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Research article

Does *Phoradendron perrottetii* (mistletoe) alter polyphenols levels of *Tapirira guianensis* (host plant)?

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ABSTRACT

The present study aimed to investigate the reciprocal effects of *Phoradendron perrottetii* (mistletoe) and *T. guianensis* (host plant) regarding their polyphenol composition. Taking into account that tannins are important molecules in plant defense and their biosynthesis tends to be enhanced when a species is exposed to stress, we address the following questions: (1) Are the tannins found in our model species important in the interaction between host and mistletoe? (2) Does the presence of mistletoe induce changes in the content of tannins and other polyphenols in the host plant? (3) Do we find differences between the tannin sub-groups in the responses of the host plant to mistletoe? (4) Could the observed differences reflect the relative importance of one tannin group over another as chemical defense against the mistletoe? Using a polyphenol and tannin group-specific MRM methods we quantified four different tannin sub-groups together with flavonoid and quinic acid derivatives by ultra-performance liquid chromatography tandem mass spectrometry together with the oxidative and protein precipitation activities of leaves and branches of *Tapirira guianensis* and *Phoradendron perrottetii.* We selected leaves and branches of six non-parasitized trees of *T. guianensis*. Leaves and branches of nine individuals of *T. guianensis* parasitized by *P. perrottetii* were also sampled. For each parasitized tree, we sampled an infested branch and its leaves, as well as a non-infested branch and its leaves. Infested branches were divided into three groups: gall (the host-parasite interface), proximal, and distal region. Both proanthocyanidins and ellagitanins seem to be important for plant-plant parasitism interaction: host infested tissues (gall and surrounding regions) have clearly less tannin contents than healthy tissues. Mistletoe showed high levels of quinic acid derivatives and flavonoids that could be important during hastorium formation and intrusion on host tissues, suggesting a defense mechanism that could promote oxidative stress together with an inhibition of mistletoe seed germination, consequently avoiding secondary infestations. Polyphenol detected in *T. guianensis-P. perrottetii* interaction could play different role as plant-mistletoe strategies of survival.

1. Introduction

Approximately 1% of all plant species parasitize the vascular system of other plants using a specialized feeding organ, the haustorium, which penetrates the living tissue of another plant (the host) (Heide-Jorgensen, 2008; Smith et al., 2013). Studies regarding plant defense mechanisms against parasitic plants showed that host plants respond and defend themselves by similar mechanisms to those used in defense against herbivores and pathogens (Hegenauer et al., 2016; Runyon et al., 2010a; Smith et al., 2009).

Major defense mechanisms reported for host-parasitic plant interactions are a reduction in the production of stimulants of parasitic plant germination (Jamil et al., 2011), hypersensitive responses such as the production of phytoalexins and lignification (Hu et al., 2017; Runyon et al., 2010b), suberization and the production of phenolic compounds (Echevarria-Zomeno et al., 2006; Lozano-Baena et al., 2007). However, there are relatively few studies regarding the defense mechanisms of host plants against mistletoes, especially when compared to studies of the plant defense mechanisms against herbivores and pathogens. Even fewer studies report secondary metabolites as part of the chemical defense mechanism during a plant-parasitic plant interaction.

Within secondary metabolites, tannins have been traditionally described as modulators in plant-herbivore and plant-pathogen interactions, due to their chemical properties such as astringent taste and

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related protein precipitation capacity. In addition, it has been shown that tannins may be oxidized in the guts of such insect species that have alkaline pH (Barbehenn et al., 2008, 2009b; 2009a; Barbehenn and Peter Constabel, 2011). Tannins are divided into phlorotannins, proanthocyanidins (PAs, condensed tannins) and hydrolysable tannins (HTs) (Salminen and Karonen, 2011). Phlorotannins are the simplest tannin group (mainly found in marine organisms), presenting two or more units of phloroglucinol attached to each other. PAs are the most common group of tannins structurally formed by two or more monomeric catechin or epicatechin units (forming the sub-group of procyanidins, PC), or gallocatechin or epigallocatechin units (forming the sub-group of prodelphinidins, PD), all found in plants as oligomers (two to ten monomer units) or polymers (> 10 monomer units). HTs are structurally the most complex group, divided in three subclasses: gallic acid derivatives (typically five or less galloyl groups esterified with *e.g.* glucose), gallotannins (six or more galloyl groups with glucose, thus forming digalloyl groups) and ellagitannins (galloyls coupled to each other forming *e.g.* hexahydroxydiphenoyl groups).

Salminen and Karonen (2011) pointed out the importance of studies concerning the oxidation of tannins and its relationship with herbivore defense. Traditionally, tannins' anti-herbivore action was correlated to their capacity of protein precipitation. In general, PAs and gallotannins have higher protein precipitation capacity than ellagitannins but this property is highly influenced by alkaline gut conditions or by surfactants present on arthropod gut, and by the difference in the structures of the individual tannins (Karonen et al., 2015). With respect to the oxidative activity at high pH, ellagitannins have been found to contain more active tannin structures than *e.g.* gallotannins or proanthocyanidins (Moilanen and Salminen, 2008). Due to their wide presence in plants and relatively high oxidative activity (Moilanen et al., 2016, 2013), ellagitannins should be more efficient than gallotannins in their anti-pathogen activity through electrophilic action of *o*-quinones towards viral proteins found in the herbivore (Feldman et al., 1999).

Taking into account that tannins are important molecules in plant defense and their biosynthesis tends to be enhanced when a species is exposed to biotic or abiotic stress, and knowing that PAs and HTs are produced via different biosynthetic pathways, we address the following questions: (1) Are the tannins found in our model species important in the interaction between host and mistletoe? (2) Does the presence of mistletoe induce changes in the content of tannins and other polyphenols in the host plant? (3) Do we find differences between the tannin sub-groups in the responses of the host plant to mistletoe? (4) Could the observed differences reflect the relative importance of one tannin group over another as chemical defense against the mistletoe?

In this paper, using a polyphenol and tannin group-specific MRM methods we quantified four different tannin sub-groups together with flavonoid and quinic acid derivatives by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) together with oxidative and protein precipitation activities of leaves and branches of *Tapirira guianensis* (host plant) and *Phoradendron perrottetii* (parasitic plant - mistletoe).

2. Material and methods

2.1. Plant material

Phoradendron perrottetii (DC.) Eichler (Santalaceae) is a xylem-tapping mistletoe that occurs in all Brazilian regions. Mistletoes are a polyphyletic group of aerial parasitic plants found in Santalales (Nickrent, 2011). The largest genus within this group of parasitic plants is *Phoradendron* (Santalaceae), which has restricted distribution to the American continent (Heide-Jorgensen, 2008).

Tapirira guianensis Aubl. (Anacardiaceae) has been reported as a host of *Phoradendron* species (Caires and Proença, 2007) and is a tree species present in many Brazilian forest formations. It is popularly known as “pau-pombo” or “peito-de-pombo” and has medicinal (Roumy et al., 2009), commercial and environment restoring applications (Da Cunha and De Albuquerque, 2006).

Plant material was collected in January 2014 in a population of *T. guianensis* parasitized by *P. perrottetii* which was located in a pasture field near a small river in Minas Gerais state within the Atlantic Forest domain (S 21° 49′ 20.06″ W 45° 25′ 03.1″). Voucher of the host-parasite interaction (G.Ceccantini 3501) was deposited in the herbarium SPF (University of São Paulo), as well as in the wood and spirit collections at the same institute (SPFw).

The level of infestation was evaluated using the model proposed by Sangüesa-Barreda et al. (2012): trees without mistletoes (ID1); moderately infested trees with mistletoe present in one or two thirds of the crown (ID2); and severely infested trees with mistletoe present throughout the crown (ID3). All samples collected had similar levels of mistletoe abundance (ID1 for control trees). We classified the population as severely infested with the mistletoe (*P. perrottetii*). Host trees baring more than one mistletoe' species were avoided during the sampling; we acknowledge the fact that mistletoe ages were not taken into account during the sampling process.

We selected leaves (L) and branches (B) of six non-parasitized trees (NP) of *T. guianensis* (groups **NP-L** and **NP-B**). Leaves and branches of nine individuals of *T. guianensis* parasitized by *P. perrottetii* were also sampled. For each of the nine parasitized trees, we sampled an infested branch (**IB**) and its leaves (**IB-L**), as well as a non-infested branch (**NIB**) and its leaves (**NIB-L**). Infested Branches were divided into three groups: gall region (**IB-G**), *i.e.* the position where the parasitic plant attaches to the host stem, proximal region (**IB-P**) and distal region (**IB-D**). For the infested branches, the proximal and distal regions were cut immediately before and after of the gall region, taking care to check if there weren't parasitic tissues in both regions. For the proximal and distal regions were cut of 4–5 cm of wood. From the same individuals, we also sampled mistletoe's (**M**) leaves and branches (groups **M-L** and **M-B**).

All material was frozen, freeze-dried, ground in a ball mill and kept at room temperature until analysis.

2.2. Polyphenol analyzes

For polyphenol analysis, 20 mg of freeze-dried and powdered plant material were extracted twice for 3 h using 1.4 mL of aqueous acetone (acetone/water 80/20, V/V) with a planar shaker. Acetone was evaporated from the pooled extract by an Eppendorf concentrator and the extract was freeze-dried, solubilized in 1 mL of water and filtered via a 0.20 μm PTFE filter. A 5-time dilution of the sample with water was prepared and 5 μL was injected in Acquity UPLC system (Waters Corp., Mildford, MA, USA) interfaced to a Xevo TQ triple-quadrupole mass spectrometer (Waters Corp., Mildford, MA, USA). In brief, the UPLC system was equipped with an autosampler, a binary solvent manager, a 100 mm × 2.1 mm i.d., 1.7 μm, Acquity UPLC BEH Phenyl column (Waters Corp., Wexford, Ireland), and a diode array detector. The elution profile used two solvents, acetonitrile (A) and 0.1% aqueous formic acid (B): 0–0.5 min, 0.1% A in B; 0.5–5.0 min, 0.1–30% A in B (linear gradient); 5.0–6.0 min, 30–35% A in B (linear gradient); 6.0–9.5 min, column wash and stabilization. UV and MS data were collected from 0 to 6 min. Negative ionization mode was used for MS analyzes. ESI conditions were as follows: capillary voltage, 2.4 kV; desolvation temperature, 650 °C; source temperature, 150 °C; desolvation and cone gas (N_2), 1000 and 100 L/h, respectively; and collision gas, argon.

Before each run, a flavonoid mix stock solution containing 4 μg mL^{-1} each of kaempferol-7-*O*-glucoside, kaempferol-7-*O*-neohesperoside, kaempferol-3-*O*-glucoside, quercetin-3-*O*-galactoside, and quercetin-3-*O*-glucoside, in a mixture of acetonitrile/0.1% aqueous formic acid (1:4 v/v), was injected twice to assess the performance of the system (stability of the UPLC retention times and *m/z* values of the MS detector). Furthermore, a catechin stock solution, containing 1 μg mL^{-1} of catechin in a mixture of acetonitrile/0.1% aqueous formic

acid (1:4 v/v) was injected five times every 10 samples, to account for possible changes in the quantitative performance of the MS/MS system for polyphenols throughout the 110 min that was required for each analysis set of 10 samples. Quantitative results were corrected for possible fluctuations in the system's quantitative performance within each analysis set, as well as among different sets.

Calibration curves for PC and PD concentrations were produced as described in Engström et al. (2014, 2015) and in Malisch et al. (2015) from purified PA stock solutions (1.0 μg mL^{-1}) of a PC-rich sample (*Salix caprea* leaves: 95% pure, as determined by thiolysis) and a PD-rich sample (*Trifolium repens* flowers: 98% pure, as determined by thiolysis), respectively, by dilution with acetonitrile/H_2O (20:80, v/v). The dilution range was from 1.0 to 0.01 μg mL^{-1}. Calibration curves were used to determine the linear range for quantification. The mDP was determined according to the method of Engström et al. (2014), by calculating the ratio of terminal and extension units for both PCs and PDs. As larger PAs tend to elute later, the maxmDP was calculated by utilizing the same method as for mDP, but only integrated the terminal units and extension units from a retention time window from 3.70 to 5.50 min, which enabled a strong enough signal for the terminal and extension units of the larger PAs. Thus, the maxmDP is not the largest polymer size found in the analyzed sample per se, but it shows reliably the mean degree of polymerization for the largest PA polymers that elute in that given retention time window.

This methodology allowed the quantitation of hydrolysable tannins (HTs) by detecting galloyl derivatives (Gall) and hexahydroxydiphenoyl derivatives (HHDP), proanthocyanidins (PAs) as procyanidins (PC) and prodelphinidins (PD), and other polyphenol subgroups as flavonols (Flav) and quinic acid derivatives (Quin).

2.3. Oxidative activity

The oxidative activity of samples was measured as reported in Salminen and Karonen (2011) with modifications. For the oxidation of phenolic compounds, 6 X 20 μL of each diluted sample were pipetted into a well-plate. For the first triplicate, 180 μL of a carbonate buffer at pH 10 (50 mM, J.T. Baker, Deventer, Holland) was applied to each well. For the second triplicate, 280 μL of a buffer containing 9 vol of buffer at pH 10 and 5 vol of 0.6% of formic acid was applied to each well; these latter samples represent the extract in its initial non-oxidized state. Samples were incubated for 30 min. After that, 100 μL of 0.6% formic acid was applied to each well of the first triplicates in order to neutralize the samples to pH 6. This allowed for direct comparisons between the initial and oxidized samples in the following analyses. From each well, 50 μL of initial and oxidized samples were taken on a new microplate. After that, 50 μL of 1N Folin-Ciocalteu reagent were added and the plate was shaken for 1 min and then, 100 μL of 20% sodium carbonate (m/v) were added to each well. The absorbance was monitored for a period of 60 min and the maximum absorbance was recorded. Gallic acid at 0, 10, 25 and 100 μg mL^{-1} was used as standard solutions. The concentrations of the total phenolics in the oxidized samples were compared to the corresponding concentrations in the initial samples resulting in the proportion of the total phenolic compounds that had been oxidized during incubation.

2.4. Protein precipitation activity

The protein precipitation activity was measured using the Radial Diffusion assay (RDA) (Hagerman, 1987), as used in Gripenberg et al. (2018). A 1% (w/v) solution of agarose was prepared in acetate buffer containing 60 mM ascorbic acid adjusted to pH 5.0, by heating the suspension of agarose to boiling while stirring. The solution was cooled to 45 °C in a water bath, and the protein [0.1% (w/v) BSA] was added while the solution was gently stirred. The solution was dispensed in 10 mL aliquots into standard plastic Petri dishes (8.5 cm diameter) and allowed to cool. Uniform wells were punched in the plates. The tannin-containing solutions (24 μL) were added in triplicate to each well. The Petri dishes were covered and sealed with ParafilmM® and were incubated at 30 °C for 3 days. The plates were photographed and the diameters of the rings were measured using the ImageJ 1.47v. Tannin concentration was calculated from the average of three diameters and a calibration curve prepared using pentagalloylglucose (1–5 mg mL^{-1}).

2.5. Statistical analysis

The plastic response parameter (PR) was used to assess how *T. guianensis* was affected by *P. perrottetii*. It was calculated according to Li et al. (2015) using the formula: PR = (VPP – MVC)/MVC, where VPP is the value of an analyzed variable in a parasitized plant, and MVC is the mean value of a variable in non-parasitized tree. As control, non-parasitized branches of *T. guianensis* were used (**NP-B**). PR values reflect the relative changes in the host caused by the mistletoe. A value less than zero ($PR < 0$) indicates a negative response, PR values greater than zero ($PR > 0$), indicate a positive response, and PR values equal to zero ($PR = 0$) indicate no response of the host plant to parasite infection (Li et al., 2015).

Analyses were performed to test the normality of the data (Shapiro-Wilk test) and homoscedasticity (Bartlett test) of the data using R software (version 3.5.1). For parametric variables, means were compared by simple variance analysis (One-Way ANOVA) followed by a post-hoc Tukey test when the ANOVA showed differences among groups (data with normal distribution and equality of variances) or Welch test followed by a Games-Howell post-hoc test (data with normal distribution but do not assume equal variances). When required, data transformation (log10) was performed to allow the use of parametric tests. For non-parametric variables, means were compared using the Kruskal-Wallis rank test followed by a post-hoc Dunn method.

Principal component analysis (PCA) was performed using Fitopac 2.1 (Software developed by G.J. Shepherd). We excluded redundant chemical variables (i.e. polyphenol groups formed by the sum of subgroups, Table 1).

3. Results

Using group-specific MRM methods we could observe a distinct polyphenol composition between host and mistletoe (SM01). Contents of different polyphenol groups, including galloyl derivatives (Gall), hexahydroxydiphenoyl derivatives (HHDP), procyanidins (PC), prodelphinidins (PD), quinic acid derivatives (Quin), and flavonoids (Flav) are showed in Tables 1 and 2 for leaves and branches, respectively. No significant differences were observed between leaves from parasitized and non-parasitized *T. guianensis* (Table 1; SM02). On the other hand, branches from *T. guianensis* showed significant difference in polyphenol amounts (Table 2).

Proximal (**IB-P**), distal (**IB-D**) and gall region (**IB-G**) of infested branches showed similarities regarding the contents of tannins. All three infested branch regions (**IB-P**, **IB-G** and **IB-D**) showed significantly lower contents of tannins than non-parasitized branches (**NP-B**). These three infested branch regions also differed significantly from not-infested branches (**NIB**) of a parasitized tree, presenting the lowest amounts of phenolic compound groups analyzed in this study, excepted for quinic acid derivatives. In general, the mistletoe' presence negatively affected the production of phenolic compounds in infested branches, especially HHDP, procyanidins, prodelphinidins, and flavonoids (Fig. 1b, c, d and f). On the other hand, it positively influenced the amounts of quinic acid derivatives in the gall region, **IB-G** (Fig. 1e).

Branches of *P. perrottetii* (**M-B**) showed the highest levels of flavonoids, the double than branches from a non-parasitized *T. guianensis* (Table 2). Interesting, flavonoids were not detected in the three infested branch regions. For *T. guianensis,* this group of polyphenols is more abundant in leaves (Table 1).

Proanthocyanidins seem to be the main type of tannin produced by

Table 1
Concentrations of phenolic groups (mg/g dw) measured in leaves of *Phoradendron perrottetii* (**M-L**, n = 9), leaves of non-parasitized *Tapirira guianensis* (**NP-L**, n = 6), leaves from not-infested branches (**NIB-L**, n = 9) and from infested branches (**IB-L**, n = 9).

Polyphenols	concentrations (mg/g dw)			
	M-L	NP-L	NIB-L	IB-L
Tannin subgroups	16.16 ± 3.00b	48.99 ± 3.25a	53.93 ± 10.27a	49.96 ± 5.95a
Hydrolysable tannins (HTs)	0.50 ± 0.10b	14.48 ± 1.68a	16.27 ± 3.14a	15.49 ± 2.23a
Galloyl derivatives (Gall)	0.50 ± 0.10b	10.92 ± 1.36a	11.34 ± 2.44a	10.58 ± 1.46a
HHDP derivatives (HHDP)	0.00 ± 0.0[#]	3.56 ± 0.86a	4.93 ± 2.51a	4.91 ± 2.09a
Proanthocyanidins (PAs)	15.66 ± 2.95b	34.51 ± 3.14a	37.66 ± 8.43a	34.47 ± 4.78a
Procyanidins (PC)	15.16 ± 2.91b	25.14 ± 3.86a	25.61 ± 6.03a	24.14 ± 6.18a
Prodelphinidins (PD)	0.50 ± 0.09b	9.36 ± 2.67a	12.05 ± 6.43a	10.33 ± 4.56a
Other polyphenol subgroups				
Quinic acid derivatives (Quin)	15.17 ± 3.16a	1.55 ± 0.54b	0.90 ± 0.66b	0.95 ± 0.41b
Flavonoids (Flav)	2.08 ± 0.52b	15.54 ± 4.73a	16.96 ± 3.07a	16.69 ± 3.29a
Total polyphenol [np]	33.41 ± 4.05b	66.07 ± 6.91a	71.79 ± 11.23a	67.61 ± 6.43a

Data are presented as the mean ± standard deviation.
Different letters on a same row indicate statistically significant differences ($P < 0.05$).
Parameters indicated with a superscript *np* ([np]) indicate non-parametrical distributions.
[#]Significant differences not tested.

both species, but *P. perrottetii* showed the lowest levels of tannin groups among all analyzed sample groups. Procyanidins (PC) were the main type of proanthocyanidins. The comparison between **NIB** and **NP-B** showed significant differences in contents of procyanidins. Not-infested branches from a parasitized tree (**NIB**) showed higher amounts of proanthocyanidins when compared to branches from a non-parasitized tree (**NP-B**) (Table 2).

Regarding tannin activities, **NIB** and **NP-B** groups that presented high contents of procyanidins, also showed high ability to precipitate proteins, but equal oxidative activity when compared to **M-B** and **IB-G**, mistletoe and gall groups (Tables 2 and 3). Mistletoe' presence negatively affected the samples ability to precipitate proteins (Fig. 1i), but for the non-infested branch from a parasitized tree (**NIB**), was observed a significantly positive effect. This group of samples showed higher precipitate protein activity than any other group here analyzed. For the oxidative activity it was observed a negative effect, *e.g.* all groups showed lower oxidative activity when compared to health trees (Fig. 1h).

Principal component analysis (PCA) of branches summarized 82% of the variability of the data within the first two axes (Fig. 2). Groups of samples were distributed along axis 1 according to their contents of quinic acid derivatives and tannins. On the positive side of the axis 1 are the groups **NIB** and **NP-B**, while on the negative side are the group formed by the samples from the parasitic plant (**M-B**). Two major groups of samples from the host plant can be observed, the first one containing samples from infested branches (**IB-P**, **IB-G** and **IB-D**), and the second one containing samples of non-infested branches (**NIB**) and of branches from not-parasitized trees (**NP-B**) (Fig. 2).

4. Discussion

Studies regarding the effects of mistletoes on secondary metabolism of a host plant are scarce. The common approach studying the secondary metabolism of host-mistletoe interaction is to compare susceptible and resistant cultivars of a host plant to analyze the defense mechanisms that could result in host resistance and/or susceptibility. Lozano-Baena et al. (2007), studying cultivars of *Medicago truncatula* observed lower levels of flavonoids and other phenolic compounds in susceptible cultivars when compared to resistant ones. Jamil et al. (2011) analyzed root exudates of cultivars of rice regarding their composition of strigolactones (a class of sesquiterpene lactones). They verified that seeds of *Striga hermonthica*, an obligate hemiparasitic weed, are dependent on strigolactones for germination, which confer resistance/susceptibilite to rice cultivars depending on their exudate

Table 2
Concentrations of phenolic groups (mg/g dw) measured in branches of *Phoradendron perrottetii* (**M-B**, n = 9) and non-parasitized *Tapirira guianensis* (**NP-B**, n = 6), not-infested branches (**NIB**, n = 9) and from proximal, gall and distal regions of infested branches (**IB-P, IB-G**, **IB-D**, n = 9).

Phenolic Groups	concentrations (mg/g dw)					
	M-B	NP-B	NIB	IB-P	IB-G	IB-D
Tannin subgroups	7.99 ± 3.13d	44.73 ± 6.35b	57.75 ± 7.77a	25.27 ± 3.82c	26.02 ± 8.02c	24.69 ± 6.51c
Hydrolysable tannins (HTs)	0.34 ± 0.07c	10.12 ± 2.64a	12.72 ± 3.54a	4.81 ± 0.53b	4.33 ± 0.92b	4.64 ± 1.15b
Galloyl derivatives (Gall)	0.33 ± 0.09b	3.56 ± 0.38a	4.03 ± 0.57a	3.93 ± 0.31a	3.21 ± 0.60a	3.48 ± 0.70a
HHDP derivatives (HHDP) [np]	0.02 ± 0.05d	6.56 ± 2.47 ab	8.69 ± 3.59a	0.88 ± 0.29cd	1.12 ± 0.35bc	1.15 ± 0.65bcd
Proanthocyanidins (PAs)	7.65 ± 3.07d	34.61 ± 4.69b	45.03 ± 5.89a	20.46 ± 3.43c	21.69 ± 7.33c	20.05 ± 5.78c
Procyanidins (PC)	7.33 ± 2.98d	28.26 ± 4.11b	37.29 ± 6.90a	16.29 ± 3.02c	18.07 ± 5.49c	16.10 ± 5.43c
Prodelphinidins (PD) [np]	0.32 ± 0.10b	6.35 ± 1.71a	7.74 ± 3.61a	4.17 ± 2.36a	3.61 ± 2.43 ab	3.95 ± 2.24a
Other polyphenol subgroups						
Quinic acid derivatives (Quin) [np]	14.10 ± 1.82a	0.02 ± 0.05b	0.00 ± 0.00[#]	0.00 ± 0.00[#]	4.77 ± 1.45a	0.03 ± 0.06b
Flavonoids	1.52 ± 0.52a	0.66 ± 0.27 b	0.70 ± 0.45b	0.00 ± 0.00[#]	0.00 ± 0.00[#]	0.00 ± 0.00[#]
Total polyphenol subgroups	23.61 ± 3.96c	45.42 ± 6.29b	58.45 ± 8.14a	25.27 ± 3.82c	30.79 ± 8.68c	24.72 ± 6.50c

Data are presented as the mean ± standard deviation.
Different letters on a same row indicate statistically significant differences ($P < 0.05$).
Parameters indicated with a superscript *np* ([np]) indicate non-parametrical distributions.
[#]Significant differences not tested.

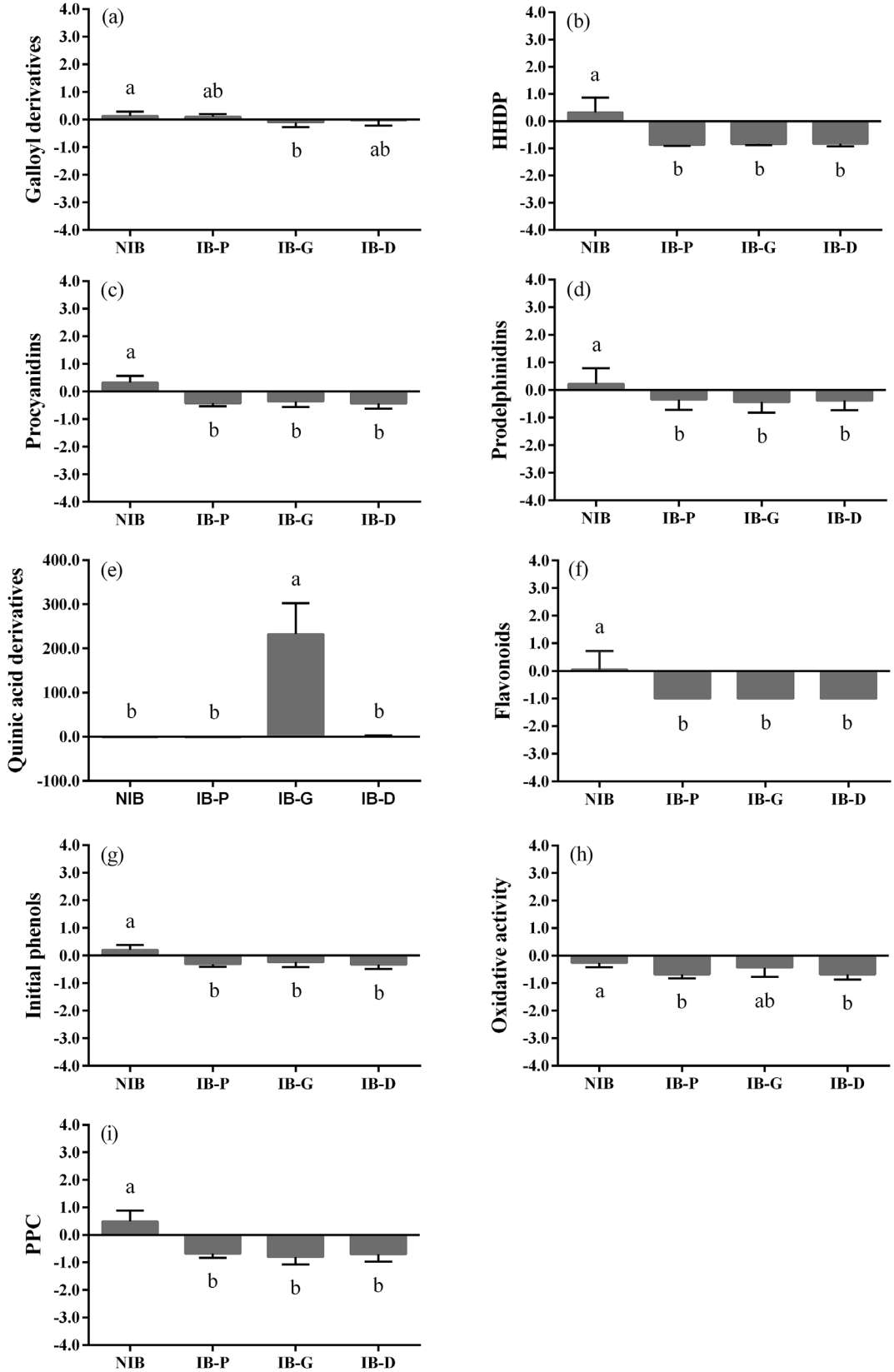


Fig. 1. Plastic response (PR) for (**a**) galloyl derivatives, (**b**) HHDP, (**c**) procyanidins, (**d**) prodelphinidins, (**e**) quinic acid derivatives, (**f**) flavonoids, (**g**) initial phenols, (**h**) oxidative activity, and (**i**) PPC (protein precipitation capacity) of the proximal, gall and distal regions from infested host branches (**IB-P**, **IB-G** and **IB-D**, n = 9) and not-infested host branches (**NIB**, n = 9). Different letters represent statistically different means ($P < 0.05$). Bars: mean ± standard deviation.

Table 3
Concentrations of initial phenolic compounds, oxidative activity and protein precipitation capacity (PPC) of leaves and branches of *Phoradendron perrottetii* (**M-L** and **M-B**, n = 9), leaves and branches of non-parasitized *Tapirira guianensis* (**NP-L** and **NP-B**, n = 6), leaves and branches from not-infested branches (**NIB-L** and **NIB**, n = 9), leaves from infested branches (**IB-L**, n = 9), and proximal, gall, and distal regions of infested branches (**IB-P, IB-G** and **IB-D**, n = 9).

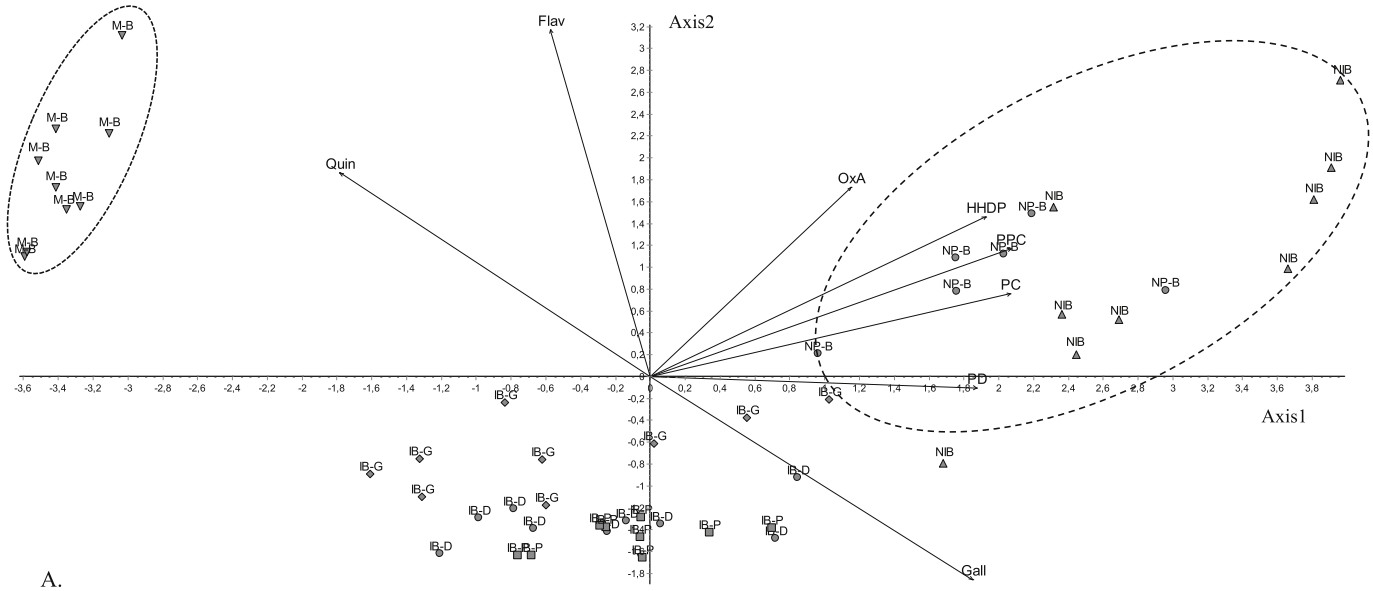
	Concentration (mg/g dw)		
	Initial phenols	Oxidative activity	PPC
M-L	73.39 ± 13.18b	15.62 ± 3.26 ^{np}	5.43 ± 5.59b ^{np}
NP-L	124.92 ± 11.53a	15.98 ± 8.63 ^{np}	32.05 ± 6.55a ^{np}
NIB-L	147.93 ± 22.35a	21.44 ± 6.51 ^{np}	37.67 ± 10.51a ^{np}
IB-L	130.58 ± 19.23a	15.79 ± 6.80 ^{np}	33.65 ± 7.22a ^{np}
M-B	51.37 ± 10.25bc	8.07 ± 2.98b	0.00 ± 0.00$^{\#}$
NP-B	56.42 ± 8.44 ab	18.23 ± 8.59 ab	15.98 ± 3.98 ab ^{np}
NIB	68.08 ± 10.05a	13.59 ± 2.97a	23.82 ± 6.39a ^{np}
IB-P	38.87 ± 4.97cd	5.93 ± 2.67b	5.12 ± 2.40bc ^{np}
IB-G	42.93 ± 10.19bd	10.37 ± 6.20 ab	3.25 ± 4.35c ^{np}
IB-D	38.13 ± 9.06d	5.91 ± 3.42b	4.81 ± 4.28bc ^{np}

Data are presented as the mean ± standard deviation.
Different letters on a same column indicate statistically significant differences ($P < 0.05$).
Parameters indicated with a superscript *np* (^{np}) indicate non-parametrical distributions.
$^{\#}$Significant differences not tested.

amounts. More recently, Pietrzak et al. (2017) analyzed contents of flavonoids and phenolic acids in *Viscum album* harvested from six different host trees. High contents of phenolics and flavonoids were reported for this mistletoe species. However, none of the above studies compared the amounts of such substances in parasitized and not-parasitized susceptible/resistant plants.

In experiments conducted by Yahraus et al. (1995) the penetration of pathogens in plant tissues was simulated, showing that plant cells were able to detect this mechanical disturbance triggering an oxidative burst. Later, Hu et al. (2017) observed that mistletoe (*Viscum album*) infestation affects the antioxidative defense system after the haustorium establishment on Scots pines by enhance hydrogen peroxide contents and the levels of glutathione. Hegenauer et al. (2016) identified a cell surface receptor-like protein essential for tomato plants to perception and resistance to *Cuscuta reflexa*, a similar mechanism to plant perception of microbial pathogens. This mechanism, called hypersensitive response (HR), is very well described for plant recognition of pathogen attack, and is characterized, *e.g.* for the generation of reactive oxygen species (ROS) and local cell death (Govrin and Levine, 2000). The oxidative burst is characterized by a marked increase in ROS, important signaling molecules that help to control several processes in plant metabolism; among them the defense response against biotic and abiotic stresses (Dinakar and Bartels, 2012; Sharma et al., 2012; War et al., 2012).

Flavonoids and chlorogenic acids have a high antioxidant capacity (Pisoschi and Pop, 2015), protecting cells from damage caused by oxidative stress; levels of these phenolic compounds can increase when



A.

Variable	Axis1	Axis2
Gall	0.3780	-0.3788
HHDP	0.3927	0.2983
PC	0.4210	0.1539
PD	0.3825	-0.0216
Quin	-0,3637	0,3797
Flav	-0.1172	0.6466
OxA	0.2353	0.3529
PPC	0.4218	0.2381

B.

PC	% variance	% acumulated variance	% expected variance
1	57.096	57.096	33.973
2	24.784	81.880	21.473
3	7.726	89.606	15.223
4	5.125	94.730	11.057

Fig. 2. Principal component analysis of variables related to polyphenol composition, protein precipitate capacity (PPC) and oxidative activity (OxA) of branches of *Phoradendron perrottetii* (**M-B**; n = 9) and branches of *Tapirira guianensis* not-parasitized (**NP-B**; n = 6), not-infested host branches (**NIB**; n = 9) and from proximal, gall and distal regions of infested branches (**IB-P, IB-G**, **IB-D**; n = 9) collected in Campanha (Minas Gerais/Brazil). Quin: quinic acid derivatives; Fla: flavonoids; Gall: gallic acid derivatives; HHDP: hexahydroxydiphenoyl derivatives; PC: procyanidins; PD: prodelphinidins.

plant is subject to different stress factors (Agati et al., 2012; Hernández et al., 2009; War et al., 2012). The present results show *P. perrottetii* presenting high levels of flavonoids and quinic acid derivatives (Tables 1 and 2). Branches of *P. perrottetii* showed three times more flavonoids than those of *T. guianensis,* suggesting a possible mechanism of quenching ROS during the haustorium penetration, gall formation and its maintenance.

Anatomical studies carried out by Teixeira-Costa and Ceccantini (2015) analyzing *T. guianensis* parasitized by *P. crassifolium* revealed that host branches undergo anatomical changes due to the parasitism. Distal branch region had the highest amount of embolized xylem vessels, suggesting a rupture of the phloem tissue during haustorium penetration and compromising the flow of nutrients in the distal-proximal direction. Moreover, Teixeira-Costa and Ceccantini (2016) investigated the 3D structure of three mistletoe species and observed that *P. perrottetii*, as other *Phoradendron* species, connects to host tissues by a single connection that frequently form the gall region. But this species shows eventual fusion of sinkers resulting in a continuous endophytic tissue connected to host xylem. In this sense, penetration of mistletoes' haustorium and its preservation could trigger the same mechanism described for plant pathogens infections. The high contents of quinic acid derivatives and flavonoids found in *P. perrottetii* tissues but also in the gall region (Table 2), could represent an important source of antioxidant substances to minimize damages caused by oxidative burst.

On the other hand, not-infested branches of parasitized individuals of *T. guianensis* (**NIB**) accumulate tannins and present higher contents of these substances when compared to branches from a not-parasitized tree (**NP-B**). Interestingly, the gall region showed significant lower amounts of tannins than non-infested branches (**NP-B** and **NIB**).

There are two different modes of biological action by tannins: one is based on the ability of tannins to precipitate proteins and by doing so they reduce the nutritive value of the plant tissue (Lokvam and Kursar, 2005); the other recognized mechanism is the oxidative activation of tannins, generating reactive oxygen species and resulting in a toxic condition to small herbivores (Lokvam et al., 2015). Hofmann et al. (2006), investigated protein binding and astringent taste procyanidin, pentagalloyl glucose, castalagin and grandinin using the half-tongue test in trained human. Procyanidins are perceived as more astringent than ellagitannins; furthermore, procyanidins showed high *in vitro* protein precipitation capacity, while ellagitannins were more effective as pro-oxidants.

Tannins were previously reported as having allelopathic potential, meaning that they inhibit seed germination (Jia et al., 2012a; Jia et al., 2012b; Zribi et al., 2014). According to Jia et al. (2012a), seed germination must be considered as a complex process involving a serious of endogenous and exogenous signals. Studying coatless seeds of *Brassica napus* with the exogenous administration of proanthocyanidins, Jia et al. (2012a) observed a reduction in seed germination and a decrease in radicle elongation. Exogenous proanthocyanidins delay seed germination by possibly promoting abscisic acid (ABA) production, which inhibits seed germination by delaying radicle protrusion.

In *Arabidopsis thaliana* proanthocyanidins-deficient mutants was observed faster seed germination than the wild-type, especially under low and moderate oxidative stress (Jia et al., 2012b). Additionally, authors found that seed accumulation of proanthocyanidins is regulated by exogenous oxidative stress by an up-regulation of almost all structural genes involving in its biosynthesis. Once more, proanthocyanidins seem to have an important role in seed germination, especially under oxidative stress.

Mistletoe seeds are dispersal, in general, by fruit-eating birds. After being deposited on a host branch, mistletoe seed will germinate and form the haustorium structure. According to Aukema and del Rio (2002), *Phoradendron californicum* parasites different leguminous tress and its differential use of hosts could be explained by three processes: bird preference for some host species, resulting in differential seed deposition; mistletoe seedlings susceptibility to some host species than others; and finally, the adult mistletoe susceptibility. Among mistletoe seedlings susceptibility to host species, host height, previous infection, and host architecture are supposed to be the main factors influencing seed deposition.

We speculate that high levels of ellagitannins, procyanidins and prodelphinidins found in not-infested branches of parasitized trees of *T. guianensis* (**NIB**) might be a host defense mechanism in order to prevent new infestations of an already parasitized tree. Different groups of organisms such as pathogens (viruses, bacteria and fungi), nematodes and insects not only induce local responses, but they also induce systemic responses in their host plants. According to Park et al. (2007), under pathogen attack a systemic defense can be activated to restrict pathogen growth. It often involves leaf necrotic lesions and subsequently a systemic acquired resistance (SAR) is developed. SAR is also compared to animal' immunity system and is important to provide plant resistance to secondary infection. However, to confirm this SAR assumption for *T. guianensis*-mistletoe interaction, further analyses of salicylic acid levels in *T. guianensis* tissues are required.

5. Conclusions

Polyphenol detected in *T. guianensis-P. perrottetii* interaction could play different role as plant-mistletoe strategies of survival. High levels of flavonoids and quinic acid derivatives characterized mistletoe tissues and could be important in quenching ROS during its establishment and survival. On the other hand, proanthocyanidins and HHDP derivatives enhanced in healthy branches of *T. guianensis* already parasitized by *P. perrottetii*, suggesting a defense mechanism that could promote oxidative stress together with an inhibition of mistletoe seed germination, consequently avoiding secondary infestations.

Regarding our initial questions both proanthocyanidins and ellagitanins seem to be important for plant-plant parasitism interaction: host infested tissues (gall and surrounding regions) have clearly less tannin contents than healthy tissues. The presence of *P. perrottetii* induces changes in polyphenol contents of *T. guianensis* and, perhaps, also induces a systemic defense mechanism.

Authors and contributions

Claudia Maria Furlan: contributed to the conception and design of the project, acquisition, analysis and interpretation of data, in writing the article and giving final approval of the version to be submitted.

Fernanda Anselmo-Moreira: contributed to the conception and design of the project, performing the experiment, contributed with editorial advice and revision of the article.

Luiza Teixeira-Costa: contributed with. logistic support for plant collection and with editorial advice.

Gregório Ceccantini: contributed with conception and design of the project.

Juha-Pekka Salminen: contributed to data interpretation, editorial advice, and critical revision of the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.01.025.

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