Plant Physiology and Biochemistry 132 (2018) 490–496

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Trans-aconitic acid inhibits the growth and photosynthesis of *Glycine max*

Tiara da Silva Coelho Bortolo, Rogério Marchiosi, Joselaine Viganó, Rita de Cássia Siqueira-Soares, Ana Paula Ferro, Gabriela Elen Barreto, Graciene de Souza Bido, Josielle Abrahão, Wanderley Dantas dos Santos, Osvaldo Ferrarese-Filho*

Laboratory of Plant Biochemistry, Department of Biochemistry, University of Maringá, Paraná, Brazil

ARTICLE INFO

Keywords:
Photosynthetic rate
Chlorophyll *a* fluorescence
Gas exchange
Reactive oxygen species

ABSTRACT

Grasses producing *trans*-aconitic acid, a geometric isomer of *cis*-aconitic acid, are often used in *Glycine max* rotation systems. However, the effects of *trans*-aconitic acid on *Glycine max* are unknown. We conducted a hydroponic experiment to evaluate the effects of 2.5–10 mM *trans*-aconitic acid on *Glycine max* growth and photosynthesis. The results revealed that the enhanced H_2O_2 production in the roots increased the membrane permeability and reduced the water uptake. These effects culminated with a reduced stomatal conductance (g_s), which seems to be the main cause for a decreased photosynthetic rate (A). Due to low g_s, the limited CO_2 assimilation may have overexcited the photosystems, as indicated by the high production of H_2O_2 in leaves. After 96 h of incubation, and due to H_2O_2-induced damage to photosystems, a probable non-stomatal limitation for photosynthesis contributed to reducing A. This is corroborated by the significant decrease in the quantum yield of electron flow through photosystem II *in vivo* (Φ_{PSII}) and the chlorophyll content. Taken together, the damage to the root system and photosynthetic apparatus caused by *trans*-aconitic acid significantly reduced the *Glycine max* plant growth.

1. Introduction

Trans-aconitic acid is a tricarboxylic acid and natural geometric isomer of *cis*-aconitic acid (Kobayashi et al., 2016; Du et al., 2017), an intermediate in the citric acid cycle and with a well-defined role in energy metabolism. Unlike its isomer, *trans*-aconitic acid does not appear to act in primary metabolism, but it has functions as an antifeeding compound (Kim et al., 1994), as a nematicide (Du et al., 2017) and an allelochemical (Voll, 2005; Voll et al., 2009; Foletto et al., 2012). Due to the negative charges conferred by the carboxylic groups, *trans*-aconitic acid can also act as a divalent cation chelator. This property makes *trans*-aconitic acid responsible for grass tetany syndrome, a nutritional disease in ruminants characterized by the deficiency of magnesium and/or calcium in the blood and urine of the animals (Thompson et al., 1997; DPIRD, 2018). However, there is also evidence that tetany in cattle is due to the toxicity of tricarballylic acid produced by rumen microorganisms through the reduction of *trans*-aconitic acid (Bohman et al., 1983).

Although *trans*-aconitic acid is widely found in many plant species at low concentrations (1% dry weight, on average), it is particularly abundant in grasses, such as *Zea mays*, *Triticum aestivum*, *Avena sativa*, *Brachiaria plantaginea* and *Saccharum officinarum* (Burau and Stout, 1965; Brauer and Teel, 1981, 1982; Foletto et al., 2012; Montoya et al., 2014; Kobayashi et al., 2016). As an example, in *Asarum europaeum*, a European native plant with medicinal properties, the *trans*-aconitic acid content reaches 11% of dry weight (Krogh, 1971; Schnitzler et al., 2007). Two routes for the biosynthesis of *trans*-aconitic acid have been described, both from intermediates of the citric acid cycle. In *Triticum aestivum*, *trans*-aconitic acid is produced through the reversible isomerization of *cis*-aconitic acid catalyzed by aconitate isomerase (Thompson et al., 1997). In *Zea mays*, a citrate dehydrase converts citric acid to *trans*-aconitic acid, and potassium (K^+) seems to regulate its accumulation in tissues (Brauer and Teel, 1981, 1982).

Worldwide, and especially in Brazil, *Glycine max* is cultivated in crop rotation systems with grasses, which produce and release *trans*-aconitic acid in soils. As an allelochemical, *trans*-aconitic acid can reduce the germination and seed bank of weeds in the soil, bringing benefits to *Glycine max* cultivation. For instance, it reduces the

Abbreviations: A, photosynthetic rate; g_s, stomatal conductance; E, Transpiration; C_i, Intercellular CO_2 concentration; PS, Photosystem; F_v/F_m, Maximum quantum yield of PSII; Φ_{PSII}, Quantum yield of electron flow through PSII *in vivo*; ROS, Reactive oxygen species; H_2O_2, Hydrogen peroxide; PPFD, Photosynthetic photon flux density

* Corresponding author.
E-mail addresses: osferrarese@gmail.com, oferrarese@uem.br (O. Ferrarese-Filho).

https://doi.org/10.1016/j.plaphy.2018.09.036
Received 2 August 2018; Received in revised form 26 September 2018; Accepted 27 September 2018
Available online 28 September 2018

germination percentage and the length of stems and roots of *Euphorbia heterophylla*, *Ipomoea grandifolia*, *Ipomoea triloba, Bidens pilosa*, and *Sida rhombifolia*; common weeds of *Glycine max* crops (Voll et al., 2010; Foletto et al., 2012). However, little is known about the effects of *trans*-aconitic acid on *Glycine max* growth. Knowledge in this regard is important for predicting impact on *Glycine max* production when it is cultivated in soils that received applications of vinasse or areas of *Saccharum officinarum* cultivation in which *trans*-aconitic acid can reach 2.5–25 mM (Voll et al., 2009). As a residue from the fractional distillation of *Saccharum officinarum* juice, vinasse has high value fertilizer since it contains significant amounts of K^+, calcium (Ca^{+2}) and magnesium (Mg^{+2}) (Voll et al., 2010). In addition, the *Glycine max* cropping systems with pasture has emerged as one of the main alternatives to the renovation and restoration of degraded pastures (Silva et al., 2005).

Because *Glycine max* planting in soils containing *trans-aconitic* acid is done often in Brazil, to evaluate its effects on growth and development of this crop is imperative. For this reason, the aim of the current work was to investigate the effects of *trans*-aconitic acid on the growth and photosynthesis (gas exchange, chlorophyll *a* fluorescence and chlorophyll content) of *Glycine max* plants. The depletion of *trans*-aconitic acid from roots, the content of hydrogen peroxide (H_2O_2) in roots and leaves, and the membrane permeability of the root cells were also evaluated.

2. Materials and methods

2.1. General procedures

Glycine max seeds (*cv*. BMX-Potencia) were sanitized with 2% sodium hypochlorite for three min, rinsed with deionized water, and dark-germinated on two sheets of moistened filter paper in a germination chamber (Tecnal TE 400, São Paulo, Brazil) at 25 °C for three days. Seedlings of uniform size were transferred to Styrofoam supports and dipped into glass containers filled with 350 mL of a 1/6-strength nutrient solution, pH 6.0 (Dong et al., 2006). Seedlings were cultivated for 16 days under a light/dark photoperiod of 14/10 h, at a temperature of 25 °C and irradiance of 300 μmol photons m^{-2} s^{-1}. The nutrient solution was replaced every other day, with its strength increasing from 1/6 to 1/3, and from 1/3 to 1/2, on the 4th and 10th days of cultivation, respectively. Nutrient solutions, with or without *trans*-aconitic acid (2.5–10 mM) were added on the 10th, 12th and 14th days. On the 16th day of cultivation, the plants were removed for determination of lengths and fresh weights of roots and stems. The dry weights were determined after dehydration of tissues in an oven at 70 °C for 96 h. The nutrient solution uptake was monitored by measuring the volume of nutrient solution consumed in the intervals between treatments. *Trans*-aconitic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA) and all other reagents used were of the purest grade available.

2.2. Depletion of trans-aconitic acid from the nutrient solution

The depletion of *trans*-aconitic acid was monitored chromatographically. Samples (1 mL) were collected at 3, 6 and 24 h after addition of nutrient solutions containing 2.5–10 mM *trans*-aconitic acid on the 14th day of cultivation. Samples were filtered using a 0.45 mm disposable syringe filter and then analyzed (20 μL) with a Prominence HPLC system (Shimadzu®, Tokyo, Japan) equipped with a quaternary gradient pump LC-20AT, photodiode array detector SPD-M20A, autosampler SIL-20A, and column oven CTO-20A. *Trans*-aconitic acid was separated using a reversed-phase Shimpack® CLC-ODS (M) column (250 × 4.6 mm, 5 μm particle size) assembled with an equivalent pre-column (10 × 4.6 mm) by using mobile phase containing 3.5 mM phosphoric acid. *Trans*-aconitic acid was measured at 220 nm and identified by comparing its retention time with that of a corresponding standard (Product number 122750, Sigma-Aldrich®, St. Louis, MO, USA). Difference between the remainder *trans*-aconitic acid at 3, 6 and 24 h, and its initial concentration in the nutrient solution was considered as absorption by the roots.

2.3. Membrane permeability of Glycine max roots

The electrical conductivity measurements were performed according to Baziramakenga et al. (1995), with modifications. On the 16th day of cultivation, the roots were completely immersed in deionized water for 10 min, which was replaced four consecutive times. Then, electrical conductivity measurements were performed after 4 h of root immersion in deionized water by using an electrical conductivity meter (Tecnal TEC-4MP, São Paulo, Brazil). The results were expressed as μS cm^{-1} g^{-1} fresh weight.

2.4. Determination of H_2O_2 from roots and leaves

The H_2O_2 content was determined according to Sergiev et al. (1997). Fresh tissues (1 g) were homogenized in an ice bath with 3 mL of 0.1% (w/v) trichloroacetic acid. The procedure was performed in the dark. After centrifugation (1660 × *g* for 20 min), 250 μL of 5 M potassium iodide and 500 μL of 10 mM phosphate buffer (pH 7.0) were added to 500 μL of supernatant. After one minute of reaction, the absorbance of the sample was read spectrophotometrically (UV-2450, Shimadzu, Kyoto, Japan) at 390 nm, using a reaction mixture without tissue extract as a blank. The results are expressed as μmol g^{-1} fresh weight.

2.5. Gas exchange, chlorophyll a fluorescence and chlorophyll index

Photosynthetic rate (A), stomatal conductance (g_s), transpiration (E) and intercellular CO_2 concentration (C_i) were determined using an Infra-Red Gas Analyzer (IRGA; ADS BioScientific LCpro+, Hertfordshire, UK). The measurements were performed under a photosynthetic photon flux density (PPFD) of 1200 μmol photons m^{-2} s^{-1}, at 25 °C, between 7:00 a.m. and 11:30 a.m. (Marchiosi et al., 2016). The maximum quantum yield of photosystem (PS) II (F_v/F_m) and the quantum yield of electron flow through PSII *in vivo* (Φ_{PSII}) were determined with the aid of a fluorometer (OS1-FL, Opti-Sciences Inc., Hudson, USA). All analyses were performed on the 12th, 14th and 16th days of cultivation using the first fully expanded trifolium. Chlorophyll content was estimated using a chlorophyll meter (SPAD-502, Konica Minolta, Ramsey, USA).

2.6. Statistical analyses

For the parameters of nutrient solution uptake, gas exchange, chlorophyll *a* fluorescence, chlorophyll content, and absorption of *trans*-aconitic acid were used in a completely randomized design, with a factorial scheme 3 × 5 (three days of evaluation × five concentrations of *trans*-aconitic acid), totaling 15 treatments. For the biometric parameters, a completely randomized design was used, with each plot represented by a glass container containing a plant. All data were expressed as the means of four independent experiments ± standard error of the mean (SEM).

The data were subjected to analysis of variance and the treatment means were compared by the method of the Scott-Knott clustering algorithm (Figs. 1 and 4) or Dunnett's multiple comparison test (Figs. 2 and 3), at 5% probability. A mean test was used due to the regression deviation being significant for all variables analyzed. Statistical analyses were performed using the Sisvar® package (Version 4.6, UFLA, Brazil). When the interaction "days of evaluation × concentrations of *trans*-aconitic acid" was significant ($P \leq 0.05$), the necessary decompositions were carried out.

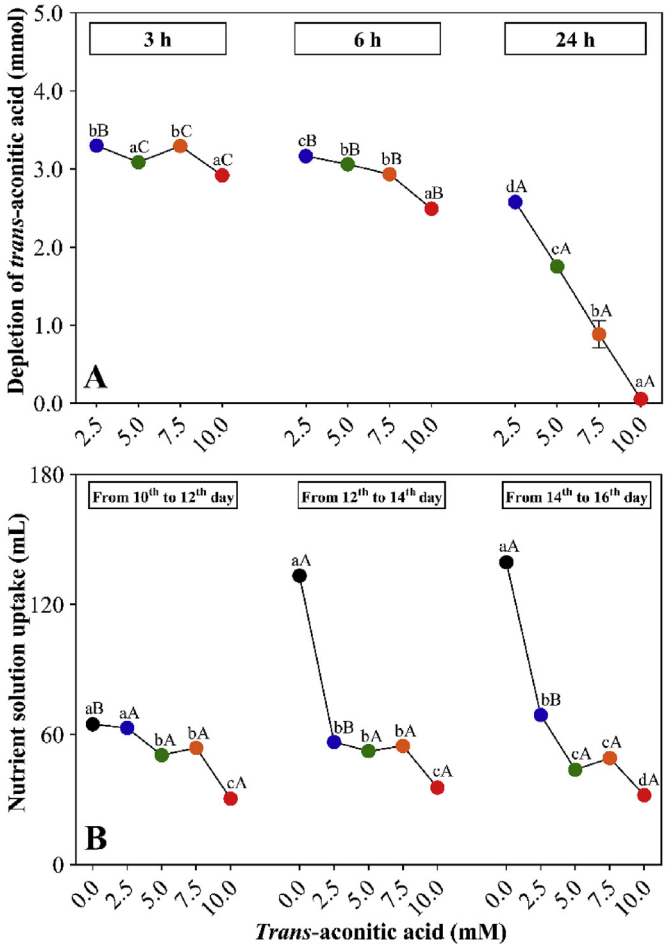

Fig. 1. Depletion of *trans*-aconitic acid from the nutrient solution (A) and nutrient solution uptake (B) by *Glycine max* plants grown hydroponically for 16 days. A) The depletion of *trans*-aconitic acid from the nutrient solution was monitored after 3, 6 and 24 h. B) The volume of the nutrient solution was measured after 2, 4 and 6 days of *trans*-aconitic acid exposure. Mean (n = 4 ± SEM) values followed by the same letter, lowercase between the doses and uppercase between the days of evaluation, are not significantly different according to the Scott-Knott multiple comparison test ($P \leq 0.05$). For most points, the error bars were shorter than the height of the symbol.

3. Results

3.1. Absorption of trans-aconitic acid by Glycine max plants

Trans-aconitic acid was not present in the nutrient solution after 24 h of incubation at any of the concentrations tested (Fig. 1A). This suggests that *trans*-aconitic acid may have been absorbed by the plants. The amount of *trans*-aconitic acid depleted from the nutrient solution was dose and time dependent, *i.e.* 0.2–0.5 mmol (at 3 h), 0.34–1 mmol (6 h) and 0.88–3.5 mmol (24 h), respectively, from 2.5 to 10 mM. As noted, one unique plant can absorb 3.5 mmol of *trans*-aconitic acid, which corresponds to about 0.61 g. This is the total amount of *trans*-aconitic acid available in 350 mL of a 10 mM solution.

3.2. Effects of trans-aconitic acid on nutrient solution uptake by Glycine max

The nutrient solution uptake by the plants was dose and time dependent under action of *trans*-aconitic acid (Fig. 1B). In the first 2 days (10^{th} to 12^{th} day of cultivation), the compound reduced the nutrient solution uptake from 22% (5 mM) to 53% (10 mM), in comparison to the control. From 2.5 to 10 mM, the uptake was reduced from 58% to 73% (12^{th} to 14^{th} day) and 50%–77% (14^{th} to 16^{th} day). As also noted, the nutrient solution uptake was exposure time dependent. Throughout the experiment (10^{th} to 16^{th} day), the uptake was reduced from 22% to 69% (at 5 mM), 17%–65% (at 7.5 mM), and 53%–77% (at 10 mM), when compared to the respective controls.

3.3. Effects of trans-aconitic acid on Glycine max growth

The biometric parameters were also negatively affected by *trans*-aconitic acid (Fig. 2). Leaf fresh weights were reduced from 8% to 62% with 2.5–10 mM of *trans*-aconitic acid, when compared to the control (Fig. 2A). A minor effect was observed on leaf dry weights; only a 38% decrease at 10 mM (Fig. 2B). The lengths of stems and roots were also reduced at all concentrations of *trans*-aconitic acid. When compared to the control, stem lengths were reduced from 19% to 41% from 2.5 to 10 mM (Fig. 2C), and root lengths were reduced from 18% to 31% within the same range of concentrations (Fig. 2F). The effects of 10 mM *trans*-aconitic acid were even more evident on fresh weights of stems (52%; Fig. 2D) and roots (64%; Fig. 2G), with respect to controls. Finally, decreased dry weights were observed from 5 to 10 mM *trans*-aconitic acid exposures; on average, 27% for stems (Fig. 2E) and 38% for roots (Fig. 2H).

3.4. Effects of trans-aconitic acid on membrane permeability and H_2O_2 content

The membrane permeability of the root cells was evaluated by determining the electrical conductivity of the solution in which the roots of the *Glycine max* plants were immersed. After 4 h of immersion, the conductivity of the solution increased about 62% regardless of the *trans*-aconitic acid concentration, in comparison to the control (Fig. 3A).

Trans-aconitic acid increased the H_2O_2 content in roots and leaves in a dose-dependent manner (Fig. 3B). Roots exposed to 2.5–10 mM *trans*-aconitic acid significantly increased H_2O_2 production from 38% to 89%, with respect to the control. A more pronounced effect occurred in leaves: H_2O_2 content increased from 97% to 168% of within the same range of concentrations.

3.5. Effects of trans-aconitic acid on gas exchange, chlorophyll a fluorescence and chlorophyll content

Fig. 4 summarizes the effects of *trans*-aconitic acid on gas exchange, chlorophyll *a* fluorescence and chlorophyll content of *Glycine max* plants. Compared with the respective controls, and after 2 days (12^{th} day of cultivation), A, g_s, E and C_i were reduced from 12% to 47%, 54%–57%, 33%–43% and 3%–9%, respectively, with increasing concentrations of *trans*-aconitic acid from 2.5 to 10 mM (Fig. 4A and B). Furthermore, the compound reduced these parameters on the last day of cultivation, which suggests an inhibitory effect related to the time of exposure. Indeed, the analysis of the decomposition of the interaction "concentrations of *trans*-aconitic acid × time of exposure" revealed a dependency between these variables.

Plants exposed to *trans*-aconitic acid showed reduced chlorophyll contents, and the time of exposure was crucial for this effect (Fig. 4A). Except on the 12^{th} day of cultivation, the chlorophyll contents were reduced from 18% to 19% (14^{th} day) and 19%–24% (16^{th} day) from 5 to 10 mM of *trans*-aconitic acid, when compared to the control. Plants submitted to *trans*-aconitic acid revealed reduced Φ_{PSII} (Fig. 4C). Similar to A, g_s, E and C_i, the effect was dose and exposure time dependent. On the 12^{th} day of cultivation, the Φ_{PSII} was reduced to 18% (at 7.5 mM) and to 9% (at 10 mM), in comparison to the control. However, on the 16^{th} day of cultivation, Φ_{PSII} was reduced from 32% to 56% from 2.5 to 10 mM of *trans*-aconitic acid. The compound was not effective on F_v/F_m (Fig. 4C). The most relevant change was observed on the 14^{th} day, when there was a 7% reduction for 10 mM of *trans*-aconitic acid,

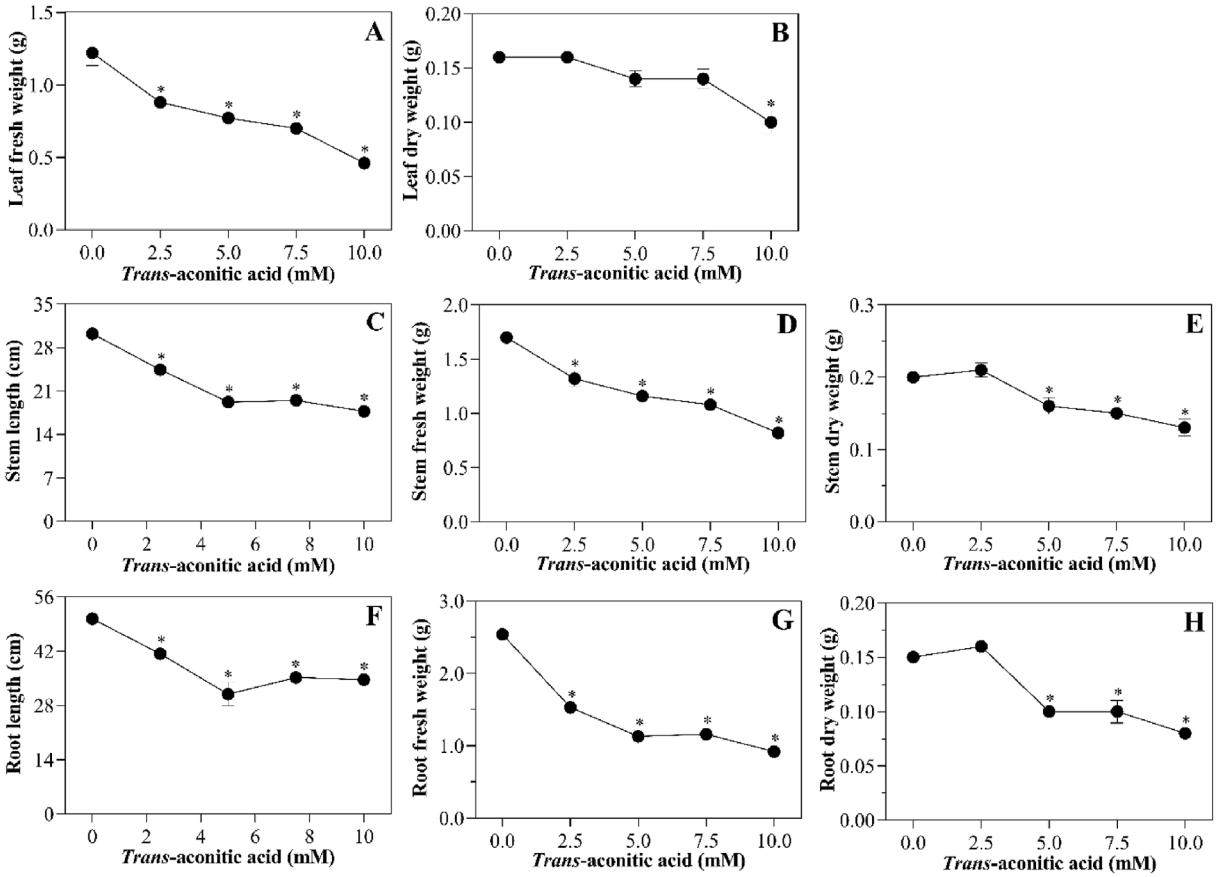

Fig. 2. Effects of a 6-day exposure to *trans*-aconitic acid on fresh (A) and dry (B) weights of leaves, length (C), fresh (D) and dry (E) weights of stems, and length (F) and fresh (G) and dry (H) weights of roots of *Glycine max* plants grown hydroponically for 16 days. *Mean (n = 4 ± SEM) values differ statistically (Dunnett's multiple comparison test) from the control ($P \leq 0.05$). For most points, the error bars were shorter than the height of the symbol.

with respect to control.

3.6. Leaf epinasty induced by trans-aconitic acid

Leaf epinasty was noted in plants exposed to 5 mM (or more) of *trans*-aconitic acid (Fig. 5). This symptom was evident after 2–3 days of treatment and mainly in older leaves.

4. Discussion

The allelochemical *trans*-aconitic acid is commonly released by grasses, such as *Triticum aestivum*, *Zea mays*, *Hordeum vulgare*, *Brachiaria ruziziensis*, *Sorghum bicolor* and *Saccharum officinarum*, which are very often cultivated in crop rotation systems with other plants, including *Glycine max* in Brazil. Because the possible impacts of *trans*-aconitic acid on *Glycine max* is unknown, we evaluated its effects on plant growth and photosynthesis. We showed here that *trans*-aconitic acid affects both processes, and this observation is supported by the literature findings as follows.

4.1. Trans-aconitic acid reduces the Glycine max growth

Previous studies have demonstrated that *trans*-aconitic acid reduces the growth of weeds, such as *E. heterophylla*, *I. grandifolia*, *I. triloba*, *B. pilosa* and *S. rhombifolia*, which are frequently found in *Glycine max* crops. Mostly, the inhibitory effect is noticeable in roots (Voll, 2005; Voll et al., 2010; Foletto et al., 2012). As noted, *trans*-aconitic acid was rapidly absorbed (Fig. 1A) and decreased the lengths of roots (Fig. 2C) and stems (Fig. 2F) of *Glycine max*. One may ask whether these effects are exclusive of the *trans*-aconitic acid itself or to products of its metabolism. Although *trans*-aconitic acid may be absorbed by roots, its possible degradation/metabolism cannot be denied, but this requires a further investigation.

The clear-cut inhibitory effect on roots is particularly important because it reveals that, when present in soils (Voll et al., 2009), *trans*-aconitic acid can limit the development of roots and compromise the crop. *Trans*-aconitic acid reduced the fresh weights of roots, stems and leaves more than the lengths of *Glycine max* plants (Fig. 2). This is a probable consequence of its action on absorption of water and nutrients, one of the first processes affected by allelochemicals (Einhellig, 1995). In addition, we highlighted that plants exposed to *trans*-aconitic acid absorb a lower volume of nutrient solution, in comparison to the control plants (Fig. 1B). Nonetheless, we cannot overlook that plants treated with *trans*-aconitic acid are smaller and therefore have a less developed root system.

The integrity of root cell membranes is essential for water and nutrient uptake by plants, and *trans*-aconitic acid likely affected this factor. We observed augmented electrical conductivity of the solution containing *trans*-aconitic acid-exposed plants (Fig. 3A). This indicates a higher ion leakage by the roots, and reveals a detrimental effect of the allelochemical on the plasma membrane followed by disturbances in water and nutrient uptake. Many allelochemicals interact directly with cell membranes or compromise metabolic functions necessary for their maintenance (Baziramakenga et al., 1995; Inderjit and Duke, 2003; Hejl and Koster, 2004; Reigosa et al., 2006; Chai et al., 2013; Soltys et al., 2013). In the specific case of *trans*-aconitic acid, changes in membrane permeability can be due to the production of reactive oxygen species (ROS) and lipid peroxidation, as noted in *I. triloba* (Foletto et al., 2012). This is plausible since we found an enhanced H_2O_2 content in roots exposed to the allelochemical (Fig. 3B). Therefore, it is reasonable to

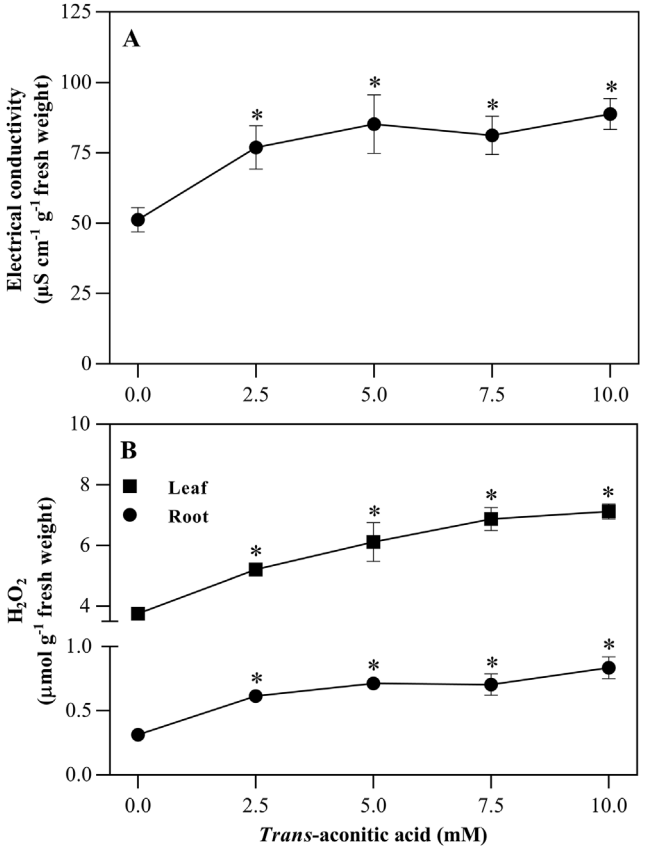

Fig. 3. Effects of a 6-day exposure to *trans*-aconitic acid on the permeability of roots (A) and H_2O_2 contents of roots and leaves (B) of *Glycine max* plants grown hydroponically for 16 days. *Mean (n = 4 ± SEM) values differ statistically (Dunnett's multiple comparison test) from the control ($P \leq 0.05$). For some points, the error bars were shorter than the height of the symbol.

believe that *trans*-aconitic acid induces the formation of ROS with subsequent damage to cell membranes.

4.2. Trans-aconitic acid affects photosynthesis

Photosynthesis is influenced by several environmental factors such as temperature, light, water, CO_2 concentration, microorganisms, and especially by chemicals (Gao et al., 2018). Among these, allelochemicals exert undesirable effects on photosynthesis by altering the CO_2 supply (controlling stomatal opening and closure), light reactions and/or carbon assimilation reactions (Zhou and Yu, 2006). For example, the allelochemical sorgoleone binds to the QB site of PSII and blocks the transport of electrons in photosynthesis (Nimbal et al., 1996; Czarnota et al., 2001). To our knowledge, there is only one study on the effect of *trans*-aconitic acid on photosynthesis, which showed that the allelochemical inhibited more photosynthesis than respiration of leaf discs of *Nicotiana rustica* and *Zea mays* (Stepanova and Shumilova, 1976), but the mechanism of action has not been elucidated.

To verify the assumption that *trans*-aconitic acid interferes in *Glycine max* photosynthesis, we evaluated its effects on gas exchange, chlorophyll *a* fluorescence and chlorophyll content of *Glycine max* plants (Fig. 4). We have found that it markedly reduced the A (−47% at 10 mM, 12th day) probably due to stomatal limitation. This finding was accompanied by the marked decline in g_s (−57%) and by a slight reduction of C_i (9%). A diminished value of C_i suggests that carbon assimilation reactions were faster than the uptake of CO_2 by stomata. In other words, stomatal closure limited the amount of CO_2 available for photosynthesis in *trans*-aconitic acid exposed plants. Moreover, it is possible that the insufficient water uptake reduced g_s (Gimenez et al.,

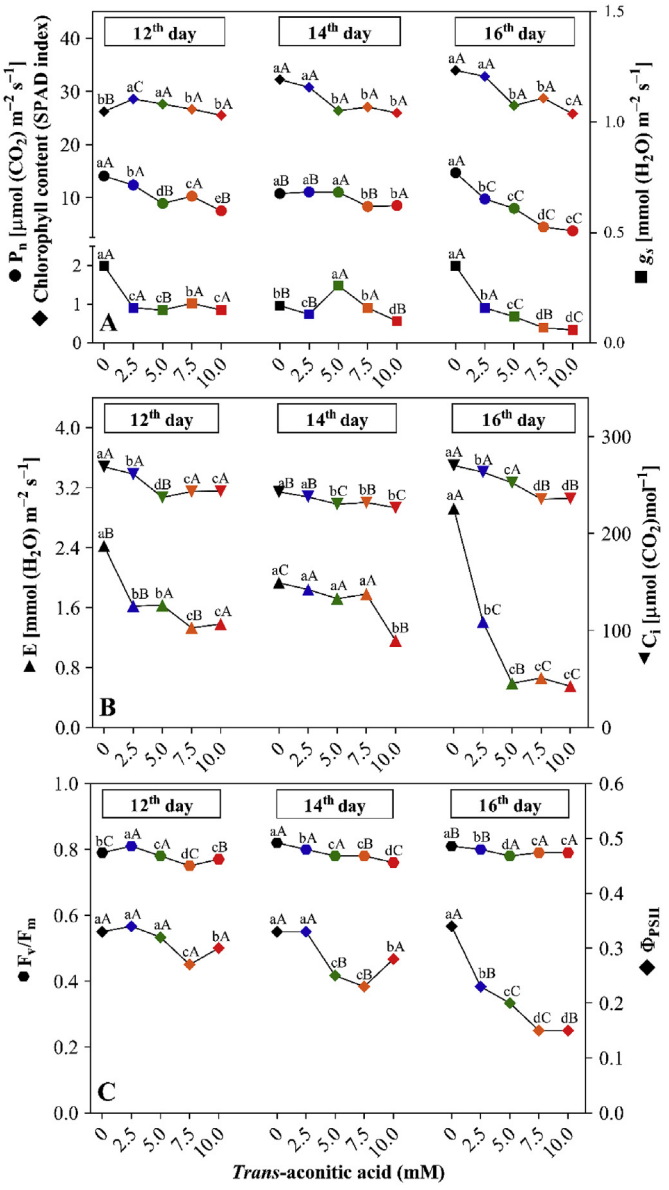

Fig. 4. Effects of a 6-day exposure to *trans*-aconitic acid on photosynthetic rate, stomatal conductance and chlorophyll content (A), transpiration and intercellular CO_2 concentration (B), and maximum quantum yield of PSII and quantum yield of electron flow through PSII *in vivo* (C) of *Glycine max* plants grown hydroponically for 16 days. Mean (n = 4 ± SEM) values followed by the same letter, lowercase between the doses and uppercase between the days of evaluation, are not significantly different according to the Scott-Knott multiple comparison test ($P \leq 0.05$). For all points, the error bars were shorter than the height of the symbol.

2005; Yan et al., 2016). We also monitored the effects of *trans*-aconitic acid on Φ_{PSII} of *Glycine max* plants. Under certain conditions, such as an absence of photorespiration, Φ_{PSII} acts as an indicator of carbon assimilation (Φ_{CO2}) *in vivo* (Baker, 2008). Unlike A, Φ_{PSII} was not markedly reduced by *trans*-aconitic acid on the 12th day of cultivation (Fig. 4C); this is indicative that reduction of A is a direct consequence of stomatal limitation. However, on the 16th day of cultivation, the remarkable decline of Φ_{PSII} suggests a non-stomatal limitation for photosynthesis. Similar results were observed in *Glycine max* plants exposed to the allelochemical benzoxazolin-2-(3*H*)-one (BOA) (Parizotto et al., 2017). In short-term experiments (24 h), BOA reduced the A due to an exclusive stomatal closure, whereas in longer-term experiments (96 h), its effects induced non-stomatal limitation for photosynthesis.

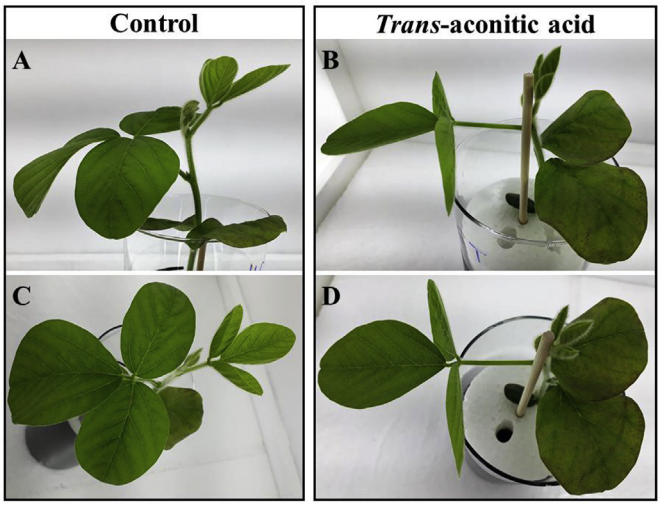

Fig. 5. Leaf epinasty induced by 5.0 mM *trans*-aconitic acid in *Glycine max* plants. Front view (A and B) and top view (C and D).

Taken together, the results indicate that stomatal closure reduced the CO_2 entry in the substomatal chamber and limited its availability for assimilation, at least for 24 h of exposure to *trans*-aconitic acid. This finding led to a decrease in the consumption of ATP and NADPH by the carbon assimilation reactions, a reduction of electron transport and, by consequence, low Φ_{PSII} (Hussain and Reigosa, 2011). In addition, these metabolic changes can lead to the overexcitation and photoinhibitory damage of the PS due to the formation of ROS. Decreased activities of CO_2 assimilatory enzymes, structural damage to PS (oxidation of proteins and lipids) and degradation of pigments (chlorophyll) are typical events of overexcitation of PSII (Papadakis et al., 2004; Souza et al., 2004; Pospíšil, 2016). Hence, damage to photosynthetic machinery can eventually occur and impose a non-stomatal limitation for photosynthesis. Corroborating this hypothesis, we have noted an increase of H_2O_2 (Fig. 3B) and reduction of chlorophyll (Fig. 4A) in leaves on the 16th day of cultivation. In agreement with our findings, Φ_{PSII}, F_v/F_m and chlorophyll content decreased in *F. arundinacea* due to photoinhibition caused by temperature stress (Cui et al., 2006).

4.3. Trans-aconitic acid induces leaves epinasty

Another observed finding was the leaf epinasty of *Glycine max* plants exposed to *trans*-aconitic acid (Fig. 5). This symptom was evident in older leaves on the 16th day of cultivation. Leaf epinasty usually results from higher growth rates of the upper (adaxial) side of the petiole, compared to the inferior (abaxial) side, and it is related to the increase in ethylene contents (Haubrick and Assmann, 2006; Lee et al., 2008; Sandalio et al., 2016). In leaves, enhanced ethylene produces cyanide, which inhibits catalase and peroxidase followed by H_2O_2 accumulation (Grossmann, 2003). Moreover, 2,4-D herbicide-induced epinasty is related to overproduction of ROS (Pazmiño et al., 2011, 2014). Like 2,4-D, a stimulus in H_2O_2 production could have led to leaf epinasty in *Glycine max* plants exposed to *trans*-aconitic acid.

5. Conclusions

Trans-aconitic acid negatively affected *Glycine max* plants. In roots, the high production of H_2O_2 changed the integrity of the membranes and impaired the water and nutrient uptake. In leaves, a stomatal limitation for photosynthesis reduced A in the first 24 h of *Glycine max* exposure to *trans*-aconitic acid. The non-stomatal limitation for photosynthesis caused by H_2O_2-induced damage to PSII appears to be involved in the reduction of A, after 96 h of exposure. In brief, damage caused to the root system and the photosynthetic apparatus reduced *Glycine max* plant growth.

Conflicts of interest

The authors declare no conflicts of interest.

Authors contribution

OFF and RM planned the experiments, analyzed the results and wrote the manuscript with the help of WDS. TSCB performed all experiments with the help of RCSS, APF, GEB, GSB and JA. JV performed the statistical analyses.

Acknowledgements

OFF is researcher fellows of National Council for Scientific and Technological Development (CNPq). TCSB thanks the Coordination of Enhancement of Higher Education Personnel (CAPES) for a fellowship. JA is a postdoctoral fellow of CAPES. The authors thank Aparecida M. D. Ramos for their technical assistance.

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