Aluminum tolerance in *Pfaffia glomerata* is associated with antioxidant defense, osmolyte accumulation, and pathway-specific transcriptional regulation

Michelle Maylla V. Almeida[a,#], Tatiane D. Silva[a,#], Quezia P. Dutra[a], Luiza Eduarda G. Monteiro[a], Marcus Dhilermando H. Souza[a], Elisandra S. Sousa[a], Joseila Maldaner[b], Sérgio Heitor S. Felipe[c], Juliane M. Henschel[d], Diego S. Batista[c,d,*], Evandro A. Fortini[a,#], Auxiliadora O. Martins[e], Wagner L. Araújo[e], Wagner C. Otoni[a,*]

[a]Departamento de Biologia Vegetal/Laboratório de Cultura de Tecidos Vegetais/BIOAGRO, Campus Universitário, Universidade Federal de Viçosa, Viçosa, MG, 36570-900, Brazil

[b]Centro Estadual de Diagnóstico e Pesquisa Florestal (CEFLOR), Departamento de Diagnóstico e Pesquisa Agropecuária, Santa Maria, RS, 97001-970, Brazil

[c]Programa de Pós-graduação em Ciências Agrárias, Universidade Estadual do Maranhão, São Luís, MA, 65055-310, Brazil

[d]Programa de Pós-graduação em Agronomia, Universidade Federal da Paraíba, Areia, PB, 58397-000, Brazil

[e]National Institute of Science and Technology on Plant Physiology under Stress Conditions, Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Viçosa, MG, 36570-900, Brazil

[#]Current Address: CTC - Centro de Tecnologia Canavieira - CTC, Piracicaba, SP, 13.400-970, Brazil

ORCIDs: MMVA (0000-0002-7193-3954), TDS (0000-0003-2253-3847), QPD (0000-0002-8294-6224), LEGM (0009-0005-8522-4437), MDHS (0000-0003-3897-5527), ESS (0000-0002-4880-8389), JM (0000-0002-3008-5047), SHSF (0000-0003-4529-9547), JMH (0000-0002-0805-5060), DSB (0000-0002-5798-7761), EAF (0000-0002-4127-8872), AOM (0000-0002-3740-0222), WLA (0000-0002-4796-2616), WCO (0000-0002-9614-9373)

***Corresponding authors**: *Diego S. Batista*, Associate Professor. Department of Agriculture, Federal University of Paraíba, University Campus III, s/n, 58220-000 Bananeiras, PB, Brazil. *Wagner C. Otoni*. Departamento de Biologia Vegetal/Laboratório de Cultura de Tecidos Vegetais/BIOAGRO, Campus Universitário, Universidade Federal de Viçosa, Viçosa, 36570-900, Brazil. E-mails: diegoesperanca@gmail.com and wotoni@ufv.br

SUPPLEMENTARY FIGURES

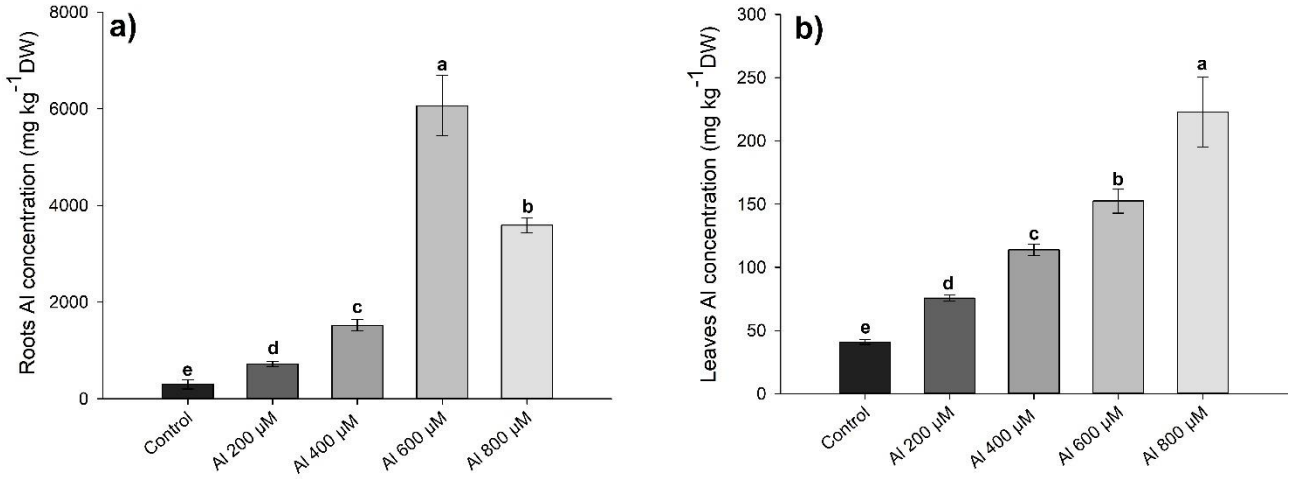

Fig. S1 Quantification of Al in *P. glomerata* after 40 days of *in vitro* cultivation with 0, 200, 400, 600, and 800 µM Al. (**a**) Al content (roots) and (**b**) Al content (leaves). Values are presented as means ± standard error (n = 4). Means followed by equal letters did not differ according to Tukey's test ($P \leq 0.05$)

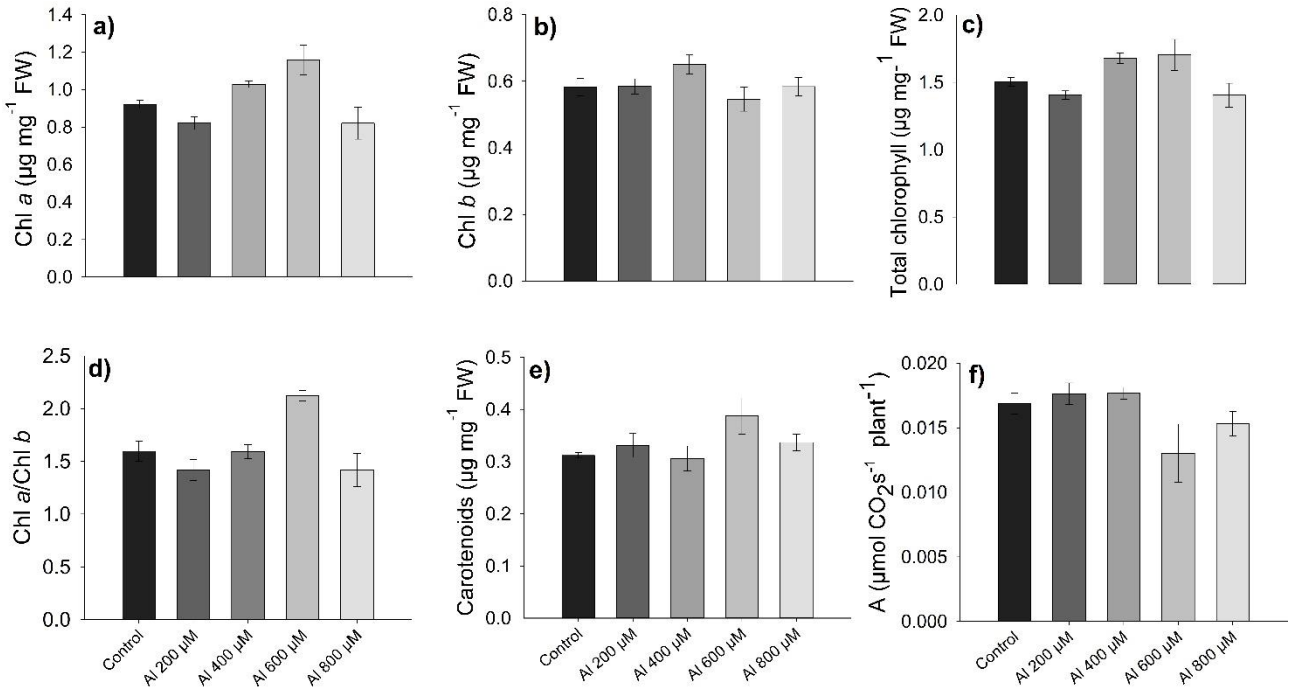

Fig. S2 Leaf pigment content and photosynthetic rate in *P. glomerata* after 40 days of *in vitro* cultivation with 0, 200, 400, 600, and 800 µM Al. (**a**) Chlorophyll *a*; (**b**) chlorophyll *b*; (**c**) total chlorophylls; (**d**) chlorophyll *a*/chlorophyll *b* ratio (Chl *a*/Chl *b*); (**e**) carotenoids; and (**f**) *in vitro* photosynthetic rate. Values are presented as means ± standard error (n = 4). Means followed by equal letters did not differ according to Tukey's test ($P \leq 0.05$)

SUPPLEMENTARY METHODS

In vitro photosynthetic rate

Gas exchange and *in vitro* photosynthetic rates were assessed according to Costa et al. (2014), with some modifications (Silva et al. 2020). Measurements were carried out using an AQ-S151 Infrared CO_2 analyzer (Qubit Systems, Kingston, ON, Canada) and data were collected using LoggerLite 1.8.1 software (Vernier Software & Technology, Beaverton, OR, USA). Reference CO_2 levels were recorded by channeling ambient air into an empty flask at a constant flow rate of 300 mL min^{-1} inside a chamber equipped with red and blue LED lights. LEDs were connected in a direct current circuit at 12 V, formed by two parallel sets of four LEDs connected in series (600 μmol m^{-2} s^{-1}). Prior to analysis, the plants were kept in the dark for 8 h to standardize metabolic conditions. After the reference CO_2 measurement, the flasks containing the plants were connected to the system and CO_2 was recorded at the point of stabilization. Gas exchange was calculated as the difference between the reference CO_2 and the CO_2 in the presence of plant samples. Air temperature and humidity inside the chamber were monitored using a Spec sensor (Thermo Recorder RS-11; Takai Spec Corp., Aichi, Japan). The *in vitro* photosynthetic rate (A) was calculated using the following equation:

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

where

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

and

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

Aluminum quantification

Al concentration was determined using 0.5 g of leaf and root tissues, dehydrated at 50 °C for 72 h, and ground in a knife mill to particles smaller than 1 mm. The extracts were obtained by digestion in a nitroperchloric solution (Malavolta et al. 1989), and Al content was determined using an inductively coupled plasma atomic emission spectrometer (Optima 7300 DV; PerkinElmer Inc., Shelton, CT, USA). Values were expressed in mg kg^{-1} of dry matter.

Carbohydrates, amino acids, and protein quantification

Leaf and root samples were collected and extracted with methanol (Lisec et al. 2006). Approximately 10 mg of plant material was frozen in liquid nitrogen, ground, lyophilized, and aliquoted in microtubes. Subsequently, 700 µL methanol was added to the samples, and the tubes were placed in a hot water bath. The samples were homogenized and incubated at 80 °C with shaking at 400 × *g* for 20 min, followed by centrifugation at 12,000 × *g* and 4 °C for 15 min. The supernatant was transferred to new microtubes, and a 150-µL sample was set aside to measure photosynthetic pigments. Then, 375 µL chloroform and 750 µL ultrapure water were added to the remaining supernatant. After thorough homogenization and centrifugation at 12,000 × *g* and 4 °C for 10 min, the upper clear aqueous phase was transferred to new microtubes and used to determine sugars and amino acids (Baxter et al. 2003; Sun et al. 2006). The precipitate from the methanolic extract was washed twice with 70% ethanol and then used to determine starch and protein content (Baxter et al. 2003; Panariello et al. 2017).

For sugar determination, 160 µL of the reaction mixture [100 mM HEPES/KOH with 3 mM $MgCl_2$, pH 7, 118 mM ATP, 48.4 mM $NADP^+$, and 56 units of glucose-6-phosphate dehydrogenase (G6PDH, 5 mg mL^{-1})] were added to each well of a 96-well microplate, followed by 25 µL methanolic extract and 25 µL methanol. Absorbance was recorded at 340 nm using a microplate reader at intervals of 1 min between readings. After the optical density stabilized, 5 µL of each enzyme was added sequentially: hexokinase (1.5 U per reaction), phosphoglucose isomerase (0.7 U per reaction), and invertase (5 U per reaction), with approximately 30 min between each addition.

Amino acid content was determined by adding 30 µL methanolic extract, 20 µL of 70% ethanol, 50 µL citrate buffer (pH 5.2) with 0.2% (w/v) ascorbic acid, and 100 µL of 1% (w/v) ninhydrin in 70% (v/v) ethanol to each well of the microplate. The microplate was incubated at 95 °C in the dark for 20 min, and absorbance was read at 570 nm. A calibration curve was constructed using leucine as the standard.

Protein content was determined by resuspending the precipitate from methanolic extraction in 400 µL of 0.1 M NaOH. The microtubes were homogenized and incubated at 95 °C with shaking at 400 × *g* for 1 h, followed by centrifugation at 12,000 × *g* and 4 °C for 10 min. A 5-µL aliquot of the supernatant was added to a microplate containing 180 µL Bradford reagent (diluted 1:5) in each well (Bradford 1976). Absorbance was measured at 595 nm, and a calibration curve was created using bovine serum albumin as standard.

Starch content was determined by adding 70 µL of 1 M acetic acid to the tubes containing the precipitate from methanolic extraction and 0.1 M NaOH to condition the pH for the reaction. A 40-µL aliquot of the suspension was transferred to a microplate containing 60 µL of a starch hydrolysis

mix comprising amyloglucosidase (0.14 units μL^{-1}) and α-amylase (0.01 U μL^{-1}). The microplate was sealed with Al tape (Model 425; 3M, St. Paul, MN, USA) and incubated overnight at 37 °C. The hydrolyzed extract (25 µL) was transferred to a new microplate containing 25 µL methanol and 160 µL reaction mixture [1 M HEPES/KOH buffer, pH 7.0, 3 mM $MgCl_2$, 118 mM ATP, 48.4 mM $NADP^+$, and 56 units G6PDH (0.7 units μL^{-1})] per well. Absorbance was measured at 340 nm using a microplate reader at 1-min intervals between readings. Once optical density stabilized, 2 µL hexokinase (2 U per reaction) was added to each well. Sugar and starch content were calculated using the Lambert-Beer equation: NADPH (µmol) = $\Delta_{OD}/(2.85 \times 6.22)$.

Quantification of oxidative stress enzymes

Catalase (EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), SOD (EC 1.15.1.1), and POD (EC1.11.1.7) activities were quantified. Fresh leaf and root material (50 mg) was homogenized in 1 mL extraction medium (0.1 M K-phosphate buffer, pH 6.8, and 0.1 mM EDTA), 1 mM phenylmethylsulfonyl fluoride, and 1% (w/v) polyvinylpyrrolidone. Centrifugation was carried out at 10,000 × *g* and 4 °C for 15 min, and the supernatant was used as the crude enzyme extract.

To determine catalase activity, 20 µL extract was combined with 200 µL reaction medium (50 mM K-phosphate buffer, pH 7.0, and 12.5 mM H_2O_2) in a microplate (Havir and McHale 1987). Absorption was measured at 240 nm every 10 s for 1 min and enzyme activity was calculated based on the decrease in absorbance. For APX activity (Nakano and Asada 1981), the reaction medium (50 mM K-phosphate buffer, pH 7.8, 0.25 mM ascorbic acid, 0.1 mM EDTA, and 0.3 mM H_2O_2) was added to 20 µL extract in a microplate and absorbance was read at 290 nm for 1 min. POD activity was determined as described by Maehly and Chance (1955). The reaction medium (13 mM guaiacol, 5 mM H_2O_2, and 50 mM Na-phosphate, pH 6.5) was added to the crude extract and absorbance was read at 470 nm for 1 min. Catalase, APX, and POD activities were expressed as μmol^{-1} min^{-1} mg^{-1} protein.

SOD activity was determined according to the protocol described by Giannopolitis and Ries (1977), by adding 190 µL reaction medium (50 mM Na-phosphate buffer, pH 7.8, 13 mM methionine, 75 µM p-nitro tetrazolium blue, 0.1 mM EDTA, and 2 µM riboflavin) and 30 µL crude extract to a microplate. The microplate was kept at 25 °C for 5 min in a reaction chamber illuminated by a 15 W fluorescent lamp. The reaction was stopped by removing the plate from the light. Photoreduction of blue tetrazolium led to a higher absorbance value, which was subtracted from the blank containing no enzyme extract. SOD activity was expressed as U min^{-1} mg^{-1} protein, with 1 U being equivalent to the concentration of SOD required to inhibit 50% of nitro blue tetrazolium photoreduction.