Evaluation of gastroprotective activity of *Passiflora alata*  

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**Abstract**

*Passiflora alata* Curtis, Passifloraceae, is a liana popularly known in Brazil as ‘maracujá-doce’ that has been used for treating different illnesses. Its leaves are described in the Brazilian Pharmacopoeia, but the gastroprotective activity has never been investigated. In the present study a freeze-dried crude 60% ethanol–water extract of *P. alata* aerial parts was prepared. Total flavonoid content, expressed as vitexin, was 0.67 ± 0.01. The hemolytic activity was 32 units for *P. alata*, using Saponin (Merck\(^a\)) as reference. *P. alata* presented EC\(_{50}\) of 108.1 ± 8.5 μg/ml in the 2,2-diphenyl-1-picrylhydrazyl assay and 1076 ± 85 μmol Trolox/g in the Oxygen Radical Absorbance Capacity assay. *P. alata*, its solvent fractions and a *P. alata* nanopreparation were investigated for gastroprotective activity. The test samples exhibited gastroprotective activity on HCl/ethanol induced gastric mucosal lesions in rats. *P. alata* at doses of 100, 200 and 400 mg/kg, using the necrotizing agent at 150 mmol/l, inhibited 100% of ulcer formation (compared to the negative control), while lansoprazole (30 mg/kg) 77%. When tested against a more concentrated necrotizing agent (300 mmol/l), fractions of *P. alata* at 100 mg/kg reduced 57% (n-hexane), 34% (ethyl acetate) and 72% (aqueous fraction) the ulcer formation. In this assay, lansoprazole (30 mg/kg) inhibited 47%. When encapsulated, *P. alata* inhibited ulcer formation at 55%, 94% and 90% for dosages of 25, 50 and 100 mg/kg. These results suggest the potential use of *P. alata* as a gastroprotective herbal medicine.

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**Introduction**

Several *Passiflora* L., Passifloraceae, species are used in the traditional medicine around the world. In Europe and United States, *Passiflora incarnata* L. has been used for treating nervous restlessness, insomnia, hysteria, most of the times in combination with other sedative extracts. Its aerial parts are officially listed as plant drug in various Pharmacopoeias. The predominant compound class in this species is C-glycosyl flavonoids; harman alkaloids, when present, occur in trace amounts (Blumenthal, 1998; Gruenwald et al., 2000; EMA, 2014).

This genus comprises more than 400 species distributed throughout tropical and subtropical areas, mainly from Central and Southern America (Plotze et al., 2005). In Brazil, there are approximately 140 species reported, from which 83 are endemic and 44 only in São Paulo State (Bernacci et al., 2015).

*Passiflora alata* Curtis is an evergreen vine, which bears an edible type of passion fruit, endemic from Brazil, occurring from the Amazon until the Atlantic Rain Forest (Bernacci et al., 2015). This species is widely cultivated for its edible fruits, appreciated in the local culinary (Loreni et al., 2006), and it has been used for treating different illnesses, e.g., nervous disturbs and gastric problems (Petry et al., 2001, Duke, 2002, Gomann et al., 2011; Noriega et al., 2011). Due to its pharmacological relevance, it had been cited in four editions of the Brazilian Pharmacopeia (Farmacopeia dos Estados Unidos do Brasil, 1926; Farmacopeia dos Estados Unidos do Brasil, 1959; Farmacopeia Brasileira, 1977, 2010).

Most of the studies about the chemical composition of *P. alata* leaves lead to the identification of several C-glycosidic flavonoids (Ulubelen et al., 1982; Doyama et al., 2005; Müller et al., 2005) and saponins (Reginatto et al., 2004; Birk et al., 2005). The antioxidative activity from *P. alata* leaf extracts was proved in different pharmacological assays and it has been related to these metabolite classes (Oga et al., 1984; De-Paris et al., 2002; Reginatto et al., 2006; Provensi et al., 2008; Klein et al., 2014). Besides the antioxidative effect, other biological activities have been detected in rodents, such as anti-inflammatory (Vargas et al., 2007), and as anti-diabetic in type 1 diabetes mellitus (Colomeu et al., 2014).

Concerning safety issues, the *P. alata* leaf extracts had no effect in female rat fertility (Amaral et al., 2001), but there was some...
indication of genotoxicity (Boeira et al., 2010), hepatotoxicity and anorexic activities in rodents (Braga et al., 2013).

Active compounds like flavonoids, tannins and other glucosides sometimes have good water solubility. However, they can also present low absorption, resulting in a loss of bioavailability and efficacy. Others may show poor water solubility and also have absorption issues. Nanostructured systems have the potential of controlling the release or reducing the amount of active compounds to obtain the desired efficacy. They can also assist on directing the molecule to its biological target (Bonífição et al., 2014).

Poly(e-caprolactone) nanocapsules are among the possibilities when using nanotechnology in the development of herbal medicines. The biodegradability and biocompatibility of this polymer make its use favorable in pharmaceuticals. These nanocapsules have a lipid core and may change pharmaceutical and pharmacological parameters of a drug, as prolonged release and enhanced stability (Pohlmann, 2013).

Concerning antiulcer activity, epidemiological data estimates that at some point in their lives, 4–12% of adults might develop a peptic ulcer (Leontiadis and Nyérn, 2014). The latest detailed epidemiology consists of 2009 when Sung et al. reported that the annual incidence of peptic ulcer in the world varies from 0.10% to 0.19%, and the prevalence from 0.12% to 1.5%. It means that tens of millions of people worldwide have this disease, making it one of the most relevant today.

Considering that most of the pharmacological research on P. alata is focused on its central nervous system activity and that to date there are no scientific data concerning the antiulcer properties of this species (Noriega et al., 2011), the present study was carried out to evaluate a possible gastroprotective activity of P. alata, based on the traditional use for gastric problems. In order to propose P. alata as a gastroprotective herbal product, we assayed its crude lyophilized extract, some solvent partition fractions and a poly(e-caprolactone) nanocapsules preparation.

Materials and methods

Plant material

Aerial parts of Passiflora alata Curtis, Passifloraceae, were provided by the Instituto Agronômico de Campinas from an Active Germplasm Bank located in Monte Alegre do Sul, SP (750 m high). The species was authenticated by Dr. Luís Carlos Bernacci. A voucher specimen was deposited at the Instituto de Biociências’ Herbarium, Universidade de São Paulo, with respective registration number VM01.

Preparation of the extract and fractions

Aerial parts were cut, washed, and dried in an air circulation oven at temperature below 50 °C for 72 h. After drying, the plant material was ground in a rotary cutter mill to obtain particles of 0.5–1.0 mm. The powdered material was percolated with 60 °C ethanol in a proportion plant:solvent of 1:10 (w/v). After extraction, the solvent was eliminated under vacuum in a rotatory evaporator at 40–45 °C and the residual water was eliminated by freeze-drying, yielding a brown-greenish powder. The freeze-dried crude P. alata extract (PA) had its total flavonoids quantified according the European Pharmacopeia quality control method for P. incarnata L. (European Pharmacopoeia, 2011) and the results are expressed as percentage of vitexin. As P. alata is also known to contain saponins, which may cause hemolysis when added to a suspension of blood, the hemolytic activity of PA was determined according the World Health Organization methodology (WHO, 2011) by comparison with that of a reference Saponin (Merck®).

PA was fractioned from 10 g of the crude extract, partitioning between water and n-hexane, followed by ethyl acetate. Each extraction was performed with 3 × 20 ml solvent. Each fraction was separately concentrated under reduced pressure.

Nanoencapsulation

Nanoencapsulated extracts of PA were obtained by a solvent displacement technique. Poly(e-caprolactone) (PCL) (0.010 g) was dissolved in 27 ml of acetone, by stirring (200 rpm) for 40 min at 40 °C (organic phase). A total of 0.333 g of capric/caprylic triacylglyceride and 0.077 g of sorbitan monostearate (span 60) were added to the organic phase. PA extract was added after the polymer dissolved and allowed to stir for 10 min in the organic phase, after this time the extract was completely dissolved in the organic phase (25, 50 and 100 mg yielding 2.5; 5.0 and 10 mg/ml concentrations). The organic phase was gently injected inside the aqueous solution (53 ml of water) containing 0.070 g of polysorbate 80. Polymer deposition at the interface of the solvents led to the instantaneous formation of a colloidal suspension. The organic solvent was removed using reduced pressure in a rotary evaporator, and the final aqueous volume was adjusted to 10 ml with water.

Characterization of the encapsulated extract

The average particle size and the polydispersity index (PDI) were measured by DLS (dynamic light scattering) using a Zetasizer ZN particle size analyzer (Malvern Instruments, Southborough, UK) equipped with vertically polarized light supplied by an argon-ion laser. The Zeta potential was also determined by DLS (Zetasizer ZN particle size analyzer, Malvern Instruments, Southborough, UK). All the measurements were determined from the mean values of three experiments.

Antioxidant activity

One assay to determine P. alata antioxidant capacity was scavenging of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as described by Mensor et al. (2001). The P. alata stock solution (2.0 mg/ml in ethanol) was diluted in ethanol to concentrations of 0.125, 0.25, 0.5, 1.0 and 2.0 mg/ml. One milliliter of DPPH solution was added to 2.5 ml of the different concentrations of the extract and the positive control. The 0.3 mmol/l DPPH (Sigma®) solution was prepared in ethanol. Rutin was employed as a positive control at concentrations of 3.125, 6.25, 12.5, 25 and 50 μg/ml. Solutions containing 1 ml ethanol and 2.5 ml of each extract were used as blanks. The absorbance of samples, positive controls, and blanks at 518 nm was determined after 30 min. The EC50 values were calculated by linear regression of plots where the absorbica represented the concentration of tested plant extracts and the ordinate the average percent of antioxidant activity from three separate tests.

The ORAC (Oxygen Radical Absorbance Capacity) assay was performed in a microtiter plate reader according to Huang et al. (2002). Briefly, AAPH (2,2’-azobis[2-amidinopropane] dihydrochloride, Sigma-Aldrich®) was used as a peroxyl radical generator, Trolox as a standard, and fluorescein as a fluorescent probe. Filters were used to select an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Twenty-five microlitres of diluted sample (P. alata extract 0.039, 0.029, 0.023, 0.019 and 0.017 mg/ml), blank, or Trolox calibration solution (6.25–100 μmol/l) were mixed with 150 μl fluorescein (4 μmol/l) and incubated for 30 min at 37 °C before injection of 25 μl AAPH (173 mmol/l) solution. The fluorescence was measured every minute for 1 h. All samples were analyzed in duplicate at three different dilutions. The final ORAC
values were calculated using the net area under the decay curves. The result is expressed as micromol Trolox equivalent/g of sample.

Animals

Female Wistar rats weighing 150–180 g from Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, were housed in standard cages at room temperature (20 ± 3°C) with 12 h dark/light cycles. They were fastened for 12 h but allowed free access to water before the experimentation. Assays were carried out using 7–9 animals per group, randomly divided. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care, and the protocol for use of animals has been approved by Comitê de Ética em Experimentação Animal, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, number 132/2006.

Gastroprotective activity

The experiment was performed according to methodology described by Mizui and Doteuchi (1983).

The solutions of PA and its fractions, plus the necrotizing agent and the control lansoprazole were freshly prepared before administration. Test solutions were administered by gavage (10 ml/kg) at the following concentrations: PA 100, 200, 400 mg/kg body weight (assay 1); PA Fractions, n-hexane, ethyl acetate and water, 100 mg/kg (assay 2); nanoencapsulated PA, 50 and 100 mg/kg (assay 3). Control rats received the vehicle (water), nanocapsules without extract or lansoprazole (30 mg/kg) suspended in vehicle, same volume as test samples. Thirty minutes after the administration of test samples, lesions were induced by administering by gavage an HCI solution in ethanol/water 60% (v/v) at doses of 10 ml/kg body weight. The administered HCl concentrations were 150 mmol/l for assay 1 and 300 mmol/l for assays 2 and 3. One hour later, the animals were killed in a CO₂ chamber. The stomachs were removed and opened along the greater curvature to determine relative lesion area (RLA, in %). The relative lesion area was determined as follows:

\[ RLA = \frac{(TLA \times 100)}{TA}, \]

where TLA = total lesion area (mm²), TA = total stomach area (mm²).

Albeit in the graphics the numbers were standardized as % of lesion area, we understand that % of inhibition facilitates understanding. The latter describes a comparison between test and negative control, bringing more meaning to these values. Now, the formula of % of inhibition was included and this relation is better explained.

\[ \% \text{ of inhibition} = 100 - \left( \frac{\% \text{ RLA of tested group}}{\% \text{ RLA of negative control}} \right) \times 100 \]

Statistical analysis

The values were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed through ANOVA followed by Tukey. Significant differences were considered for \( p < 0.05 \).

Results and discussion

The PA yield after freeze-drying was 16%, and the total flavonoid content, expressed as vitexin, was 0.67% ± 0.01. The plant material employed contained a lower concentration of flavonoids than it was expected; accordingly the Brazilian Pharmacopoeia the P. alata plant drug should present a minimum of 1% of flavonoids (Farmacopeia Brasileira, 2010) for leaves only. Previous flavonoid quantifications of aqueous and hydro-alcoholic extracts from P. alata leaves also presented higher concentrations, such as 2.6% (De-Paris et al., 2002) and 2.9% (Petry et al., 2001). However, values of 0.47% and 0.39% were also reported for P. alata leaves extracts (Müller et al., 2005).

P. alata is known to present saponins as major secondary metabolites, different from other Passiflora species in which flavonoids are the main metabolites (Birk et al., 2005). The main characteristic property of saponins is their ability to cause hemolysis. When added to a suspension of blood, saponins produce changes in erythrocyte membranes, causing hemoglobin to diffuse into the surrounding medium. The hemolytic activity of plant extracts containing saponins was determined by comparison with that of a reference saponin, which has its hemolytic activity established at 1000 units per g (WHO, 2011). The obtained PA extract, indicated the presence of saponins by the persisting foam produced, when tested. The measured hemolytic activity was 32 units for PA, using Saponin (Merck®) as reference. This activity was not very high when compared with the reference substance, but the hemolysis might be increased if an enriched saponin fraction is analyzed (Yuldasheva et al., 2005; Rocha et al., 2012).

Encapsulated extract characterization

Encapsulated extract showed particle size (diameter) of 359.3 ± 21.5 nm with polydispersity index of 0.288 ± 0.062 and zeta potential of −36.2 ± 6.1 mV. Nanocapsules vehicle (same formulation with no extract added) exhibited diameter of 259.7 ± 16.4 nm, polydispersity index of 0.241 ± 0.060 and zeta potential of −32.7 ± 7.7 mV (results expressed by mean ± standard error).

Mayer (2005) reported that the optimum radii should be between 100 and 500 nm. Sinha et al. (2004) consider that diameter may range between 10 and 1000 nm. Mora-Huertas et al. (2010) reviewed parameters of polymeric nanocapsules and concluded that there are no expected rules for zeta potential values, as it depends on polymer chemical properties, stabilizing agents and medium’s pH. In most cases values below −10 mV are obtained (generally between −25 and −30 mV). This is considered a prediction for good stability due to a high energy barrier between the particles.

Antioxidant activity

Antioxidants can prevent or slow the oxidative damage to human body by scavenging reactive oxygen species (ROS). Superoxide and hydroxyl radicals are major contributors to oxidative damage of gastric mucosa, and are involved in the complex pathogenesis of ethanol induced mucosal lesions. Plant phenolics, including flavonoids, are examples of compounds that can reduce the damages caused by ROS, resulting in gastroprotective properties (Priya et al., 2012).

Several assays have frequently been used to estimate antioxidant capacities of plant extracts, including DPPH scavenging activity and the oxygen radical absorption capacity (ORAC). The ORAC assay is considered more relevant because it utilizes a biologically relevant radical source (Prior et al., 2003). The antioxidant capacity of PA was measured by DPPH and ORAC, presenting an EC₅₀ of 1061.2 ± 8.5 µg/ml and 1076 ± 85 μmol Trolox/g, respectively. In order to assess the PA antioxidant potential rutin was used as reference and presented an EC₅₀ of 18.3 ± 0.9 µg/ml in the DPPH assay, while literature data of EC₅₀ for some of the flavonoids found in P. alata varied from 290 to 1000 µg/ml (Okawa et al., 2001). The ORAC assay also indicated that the antioxidant potential of PA is not high, since plant extracts considered with a promising activity in this assay presented values from 3000 to 8500 µmol Trolox/g (Dudonné et al., 2009). Although the low antioxidant potential from
PA, the presence of flavonoids in the crude extract might explain the lower hemolytic activity found for the crude extract when compared with the isolated saponins. It is known that some flavonoids and their glycosides are effective antioxidants protecting human red blood cells from free radical-induced oxidative hemolysis (Dai et al., 2006).

**Gastroprotective activity**

Hemorrhagic ethanol-induced ulcers show nonspecific mechanisms, acting over several homeostasis regulators. In a review about laboratory ulcer models, Glavin and Szabo (1992) summarize some factors involved in this kind of gastric mucosal injury. Among other factors, we can mention a decrease of: gastric mucosal blood flow, mucus, glutathione (GSH) and prostaglandin production; and an increase of: free radical generation, histamine release, ischemia and vascular permeability. Kawano and Tsuji (2000) reviewed endotelin-1 and nitric oxide (NO) roles on gastric ulcer, including on ethanol-induced lesions, concluding that these substances are essential to the mucosal blood flow balance. Ethanol stimulates endotelin-1 release from gastric vessels, resulting in vasoconstriction by interacting with ETa receptors. This process disturbs gastric microcirculation, which contributes to lesion formation. Endogenous nitric oxide has a vasodilator effect and reduces the generation of hemorrhagic lesions. Interactions between these two substances are important to the regulation of local vascular tone. Kwicier et al. (2002) also found evidences that reactive oxygen species (ROS) also play a major role in ethanol-induced lesions due to a decrease in the gastric blood flow, increment of inflammatory changes (increase of IL-1β and TNFα levels), as well as generation of ROS and decrease of superoxide dismutase activity in gastric mucosa, leading to lipid peroxidation and consequently oxidative stress and tissue damage. Lansoprazole, used as positive control in the ethanol induced ulceration methodology, plays an important role on various gastric ulcer mechanisms. Albeit its main activity is over proton pump, it also may show several acid-independent gastroprotective effects, as histamine-stimulated acid secretion inhibition; increase of blood flow and NO production; enhancement of bicarbonate secretion stimulated by acid; increase of gastric mucus content, sulphydryl compounds and prostaglandin levels; antioxidant and anti-inflammatory effects. This nonspecific behavior of lansoprazole could explain why it can inhibit ulcer lesions caused by nonspecific mechanisms of the employed harmful agent (Sato et al., 1989; Inatomi et al., 1991; Brunton, 2006; Satoh, 2013).

In order to evaluate which compound classes could present gastroprotective activity, PA was fractioned with increasing polarity solvents (n-hexane, ethyl acetate and water). Previously, almost all biological assays performed with *P. alata* used aqueous or hydroethanolic extracts (Petry et al., 2001; Noriega et al., 2011; Colomeu et al., 2014). Hexane fraction generally contains mostly fatty acids, pigments, phytosterols and terpenes (Harborne, 1978). In *P. alata* ethyl acetate fraction, simple phenolic compounds and non-glycoside flavonoids were previously found (Vasic et al., 2012). *P. alata* aqueous fraction is known to contain the most polar compounds, saponins and C-glycoside flavonoids (Reginatto et al., 2001; Doyama et al., 2005). As the gastroprotective activity was lower in the fractions it indicates that the activity observed was not caused by one specific metabolite group.

In assay 1 (Fig. 1), using the nectrotizing agent at 150 mmol/l, PA was able to inhibit completely the ulcer formation (compared to the negative control) for all tested dosages, while lansoprazole (30 mg/kg) promoted 77% of inhibition. These results showed significant gastroprotective effect for PA in the model of acute gastric ulcer induced by HCl/ethanol. After the acute antilucre test, at the dose of 150 mmol/l HCl the relative area of lesion was 4% for the water control group. The proposal was to increase the concentration of the ulcerative agent (300 mmol/l HCl), to increase the ulceration area for the negative control (8%), in order to verify if at this concentration the extract and the fractions would still be able to prevent the ulceration in the tested model. In this assay, PA fractions reduced the ulcer formation in 57% (n-hexane), 34% (ethyl acetate) and 72% (aqueous fraction) of lesion area at 100 mg/kg, and lansoprazole (30 mg/kg) 47% (Fig. 2). The most active fraction was the aqueous one exhibiting only 29% of ulcer formation. However, none of the fractions exhibited higher activity than the crude extract; further experiments were only performed with PA.

Using the higher concentration of the necrotizing agent, even lower PA dosages (50 and 100 mg/kg) were also able to inhibit ulcer formation, a decrease of 27% and 84% of lesion area was observed (Fig. 3, assay 3), the control drug had a similar performance compared to the higher PA dosage (84%). In the same assay, PA nanocapsules exhibited stronger activity with 55%, 94% and 90% inhibition for the respective doses of 25, 50 and 100 mg/kg (Fig. 3). As there was no significant difference between nanocapsulated 50 and 100 mg/kg dosages, it suggests a dose-response curve slightly above these doses for this formulation on this model. Concerning the anti-ulcer test with the extract (PA) incorporated and not incorporated in nanoparticles, there is a significant difference between nano and PA at 50 mg/kg, but not at the 100 mg/kg dose. Analyzing the graphs, it is possible to see that the maximum
ulcer protection occurred at 50 mg/kg with the nanoencapsulated extract. For this reason, we suggest approximately this dosage, for future studies for developing a gastroprotective formulation using the *P. alata* nanoencapsulated extract.

Plant extracts containing flavonoids can inhibit the proton pump activity or increase the secretion of mucus and PGE2 (prostaglandin E2), what explains its gastroprotective activity. Gracioso et al. (2002), investigating the antiulcer activity of *Turnera ulmifolia* L. showed that the active fraction presented C-glycosylated flavones, derived from apigenin and luteolin. Baggio et al. (2007) suggested that the flavonoid-rich fraction of *Maytenus ilicifolia* Mart. ex Reissek possess antiulcer activity. As the PA extract contained 0.67% flavonoids, most of them C-glycosides, these compounds might have some influence in the activity measured. Although flavonoids are known to present free radical scavenger activity, which might have a clinical application as antiulcer drugs (Yamaguchi et al., 2000), the low antioxidant activity measured in PA indicates that this was not the main gastroprotection mechanism involved in the results observed.

Moreover, dried aqueous *P. alata* extracts are also known to contain 0.8% (w/w) saponins (Reginatto et al., 2004); these metabolites can also be involved in the gastroprotection once we determined the presence of non-hemolytic saponins in PA. In literature, among the pharmacologic activities described for saponins, we find the antiulcer activity (Hostettmann and Marston, 1995). In fact, one of the most knowledgeable antiulcerogenic crude drugs of this metabolite class is liquorice (Hostettmann and Marston, 1995). Although along with glycyr rhizin, other groups of pharmacologically active substances are present in *Glycyrrhiza glabra* roots and rhizomes in which flavonoids are included (Saitoh et al., 1976). Despite the historic use of this plant, contradictory results are found in its pharmacological efficacy (Isbrucker and Burdock, 2006). Moreover, gastroprotective effect of tea saponin (*Camellia sinensis*) on ethanol induced gastric mucosal lesions in rats, is more potent than that of omeprazole, as observed by Morikawa et al. (2006).

Concluding, our results provide scientific evidence for the use of *P. alata* as a gastroprotective medicine, a disease that needs new and more effective drugs. It is also important to note the fact that *P. alata* is included in Requisis, a list of medicinal plants, which research and development of derived products are of interest to the official health system of Brazil. Furthermore, the nanoencapsulation seems to be an effective technology to improve the activity of *P. alata* extracts to produce a gastroprotective herbal product.

**Conflicts of interest**

The authors declare no conflicts of interest.

**Authors’ contribution**

MY is the coordinator of the *Passiflora* project; PRHM, EMB and ETMK are participants of the *Passiflora* project; AW and AV-N conducted the experimental study; LSH is the main responsible for the organization and writing of the manuscript.

**Acknowledgments**

This project (number 04/07933-3) was supported by FAPESP. A. Wasicky thanks CNPq for scholarship (134740/2005-0) during the period of his M.Sc. The authors thank M.O.M. Marques, L.M.M. Melletti, L.C. Bernacci and M.D.S. Scott (Instituto Agronômico de Campinas) for the plant material used in this research and I.M. Cuccovia for the nanoparticles analysis by Zetasizer.

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