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Mechanisms of storage and detoxification of Al in two tropical mistletoes

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ABSTRACT

Passovia ovata (Pohl ex DC.) Kuijt and *Struthanthus polyanthus* Mart. are hemiparasitic mistletoes that can grow on Al-accumulating and Al-excluding woody species in the Brazilian Cerrado. It is unclear to what extent this hemiparasitic lifestyle implies facultative Al accumulation by the mistletoes and if so, what are the Al tolerance mechanisms in these species. Using a field-work experiment composed by two facultative Al-accumulating mistletoe species (*P. ovada* and *S. polyanthus*) infecting Al-accumulating (*Miconia albicans* (Sw.) Steud.) and Al-excluding (*Byrsonima verbascifolia* (L.) DC.) hosts, we (1) investigated Al accumulation and leaf nutritional status of the mistletoes, (2) characterized the storage sites and the forms of Al accumulated in their leaves, and (3) determined differences in levels of simple organic acids associated with Al detoxification. Leaf nutrients and Al accumulation in mistletoes followed the host elements availability. In both mistletoe species infecting the Al-accumulating host *M. albicans*, Al was mainly allocated to the phloem fibres, and Al-citrate was the main form of Al. Contrastingly, in the hosts' leaves Al was present mainly in the form of oxalate complexes. When growing on *B. verbascifolia*, the Al excluding host, the two mistletoes neither showed symptoms of Al storage nor formation of Al-organic acid complexes. In conclusion, mistletoes growing on Al-accumulating trees tolerate high Al tissue levels by allocating Al in phloem fibres and by its chelation with citrate.

1. Introduction

Aluminium, mainly in the Al^{3+} form that occurs in soils with low pH (< 4.5), is considered a phytotoxic element that injures sensitive species (mostly crops) by disrupting root growth and impairing water and nutrient uptake (Poschenrieder et al., 2008; Brunner and Sperisen, 2013). Low productivity is a well-known consequence of high Al availability in poorly adapted crop plants (Fageria and Nascente, 2014).

Different from sensitive species, Al tolerant plants have developed two strategies to detoxify Al. Generally, the Al-excluding species secrete organic acids via the root system to avoid Al uptake by roots, while the Al-accumulating species detoxify Al internally, chelating it with organic acids and storing this complex in vacuoles, epidermal cell walls, phloem tissue and chloroplasts (Kochian et al., 2004; Tolrà et al., 2011; Andrade et al., 2011; Bressan et al., 2016; Malta et al., 2016). Among the organic acids produced by plants, citrate, malate and oxalate are the predominant compounds for internal detoxification by Al-accumulating species (Brunner and Sperisen, 2013). Citrate is the most common organic acid used by plants to detoxify Al (e.g. *Hydrangea macrophylla* (Thunb.) Ser.) (Ma et al., 1997a), while oxalate is frequently used by calcifuge species to detoxify Al (e.g. *Melastoma malabathricum* L.) (Watanabe et al., 1998). Despite the literature about Al detoxification in plants, nothing is known about the mechanisms used by native Al-accumulating species from the Brazilian savanna (known as Cerrado) to detoxify Al internally.

Approximately, 35% of the Cerrado tree species are Al-accumulating (1000 > 15,000 mg Al kg^{-1} leaf dry mass, or 37 > 550 mmol Al kg^{-1} leaf dry mass) (Haridasan, 1982). Soils from Cerrado are acidic, pH < 5.0, rich in exchangeable Al and poor in nutrients. However, a few Cerrado sites have calcareous soils with pH > 6.0. At these sites soils have very low exchangeable Al and are richer in nutrients compared to the acid soils (Haridasan and Araújo, 1988). Despite the high abundance of species common in both acidic and calcareous soils, some Al-accumulating species, such as, *Miconia albicans* (Sw.) Steud. (Melastomataceae), *Vochysia thyrsoidea* Pohl., and *Vochysia tucanorum* Pohl. (Vochysiaceae) are endemic from acidic soils and may exhibit abnormal development with symptoms of mineral deficiencies (chlorotic and necrotic leaves) when growing under very low Al availability

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Table 1
Element levels in leaves of *Miconia albicans* (Al-accumulating host) and *Byrsonima verbascifolia* (Al-excluding host).

Leaf traits (mmol kg^{-1} dry mass)	*Byrsonima verbascifolia*	*Miconia albicans*	*P*
Aluminium	4.68 ± 1.51	280.76 ± 31.88	< 0.001
Nitrogen	688.26 ± 91.40	1182.82 ± 183.89	< 0.001
Phosphorus	13.98 ± 1.53	17.28 ± 3.06	0.02
Potassium	119.47 ± 17.19	75.81 ± 7.14	< 0.001
Calcium	199.10 ± 30.13	139.72 ± 8.86	< 0.001
Magnesium	75.19 ± 14.97	63.10 ± 4.68	0.05
Sulphur	18.73 ± 2.83	37.96 ± 3.02	< 0.001
Iron	1.68 ± 0.56	1.39 ± 0.14	0.18
Manganese	0.88 ± 0.16	2.05 ± 0.54	< 0.001
Boron	16.52 ± 0.77	15.24 ± 1.82	ns

Table 2
Element levels in leaves of *Passovia ovata* and *Struthanthus polyanthus* (mistletoes with facultative Al-accumulating behaviour) on *Miconia albicans* (Al-accumulating host) and in *Byrsonima verbascifolia* (Al-excluding host) species.

Leaf traits (mmol kg^{-1} dry mass)	*Passovia ovata* on			*Struthanthus polyanthus* on		
	M. albicans	*B. verbascifolia*	*P*	*M. albicans*	*B. verbascifolia*	*P*
Aluminium	413.77 ± 27.71	3.34 ± 0.44	< 0.001	359.92 ± 19.32	2.44 ± 0.30	< 0.001
Nitrogen	1473.77 ± 393.37	870.81 ± 195.56	< 0.05	1368.67 ± 314.72	863.13 ± 59.59	< 0.05
Phosphorus	25.60 ± 1.87	20.77 ± 1.69	< 0.01	27.09 ± 1.02	13.96 ± 1.09	< 0.001
Potassium	374.43 ± 23.75	621.91 ± 7.81	< 0.001	332.98 ± 15.86	506.89 ± 15.56	< 0.001
Calcium	96.67 ± 15.19	182.71 ± 10.62	< 0.001	128.96 ± 2.38	265.57 ± 13.21	< 0.001
Magnesium	78.56 ± 4.97	121.44 ± 4.51	< 0.001	86.54 ± 4.27	144.38 ± 5.36	< 0.001
Sulphur	47.49 ± 3.82	26.87 ± 2.05	< 0.001	53.81 ± 3.35	31.89 ± 0.51	< 0.001
Iron	1.29 ± 0.10	0.92 ± 0.16	0.01	1.04 ± ± 0.09	0.85 ± 0.04	0.02
Manganese	3.34 ± 0.27	1.47 ± 0.08	< 0.001	2.70 ± 0.23	1.11 ± 0.06	< 0.001
Boron	16.63 ± 2.43	16.36 ± 2.21	ns	16.00 ± 1.73	17.57 ± 3.50	ns

(calcareous soils) (Haridasan, 2008; Souza et al., 2017). These species were classified as Al-accumulating with a calcifuge behaviour (Haridasan, 2008; Souza et al., 2017).

A remarkable characteristic of Cerrado woody species is the high frequency of mistletoe bearing individuals, especially among Al-accumulating species from the *Vochysia* genus (Teodoro et al., 2010). Mistletoes are photosynthetically active hemiparasitic species worldwide distributed. These plants invade the xylem of branches and twigs of host trees and shrubs using the haustorium to acquire water and mineral nutrients essential to survive (Lamont and Southall, 1982). This semi-parasitic lifestyle may provide evolutionary advantages, especially in stressful environments, such as the Cerrado. In this habitat, low soil fertility, Al toxicity, and drought are main constraints for the performance, especially of plants with shallow root systems (Lopes and Guilherme, 2016). Among the mistletoes in Cerrado, the Loranthaceae species *Passovia ovata* (Pohl ex DC.) Kuijt (Scalon et al., 2013) and *Struthanthus polyanthus* Mart. (Arruda et al., 2006) have been found infesting both Al-accumulating (*Miconia* sp., *Qualea* sp., and *Vochysia* sp.) and Al-excluding (*Byrsonima* sp., *Pouteria* sp., and *Styrax* sp.) hosts (Lüttge et al., 1998, Arruda et al., 2006). These mistletoes can be classified as facultative Al-accumulating species.

Despite the convergent facultative Al-accumulating behaviour, these two mistletoe species have different lifestyle due to the variations of the haustoria structure, and epiphytic growth on the host's branches. *Passovia ovata* develops a single epicortical root with relatively few secondary haustoria connections, parasitizing a single host plant, while *S. polyanthus* shows sinuous growth of epicortical roots on stems, leaves and fruits of a single or multiple host plants (Calvin and Wilson, 2006; Souza MC and Scalon MC personal observations). These mistletoes represent a singular, highly interesting model to investigate the mechanisms of Al detoxification in vascular plants, since they are naturally found infesting both Al-accumulating and Al-excluding hosts growing side by side in the Cerrado under the same environmental filters (soil and climate) (Scalon et al., 2013). As far as we know (Lüttge et al., 1998; Arruda et al., 2006; Haridasan, 2008; Teodoro et al., 2010; Scalon et al., 2013), *P. ovata* and *S. polyanthus* are the only vascular plant species that exhibit a facultative Al-accumulating behaviour in Cerrado. However, to the best of our knowledge, the speciation of Al in the mistletoes, more specifically in facultative Al-accumulating mistletoes, infesting Al-accumulating hosts has not previously been investigated worldwide.

Using a field-work experiment, the aim here was to characterize mechanisms of Al tolerance in two facultative Al-accumulating mistletoes (*P. ovata* and *S. polyanthus*) of Cerrado infecting Al-accumulating and Al-excluding host species. We choose these species because they occur abundantly in the same site and parasiting the same host species. For this purpose, in this study, changes in the level of potential Al chelators and simple organic acids in mistletoes from both Al-accumulating and Al-excluding hosts were analysed for the first time, along with the corresponding ^{27}Al-NMR spectra and the histochemical localization of Al in the mistletoes. We hypothesized (i) that both facultative Al-accumulating mistletoes tolerate high Al tissue level by chelating with organic acids and allocating Al in phloem fibres. (ii) Due to the facultative Al-accumulating behaviour of both mistletoe species, we expect that Al will be chelate with citrate, while due to the calcifuge behaviour of *M. albicans* (Al-accumulating host) Al will be chelate with oxalate. (iii) We expect no signal of storage or detoxification of Al in mistletoes infecting *B. verbascifolia* (Al-excluding host).

2. Materials and methods

2.1. Site description

Leaf samples were collected in a Cerrado site at the Natural Reserve of the Roncador (RECOR/IBGE), located 35 km south of Brasilia in the Federal District of Brazil (15°56′S, 47°53′W, altitude 1000 m). The climate in this region is seasonal with mean annual rainfall of 1500 mm. Soils are nutrient limited, mainly P, Ca and Mg, and acidic (pH ~ 4.5) with high concentration of exchangeable Al (~8.5 mmol dm^{-3}) (Scalon et al., 2013).

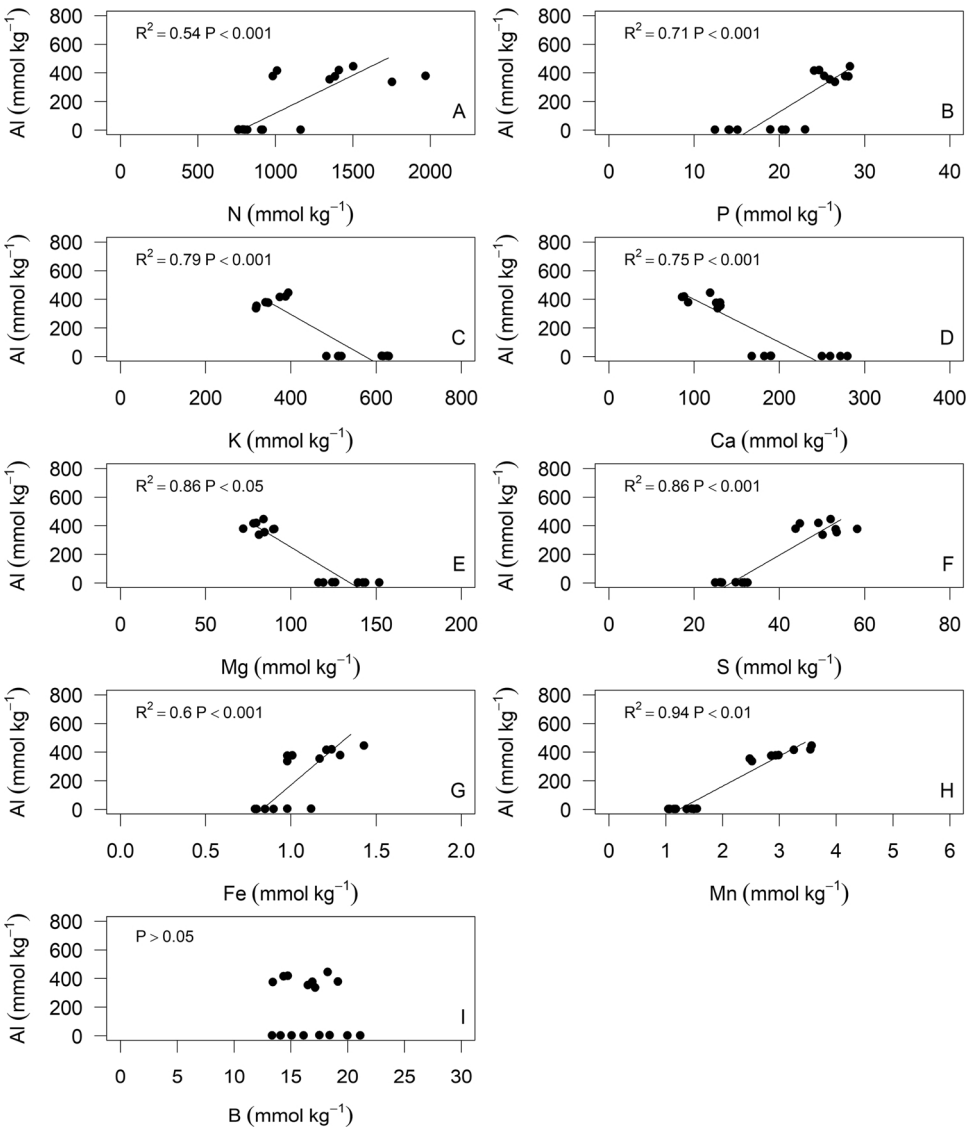

Fig. 1. Linear regressions between element levels in mistletoes leaves parasiting Al-accumulating and non-accumulating hosts.

2.2. Experimental design and plant samples

We used a field-work experiment to explore the response of two tropical mistletoes with a facultative Al-accumulating behaviour (*Passovia ovata* and *Struthanthus polyanthus*) to Al toxicity. The "treatments" of high and low Al availability to mistletoes were stablished based on the levels of Al in the leaves of *Miconia albicans* (Melastomataceae, Al-accumulating host species); and *Byrsonima verbascifolia* (Malpighiaceae, Al-excluding host species) (Scalon et al., 2013). Due to the strong correlation of leaf Al levels between mistletoes and hosts (Scalon et al., 2013), we considered the leaf Al concentration on the hosts as a proxy to available Al to mistletoes. Mature fully expanded sun leaves of eight individuals of *P. ovata* and eight individuals of *S. polyanthus* were sampled. For both species, four of them were parasiting an Al-accumulating species (*M. albicans*), and four parasiting an Al-excluding species (*B. verbascifolia*). Leaves of the hosts were also sampled.

2.3. Leaf nutrients and Al accumulation

Leaves were oven-dried at 60 °C for 72 h and ground in a Wiley mill. For P, K, Ca, Mg, S, Fe, Mn, B and Al determination, 0.1 g of the dried samples were overnight digested in a solution of 5 mL HNO_3 69% and 2 mL of H_2O_2 30% at 110 °C followed by determination using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Perkin Elmer – Elan 6000). Nitrogen was determined by the micro-Kjeldahl method (Allen, 1989).

2.4. Localization of Al in leaves

The leaves were fixed in buffered formaldehyde (Lillie, 1965), dehydrated in an ethanol series, and preserved in 70% ethanol (Johansen, 1940). Consecutively-sliced cross sections (1 cm^2) were made manually with a razor blade. Sections were immersed for 45 min in acidic distilled water (pH 4.0), stained for 45 min (at room temperature, *ca.* 25 °C) in Chrome Azurol S solution (50% purity, Sigma-Aldrich) at final concentration of 8.3 mM in pH 4.0, washed three times (15 min each) in acidic distilled water (pH 4.0), deposited on a glass slide, and observed in a photomicroscope (Leica DM 5000 B) coupled to a digital camera (Leica DFC295) (Bressan et al., 2016).

2.5. Forms of al on leaves using ^{27}Al-NMR

The qualitative forms of Al in the leaves of mistletoes and hosts were determined by ^{27}Al-NMR analyses using a solid state probe head under high resolution magic angle spinning (HR-MAS) conditions. This is the

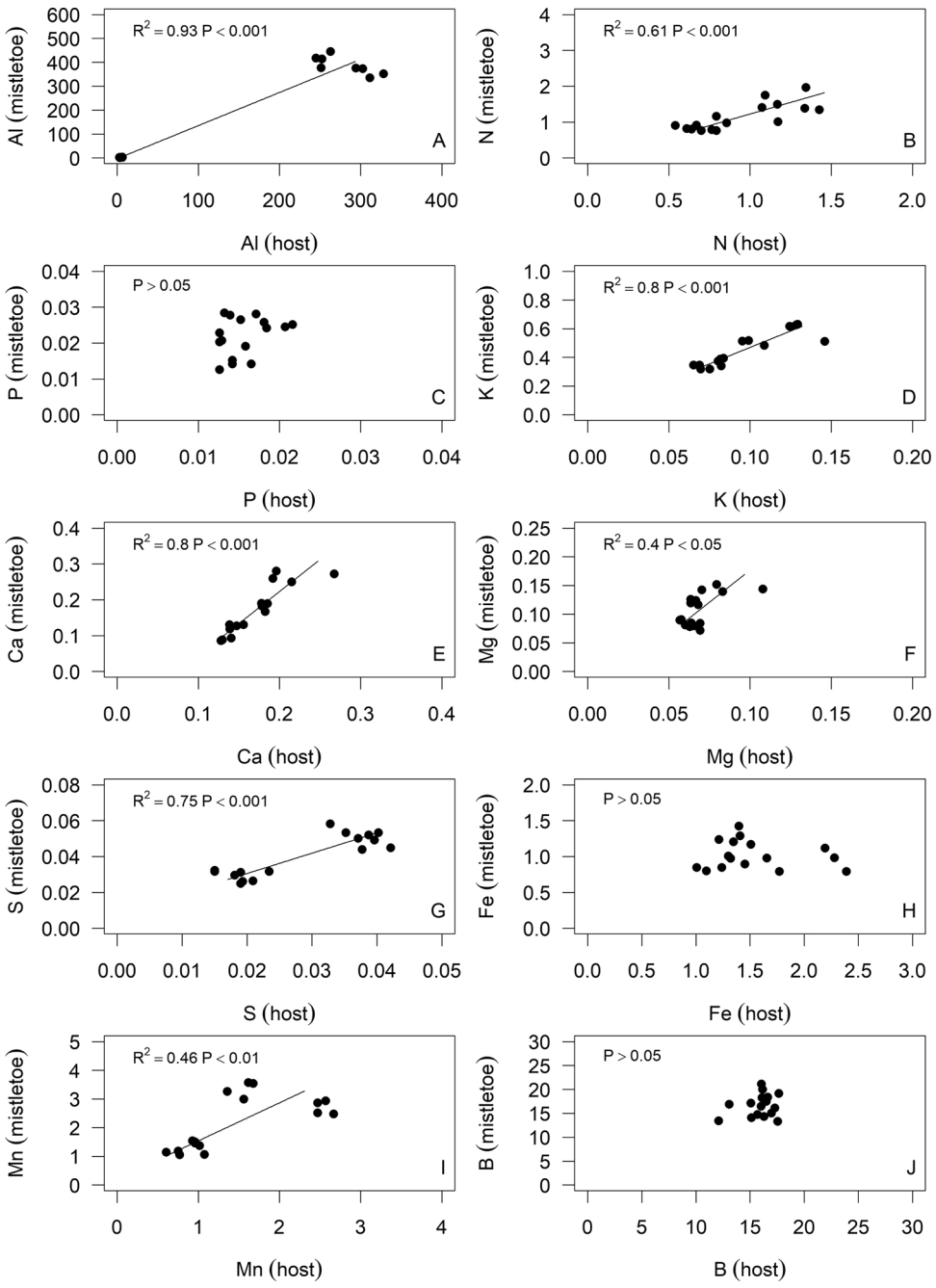

Fig. 2. Linear regressions of element levels between mistletoes and hosts. (Al, N, P, K, Ca, Mg, S, Fe, Mn and B are expressed in mmol kg^{-1} leaf dry mass).

first report using HR-MAS to detect forms of Al in tress and mistletoes from Cerrado.

Intact fresh mature leaves were cut in 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 1 s 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 the chemical shifts (ppm) available in the literature (Ma et al., 1997a; Morita et al., 2008 for references).

2.6. Extraction and analysis of simple organic acids

Immediately after sampling, leaves were wrapped into two layers of Al foil, stocked in a cooler box with dry ice, and stored in −80 °C until use. Frozen leaves were ground in porcelain mortar with pestle. Powdered leaves (100 mg) were mixed with 5 mL of 0.025 N HCl, vortexed, centrifuged at 10,000 rpm for 20 min at 4 °C and the supernatant was recovered. Then, it was passed through a Sep-pack Plus C18 cartridge (Waters) 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 (Millipore corp) 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 × 150.0 mm, 5.0 m, Waters Corporation). Analytical conditions were as follows: injection volume of

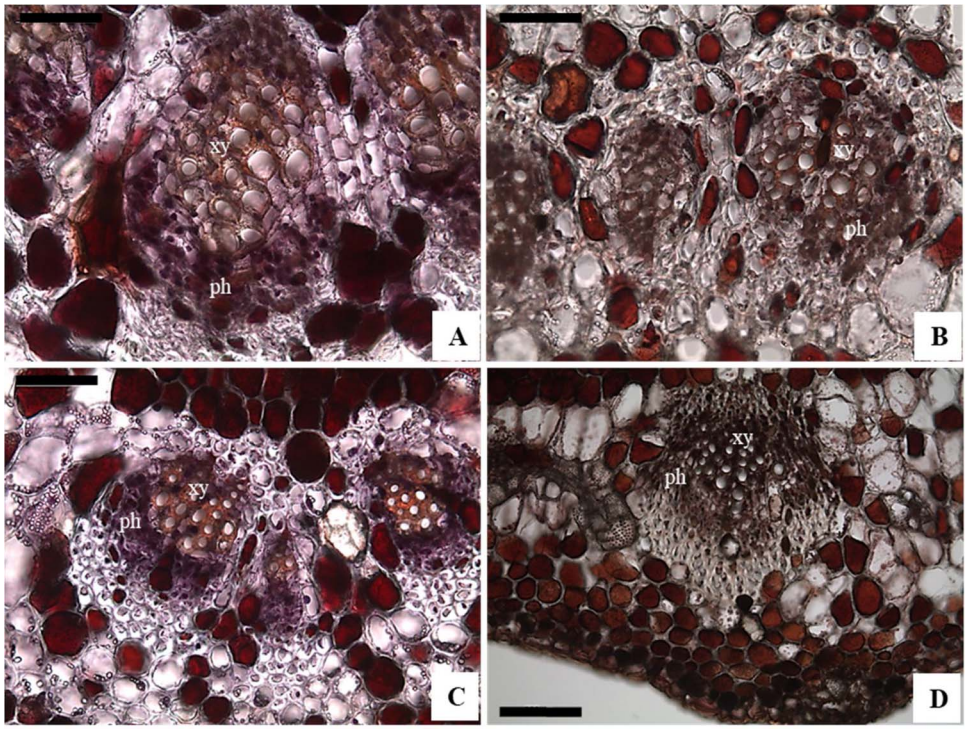

Fig. 3. Cross-section of leaf blade of **(A)** *Passovia ovata* on *Miconia albicans*. (**B**) *P. ovata* on *Byrsonima verbascifolia*. **(C)** *Struthanthus polyanthus* on *M. albicans* and **(D)** *S. polyanthus* on *B. verbascifolia* after staining with Chrome Azurol S. The presence of Al in the tissues is indicated by a purple colour, while dark red spots observed in all photomicrographs represent phenolic compounds. xy = xylem. ph = phloem. Scale bars: **A. B. C** = 100 µm; **D** = 200 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

10 µL; flow rate 0.5 mL min^{-1}; column T of 30 °C; mobile phase: NaH_2PO_4 (pH 2.7); run time of 20 min; detection wavelength at 210 nm (see Tolrà et al., 2005 for more details). 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 in the extracts, the integrate peak area of known concentrations of the standards were used to build a calibration curve.

2.7. Statistical procedures

Normality was tested with the Shapiro-Wilk test. When necessary, data were log_{10} transformed for normal distribution. A multivariate analysis of variance (MANOVA) was applied to check for differences on the nutritional status between the hosts, and to check for leaf traits differences (nutrients, Al accumulation and organic acids) between mistletoes infecting Al-accumulating and Al-excluding hosts. When MANOVA was significant, individual univariate ANOVAs were performed per species of hosts and mistletoes as *post hoc* test ($\alpha = 0.05$) to determine which response variable differed between plants. The host-to-parasite transfer factors (HPTFs) were obtained by dividing the mistletoe-leaf nutrient level by the host-leaf nutrient level (Llugany et al., 2009). We applied linear regressions to test (1) the effects of Al accumulation on the leaf nutrients level (N, P, K, Ca, S, Fe, Mn and B) in mistletoes; (2) the effects of host Al and nutrients availability on Al accumulation and nutritional status of mistletoes. Pearson correlation were applied to test (3) the correlation among simple organic acids; (4) correlation of organic acids between mistletoes and hosts; and (5) the relationship between simple organic acids and the levels of leaf Al and Ca in mistletoes. Statistical procedures (MANOVA, ANOVA, Linear regressions and Pearson correlation) were performed in R 3.3.2 (R Core Team, 2016).

3. Results

As expected, host species differed in terms of Al accumulation and leaf nutritional status (MANOVA $\lambda = 0.00098898$, $F = 505.07$, $P < 0.001$). Leaf levels of Al, N, P, S, and Mn were, respectively 98, 42, 19, 51, and 57% higher in leaves of *M. albicans* (Al-accumulating), while K, Ca, and Mg were 37, 30, and 16% higher in leaves of *B. verbascifolia* (Al-excluding). Iron (Fe) and B did not differ between the hosts (Table 1).

These host nutritional status differences were reflected in the leaf Al and nutrient levels of the associated mistletoe species (MANOVA $\lambda = 0.0009915$, $F = 503.79$, $P < 0.001$). In both *P. ovata* and *S. polyanthus* the levels of Al were 99% higher when infesting Al-accumulating hosts than in the same species on the Al-excluding host (Table 2). In *P. ovata*, leaf levels of N, P, S, Fe, and Mn were, respectively, 73, 19, 43, 23, and 56% higher when growing on the Al-accumulating host. Leaf N, P, S, Fe, and Mn levels were 37, 48, 41, 18, and 59% higher in *S. polyanthus* plants parasitizing the Al-accumulating species than on the Al-excluding. The contrary holds true for leaf levels of K, Ca and Mg. Leaf levels of these nutrients were higher in individuals of *P. ovata* (40, 47, and 35%) and *S. polyanthus* (34, 51, and 40%) parasitizing the Al-excluding host. Among all analysed nutrients, only boron levels did not differ between the mistletoes parasiting Al-accumulating and Al-excluding hosts (Table 2).

Potassium was preferentially translocated to the mistletoes, as indicated by a parasite/host molar ratio (host parasite transfer factor HPTF) of more than 4, while the molar ratios for other nutrients were close to 1, with some noteworthy exceptions. Transfer of Al and Fe from *B. verbascifolia* to the mistletoes was inhibited (HPTTF < 1), regardless the parasitizing species. *Passovia ovata* attained the highest Al transfer on *M. albicans*, in combination with the lowest Ca and Mg transfers. The Al-accumulating *M. albicans* transferred proportionally more P to either of the mistletoe species (HPTTF > 1.8) than the Al-excluding *B. verbascifolia* (HPTTF < 1.2).

Analysing together all mistletoe nutrient data, we observed a positive correlation between Al accumulation and the leaf levels of N, P, S, Fe and Mn, but a negative correlation with K, Ca and Mg (Fig. 1). No significant interaction between B and Al accumulation was observed. Considering that nutrient levels in mistletoes leaves are directly linked to the host nutrient availability, we correlated mistletoe and host Al accumulation and leaf nutrient levels. A statistically significant positive correlation for Al, N, K, Ca, Mg, S and Mn was found, while no significant correlations were observed for P, Fe and B (Fig. 2).

Histochemical analysis of Chrome Azurol S stained mistletoe leaves

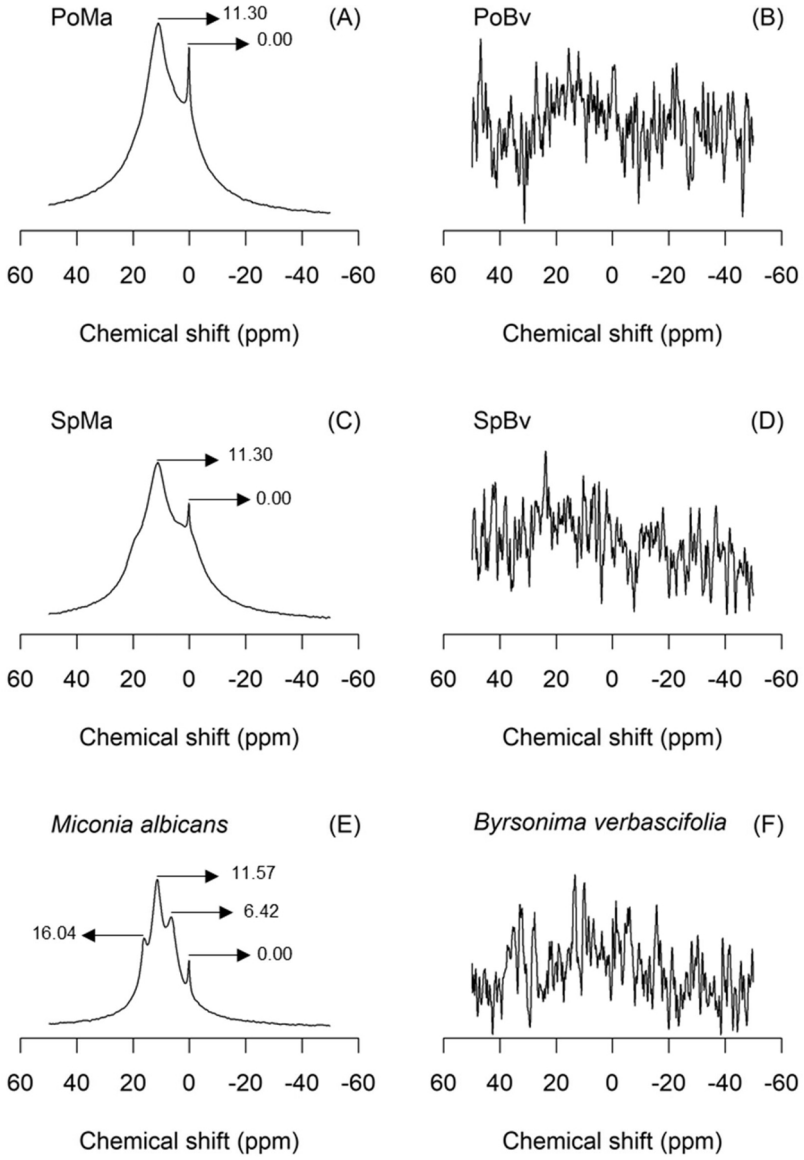

Fig. 4. ^{27}Al-NMR spectra of fresh leaves (a) *Passovia ovata* on *Miconia albicans* (PoMA), (b) *P. ovata* on *Byrsonima verbascifolia* (PoBv), (c) *Struthanthus polyanthus* on *M. albicans* (SpMa), (d) *S. polyanthus* on *B. verbascifolia* (SpBv), (e) *M. albicans* and (f) *B. verbascifolia*. (Free Al^{3+} (0.0 ppm), Al-oxalate(1) (6.42 ppm), Al-oxalate(2) (11.57 ppm), Al-oxalate(3) (16.04 ppm) and Al-citrate (11.30 ppm) complexes. No signals of Al^{3+} or Al-complexes were observed for PoBv, SpBv and *B. verbascifolia*.

from plants on the Al-accumulating host revealed purple colour stain on phloematic fibres indicating Al accumulation (Fig. 3A,C). Contrastingly, leaf samples from plants growing on the Al-excluding species did not stain (Fig. 3B,D). ^{27}Al-NMR did not yield spectra for the Al-excluding host (Fig. 4F). On the other hand, ^{27}Al-NMR from the Al accumulating *M. albicans* revealed the free form of Al^{3+} (0.0 ppm), and 3 major forms of Al in the leaves of this host tree: Al-oxalate(1) (6.42 ppm), Al-oxalate (2) (11.57 ppm) and Al-oxalate(3) (16.04 ppm) (Fig. 4E). The free form of Al^{3+} (0.0 ppm) and a major form of Al-citrate (11.30 ppm) were detected in both mistletoe species when growing on the Al-accumulating *M. albicans* (Fig. 4A,C). No signals of Al were observed for mistletoes infecting the Al-excluding species (Fig. 4B,D).

Malate, citrate, and oxalate were the main simple organic acids detected in the mistletoes. For both mistletoe species the levels of organic acids differed significantly between mistletoes infecting Al-accumulating and Al-excluding hosts (MANOVA $\lambda = 0.50889$, $F = 3.86$, $P < 0.05$). Citrate levels were 45% higher in *P. ovata* and 58% higher in *S. polyanthus* individuals infecting the Al-accumulating species (*M. albicans*) (Fig. 5A). On the contrary, the level of malate was 13% higher in plants of *P. ovata* and 46% higher in plants of *S. polyanthus* infecting the Al-excluding host (Fig. 5B). The level of oxalate was 50% higher in *P. ovata* and 13% higher in *S. polyanthus* infecting the Al-excluding species (Fig. 5C).

Considering only the mistletoes, we observed a positive correlation between malate and oxalate ($r = 0.55$, $P < 0.05$) and a negative correlation between malate and citrate ($r = -0.77$, $P < 0.001$). There was no significant correlation between citrate and oxalate ($P > 0.05$). We also found that citrate was positively correlated with Al ($r = 0.74$, $P = 0.001$) but negatively correlated with Ca ($r = -0.87$, $P < 0.001$), while malate correlated negatively with Al ($r = -0.57$, $P = 0.02$) but positively with Ca ($r = 0.88$, $P < 0.001$). No correlations were found between oxalate with either Al or Ca.

The correlation of leaf organic acids between mistletoes and hosts, were positive for malate ($r = 0.57$, $P = 0.02$) and citrate ($r = 0.81$, $P < 0.001$). No correlation was observed for oxalate ($P > 0.05$).

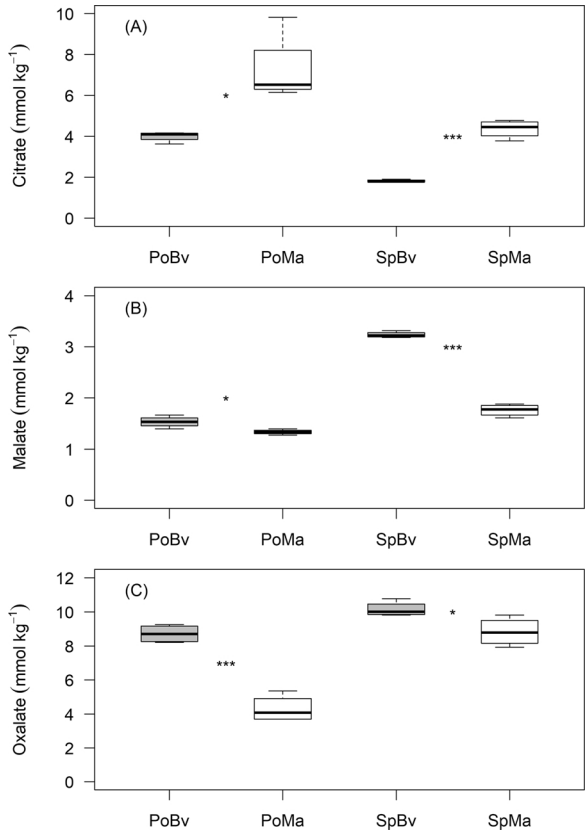

Fig. 5. Levels of small organic acid on leaves (mmol kg^{-1} dry mass) of (PoMA) *Passovia ovata* in *Miconia albicans*; (PoBv) *P. ovata* in *Byrsonima verbascifolia*; (SpMa) *Struthanthus polyanthus* in *M. albicans*; and (SpBv) *S. polyanthus* on *B. verbascifolia*. Hemiparasites with a facultative-Al-accumulating behaviour = *P. ovata* and *S. polyanthus*. Hosts = *M. albicans* (Al-accumulating species) and *S. polyanthus* (non-accumulating species).

4. Discussion

4.1. Leaf nutrients and Al accumulation

As expected, Al-accumulating and Al-excluding host species not only delivered different levels of Al to the mistletoes, but also provided different levels of essential nutrients. Contrasting nutrient availability reflected in different leaf nutrient concentrations has also been showed by Haridasan (1988) and by Souza et al. (2017) when studying the performance of *M. albicans* and *V. tucanorum* in soils with high and low exchangeable Al^{3+} in Cerrado, and by Andrade et al. (2011) when investigating variations of Al storage in Vochysiaceae species from Cerrado, Gallery forest and Semideciduous forest in Brazil. This is a common situation in field-work experiments, because both sap and soils are complex matrices being difficult to stablish a contrast of a single element without human manipulation. In addition, the levels of essential nutrients in *P. ovata* and *S. polyanthus* infecting the Al-accumulating host is in accordance with the level of nutrients reported for most Al-accumulating and Al-excluding tree species from Cerrado (Haridasan and Araújo, 1988).

Mistletoes rely on nutrient supply through the xylem from the host. Therefore, mistletoes' nutrient relationships reflect the element levels of their hosts (Glatzel and Geils, 2009; Gebauer et al., 2012; Scalon and Wright, 2015). The significant correlations between mistletoes and hosts mineral nutrients confirm this view, except for P, Fe, and B. Host to parasite transfer factors (HPTF) are excellent indicators for the availability of host nutrients to xylem tapping hemiparasitic plants (Llugany et al., 2009). Values lower than unity indicate restriction of transfer, as expectedly observed for Al in mistletoes on the excluder tree *B. verbascifolia*. However, HPTF values far below unity were also observed for Fe in both tree species and for Ca in *P. ovata* on *M. albicans*. Low host-parasite transfer of Fe, a poorly mobile element, seems to be a common characteristic for xylem tapping hemiparasitic plants (Llugany et al., 2009; Mutlu et al., 2016) and apparently mistletoes have high Fe use efficiency, for yet unknown reasons.

Both mistletoes *P. ovata* and *S. polyanthus* were able to accumulate higher P levels when growing on the Al-accumulating *M. albicans* than on the Al-excluding *B. verbascifolia.* This may indicate either or both higher xylem P transport in the accumulator, albeit the high xylem Al translocation in this accumulating host and a more efficient resorption process of P from senescing leaves when infesting *M. albicans* (Scalon et al., 2017). In fact, leaves of *P. ovata* displayed almost the same Al/P molar ratio (16.2) as *M. albicans* (16.1), while *S. polyantus* with an Al/P molar ratio of 13.3 was even more efficient in P acquisition from this host. Not only P, but also N, S, Fe and Mn leaf levels of the mistletoes were positively correlated to their leaf Al accumulation. This result further supports the view that Al does not interfere with the transport of these nutrients in these highly Al tolerant Cerrado species.

Contrastingly, Al accumulation in the mistletoes apparently reduced translocation of Ca, Mg and K from host to the parasite as suggested by both the negative correlation coefficients and the lower HPTF values for *M. albicans* than for *B. verbascifolia*. However, considering that the levels of Ca and Mg were significantly lower in the Al-accumulating host than in the Al-excluding host, we cannot assert whether the high Al leaf levels in the mistletoes infecting the Al-accumulating host affected the absorption of Ca and Mg from hosts. Nonetheless, K levels in mistletoe leaves were 4 to 5 times higher than in the hosts. This preferential accumulation of K is a common feature of hemiparasitic plants tapping the host xylem. Mobile elements like K, that are recycled between xylem and phloem, are trapped in the mistletoe because of the absence of phloem retranslocation in this hemiparasite-host system (Lo Gullo et al., 2012). Lower HPTFs values, but still clearly above unity were also observed for other phloem mobile nutrients, such as S, N, P, Mg, and Mn (Rengel, 1999).

4.2. Localization of Al on leaves

Chrome azurol S stained samples of *P. ovata* and *S. polyanthus* growing on *M. albicans* revealed purple staining of the phloem tissue. This distribution is similar to what was earlier reported for the host tree *M. albicans* and also for other Al-accumulating shrubs and trees from the Cerrado (*Miconia ferruginata* DC., *Miconia pohliana* Cogn., *Palicourea rigida* Kunth, *Qualea grandiflora* Mart., *Qualea parviflora* Mart., *Qualea multiflora* Mart., *Vochysia elliptica* Mart., *Vochysia tyrsoidea* Pohl. and *Vochysia rufa* (Spr.) Mart) (Haridasan et al., 1986; Bressan et al., 2016).

Compartmentalization of Al in the cell wall is an important mechanism used by a number of species (e.g., *Camellia sinensis* (L.) Kuntze) to reduce the cytotoxic effects of Al (Taylor et al., 2000; Horst et al., 2010; Tolrà et al., 2011; Kopittke et al., 2015). Some widely used stains for Al detection in plant tissues are apparently unable to visualize Al bound to the pectin fraction of cell walls (Eticha et al., 2005). This is not the case for the Chrome azurol S dye used here. This Al-specific dye clearly stains pectin–bound Al with a purple colour (Wehr et al., 2010), such as revealed here in the phloem tissue of the mistletoes.

4.3. Forms of Al on leaves and organic acids

Despite intrinsic differences in the number of epicortical roots and growth forms over the hosts, both mistletoe species showed the same form of Al in their leaves (Al-citrate complex). The presence of Al in the form of citrate in both mistletoe species contrasts with the oxalate complexes used by the host *M. albicans* to detoxify this element [Al-oxalate(1), Al-oxalate(2) and Al-oxalate(3)]. The different forms of complexed Al identified by the ^{27}Al-NMR analysis suggests that facultative Al-accumulating (*P. ovata* and *S. polyanthus*) and obliged Al-accumulating species (*M. albicans*) used different strategies to deal with

Al-toxicity. These observations may indicate a convergent mechanism to detoxify Al among the facultative Al-accumulating mistletoes from the Loranthaceae, while the obligate Al-accumulating hosts have developed a different mechanism. Therefore, it is likely that Loranthaceae mistletoes and the Melastomataceae host evolved independently in relation to their strategy to deal with Al phytotoxicity.

A number of studies have reported the use of simple organic acids to detoxify Al (Nagata et al., 1991; Ma et al., 1997a,b; Watanabe et al., 1998; Brunner and Sperisen, 2013). Our observation that *M. albicans* uses Al-oxalate (1:1, 1:2, 1:3) complexes to detoxify Al is in line with previous findings in other Melastomataceae species (*M. malabathricum*) (Watanabe et al., 1998).

However, this is the first report characterizing unambiguously the forms of Al in mistletoes and woody species from Cerrado, and also connecting the forms of Al with the histochemical Al storage in their leaves. Both *P. ovata* and *S. polyanthus* exhibited similar patterns of the production of citrate (higher in high Al concentration), oxalate and malate (both were lower in high Al concentration). These observations combined with the identification of the Al-citrate complex by ^{27}Al-NMR confirm that Al was bound by citrate in these two facultative Al-accumulating species (Ma et al., 1997a; Morita et al., 2008).

The use of Al-citrate complex to detoxify Al in plants has previously been observed in leaves of *H. macrophylla* (Ma et al., 1997a) and *Fagopyrum esculentum* Moench. (Shen et al., 2004). Moreover, in *F. esculentum* an Al-oxalate (1:3) complex seems also involved in Al tolerance (Ma et al., 1997b). The observation of two strategies to bind Al in leaves of *F. esculentum* was attributed to the significant differences in the leaf Al accumulation between plants from both studies, being the Al-citrate complex observed in plants with higher levels of Al (Shen et al., 2004).

Among the small organic acids-Al complex, Al-citrate complex is characterized by a broad spectrum (Shen et al., 2004), being frequently observed in Al-excluding and in Al-accumulating species (Brunner and Sperisen, 2013), while Al-oxalate complex is characterized by a sharp spectrum (Ma et al., 1997b) being frequently observed in calcifuge species (Lee, 1999; Brunner and Sperisen, 2013). There is not a clear explanation about the specificity of the organic acids used by each species to detoxify Al. The most probable explanation for the broad spectrum of species using citrate is the fact that this organic acid is a tricarboxylic anion while oxalate is a dicarboxylic anion. Thus, citrate forms a more stable complex with Al^{3+} than oxalate (Lee, 1999; Brunner and Sperisen, 2013). For calcifuge species, oxalate accumulation can be advantageous because it not only binds Al but also reduce the Ca^{2+} toxicity (Lee, 1999).

Analysing the stoichiometry between the levels of organic acids and Al in leaves of mistletoes and hosts, we observed an insufficient amount of citrate in the two mistletoe species, and also and insufficient amount of oxalate in *M. albicans* (Al-accumulating host) to chelate 100% of the accumulated Al in these species. These observations are in accordance with the peaks of free Al^{3+} (0.0 ppm) observed on the ^{27}Al-NMR spectra. The presence of Al in the phloematic fibres suggests that this element can be translocated to other organs, such as seeds, as observed in species from the Loranthaceae (*Psittacanthus robustus* (Mart.) Marloth), Melastomataceae (*M. albicans* and *M. pohliana*), Rubiaceae (*P. rigida*), and Vochysiaceae (*Q. parviflora*, *Q. multiflora*, *Salvertia convallariodora* St. Hil., *V. rufa*, *V. thyrsoidea* and *V. tucanorum*) families (Haridasan, 2008; Scalon et al., 2013; Bressan et al., 2016). However, the biological function of Al in the seeds of Al-accumulating plants is still unknown.

We also observed that individuals of *M. albicans* and *B. verbascifolia* infected by *P. ovata* exhibited higher levels of citrate in leaves (0.41 ± 0.05 and 0.87 ± 0.07 mmol kg^{-1} leaf dry mass, respectively) in relation to the same plant species infected by *S. polyanthus* (< 0.001 mmol kg^{-1}). To what extent the parasitic species influences the carbon metabolism and, in consequence, the Al detoxification ability of the hosts, still remains to be established.

5. Conclusions

The levels of essential and non-essential elements in the two facultative Al-accumulating mistletoe species reflected the nutritional status of host species, regardless the level of available Al on the hosts. Despite the high accumulation of Al and the low levels of Ca and Mg in leaves of the hemiparasitic plants *P. ovata* and *S. polyanthus*, we cannot assert that Al interfered with nutrient uptake. However, high levels of Al increased the amount of citrate in leaves of mistletoes infecting Al-accumulating hosts. The use of ^{27}Al-NMR confirmed that Al was detoxified by citrate (Al-citrate complex) and the histochemical analysis suggested that Al is stored in phloematic fibres in these species. We also observed that both mistletoe species converged to a similar mechanism to deal with the Al-toxicity, and this mechanism differed from that used by the Al-accumulating host.

Author contributions

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