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Aluminium detoxification in facultative (*Passovia ovata* (Pohl ex DC.) Kuijt and *Struthanthus polyanthus* Mart. - Loranthaceae) and dependent (*Psittacanthus robustus* (Mart.) Marloth - Loranthaceae) Al-accumulating mistletoe species from the Brazilian savanna

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ABSTRACT

Mechanisms to detoxify aluminium (Al) is a hot topic for cultivated plants. However, little information is known about the mechanisms used by native plants to deal with Al-toxicity. In Cerrado, some generalist mistletoe species, such as *Passovia ovata* (Pohl ex DC.) Kuijt and *Struthanthus polyanthus* Mart. can parasitize Al-accumulating and Al-excluding plant species without any clear symptoms of toxicity and mineral deficiency, while *Psittacanthus robustus* (Mart.) Marloth, a more specialist mistletoe, seems to be an Al-dependent species, parasitizing only Al-accumulating hosts. Here we (i) characterized the forms and compartmentalization of Al in leaves of *P. robustus*; (ii) compared Ca and Al leaf concentration, and leaf concentration of organic acids and polyphenols between facultative Al-accumulating (*P. ovata* and *S. polyanthus*) and Al-dependent (*P. robustus*) mistletoe species infecting *Miconia albicans* (Sw.) Steud. (Al-accumulating species). *P. robustus* chelated Al^{3+} with oxalate and stored it in the phloematic and epidermic leaf tissues. Leaf Ca and Al concentration did not differ among species. Leaf oxalate concentration was higher in the Al-dependent species. Concentrations of citrate and phenolic compounds were higher in the leaves of the facultative Al-accumulating species. These results show that facultative Al-accumulating and Al-dependent species use different mechanisms to detoxify Al. Moreover, this is the first report on a mistletoes species (*P. robustus*) with a potential calcifuge behaviour in Cerrado.

1. Introduction

Aluminium trivalent (Al^{3+}) is a toxic element for many cultivated and native plants in acidic soils with pH < 4.5 (Kochian et al., 2015). Even at concentrations lower than micromolar, Al^{3+} disrupts the root system, reduces water and nutrient uptake and compromises the plant development (Barceló and Poschenrieder, 2002; Brunner and Sperisen, 2013). Nevertheless, some native and cultivated plants are tolerant to Al-toxicity due to efficient mechanisms for detoxifying Al externally (Al-excluders) or internally (Al-accumulating).

The Al-excluders release small organic acids and phenolic compounds into the soil through their roots avoiding Al uptake (Barceló and Poschenrieder, 2002), while Al-accumulating species uptakes and accumulate high concentrations of Al in leaves and roots (> 1000 mg Al kg^{-1} leaf dry mass) (Haridasan, 2008) without symptoms of toxicity. For some Al-accumulating species (*Miconia albicans* (Sw.) Steud - Metastomataceae, *Vochysia thyrsoidea* Pohl and *V. tucanorum* Mart. - Vochysiaceae) from the Brazilian savanna (Cerrado) with a calcifuge behaviour (i.e., sensitive species to high Ca availability in the soil), Al may be considered a beneficial element reducing the toxic effects of high Ca concentration (Haridasan, 1988, 2008; Souza et al., 2017).

During the last thirty years a massive investment in research was devoted to understand the mechanisms used by Al-excluders (mostly crops) to deal with Al-toxicity (Barceló and Poschenrieder, 2002; Brunner and Sperisen, 2013; Kochian et al., 2015). However, a reduced number of studies was conducted to understand the mechanisms used

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by native Al-accumulating plants to deal with Al-toxicity. In Al-accumulating plants, the contribution of low-molecular organic acids (e.g. citrate, malate and oxalate) and phenolic compounds (e.g. catechin) to detoxify Al were basically investigated in cultivated plants such as tea (*Camellia sinensis* (L.) Kuntze - Theaceae), buckwheat (*Fagopyrum esculentum* Moench - Polygonaceae) and *Hydrangea macrophylla* (Thunb.) Ser. (Hydrangeaceae) (Barceló and Poschenrieder, 2002; Brunner and Sperisen, 2013). An exception are investigations on some Al-accumulating members of the Myrtales and Santalales (Maejima et al., 2014, 2017; Souza et al. 2017, 2018a). The first study investigating the mechanisms of Al-detoxification in Cerrado species was recently published. The authors reported that, while the mistletoes *Passovia ovata* (DC.) Kuijt and *Struthanthus polyanthus* Mart. (Loranthaceae) chelate Al with citrate, the host *M. albicans* chelate Al with oxalate (Souza et al., 2018b). They also observed significant variation on leaf concentration of organic acids between mistletoes infecting an Al-accumulating and Al-excluding host.

Mistletoes are parasitic plants that penetrate the bark of the hosts' branches through a modified root, the haustorium, connecting to their host xylem (Lamont and Southall, 1982). Therefore, the mistletoe-host system is a very interesting model to understand nutrient relations in plants, especially Al toxicity, since the same mistletoe species can be found parasitizing different hosts with contrasting Al and nutrient concentrations in the xylem. Tropical mistletoes from the Loranthaceae family are highly diverse, represented by 73 genera and over 1500 species (Nickrent et al., 2010; Nickrent, 2011), and varying widely in the degree of host specificity. In the Brazilian Savanna (Cerrado), some mistletoes from the Loranthaceae family can be classified as facultative Al-accumulating (*P. ovata and S. polyanthus*) and Al-dependent (*Psittacanthus robustus* Mart.) species (Scalon et al., 2013; Souza et al., 2018b), depending on the host relative specificity. Facultative Al-dependent species are found parasitizing a large number of species, including Al-accumulating and Al-excluding, while the Al-dependent species is restricted to Al-accumulating hosts (Monteiro et al., 1992; Scalon et al., 2013). Despite the report of Al-accumulating tree species from Cerrado with a calcifuge behaviour (Haridasan, 1988, 2008; Souza et al., 2017), until date there are no report of tropical mistletoe species with a calcifuge behaviour.

The aims of this study was to (1) compare the leaf concentration of Ca and Al between facultative Al-accumulating (*Passovia ovata* and *Struthanthus polyanthus*) and Al-dependent species (*Psittacanthus robustus*) infecting *Miconia albicans* (calcifuge and Al-accumulating tree species); (2) characterize the forms of Al and their compartmentalization on leaves of *P. robustus* (Al-dependent species) infecting *M. albicans*; (3) compare the leaf concentration of organic acids and phenolic compounds between facultative Al-accumulating (*P. ovata* and *S. polyanthus*) and Al-dependent species (*P. robustus*) parasiting *M. albicans*. We expect to find significant differences on the mechanisms to detoxify Al between facultative Al-accumulating and Al-dependent species, reflecting the distinct strategies to deal with high Al availability on the host xylem. Based on the mechanisms used by the Al-dependent species, we expect to propose the first case of calcifuge behaviour in tropical mistletoes.

2. Results

We observed no significant variation on the leaf concentration of Ca ($x^2 = 2.72$, $p = 0.44$) and Al accumulation ($x^2 = 4.11$, $p = 0.25$) between the facultative and dependent Al-accumulating mistletoe species (Fig. 1a and b). Putting all data together, we observed that leaf Ca ($r = 0.88$, $p < 0.001$) and Al ($r = 0.58$, $p < 0.01$) levels correlated positively between mistletoes and hots (Fig. 2).

The ^{27}Al-NMR analyses revealed that *P. robustus* detoxify Al with Al-oxalate complexes (Al-oxalate(1) - 6.4 ppm, Al-oxalate(2) - 11.6 ppm and Al-oxalate(3) - 16.1 ppm). We also observed a peak indicating the presence of free Al^{3+} (0.0 ppm) in leaves of *P. robustus* (Fig. 3). The histochemical analyses revealed that *P. robustus* stored Al in the phloematic tissues and the epidermis (Fig. 4).

Leaf concentrations of organic acids differed significantly between facultative and dependent Al-accumulating species. *P. robustus* (Al-dependent) had four times more oxalate leaf concentrations than *P. ovata* and *S. polyanthus* (facultative Al-accumulating) ($x^2 = 7.42$, $p = 0.02$) (Fig. 5a). On the contrary, the lowest level of citrate was observed in *P. robustus* (Al-dependent) and the highest level in *P. ovata* (facultative Al-accumulating) ($x^2 = 9.85$, $p < 0.01$) (Fig. 5b).

Leaf concentrations of phenolic compounds varied significantly between facultative and dependent Al-accumulating species ($x^2 = 7.73$, $p = 0.02$). The concentration of phenolic compounds was three-fold higher in the facultative Al-accumulating species (*P. ovata* and *S. polyanthus*) than in Al-dependent (*P. robustus*) (Fig. 6).

3. Discussion

Similar leaf Ca and Al concentrations between facultative and dependent Al-accumulating species reflected the nutrient availability in the host. This similarity was expected since hemiparasites sink nutrients from the host's xylem sap by the haustorium (Scalon et al., 2013). Correlations of leaf nutrient concentrations between mistletoes and hosts were previously reported for tropical and subtropical plants, including Al-accumulating and Al-excluding species (Glatzel and Geils, 2009; Scalon et al., 2013; Scalon and Wright, 2015; Souza et al., 2018b). Despite similar leaf concentrations of Ca and Al among the species, the ratio of Ca:Al was higher in the facultative Al-accumulating species (1.05 - *P. ovata*; 0.97 - *S. polyanthus*) than in the Al-dependent species (0.58 - *P. robustus*) suggesting that these two groups may use different mechanisms to deal with Al and Ca toxicity.

Chelation with low-molecular organic acids is one of the most important mechanisms to detoxify Al and Ca in the leaves of both Al-accumulating species and calcifuge species (Lee, 1998; Brunner and Sperisen, 2013). In the present study we observed that the Al-dependent mistletoe species (*P. robustus*) detoxify Al with oxalate, while Souza et al. (2018b) found that facultative Al-accumulating mistletoe species (*P. ovata* and *S. polyanthus*) chelate Al with citrate. The Al-citrate complex observed in the facultative Al-accumulating mistletoes (Souza et al., 2018b) is considered the generalist complex for Al detoxification being used for both internal and external Al detoxification for Al-accumulating and Al-excluding species (Ma et al., 1997; Brunner and Sperisen, 2013; Kochian et al., 2015). The broad use of citrate to detoxify Al^{3+} is attributed to the tricarboxylic anions that forms a more stable complex with Al^{3+} than oxalate and malate that are dicarboxylic anions (Kochian et al., 2015).

Furthermore, the Al-oxalate complexes observed in *P. robustus* are frequently found in Al-accumulating species with a calcifuge behaviour, such as *M. albicans* (Souza et al., 2018b), *Melastoma malabathricum* L. (Watanabe et al., 1998; Maejima et al., 2017) (Metastomataceae) and *Symplocos chinensis* (Lour.) Druce - Symplocaceae (Maejima et al., 2014). Oxalate not only detoxify Al^{3+} but also acts on the regulation of Ca concentrations by balancing soluble and insoluble forms, reducing the toxic effects of high Ca^{2+} concentrations (Lee, 1998; Nakata, 2012). This is the first report of Al-oxalate complexes to detoxify Al and Ca in Loranthaceae family. The calcifuge behaviour, combined with the dependency of high Al-concentrations in the host xylem, may influence host selection by this mistletoe species and may explain the high specificity exhibited by *P. robustus* for the Al-accumulating hosts within the Vochysiaceae and Melastomataceae families (Scalon et al., 2013; Guerra et al., 2017).

In contrast to the facultative Al-accumulating mistletoes (*P. ovata* and *S. polyanthus*) that store Al exclusively in the phloematic fibres (Souza et al., 2018b), the histochemical analyses showed that *P. robustus* (Al-dependent species) stores Al in both phloematic tissues and epidermis. The compartmentalization of Al ion in the epidermis has previously been observed in other Al-dependent species belonging to

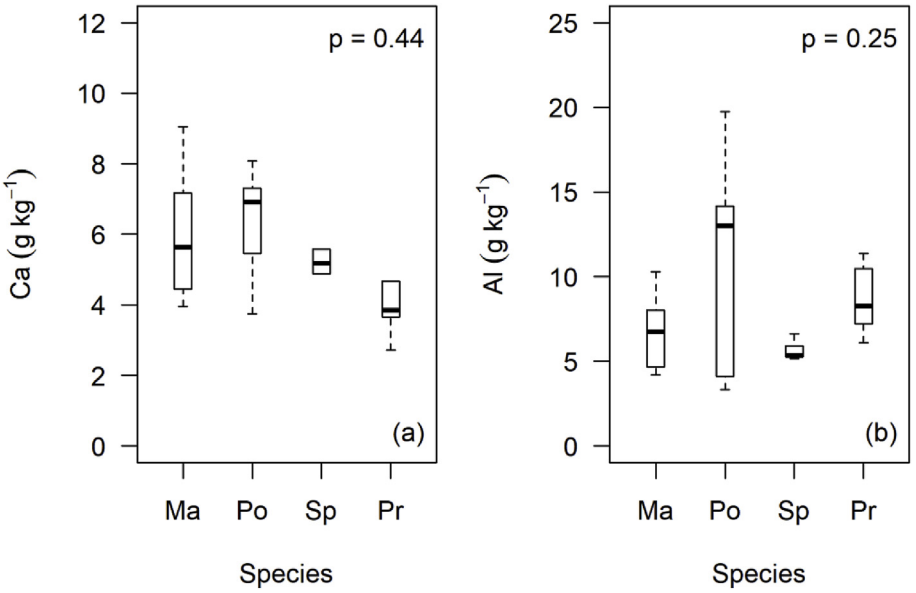

Fig. 1. Leaf calcium concentration and aluminium accumulation in (Ma) *Miconia albicans* (host), (Po) *Passovia ovata*, (Sp) *Struthanthus polyanthus* (facultative Al-accumulating) and (Pr) *Psittacanthus robustus* (Al-dependent). The box extends from the 25th to 75th percentiles, continuous line within the box shows the median, and error bars represent the 5th and 95th percentiles (n = 4).

the Melastomataceae, Rubiaceae and Vochysiaceae families (Haridasan et al., 1986; Britez et al., 2002; Bressan et al., 2016). Considering that epidermis does not contribute directly to photosynthesis, the compartmentalization of Al in epidermis may be considered part of the strategies used by Al-dependent species to deal with Al-toxicity. Until date, there are no report of Al compartmentalization in the epidermis of facultative Al-accumulating species.

The histochemical analyses not only confirmed Al compartmentalization in leaves, but also evidenced a greater deposition of phenolic compounds in leaves of the facultative Al-accumulating species compared to the Al-dependent species (Souza et al., 2018b). In acidic environments, isolated simple phenols are not considered important for Al detoxification in plants (Barceló and Poschenrieder, 2002). However, the protonation reaction of the phenol in presence of carboxylic groups from organic acids can strength the interaction between Al^{3+} and the organic acid anion ligand, increasing the effective stability of the Al-organic acid complex (Barceló and Poschenrieder, 2002). Despite the non-observation of Al-phenolic complexes neither in facultative Al-accumulating (Souza et al., 2018b) nor in Al-dependent mistletoes by the ^{27}Al NMR, leaf concentration of phenolic compounds were three-fold higher in the facultative Al-accumulating species than in the Al-dependent species, suggesting that some phenolic compounds could be essential for Al-detoxification on facultative Al-accumulating species.

To conclude, we demonstrated that facultative Al-accumulating and Al-dependent mistletoe species have different mechanisms to detoxify Al in their leaves. Citrate is the main organic acid used by the facultative Al-accumulating species to detoxify Al, while oxalate is the main organic acid used by the Al-dependent species to detoxify Al. Histochemical analysis showed significant differences of Al and polyphenol compartmentalization in leaves between the facultative and dependent Al-accumulating species. Based on the forms and compartmentalization of Al in the leaves of *P. robustus*, we propose for the first time a mistletoe with a calcifuge behaviour.

4. Experimental

4.1. Plant samples

Fully expanded leaves of four individuals of *Passovia ovata* (Pohl ex DC.) Kuijt, *Struthanthus polyanthus* Mart. (facultative Al-accumulating) and *Psittacanthus robustus* (Mart.) Marloth (Al-dependent) (Loranthaceae) parasitizing *Miconia albicans* (Al-accumulating) were collected at a Cerrado site in the Natural Reserve of the Roncador (RECOR/IBGE) (15°56′S, 47°53′W), Brazil, during the peak of the wet

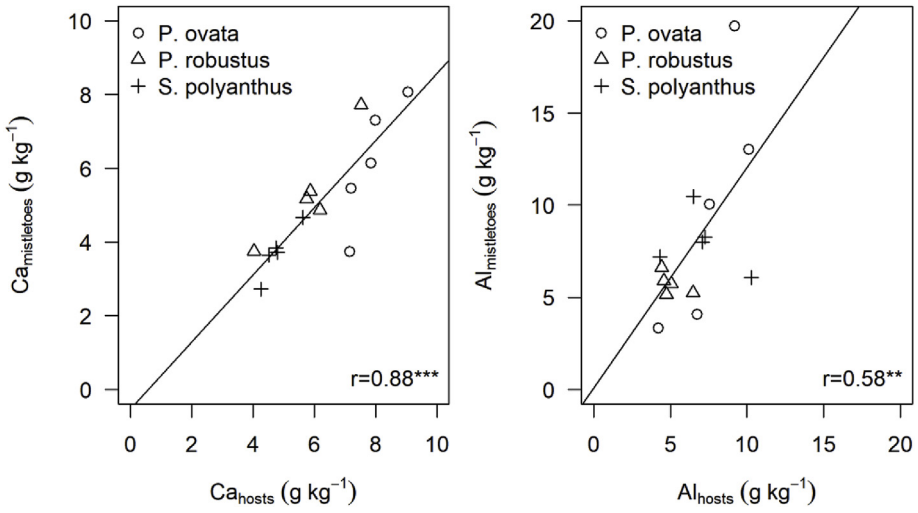

Fig. 2. Pearson's correlation of leaf Ca and Al levels between mistletoes and hosts.

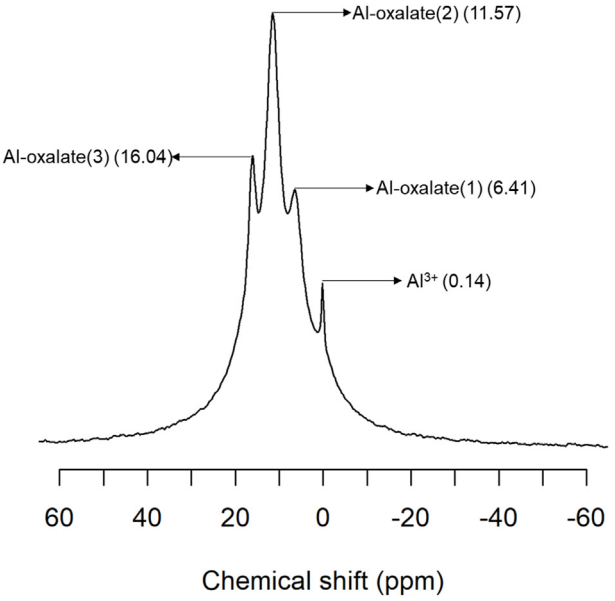

Fig. 3. ^{27}Al NMR spectrum of *Psittacanthus robustus* (Al-dependent) parasiting *Miconia albicans*. These spectra were processed in "software-version" using exponential multiplication with a linebroadening (lb) factor of 50.

season (February 2015).

4.2. Leaf Ca concentration and Al-accumulation in facultative and dependent Al-accumulating mistletoes

Leaves were oven dried for 72 h at 60 °C, ground in Wiley mill and digested in a nitric:sulphuric:perchloric (10:1:2 v/v/v) acids solution. Ca concentration was determined by atomic absorption spectrophotometry and Al was determined colorimetrically (Allen, 1989).

4.3. Forms of Al on leaves of P. robustus using ^{27}Al-NMR

Intact mature-fresh-leaves were cut into small pieces, inserted in a 4 mm zirconia rotor and sealed with a Kel-F cap. The ^{27}Al-NMR spectra were recorded using a spinning speed of 10000 Hz on a prototype MAS 4 mm, at 104.21 MHz (Bruker Avance III - 400 NMR spectrometer, Germany) with 1024 scans and repetition delay time of 1s per 1 scan. Al $(NO_3)_3$ was used as an external reference for calibration of the chemical shift (0.0 ppm) (Sanz et al., 1988). A pulse sequence described by a single 5 μs pulse followed by an acquisition time (34 ms) was used to acquire the NMR spectra. Identification of the forms of Al on leaves of mistletoes was based on literature reports (Morita et al., 2008; Souza et al., 2018b).

4.4. Localization of Al on leaves of P. robustus

The leaves were fixed in buffered formaldehyde (Lillie, 1965), dehydrated in an ethanol series, and stored in 70% ethanol (Johansen, 1940). Consecutively-sliced cross sections were made manually with a razor blade. Cuts were immersed for 45 min in acidic deionized water (pH 4.0) before staining, stained for 45 min (at room temperature) in Chrome Azurol S solution (50% purity) at final concentration of 8.3 mM in pH 4.0, washed thrice (15 min each) in acidic deionized water (pH 4.0), mounted between a glass slide and cover slip, and observed under a Leica DM 500 B light microscope coupled to a digital camera (Leica DFC295) (Bressan et al., 2016). Al was stained in purple, while phenolic compounds were stained in dark red.

4.5. Determination of the organic acids concentration in facultative and dependent Al-accumulating mistletoes

Powdered leaves (100 mg) were mixed with 5 mL of 0.025 N HCl, vortexed, centrifuged at 10000 rpm for 20 min at 4 °C and the supernatant was recovered. Then, it was passed through a Sep-pack Plus C18 cartridges previously activated with 1.4 mL MeOH, 0.7 mL Milli-Q H_2O and 1.4 mL HCl 0.025 N, and filtered using a Millex 0.22 μm directly into a glass HPLC vial (Tolrà et al., 1996).

Extracts were analysed by HPLC (LC-10 AT, Shimadzu Corporation) coupled with a C18 Atlantis™ column (4.6 × 150 mm, 5 μm). Analytical conditions were as follows: injection volume: 10 μL; flux: 0.5 mL min^{-1}; column T: 30 °C; mobile phase: NaH_2PO_4 (buffer solution) (pH 2.7); run time: 20 min; detection wavelength: 210 nm (Tolrà et al., 2005). Identification of the small organic acids in the samples was achieved by comparison of component retention times in standard solutions. To quantify the organic acids on the extracts, the integrated peak area of known concentration of the standards were used to build a calibration

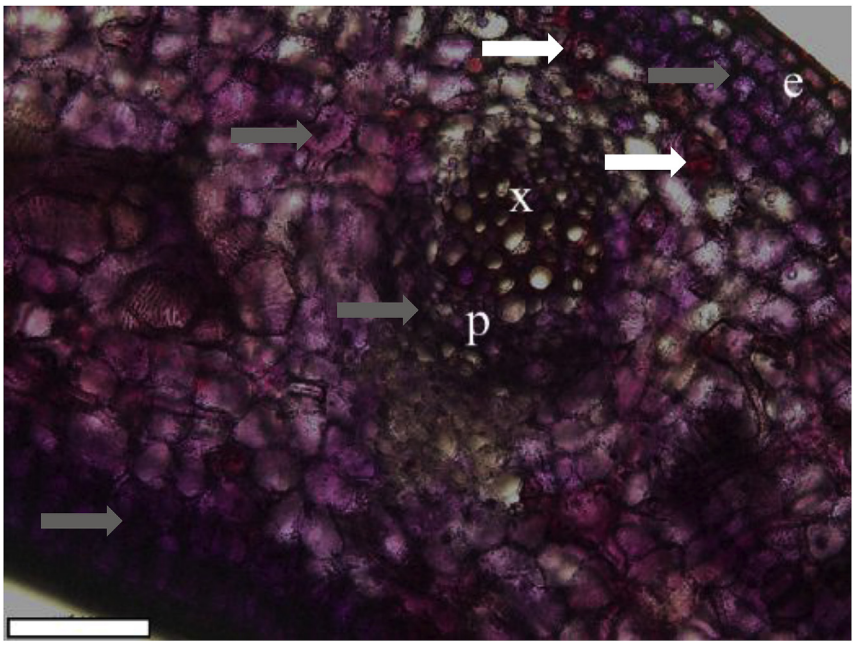

Fig. 4. Leaf section of *Psittacanthus robustus* (Al-dependent) parasitizing *Miconia albicans*. Aluminium presence is stained in purple (grey arrows) while polyphenols are stained in dark red (white arrows); x = xylem, p = phloem, e = epidermis. Scale bars: 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

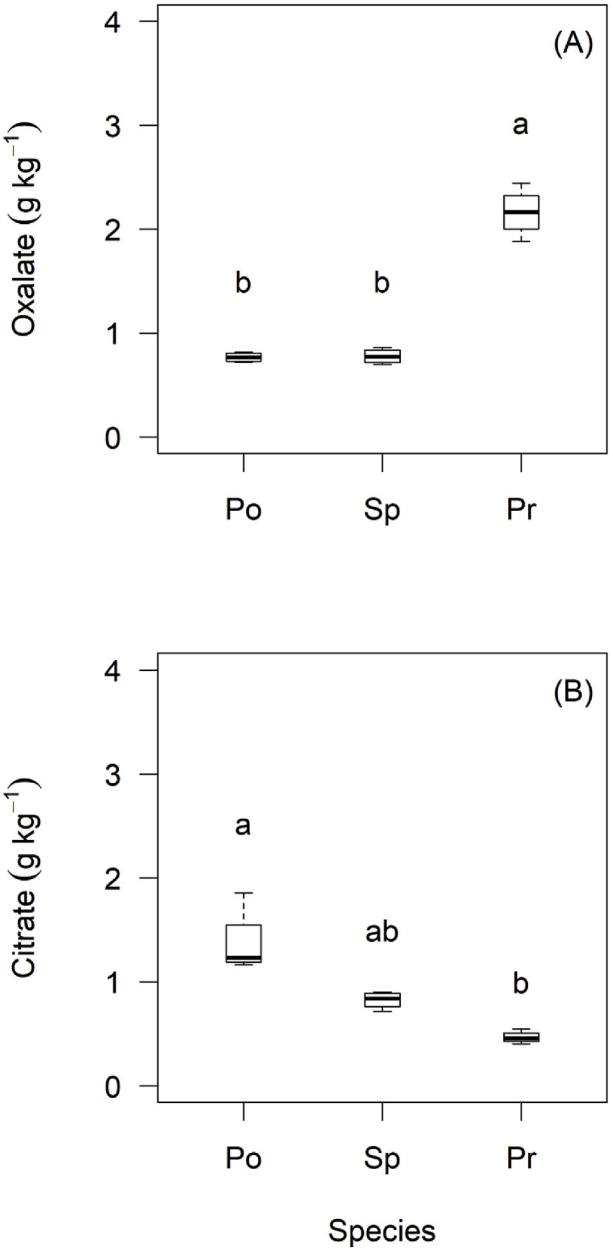

Fig. 5. Leaf organic acids concentration in (Po) *Passovia ovata*, (Sp) *Struthanthus polyanthus* (facultative Al-accumulating) and (Pr) *Psittacanthus robustus* (Al-dependent) parasitizing *Miconia albicans*. The box extends from the 25th to 75th percentiles, continuous line within the box shows the median, and error bars represent the 5th and 95th percentiles (n = 4).

curve.

4.6. Leaf phenolic compounds concentration in facultative and dependent Al-accumulating mistletoes

Leaves were dried at 50 °C for 96 h, ground in a mortar with pestle and 50 mg were extracted in a 2.5 mL methanol:water (7:3) solution, after 1 h in ultrasound bath. The crude extract was diluted 10 times, and 155 µL of distilled water, 20 µL of Folin-Denis reagent, 20 µL of Na_2CO_3 saturated at 35%, and 5 µL of the crude extract were deposited into each well of a 96-well microtiter plate (Biochrom EZ Read 2000). Absorbance values were measured at 760 nm using a microtiter plate reader, after 30 min of incubation in the dark at room temperature (Cortés-Rojas et al., 2012).

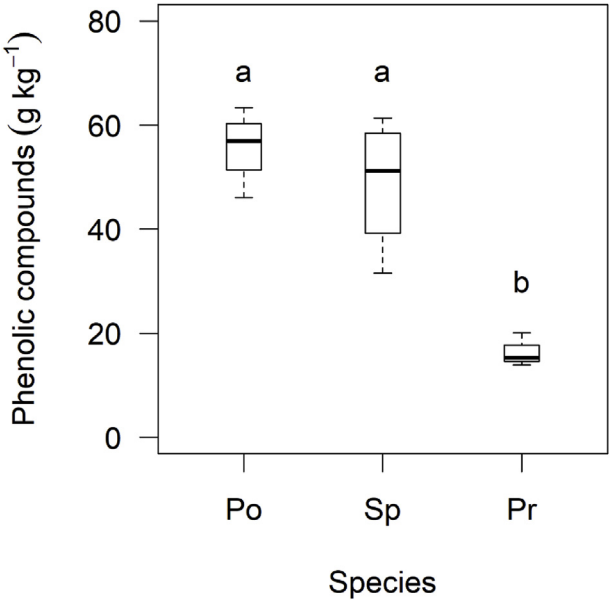

Fig. 6. Leaf concentrations of phenolic compounds in (Po) *Passovia ovata*, (Sp) *Struthanthus polyanthus* (facultative Al-accumulating) and (Pr) *Psittacanthus robustus* (Al-dependent) parasiting *Miconia albicans*. The box extends from the 25th to 75th percentiles, continuous line within the box shows the median, and error bars represent the 5th and 95th percentiles (n = 4).

4.7. Statistical procedures

Normality was tested with the Shapiro-Wilk test. Variations on leaf Ca concentrations, Al-accumulation, concentrations of organic acids and phenolic compounds were tested using the Dunn's test with the Bonferroni *p* value adjustment. Pearson correlations were used to assess bivariate relationships of Ca and Al levels between mistletoes and hosts (Zar, 2010). Statistical procedures were performed in R 3.3.3 (R Development Core Team, 2016), using the packages *dunn.test* (Dinno, 2017).

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