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Fingerprinting metabolomics in tropical mistletoes: A case study with facultative aluminum-accumulating species

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ABSTRACT

Aluminum (Al) toxicity is a hot topic due to the high sensitivity of cultivated plants. Despite the large investment in the last century in understanding the mechanisms used by sensitive and Al-excluding species to avoid Al uptake from soil, little attention has been devoted to understanding the mechanisms used by native Al-accumulating species to deal with Al-toxicity. *Passovia ovata* (Pohl ex DC.) Kuijt and *Struthanthus polyanthus* Mart. are mistletoes with a facultative Al-accumulating behavior. In the Brazilian Cerrado they are commonly found infecting Al-accumulating (*Miconia albicans* (Sw.) Steud.) and non-accumulating (*Byrsonima verbascifolia* (L.) DC.) species. Taking into account the importance of organic complexes in the detoxification of the highly toxic Al^{3+} ions, it is to be expected that mistletoes differ in their metabolomic profile when feeding on species differing in Al accumulation. Here we tested this hypothesis using an untargeted LC–MS approach to investigate the influence of Al on the metabolome of *P. ovata* and *S. polyanthus* infecting *M. albicans* and *B. verbascifolia* under field conditions. We observed differences in the metabolic profiles between mistletoes growing on Al-accumulating and Al-excluding hosts, and also observed a positive correlation between Al leaf-accumulation and the metabolic profile. Using the OPLS-DA, we identified quinic acid (phenolic compound) as a metabolic biomarker distinguishing mistletoes grown under high and low Al availability.

1. Introduction

Aluminum toxicity is an important stress factor for many plants worldwide. In acidic soils (pH < 4.5), the toxic form of Al^{3+} can cause injury to the root system of sensitive plants, reducing water and nutrient uptake (Brunner and Sperisen, 2013). In cultivated plants, Al-toxicity results in low yield (Fageria and Nascente, 2014).

In contrast to Al-sensitive species, the Al-tolerant (Al-excluding and Al-accumulating) species developed mechanisms to deal with Al-toxicity. Al-excluding species release organic acids and phenolic compounds from their roots in order to avoid uptake of Al from the soil (external detoxification) (e.g. *Urochloa decumbens* Stapf – Poaceae) (Arroyave et al., 2018). However, impairment of lateral root development, reduced water uptake, and reduced gas exchange rates were reported in *Styrax camporum* Pohl. (Styracaceae) (Al-excluding species native to acidic soils) grown in a nutrient solution with high Al availability (> 1400 µM Al) (Banhos et al., 2016).

On the other hand, Al-accumulating trees (e.g. *Camellia sinensis* (L.) Kuntze, *Fagopyrum esculentum* Moench, *Hydrangea macrophylla* (Thunb.) Ser., and *Melastoma malabathricum* L.), and mistletoe species (*Passovia ovata* (Pohl ex DC.) Kuijt, and *Struthanthus polyanthus* Mart.) take up high Al concentrations from soils and host xylem sap, respectively. In these species internal detoxification is based on efficient chelation of Al by organic acids (e.g. citrate, malate and oxalate) and phenolic compounds (e.g. catechin), in combination with compartmentation mechanisms (vacuoles and cell walls) and high levels of antioxidant defense (Nagata et al., 1992; Kochian et al., 2004; Foyer and Noctor, 2005; Souza et al., 2018). The biochemical and molecular basis of the mechanisms related to Al-detoxification have been intensively studied in several cultivated species and in the model plant *Arabidopsis thaliana* (L.) Heynh. (Brunner and Sperisen, 2013). Contrastingly, the chemistry of Al and its relationship with secondary metabolites in native Al-accumulating species is still poorly understood.

Due to the scarce genomic information available for native Al-

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accumulating species from the Brazilian Cerrado, untargeted analyses of metabolism can be useful in investigations of the effects of aluminum. Fingerprinting metabolomics using high resolution mass spectrometry coupled with liquid chromatography (LC–MS) is an emerging technique used to detect multiple metabolites in a single run without previous identification. Recently, this technique was successfully used to elucidate the calcifuge behavior of *Vochysia tucanorum* Mart., an Al-accumulating species from the Cerrado (Souza et al., 2017). Similar approaches were also applied to understand soil-climate effects on the metabolome of *Tithonia diversifolia* (Hemsl.) A. Gray (Sampaio et al., 2016), and to investigate biogeographical effects on the metabolic evolution of the genus *Espeletia* (Padilla-González et al., 2017).

The Cerrado comprises the richest flora among savannas and, due to the high number of endemic species, it is considered one of the 25 hotspots sites for conservation (Myers et al., 2000). A remarkable characteristic of the Cerrado vegetation is the presence of mistletoes growing on shrubs and trees (Lüttge et al., 1998, Arruda et al., 2006). These hemiparasites take up water, nutrients, and metals from the host's xylem sap using a modified root system called a haustorium (Scalon et al., 2013 for references). Despite the fact that mistletoes photosynthesize, they are considered pests in agricultural settings worldwide since a high incidence of mistletoes can cause damage to the hosts (Arruda et al., 2006 for references).

The Loranthaceae family comprises approximately 950 species, occurring mainly in tropical regions (Vidal-Russell and Nickrent, 2007). *Passovia ovata* (Pohl ex DC.) Kuijt (Scalon et al., 2013) and *Struthanthus polyanthus* Mart. (Arruda et al., 2006) (Lorantaceae) have been found infesting both Al-accumulating and non-accumulating hosts in the Cerrado (Lüttge et al., 1998, Arruda et al., 2006). Due to this behavior, these species can be classified as facultative Al-accumulators (Scalon et al., 2013; Souza et al., 2018). Since they are naturally found infesting both Al-accumulating and Al-excluding hosts growing side by side under the same environmental filters (soil and climate) in the Cerrado (Scalon et al., 2013; Souza et al., 2018), these species represent an interesting model for the investigation of the effects of high and low Al availability on the metabolome of vascular plants under field conditions.

Considering the importance of organic complexes in the detoxification of the highly toxic Al^{3+} ions in mistletoes (Souza et al., 2018), it is to be expected that mistletoes differ in their metabolomic profile when feeding on species differing in Al accumulation. Here we tested the effects of Al-accumulation on multiple metabolites in mistletoes with a facultative Al-accumulating behavior. This was carried out using an LC–MS approach to identify one or more indicator metabolites (biomarkers) associated with Al-accumulation. Based on Souza et al. (2017), we expected to detect a phenolic compound as metabolic biomarker for Al-accumulation in mistletoes.

2. Material and methods

2.1. Plant samples and site description

Mature leaf samples of four plants of *Passovia ovata* and four plants of *Struthanthus polyanthus* were collected from eight plants of *Miconia albicans* (Al-accumulating host), and from eight plants of *Byrsonima verbascifolia* (Al-excluding host) species at a Cerrado site in the Natural Reserve of the Roncador (RECOR/IBGE), Federal District of Brazil (15°56′S, 47°53′W) (Fig. 1). Leaves of mistletoes and hosts were sampled during the late dry season of 2015. The climate in this region is seasonal with a mean annual rainfall of 1500 mm. Soils are nutrient limited, mainly in P, Ca and Mg, and acidic (pH ~ 4.5) with high a concentration of exchangeable Al (~8.5 mmol dm^{-3}) (Scalon et al., 2013).

2.2. Leaf Al determination

Leaves were oven-dried at 60 °C for 72 h then ground in a Wiley mill. The samples were digested overnight in a solution of HNO_3 69% and H_2O_2 30% at 110 °C followed by determination of Al levels using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Perkin Elmer – Elan 6000).

2.3. Fingerprinting metabolomics approach

Immediately after sampling, leaves of mistletoes were stocked in a cooler box with dry ice, transported to the lab, and stored in a deep freezer (−80 °C) until use. Frozen leaves were ground in porcelain mortar and pestle. Powdered leaves were extracted in 80% ethanol (HPLC grade) using an ultrasonic bath and centrifuged at 14,000*g*. The clean-up was done using 95% *n*-hexane. The hydroalcoholic fraction was collected and dried using a vacuum concentrator. The extracts were diluted in acetonitrile:water (1:1) + 0.05% of formic acid and filtered through a 0.22 µm PTFE membrane (Millipore). The samples were injected into a UHPLC-DAD-(ESI)-HRMS system equipped with an Orbitrap mass spectrometer (Thermo Scientific), using a Kinetex xb-C18 column (Phenomenex) and a gradient elution method. Eluent "A" was ultrapure water with 0.05% of formic acid (v:v) and eluent "B" was acetonitrile (LC–MS grade) containing 0.05% of formic acid (v:v). The injection volume was 4 µL. For the mass data acquisition, a full scan in the positive and negative ionization modes was performed using spray voltage at 3.6 and 3.2 kV, heater temperature of 300 °C, capillary temperature at 320 °C, sheath gas at 30 and aux gas 11. Methods are detailed in Souza et al. (2017).

2.4. Data treatment and statistical procedures

Shapiro Wilk test was applied to test normality. A *t*-test was applied to test variations in leaf Al concentration in hosts and mistletoes. Peak area of the chromatograms in the negative ionization mode, were deconvoluted and aligned using MZmine 2.21 (Sampaio et al., 2016). LC–MS data were transformed using Pareto transformation (van den Berg et al., 2006) and the matrices were evaluated by Principal Component Analysis (PCA). Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used to identify the discriminant compounds between plants infecting Al-accumulating and Al-excluding species (Sampaio et al., 2016).

For the putative compound-identification of the extracts by LC–MS, the values of the accurate masses and the profiles of UV/Vis absorbance for the detected peaks were compared with the values of compounds available in the literature or databases (Simirgiotis et al., 2016; Sampaio et al., 2016). For this purpose we used our *in-house* chemical structures database AsterDB (Asteraceae Database, http://www.asterbiochem.org/asterdb) and the online databases DNP (Dictionary of Natural Products, http://dnp.chemnetbase.com) and MZedDB (http://maltese.dbs.aber.ac.uk:8888/hrmet/index.html). After putative identification, compounds identified as biomarkers were confirmed using authentic standards.

Pearson correlation was applied to verify the relationship between the first principal component (PC1) from the PCA of the metabolomic approach and Al leaf concentration in mistletoes (Okazaki et al., 2012). Statistical procedures were performed in R 3.3.2 (R Core Team, 2016). Pareto transformation was performed using the package *pcaMethods* (Stacklies et al., 2007) and PCA using the package *vegan* (Oksanen et al., 2016). OPLS-DA procedures were performed in SINCA-P (Umetrics®).

3. Results

Leaf Al concentration was significantly higher in the leaves of *M. albicans* (6.64 ± 2.14 g kg^{-1}) than in *B. verbascifolia* (0.26 ± 0.14 g kg^{-1}). As expected, leaf Al-accumulation was higher in