



Physiological responses of soybean (*Glycine max* (L.) Merrill) cultivars to copper excess

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Abstract: Successive applications of copper fungicides on vines have resulted in increased copper content in vineyard soils over the years. This high copper content has affected the growth of young vines in eradicated vineyards. Thus, the cultivation of annual species for a few years is an alternative to copper phytostabilization, because it would be a good way to decrease copper availability to plants. The aim of this study was to evaluate the physiological responses of different soybean cultivars to copper concentration increase. Four different soybean cultivars were grown under three copper concentrations: 0.5, 20 and 40 μM in nutrient solution. The main outcomes of this study were: i) Cultivar M 6410 IPRO recorded the highest photosynthetic rate when plants were exposed to 40 μM of copper in the nutrient solution; ii) plants in cultivar M 6410 IPRO accumulated large copper concentrations in their roots although did not decrease the root dry mass, possibly due to the higher superoxide dismutase activity; iii) cultivar DM 5958 RSF IPRO recorded drastically reduced photosynthetic rate and dry mass production due to copper excess. We conclude that each cultivar responded differently to the excess of copper, but none of them showed tolerance to it.

Key words: antioxidant system, cash crops, heavy metal phytostabilization, photosynthesis.

INTRODUCTION

Successive applications of copper (Cu) fungicides on vines have resulted in increased Cu content in vineyard soils over the years (Brunetto et al. 2014). Grape yield decreases due to vine aging,

and this process leads to vineyard eradication. Soil organic matter (SOM) oxidation is increased by soil mobilization at vineyard replanting, besides increasing Cu availability in the soil (Campos et al. 2013), especially in soils presenting low physical SOM protection and low Cu sorption ability (Brunetto et al. 2014). It is known that Cu excess in soil is toxic to transplanted young vines in ancient vineyards (Miotto et al. 2014).

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Cu is an essential element for plants, but if its concentration in the soil is too high it can lead to Cu accumulation in plant tissue. Moreover, Cu excess causes negative plant response at physiological level and help inhibiting plant growth and development (Kabata-Pendias 2011). Cu excess can inhibit the flow of electrons in photosynthesis and change the composition of chloroplast membranes. Likewise, Cu excess can inhibit the synthesis of photosynthetic pigments such as chlorophylls and carotenoids and/or change their structure (González-Mendoza et al. 2013, John et al. 2009).

Cu can also catalyze the production of reactive oxygen species (ROS), such as hydroxyl radical (OH•) formed in Fenton reactions (Gill and Tuteja 2010). ROS have one unpaired electron in their structures, thus they are highly reactive. Balance between ROS generation and elimination is controlled by the activity of antioxidant defense system (Becana et al. 2010). Oxidative stress is observed when the activity of antioxidant defense system is lower than the ROS production (Giroto et al. 2013). This phenomenon occurs because ROS damages the structures of fatty acids, such as lipid peroxidation in cell membranes, which, in turn, produce carbon compounds such as malondialdehyde (Soto et al. 2011). Thus, the balance between ROS production and antioxidant system activity is crucial for the survival and adaptation of plants growing in soils presenting high levels of heavy metals (Słomka et al. 2008). Superoxide dismutase (SOD) is the first defense line of the plant antioxidant system against ROS. These enzymes act in superoxide radical ($O_2^{\cdot-}$) dismutation into less toxic forms such as H_2O_2 and O_2 (Zouari et al. 2016). The SOD activity often increases under high concentration of metals such as Cu (Zhang et al. 2010).

Recent studies have been documented using cash crops on heavy metal phytoremediation on vineyards (Giroto et al. 2016, Marastoni et al. 2019, Tiecher et al. 2016a, b). The introduction of

cash crops on this scenario has an additional benefit due to the financial return that can be obtained, besides promoting the maintenance of vegetal cover. Annual crops, such as soybean, can be cultivated for few years and become an alternative to Cu phytostabilization because it can make the future reinsertion of vines in previous soybean crop areas possible (Fellet et al. 2007, Murakami and Ae 2009, Pierzynski et al. 1994). Soybean is the most economically important grain legume in the global agricultural scenario (Sánchez-Pardo et al. 2012). Furthermore, the inclusion of plants that symbiotically fix N_2 , such as soybean, can contribute to the ecosystem improvement by increasing the nitrogen available content in the soil, especially in vineyards soils that usually have low SOM content (Whiting et al. 2004, Conrad et al. 2018). However, studies on soybean tolerance to Cu excess in contaminated soils are still scarce, suggesting that room exists to further research on this topic (Silva et al. 2014).

The physiological responses of plants grown in soils presenting Cu excess can vary between species and/or between cultivars within a single species (Massocatto et al. 2013). Evaluating these responses can help uncovering strategies used by plants to uptake, accumulate and tolerate heavy metals. These responses also help detecting the cultivars that best adapt to Cu contaminated soils (Kabata-Pendias 2011, Cambrollé et al. 2013). The present study aimed to evaluate the physiological responses of different soybean cultivars to Cu excess. Therefore, we performed tissue composition, photosynthetic, biochemical, and growth measurements in plants cultivated under Cu increase concentrations in nutrient solution.

MATERIALS AND METHODS

DESCRIPTION OF THE EXPERIMENT

The study was carried out in a greenhouse located in Santa Maria, Rio Grande do Sul State, Southern

Brazil (29°42'56.44" S and 53°43'12.57" W). Mean air temperature inside the greenhouse was set at 26 °C and relative humidity was 50%. The experiment followed a completely randomized design, with 4 replicates.

The following soybean cultivars were assessed in the current study: NA 5909 RG (5909), DM 5958 RSF IPRO (5958), M 6410 IPRO (6410) and DM 6563 RSF IPRO (6563). According to the national register of cultivars (RNC, 2019), these cultivars were chosen, given their adaptability to climate and soil conditions in Southern Brazil (Embrapa 2014). Seeds were disinfested with 10% (v/v) sodium hypochlorite for 15 minutes (Sánchez-Pardo et al. 2012), sown in moistened filter paper (2.5 times the weight of the dry paper) and stored in germinator type BOD (Box Organism Development) at 20 °C, under 24-h photoperiod.

Three seedlings of each soybean cultivar were transferred to plastic containers covered with polystyrene plates with holes, which were used as physical support for plants. Roots were immersed in an aerated full-nutrient solution expressed in mg L⁻¹: 85.31 N; 7.54 P; 11.54 S; 97.64 Ca; 23.68 Mg; 104.75 K; 176.76 Cl; 0.27 B; 0.05 Mo; 0.01 Ni; 0.13 Zn; 0.03 Cu; 0.11 Mn and 2.68 Fe.

Three Cu concentrations (CuSO₄ - 0.5 µM, which corresponds to the original concentration of the nutrient solution, 20 and 40 µM) were added to the nutrient solution after seven plant-acclimation days. Nutrient solution was replaced every four days during the experimental period - pH was adjusted to 5.5. Gas exchange evaluations were carried out and plants were collected 21 days after cultivation.

DRY MATTER PRODUCTION AND TOTAL Cu CONCENTRATION IN PLANT TISSUES

Two plants from each replicate were harvested and roots and shoots were divided. The roots were washed and dried with absorbent paper and reserved. Root and shoot samples were dried in

forced air-circulation oven at ± 65 °C, until they reached constant mass. Root (MSR) and shoot dry masses (MSPA) were measured in precision scale (Shimadzu, AY220, Philippines). Root and shoot samples were ground in Wiley mill after drying and passed through 2 mm mesh sieve. 0.25 g of plant tissue was subjected to nitroperchloric digestion (3.0 mL of HNO₃ 65% P.A and 1 mL of HClO₄ 70% P.A.) (Embrapa 2009). Total Cu concentration was analyzed in atomic absorption spectrophotometer (AAS, Perkin Elmer Analyst 200, USA).

GAS EXCHANGES

Gas exchanges were measured 21 days after transplantation (DAT) in Infrared Gas Analyzer (IRGA- Li-COR[®] 6400 XT, Lincoln, NE, EUA). Measurements were performed between 8:00 and 10:00 a.m. at CO₂ concentration 400 µmol mol⁻¹ and photon flux density 1500 µmol m⁻² s⁻¹. One plant from each replicate was selected and the last fully expanded trifoliated leaf was used to measure gas exchange. The following parameters were observed: net photosynthetic rate (A), intercellular CO₂ concentration (C_i), transpiration rate (E), stomatal conductance (G_s), water-use efficiency (WUE) and RuBisCo instantaneous carboxylation efficiency (A/C_i). WUE was the ratio between the amount of CO₂ fixed through photosynthesis and the amount of transpired water. A/C_i was the ratio between the amount of CO₂ fixed through photosynthesis and the intercellular CO₂ concentration.

The leaves and roots of each plant were collected immediately after the end of the measurements and frozen in liquid nitrogen. Samples were macerated in liquid nitrogen to determine photosynthetic pigments, SOD activity, H₂O₂ concentration and lipid peroxidation.

EXTRACTION AND QUANTIFICATION OF PHOTOSYNTHETIC PIGMENTS

The concentration of photosynthetic pigments was determined based on the methodology

proposed by Hiscox and Israelstam (1979), by using Lichtenthaler (1987) formulae. 0.05 g of plant material were weighed and, then, incubated for 5 min at 65 °C in 40 mL of dimethylsulfoxide (DMSO) until the pigments were fully removed from the assessed tissues. Supernatant solution absorbance was determined in spectrophotometer (Bel Photonics, 1105, Brazil) at wavelengths 663, 645 and 470 nm in order to determine chlorophyll a, b, and carotenoids, respectively. Total chlorophyll results from the sum of chlorophyll a and b values.

SOD ACTIVITY

Half gram of plant material was homogenized in 3 mL of sodium phosphate buffer solution (0.05 mol L⁻¹) at pH 7.8, including 1 mM EDTA and 0.5% Triton X-100. The homogenate extract was centrifuged at 13000 g for 20 min at 4 °C. Enzymatic activity and protein content were determined by using the supernatant (Bradford 1976, Zhu et al. 2004).

SOD activity was determined based on the spectrophotometric method described by Giannopolitis and Ries (1977). 3 mL of a mixture containing 50 mM of potassium phosphate buffer (pH 7.8), methionine (13 mM), EDTA (0.1 μM), NBT (75 μM) and riboflavin (2 μM) were added to the tubes, which were incubated under fluorescent lamps (15 watts) for 15 min. Absorbance was determined in spectrophotometer (Bel Photonics, 1105, Brazil) at wavelength 560 nm. NBT (p-nitro blue tetrazolium) reduction inhibition by enzymatic extract was determined based on this spectrophotometric method. Extract-free tubes exposed, or not, to light were used as blanks for the reaction. The SOD enzymatic activity unit (U) was defined as the amount of enzyme required to find 50% NBT reduction inhibition through SOD, which was observed in the enzyme extract. SOD activity was determined by calculating the amount

of extract inhibiting 50% NBT reaction and was expressed as U mg⁻¹ protein.

HYDROGEN PEROXIDE (H₂O₂)

H₂O₂ concentration was determined based on the methodology by Loreto and Velikova (2001). Therefore, 0.1 g of plant material was homogenized in 2.0 mL of 0.1% (w/v) trichloroacetic acid (TCA) and, subsequently, centrifuged at 12000 g for 15 min at 4 °C. 0.5 mL of supernatant was added with 0.5 mL of potassium phosphate buffer (10 mM) (pH 7.0) and 1 mL of KI (1 mol L⁻¹). Absorbance was determined in spectrophotometer (Bel Photonics, 1105, Brazil) at wavelength 390 nm.

LIPID PEROXIDATION

Thiobarbituric acid reactive substances (TBARS) were determined based on the methodology by El-Moshaty et al. (1993), which quantifies malondialdehyde (MDA) accumulation as the result from lipid peroxidation. Half gram of plant material was added with 4.0 mL of citrate phosphate buffer TFK 0.2 M (pH 6.5). Samples were centrifuged in refrigerated centrifuge at 4 °C for 15 min at 20000 g.

Aliquots of 1.5 mL of supernatant were collected for TBARS determination. The same volume of thiobarbituric acid (TBA) - 0.5% (w/v) - and trichloroacetic acid (TCA) - 20% (w/v) - was added to the samples; which were incubated in water bath at 95 °C for 40 min. The reaction was stopped in ice bath for 15 min and samples were centrifuged at 10000 g for 5 min. Absorbance was determined in spectrophotometer (Bel Photonics, 1105, Brazil) at 532 nm, by subtracting the non-specific absorbance at 600 nm.

STATISTICAL ANALYSIS

Residue normality was subjected to Shapiro-Wilk test. Different homocedastic and heterocedastic models were selected based on the Akaike

information criterion (AIC) and Bayesian information criterion (BIC). All models were adjusted in the “nlme” package (Pinheiro et al. 2017) of the R statistical environment (R Core Team 2017). Results were subjected to analysis of variance and tested through F test. Means were compared through Scott Knott test, at 5% error probability. The principal component analysis (PCA) was performed to assess correlation structure and the degree of association between different variables that were taken into consideration in the current study. PCA allowed identifying more complex associations between the evaluated variables and the identification of variables responsible for the greatest contributions to differences between treatments. Only components presenting eigenvalue higher than 1 were taken into account in the present study. The principal component analysis was performed in “FactoMineR” (Lê et al. 2008), which is a package of the R statistical environment (R Core Team 2017).

RESULTS

DRY MATTER PRODUCTION AND TOTAL Cu CONCENTRATION IN PLANT TISSUE

All soybean cultivars tested in the current study recorded decreased shoot dry mass (SDM) production as Cu concentration in the nutrient solution increased (Table I); however, SDM production did not differ between cultivars. Cultivar 5958 (grown at Cu concentrations 20 and 40 μM) presented 24% and 45% root dry mass (RDM) decrease, respectively, in comparison to the control concentration (0.5 μM Cu).

The RDM/SDM ratio in this cultivar did not differ between Cu concentrations; this outcome evidences that the Cu excess had similar effect on shoots and roots. Cu concentrations did not affect RDM production in cultivars 6410 and 6563. RDM/SDM ratio was 55% higher in these cultivars

grown at Cu concentration 40 μM than in the Cu control concentration (Table I).

Cu concentrations in shoot and root tissues increased in all soybean cultivars as Cu concentration in the nutrient solution also increased (Figure 1). Cu concentration in shoot and root tissues did not differ between cultivars at Cu control concentration (0.5 μM). Cultivar 6563 recorded the highest Cu concentration in plant shoots at Cu concentration 20 μM. Cu concentration in the roots did not statistically differ between cultivars in this treatment. Cultivar 5909 presented the highest Cu concentration in the shoots of plants grown at Cu concentration 40 μM, approximately 10 times higher than that observed in the control concentration. Besides, cultivar 5909 presented the

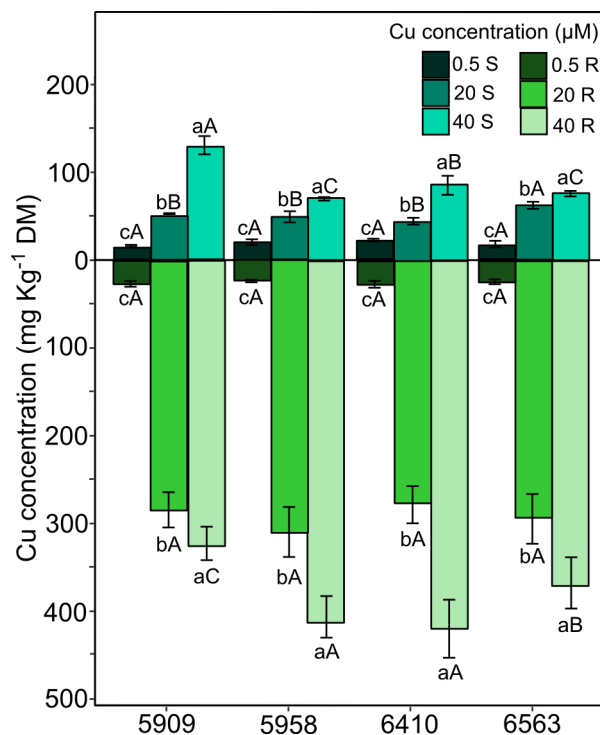


Figure 1 - Cu concentration in the shoots (S) and roots (R) of four soybean cultivars (5909, 5958, 6410 and 6563) grown in nutrient solution at three Cu concentrations (0.5, 20 and 40 μM). Capital letters indicate comparison between cultivars in the same Cu concentration and plant part. Lowercase letters indicate comparisons between Cu concentrations in the same cultivar and plant part (p <0.05). Vertical bars represent ± standard deviation.

TABLE I
Root dry mass, root dry mass/ shoot dry mass ratio and shoot dry mass of four soybean cultivars (5909, 5958, 6410 and 6563) grown in nutrient solution at three Cu concentrations (0.5, 20 and 40 μM).

Cu concentration (μM)	Root dry mass (g)*			
	5909	5958	6410	6563
0.5	0.22 \pm 0.01 aC	0.37 \pm 0.03 aA	0.27 \pm 0.03 aB	0.25 \pm 0.03 aB
20	0.24 \pm 0.04 aB	0.28 \pm 0.02 bA	0.24 \pm 0.00 aB	0.27 \pm 0.02 aA
40	0.14 \pm 0.01 bC	0.20 \pm 0.01 cB	0.24 \pm 0.01 aA	0.27 \pm 0.02 aA
Cu concentration (μM)	Root dry mass/ shoot dry mass ratio (g)*			
	5909	5958	6410	6563
0.5	0.11 \pm 0.01 bC	0.17 \pm 0.02 aA	0.14 \pm 0.01 bB	0.12 \pm 0.01 cC
20	0.14 \pm 0.02 aB	0.16 \pm 0.02 aA	0.13 \pm 0.01 bB	0.15 \pm 0.01 bB
40	0.11 \pm 0.01 bC	0.19 \pm 0.02 aB	0.21 \pm 0.03 aA	0.18 \pm 0.02 aB
Cu concentration (μM)	Shoot dry mass (g)*			
	5909	5958	6410	6563
0.5	2.03 \pm 0.17 a	2.15 \pm 0.36 a	1.98 \pm 0.12 a	2.12 \pm 0.22 a
20	1.77 \pm 0.25 b	1.67 \pm 0.13 b	1.80 \pm 0.17 b	1.80 \pm 0.08 b
40	1.34 \pm 0.06 c	1.10 \pm 0.08 c	1.13 \pm 0.10 c	1.47 \pm 0.08 c

Values from two plants. * Values represent the means \pm standard deviation of four repetitions. Capital letters indicate comparison between cultivars in the same Cu concentration, whereas lowercase letters indicate a comparison between Cu concentrations in the same cultivar ($p < 0.05$).

lowest Cu concentration in the roots. Cultivars 5958 and 6410 recorded the highest Cu concentrations in the roots of plants grown at Cu concentration 40 μM (Figure 1).

GAS EXCHANGES

Cu excess in the nutrient solution changed the photosynthetic parameters of soybean cultivars. Cultivar 6563 was the only one presenting lower net photosynthetic rate (A) at Cu concentration 20 μM than that observed in the control treatment (Figure 2a). The other three cultivars presented the highest A and A/Ci values at Cu concentration 20 μM (Figures 2a, b). Intercellular CO_2 concentration (C_i) and transpiration rate (E) decreased in all cultivars subjected to the aforementioned Cu concentration (Figures 2c, d). Except for cultivar 6410, whose plants did not differ from the control treatment in this variable, the same result was observed for stomatal conductance (G_s) (Figure 2e).

All soybean cultivars grown at Cu concentration 40 μM presented the lowest A, A/Ci, C_i , E and G_s values, which were compared to values observed at different Cu concentrations. However, cultivar 6410 was less sensitive to Cu excess, since it showed 70% A in the control (Figure 2). On the other hand, cultivar 5958 was the most sensitive to Cu concentration 40 μM , since it maintained only 23% A in the control.

Water use efficiency (WUE) was lower in all soybean cultivars grown at Cu control concentration in comparison to other concentrations. There was no variation in WUE at Cu concentrations 20 or 40 μM in all cultivars, except for cultivar 6410, which showed maximum WUE at 20 μM of Cu (Figure 2f).

PHOTOSYNTHETIC PIGMENTS

All soybean cultivars grown at Cu concentration 40 μM presented lower concentration of photosynthetic pigments than the other Cu concentrations (Table

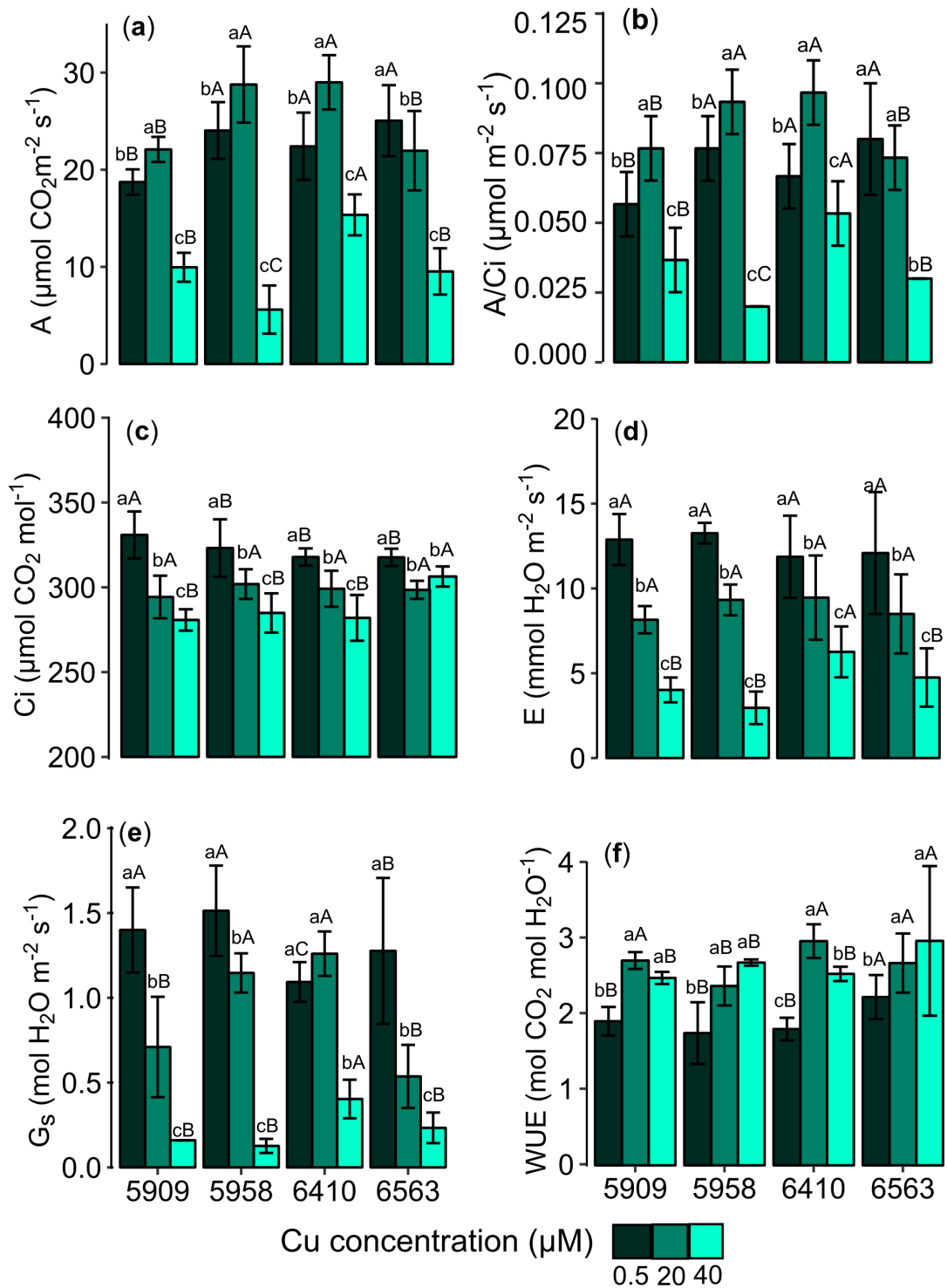


Figure 2 - Net photosynthetic rate (a), RuBisCo instantaneous carboxylation efficiency (b), intercellular CO₂ concentration (c), transpiration rate (d), stomatal conductance (e) and water use efficiency (f) of four soybean cultivars (5909, 5958, 6410 and 6563) grown in nutrient solution at three different Cu concentrations (0.5, 20 and 40 μM). Capital letters indicate comparison between cultivars in the same Cu concentration. Lowercase letters indicate comparison between Cu concentrations in the same cultivar ($p < 0.05$). Vertical bars represent ± standard deviation.

II). Cultivars 6410 and 5958 showed chlorophyll concentration increase at Cu concentration 20 μM , and cultivar 5958 recorded chlorophyll b concentration and total chlorophyll increase (Table II).

SOD ACTIVITY

The lowest SOD activity in plant roots and shoots, in all soybean cultivars, was recorded for plants grown at low Cu concentration (0.5 μM). SOD activity in plant roots and shoots increased at Cu concentrations 20 or 40 μM in the nutrient solution. SOD activity was often higher in the shoots than in the roots; SOD activity in the roots was similar in soybean cultivars at Cu concentrations 0.5 and 20 μM . However, cultivar 6410 showed the highest SOD activity in the roots of plants cultivated at Cu concentration 40 μM than in other cultivars (Figure 3a).

HYDROGEN PEROXIDE (H_2O_2)

H_2O_2 concentration in the shoots and roots was similar in all soybean cultivars grown at Cu concentration 0.5 μM . H_2O_2 concentrations in the roots and shoots of all cultivars grown at Cu concentration 40 μM were often higher than the control. Cultivar 5909 presented the highest H_2O_2 concentration in the shoots at the aforementioned Cu concentration in nutrient solution. Likewise, cultivars 6563 and 5958 presented the highest and the lowest H_2O_2 concentrations in the roots, respectively (Figure 3b).

LIPID PEROXIDATION

Malondialdehyde (MDA) concentrations in the shoots showed differences in cultivar 6563, which recorded MDA level increase by 45% when plants were cultivated at Cu concentration

TABLE II
Chlorophyll and carotenoid concentration in the leaves of the four soybean cultivars (5909, 5958, 6410 and 6563) grown in nutrient solution at Cu concentrations: 0.5, 20 and 40 μM .

Cu concentration (μM)	5909	5958	6410	6563
<i>Chlorophyll a (mg g⁻¹ FW)</i>				
0.5	1.20 ± 0.12 aA*	1.09 ± 0.16 bB	1.03 ± 0.01 bB	1.05 ± 0.03 aB
20	1.04 ± 0.11 bB	1.34 ± 0.05 aA	1.04 ± 0.02 aB	1.16 ± 0.11 aB
40	0.78 ± 0.05 cA	0.67 ± 0.03 cA	0.46 ± 0.06 cB	0.59 ± 0.05 bB
<i>Chlorophyll b (mg g⁻¹ FW)</i>				
0.5	0.36 ± 0.04 aA	0.33 ± 0.03 bA	0.29 ± 0.02 aB	0.29 ± 0.01 aB
20	0.28 ± 0.03 bC	0.40 ± 0.02 aA	0.26 ± 0.01 aC	0.32 ± 0.04 aB
40	0.21 ± 0.03 cA	0.18 ± 0.02 cA	0.11 ± 0.02 bB	0.15 ± 0.01 bB
<i>Total chlorophyll (mg g⁻¹ FW)</i>				
0.5	1.56 ± 0.03 aA	1.42 ± 0.02 bB	1.32 ± 0.02 aB	1.34 ± 0.01 aB
20	1.32 ± 0.01 bC	1.75 ± 0.01 aA	1.30 ± 0.01 aC	1.49 ± 0.01 aB
40	0.99 ± 0.01 cA	0.86 ± 0.03 cA	0.58 ± 0.03 bB	0.74 ± 0.01 bB
<i>Carotenoids (mg g⁻¹ FW)</i>				
0.5	0.33 ± 0.16 aA	0.35 ± 0.17 aA	0.28 ± 0.01 aB	0.31 ± 0.05 aB
20	0.29 ± 0.14 bC	0.37 ± 0.07 aA	0.26 ± 0.03 aD	0.33 ± 0.15 aB
40	0.21 ± 0.07 cA	0.19 ± 0.05 bA	0.12 ± 0.06 bC	0.16 ± 0.06 bB

*Values represent means ± standard deviation of the four repetitions. Capital letters indicate comparison between cultivars at the same Cu concentration. Lowercase letters indicate comparison between Cu concentrations in the same cultivar ($p < 0.05$).

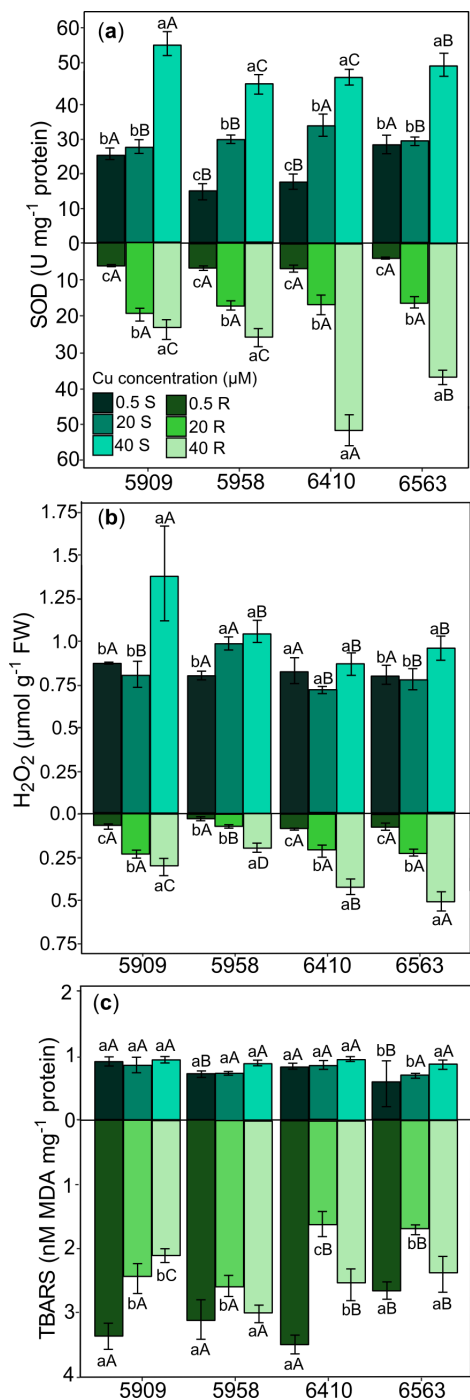


Figure 3 - Enzymatic activity of superoxide dismutase (a), hydrogen peroxide concentration (b) and malondialdehyde concentration (c) of four soybean cultivars (5909, 5958, 6410 and 6563) grown in nutrient solution at three different Cu concentrations (0.5, 20 and 40 μM). Capital letters indicate comparison between cultivars in the same Cu concentration. Lowercase letters indicate comparison between Cu concentrations in the same cultivar (p < 0.05). Vertical bars represent ± standard deviation.

40 μM in comparison to the control. The roots of plants grown at Cu concentration 20 μM recorded decreased MDA concentrations in comparison to the control (Figure 3c).

PRINCIPAL COMPONENT ANALYSIS

According to the eigenvalue threshold (>1), only the two first components were retained, and it explains the 78.84% variation in the original results (Figure 4a). Of this total, 66.46% variation was explained by principal component 1 (PC 1) and 12.38% was justified by principal component 2 (PC 2). PC 1 separated Cu concentrations 0.5 and 20 μM from the treatment at Cu concentration 40 μM. The most influent variables in the group at Cu concentrations 0.5 and 20 μM in this separation were photosynthetic pigment concentrations, photosynthetic variables (except for WUE), dry mass and MDA concentration in the roots. Variables in the group subjected to the highest Cu concentration, that have mostly contributed to this separation were Cu concentration in plant tissue, SOD activity and H₂O₂ concentration in the roots and shoots, MDA concentration in shoots and WUE (Figure 4).

DISCUSSION

Cu concentrations observed in soybean plants grown at Cu concentration 40 μM can be toxic (Kabata-Pendias 2011); moreover, they showed negative correlation to dry mass production (Figure 4). The lower SDM observed at Cu concentration 40 μM (Table I) can be attributed to lower photosynthesis (A) (Figure 2a), which was also suggested by Cambrollé et al. (2013). This may occur because the high Cu concentration in the shoots can cause disorders in photosynthetic processes (Zhang et al. 2014) by (i) preventing the flow of electrons in the photochemical phase of the photosynthesis, (ii) by altering the composition of thylakoid membranes and of photosynthetic pigments, and (iii) by

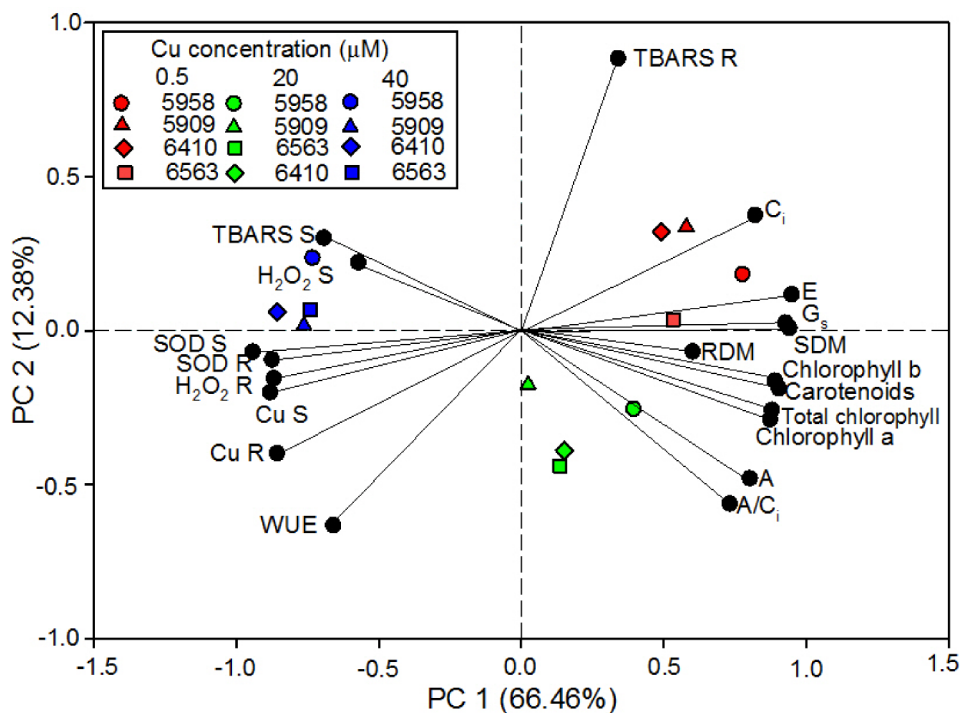


Figure 4 - Relationship between principal component 1 (PC1) and principal component 2 (PC2) in the variables such as groups of photosynthetic parameters (A, A/C_i, C_i, E, G_s and WUE), photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) shoot dry mass (SDM), root dry mass (RDM), Cu concentration in shoots and roots (Cu S and Cu R), enzymatic activity of superoxide dismutase in shoots and roots (SOD S and SOD R), hydrogen peroxide concentration in shoots and roots (H₂O₂ S and H₂O₂ R) and lipid peroxidation in shoots and roots (TBARS S and TBARS R) in the four soybean cultivars (5909, 5958, 6410 and 6563) subjected to different Cu concentrations in nutrient solution (0.5, 20 and 40 μM).

inhibiting the synthesis or activity of Calvin cycle enzymes (John et al. 2009, González-Mendoza et al. 2013).

SDM production decrease in cultivars 5958, 5909 and 6410 observed at Cu concentration 20 μM did not affect the photosynthetic rate of these cultivars. Such outcome can be related to the adoption of some tolerance mechanisms triggered by plants, which have used photosynthetic products instead of growth (Bazihizina et al. 2015). Such tolerance mechanisms to Cu excess can be the synthesis of metal binders to sequester excess Cu in cell cytoplasm and/or metal compartmentalization in the vacuole (Hall 2002). In addition, the generation of reactive oxygen species (ROS) can be responsible for the decrease in dry biomass in soybean plants, because of the negative

correlation between H₂O₂ concentration and dry mass production (SDM and RDM) (Figure 4). This process is based on the principle that organisms can mobilize their energy reserves to withstand stress conditions, such as the detoxification process influencing costs with biological functions such as growth (Calow 1991).

Cu concentration in plant tissues increased, as metal concentration in the nutrient solution also increased (Figure 1), but it was higher in plant roots than in shoots. This behavior derives from Cu immobilization due to extracellular carbohydrates in the cell wall of roots. This process allows less ions to remain free in the cytoplasm and to be carried to the shoots (Lasat 2002). This restriction in Cu translocation to the shoots seems to be a survival strategy adopted by plants, which seek to

maintain lower concentration of metals in their most photosynthetic-sensitive organs. Cu accumulation is higher in the least sensitive organs, such as the roots (Yang et al. 2011); cultivars 6563 and 6410 showed greater ability to accumulate Cu in their roots (Figure 1), without leading to decrease on RDM production (Table I). On the other hand, these cultivars recorded decrease on SDM production when plants were grown in medium presenting Cu excess (Table I). This outcome resulted in MSR/MSPA ratio increase due to Cu excess. According to Zhang et al. (2014), this MSR/MSPA ratio increase can be a mechanism to improve the uptake of other nutrients, or of some other limiting resources affected by high Cu concentrations.

Cu concentration 20 μM increased the net photosynthetic rate (A) and RuBisCo instantaneous carboxylation efficiency (A/C_i) of cultivars 5909, 5958 and 6410. This result was observed, although there was small restriction in stomatal conductance (G_s) and decrease in intercellular CO_2 concentration (C_i) (Figure 1). Cu is a structural component of plastocyanin, which plays a key role in the transportation of the electron chain in photosynthesis (Yruela 2013) this outcome helps explaining the A and A/C_i increase. Low Cu concentrations in the medium can stimulate oxygen evolution in PSII and, consequently, the flow of electrons (Burda et al. 2002). On the other hand, cultivar 6563 was the only one that had reduced net photosynthetic rate at Cu concentration 20 μM (Figure 2a). Likewise, cultivar 6563 accumulated a large amount of Cu in plant shoots (Figure 1). This result is justified by the negative correlation between net photosynthetic rate (A) and Cu concentration in shoots (Cu S) (Figure 4). Cu concentration in shoots of cultivar 6563 grown at Cu concentration 20 μM may have induced the formation of reactive oxygen species (ROS), fact that have indirectly compromised the photosynthetic apparatus by inhibiting repairs in the fundamental protein of PSII (Murata et al. 2007).

G_s decreased at higher Cu concentrations, and such decrease is likely responsible for transpiration rate (E) decrease, because these two variables (G_s and E) was positively correlated (Figure 4). The lower the stomatal conductance, the lower the water loss; therefore, the greater the water use efficiency. This result was similar in all cultivars grown at Cu concentrations 20 and 40 μM in comparison to the control (Figure 2). However, G_s decrease seemed not to be the main cause of A and A/C_i decrease when plants were grown at Cu concentration 40 μM , because C_i remained close to 90% of that observed in the control treatment. Therefore, photosynthesis decrease at Cu concentration 40 μM may have other reasons. These reasons can be lower concentration of photosynthetic pigments (Table II) or ROS formation caused by Cu excess (Halliwell and Gutteridge 2015), since these variables had positive and negative correlation to photosynthesis, respectively (Figure 4).

The toxic effect of Cu concentration 40 μM on the concentration of photosynthetic pigments can be attributed to (i) changes in the composition of thylakoids (John et al. 2009); (ii) peroxidation of chloroplast membranes by ROS (Gill and Tuteja 2010); and (iii) formation of metal-pigment complexes (Bazihizina et al. 2015) such as Mg replacement by Cu in chlorophyll molecules (Küpper et al. 2002). Besides the function of carotenoids in light absorption as accessory pigments in light-trap complexes, they also act as photo-protective agents of the photochemical apparatus and prevent photo-oxidative damage in chlorophyll molecules (Raven et al. 2007). Assumingly, the chlorophylls concentration decrease at Cu concentration 40 μM (Table II) may have partially resulted from the reduced concentration of carotenoids (Table II).

Plants grown at Cu concentration 20 or 40 μM in the current study increased SOD activity (Figure 3a) and it happened due to $\text{O}_2^{\cdot-}$ production, since SOD is responsible for its detoxification; besides, SOD is the first cell defense line against

ROS (Thounaojam et al. 2012). SOD activity had positive correlation to Cu concentration (Figure 4), because SOD, and other antioxidant enzymes, often present increased activity at high Cu concentrations (Zhang et al. 2010). The SOD activity was higher in the shoots than in the roots likely due to the SOD isoforms (SOD Cu/Zn and SOD Fe) found in chloroplasts (Pilon et al. 2011).

H₂O₂ concentrations progressively increased in the shoots and roots of most cultivars (Figure 3b), as observed by Thounaojam et al. (2012). We verified a positive correlation between variables: H₂O₂ concentrations, Cu accumulation and SOD activity (Figure 4). Assumingly, Cu accumulation in plants had caused O₂^{•-} production, and it led to SOD activity increase and to consequent H₂O₂ concentration increase due to the SOD reaction. It is possible that some other defense line of antioxidant system in cultivar 6410, such as catalase or peroxides, have detoxified H₂O₂ in the plant shoots (Choudhary et al. 2007, Gill and Tuteja 2010). According to the MDA concentrations detected in the shoots of cultivars 5958, 5909 and 6410 (Figure 3c), ROS apparently did not cause membrane peroxidation. Chen et al. (2015) observed similar results, they related such outcome to low Cu transportation from the roots to the shoot. The same results were not recorded for cultivar 6563, which showed high MDA concentration in the shoot of plants grown at Cu concentration 40 µM (Figure 3c). This outcome indicates the possible occurrence of lipid peroxidation. It is possible saying that H₂O₂ detoxification was not efficient, since it became OH[•] in the presence of Cu in the Fenton reaction (Gill and Tuteja 2010).

CONCLUSIONS

High Cu concentrations in the nutrient solution were harmful to soybean plants. Although each cultivar responded differently to the excess of Cu, none of them showed tolerance to it.

Cultivar M 6410 IPRO was the least affected by the excess of Cu (Cu concentration 40 µM), showing the highest photosynthetic rate and the accumulation of Cu in the roots, without recording a decrease in the root biomass.

Cultivar DM 6563 RSF IPRO showed similar results to M 6410 IPRO; however, its photosynthetic rate recorded stronger decrease and oxidative damage in plant shoots.

Cultivar NA 5909 RG recorded the highest Cu translocation to plant shoots; however, the antioxidant system of the plants avoided lipid peroxidation.

DM 5958 RSF IPRO appeared to be the most sensitive cultivar to Cu excess, its photosynthetic rate and biomass production were significantly lower than in the other cultivars.

Based on these results, we conclude that cultivar M 6410 IPRO has greater potential to be cultivated in environments contaminated by Cu. On the other hand, cultivar DM 5958 RSF IPRO appears not to be recommended for this purpose. However, studies involving field conditions are required to validate such speculations.

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AUTHOR CONTRIBUTIONS

Raissa Schwalbert led the study, performed the greenhouse work and laboratory analysis and wrote the paper; Lincon Oliveira Stefanello da Silva, Gillian dos Santos Fernandes and Rodrigo Knevez Hammerschmitt participated on plant growing and copper determination in plants and also contributed

to the data discussion; Rai Augusto Schwalbert contributed to the data analysis and writing of the paper; Anderson César Ramos Marques, Camila Cavalheiro Costa, and Camila Peligrinotti Tarouco contributed to the photosynthetic and biochemical analysis and data discussion; Gustavo Brunetto and Fernando Teixeira Nicoloso led the study and contributed to the data discussion and writing of the paper.

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