each canonical component is indicated in parentheses; treatments indicated by the same color were assembled into the same group by the Tocher optimization method and the generalized squared interpoint distance of Mahalanobis). (**c**) Relative contributions of original variables, calculated using the Singh method, to the canonical variables

(Fig. 5a, c). Comparisons between Al/Control; MET/Control; Al+MET/Control and Al/MET, revealed 11, 23, 24 and 20 proteins up-accumulated, respectively; and 24, 22, 26 and 24 proteins down-accumulated, respectively (Fig. 5a, c). The first two principal components (PC1 and PC2) for the proteomic data explained 48.6% of the total variability, revealing a similar distribution between Control and MET, which were distributed separately from Al+MET (Fig. 5b). Al treatment, in turn, had a broader distribution among the two components, pointing that Al alone and combined with MET modulate the protein profile in *P. glomerata*.

The gene ontology (GO) analyses of DAPs were performed by using OmicsBox software (Fig. 5d-g). The proteins were identified and categorized into Biological Process and Cellular Component, Molecular Function and Metabolic Pathways, with proteins of all categories being altered in response to treatments. Al+MET was the treatment that most altered proteins involved in biological processes,

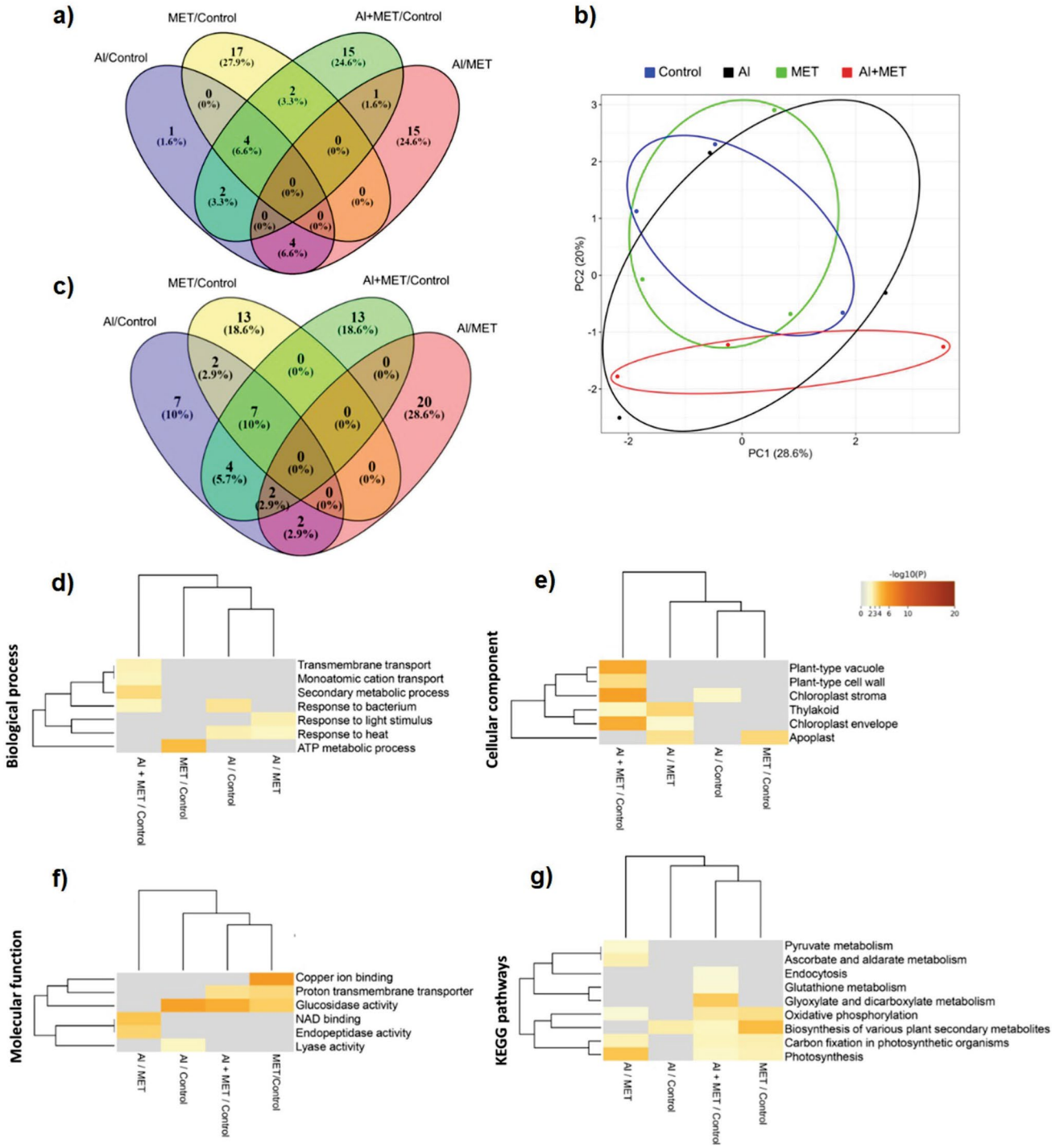


Fig. 5 Analysis of differentially accumulated proteins (DAPs) and functional classification based on the gene ontology (GO) in leaves of *Pfaffia glomerata* plants grown in vitro for 40 days on the effect of aluminum (Al), melatonin (MET), both combined (Al + MET) and neither of them (Control). Venn diagram showing common DAPs proteins (**a**) up- and (**b**) down-accumulated; (**c**) Principal component analysis (PCA) score plot of proteome profiles; (**d**) biological process; (**e**) cellular component; (**f**) molecular function; and (**g**) KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways analysis

cellular components, and KEGG pathways. Al increased the accumulation of proteins involved in responses to heat, light, and bacterium, which were mainly localized in the chloroplast and apoplast, functioned as glucosidase, endopeptidase, lyase and NAD-binding proteins, in pathways related to photosynthesis, pyruvate, ascorbate, and secondary metabolism (Fig. 5d-g). MET, in turn, increased the accumulation of glucosidases, proton transporters, and Cu binding proteins related to ATP metabolic processes, photosynthesis, secondary metabolism, and respiration. The DAPs were predominantly present in the vacuoles, cell wall and chloroplasts and apoplasts (Fig. 5e).

Among specific protein changes, Al+MET up-accumulated the photosynthesis-related chloroplastidic transketolase (TKL-1), rubisco activase (F3N23.32), photosystem I P700 apoprotein A2 (psaB), and a plastidal glycolate/glycerate translocator (PLGG1), as well as a heat shock protein (HSP70-6), which was also up-accumulated by MET alone (Table S1). Al, in turn, down-accumulated an aspartic proteinase CDR1-like (F13O11.13) involved in salicylic acid defense responses, and a chloroplastic chorismate synthase 1 (EMB1144) and aspartokinase/homoserine dehydrogenase (AKHSDH2), both involved in amino acids biosynthesis.

Al Decreases the DNA Ploidy in the Roots and Positively Regulates Endoreduplication

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 leaf tissues and root apices. Our results indicated a reduction in nuclear DNA content in root apex cells under Al toxicity, while no changes were observed in leaves (Fig. 6a, b). Interestingly, MET partially mitigated this effect, as shown by root DNA content in Al+MET (Fig. 6b).

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, with no signal of MET-mediated repair of proliferative capacity under stress (Fig. 6c). Our results also revealed a pronounced increase in the endoreduplication index in the presence of Al, which was only slightly decreased by MET in the Al+MET treatment (Fig. 6d). MET alone also induced polyploidy in root cells, increasing the endoreduplication in relation to the control (Fig. 6d).

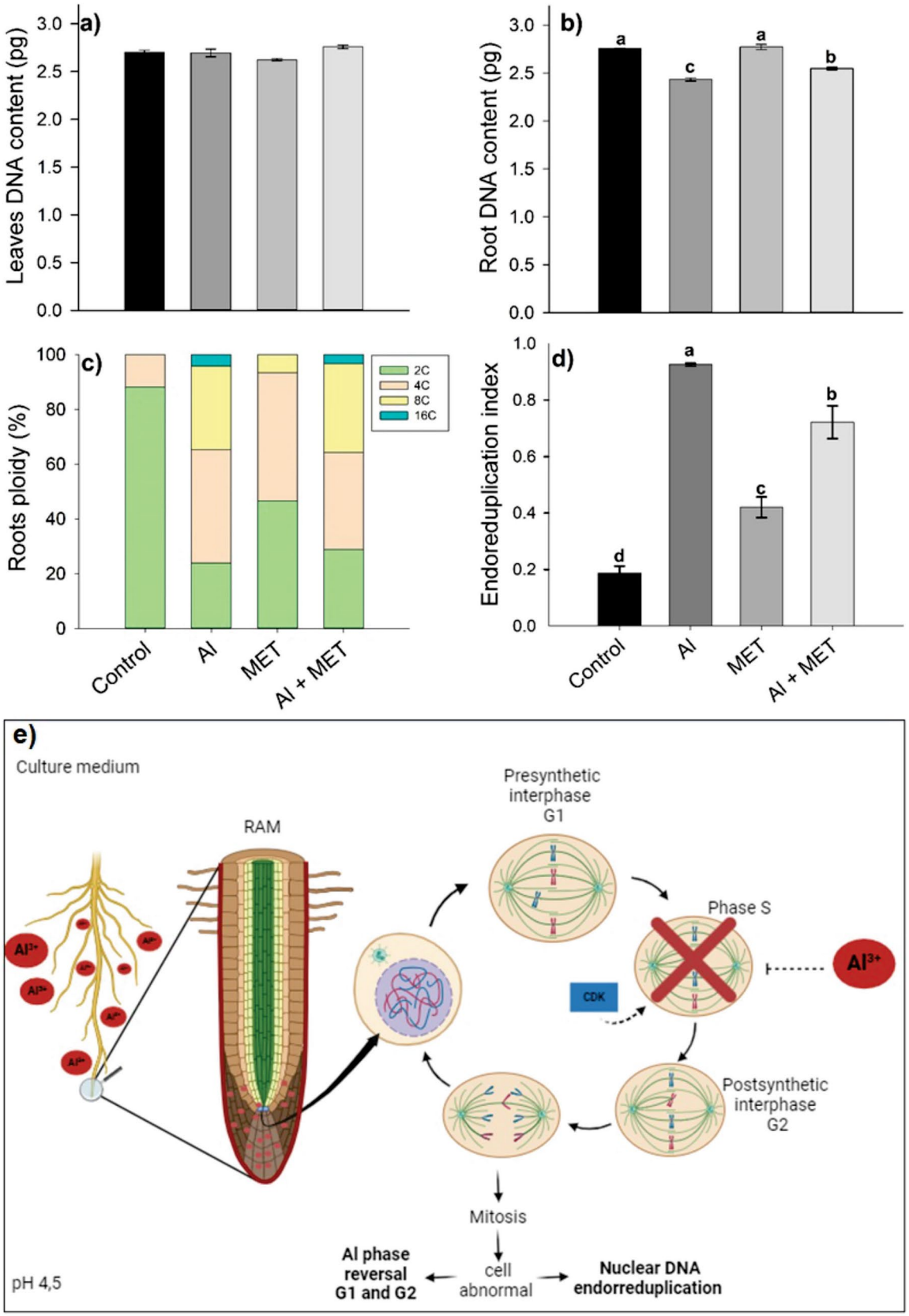
Discussion

Melatonin (MET) is a regulatory molecule that plays various biological roles in plant growth and development, as well as in response to abiotic stresses, including the mitigation of Al stress (Hassan et al. 2022; Zeng et al. 2022). In the present work, we addressed the alleviation of Al toxicity promoted by melatonin in *Pfaffia glomerata* plants grown in vitro.

Fig. 6 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 on the effect of aluminum (Al), melatonin (MET), both combined (Al+MET) and neither of them (Control). (**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 (0–1). Values are presented as means ($n=4$)±standard error. Means followed by equal letters did not differ by the Tukey's test ($P \leq 0.05$). (**e**) 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

Here, Al stress impaired overall growth of *P. glomerata*, reducing the shoot length and leaf area, as well as the length, diameter, total length and volume of the roots. As a consequence of smaller roots and photosynthetic area, less water and essential nutrients are absorbed, and less biomass is produced (Xu et al. 2020). Conversely, exogenous MET maintained the growth of *P. glomerata* and partially recovered biomass accumulation under Al stress, as indicated by the root length and leaf area, which remained similar to the control, allowing greater photosynthetically active area and, ultimately, biomass production (Xu et al. 2020). This is in accordance with our results for photosynthetic pigments and photosynthetic rate, that were decreased by Al, but recovered upon exogenous MET. These results are in accordance with proteomic findings, which show that Al resulted in the down-accumulation of delta-aminolevulinic acid dehydratase (HEMB1) and photosystem II 22 kDa protein, chloroplastic isoform X1 (PSBS), as these proteins are involved in the biosynthesis of chlorophylls and light harvesting in photosystem II.

The alleviation effect of MET on the photosynthetic capacity in plants under abiotic stresses has been previously reported in other species (Wang et al. 2020; Hassan et al. 2022). According to the protein profile of *P. glomerata* analyzed here, MET-induced alleviation of Al toxicity on photosynthesis may be related to the up-accumulation of a chloroplast transketolase (TKL-1; Table S1), 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. It also points to its effects on substrate saturation kinetics at specific physiological pH values, favoring carbon allocation and thus promoting the maintenance of photosynthetic activity (Hassan et al. 2022). Moreover, Al+MET resulted in the up-accumulation of a rubisco activase (F3N23.32), and a photosystem I P700 apoprotein A2 (psaB), which may be involved in the increase in photosynthetic rate under this condition (Table S1). Our results are in accordance with other studies, which report that MET induces the expression


a)
Leaves DNA content (pg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b)
Root DNA content (pg)
a
c
a
b
c)
Roots ploidy (%)
100
80
60
40
20
0
2C
4C
8C
16C
Control
Al
MET
Al + MET
d)
Endoreduplication index
1.0
0.8
0.6
0.4
0.2
0.0
d
e)
Culture medium
RAM
Presynthetic interphase G1
Phase S
CDK
Postsynthetic interphase G2
Mitosis
cell abnormal
Al phase reversal G1 and G2
Nuclear DNA endorreduplication
pH 4,5

of genes and the accumulation of Rubisco activase in different species, thereby improving their photosynthetic capacity (Erdal 2019). Nevertheless, here, this protein was up-accumulated only by Al + MET, and not by MET alone, suggesting that this is a response induced specifically under stress conditions. Taken together, our results point to the function of MET in improving plant photosynthesis in the presence of Al at the protein level, sustaining the initial growth of *P. glomerata*.

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), with all these processes associated with loss of DNA integrity (Eekhout et al. 2017; Yang et al. 2019). Accordingly, we found decreases in the DNA content and increases in endoreduplication in roots under Al toxicity, which was partially alleviated by MET, pointing to a possible defense mechanism by which MET alleviated root growth arrest. Another aspect that may be involved in MET-induced maintenance of *P. glomerata* growth under Al toxicity was the up-accumulation of proteins involved in cell division and differentiation, such as protein rough sheath 2 (RS2) and beta tubulin b6 (TUBB1), in Al + MET treated plants, as well as the reversion of down-accumulation of a tubulin beta chain-like (TUBB3) induced by Al (Table S1).

Despite the alleviation of Al toxicity, MET alone also impaired the growth of *P. glomerata*. As MET has been previously reported to inhibit brassinosteroids, preventing cell expansion (Xiong et al. 2019), the MET-induced growth inhibition observed here may be related to its crosstalk with other hormones. In fact, we found a down-accumulation of EXORDIUM-like 2 protein (EXL2-2), a regulator of BR-responsive genes that promote plant growth (Coll-Garcia et al. 2004). Furthermore, MET induced the down-accumulation of α-expansin (EXPA8), also involved in cell expansion (Wang et al. 2020).

The decreased biomass production under Al stress was accompanied by a lower concentration of non-structural carbohydrates (glucose and fructose), which is consistent with the observed inhibition of photosynthesis. In addition, Al toxicity significantly reduced protein and total amino acid contents, as well as the relative content of several amino acids, such as glutamine, ornithine, and tyramine, which was, at least partially, recovered by MET. This could be related to the down-accumulation of urea, indicating low nitrogen availability for protein and amino acid biosynthesis; however, the decrease was also observed in the other treatments that had contents similar to the control. Thus, the modulation of amino compounds may have occurred through biochemical pathways. In fact, we found that Al induced the down-accumulation of chloroplastic chorismate synthase 1 (EMB1144) – an enzyme involved in the biosynthesis of several amino acids –, with this effect being reversed by MET. Despite the well-described role of MET in proline metabolism and the importance of these osmoprotectants in Al stress tolerance (Rahman and Upadhyaya 2021; Ghorbani et al. 2023), the content of this amino acid was not affected by Al or MET. Regarding protein profile, it is also 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.

The modulation of Al uptake, accumulation, and transport through plant tissues are major tolerance responses (Rahman and Upadhyaya 2021). For instance, plants can reduce Al uptake by inducing exudation of organic acids to the rhizosphere and modifying the cell wall, as well as maintain low levels of free Al in the cytosol by complexation with organic acids and phenolics, and by sequestration in the vacuole (Nunes-Nesi et al. 2014; Yang et al. 2019; Rahman and Upadhyaya 2021; Hajiboland et al. 2023). Here, *P. glomerata* plants accumulated Al mainly in their root system, also translocating it to the aerial parts, while MET did not decrease Al accumulation in shoots, but only slightly reduced it in root tissues. Despite that, MET did decrease Al adherence to the roots, as shown by the low detection of Al in root tissues of Al + MET (Online Resource 1, Supplementary Fig. 1). This low adherence may be related to proteins of the ATP-binding cassette (ABC) family, a group of proteins comprising transporters that play a vital role in Al tolerance (Wen et al. 2021). For instance, STAR1 and STAR2 are ABC members that transport UDP-glucose to the apoplast, masking Al binding sites and decreasing its adhesion to roots (Hajiboland et al. 2023). In this study, ABCI17, as well as total phenols, citric acid, malic acid, and oxalic acid, were up-accumulated by Al, pointing to the occurrence of complexation and compartmentalization of Al. On the other hand, Al + MET did not alter the abundance of ABCI17, but rather increased the accumulation of organic acids to a lesser extent than Al alone. Despite that, Al + MET induced the up-accumulation of GABA and a proton ATP-ase (AHA2), which are known to participate in malate and citrate exudation to the rhizosphere during Al toxicity (Hajiboland et al. 2023). This, together with the reported role of MET in modifying the cell wall polysaccharides composition (Sun et al. 2020), may help to explain the Al exclusion induced by MET observed here.

MET is a free amphiphilic molecule that crosses cell membranes, directly scavenging ROS, and increasing the activity of antioxidant enzymes and accumulation of non-enzymatic antioxidants under several stresses (Imran et al. 2021; Hassan et al. 2022; Iqbal et al. 2024). Under Al stress conditions, MET was reported to increase SOD, POD and CAT activity in roots and leaves of *Zea mays* (Ren et

al. 2022), which is in accordance with our results for CAT and SOD activity in roots and shoots of *P. glomerata*. Furthermore, as GABA is also involved in Al toxicity tolerance by modulating hormonal responses, plant signaling, and ROS metabolism (Hajiboland et al. 2023; Kabała and Janicka 2024), the increase in the relative content of GABA in Al+MET supports the modulation of the antioxidant defenses by MET. It is also important to note here that the expression of genes related to 20E biosynthesis – the main secondary compound conferring medicinal properties to P. *glomerata* – was modulated by Al, independently of exogenous MET.

Upon reaching the root, Al interacts with apoplastic targets, such as the cell wall and plasma membrane, whereas in the symplast, it binds mainly to the phosphodiester backbone of DNA, impairing the maintenance of QC integrity, especially in the root apical meristem (RAM) (Eekhout et al. 2017). Al toxicity can disrupt cell cycle progression and DNA replication, two crucial cellular processes that impact genome integrity maintenance, particularly in the root apex (Szurman-Zubrzycka et al. 2023). During Al toxicity, cell cycle arrest occurs, inducing the differentiation of the root apical meristem (RAM), which progresses to loss of identity of the QC and, ultimately, alters DNA ploidy, compromising root elongation (Sjogren 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. 6). 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 emergence 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 (Sjogren et al. 2015). In this sense, 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. 6d), 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, primarily through reductions in cell content induced 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.

Taken together, our results provide integrated information on the responses of *P. glomerata* to Al stress and its alleviation by MET, from which we proposed a regulation model (Fig. 7). Briefly, our results indicate that MET decreases the Al uptake and induces the formation of complexes between Al and organic acids, also modifying the cell wall affinity to Al. Moreover, MET stimulated antioxidant metabolism by increasing the activity of CAT and SOD, and the relative GABA content. MET also reversed Al-induced growth arrest by maintaining photosynthetic rates, carbon metabolism, and cell expansion through the up-accumulation of enzymes involved in Rubisco activity, chlorophyll biosynthesis, and cell expansion and division. Specifically in roots, MET enabled elongation by alleviating DNA degradation and endoreduplication, which allowed cell cycle progression, and protected the QC identity.

Conclusion

Aluminum toxicity severely compromises the growth and physiology of *P. glomerata* by impairing photosynthesis, carbon and nitrogen metabolism, antioxidant balance, and root meristem integrity. Our integrative analyses demonstrate that MET partially counteracts these deleterious effects through a coordinated set of physiological, biochemical, and molecular responses. MET improved photosynthetic performance by sustaining pigment content, restoring photosynthetic rate, and promoting the accumulation of key proteins involved in carbon fixation and energy metabolism. In parallel, MET enhanced antioxidant defenses (elevating CAT, SOD, and GABA) thereby reducing oxidative damage. MET also modulated aluminum handling by decreasing its adherence to roots, promoting complexation with organic acids, and contributing to Al exclusion processes. At the cellular level, MET alleviated Al-induced DNA degradation and excessive endoreduplication in the root apical meristem, helping preserve QC identity, maintain cell cycle progression, and enable root elongation. Overall, our findings indicate that melatonin acts through multiple, interconnected pathways to enhance Al stress tolerance in *P. glomerata*, supporting its potential as an effective protective molecule for mitigating aluminum toxicity in medicinal plants.

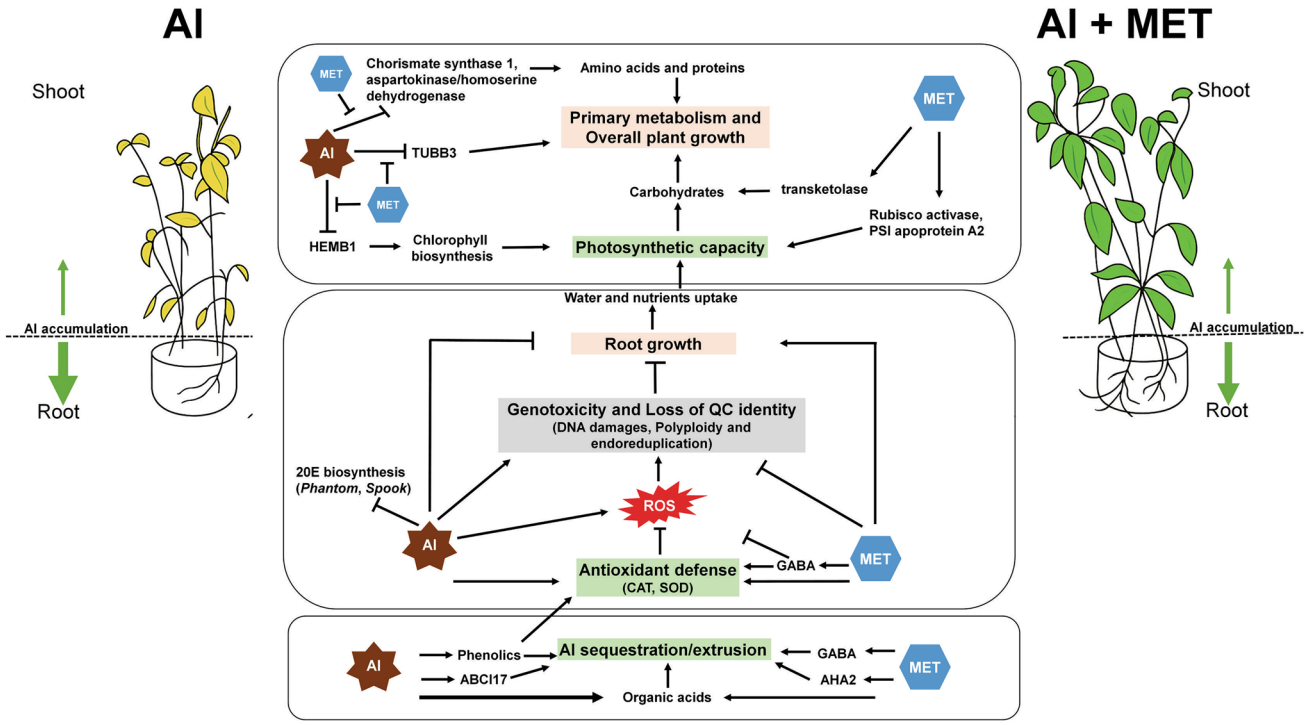


Fig. 7 General scheme of the effects of melatonin and aluminum on *Pfaffia glomerata* plants, and the underlying regulation mechanisms on Al sequestration and extrusion, antioxidant defense, and photosynthetic capacity, which ultimately leads to the maintenance of overall growth and primary metabolism

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Data availability Data will be made available at reasonable requests to the corresponding authors.

Declarations

Competing Interests The authors declare that they have no conflicts of interest to this work.

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