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Mistletoe effects on the host tree *Tapirira guianensis*: insights from primary and secondary metabolites

Fernanda Anselmo-Moreira[1] · **Luíza Teixeira-Costa**[1] · **Gregório Ceccantini**[1] · **Cláudia Maria Furlan**[1]

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Abstract

Mistletoes are parasitic plants that are capable of penetrating the living tissue of another plant's stems and branches and extracting the necessary resources for their survival. This study aimed to compare the leaves and branches of parasitized and non-parasitized *Tapirira guianensis* host trees to gain insights of reciprocal effects of *Phoradendron perrottetii* (mistletoe) infection and profiles of primary metabolites and phenolic compounds of *T. guianensis*. Our hypothesis was that either the host's chemical profile determines mistletoe infestation, or that the mistletoe infestation leads to fundamental changes in the metabolite profile of the host. Plant material was collected from *T. guianensis* parasitized by *P. perrottetii*, yielding samples from infested and non-infested host branches and their respective leaves. Infested branches were divided into two regions, the proximal region and the host-parasite interface (gall) region. Leaves and branches of non-parasitized plants were also collected. Statistical analyses revealed negative effects of the parasite on infested branches regarding most of the analyzed primary metabolites, especially soluble carbohydrates. This suggests a flow of carbohydrates towards the mistletoe, indicating a partially heterotrophic nutrition. Additionally, we observed a positive effect on the tannin contents of non-infested host branches caused by the mistletoe, which might suggest that this parasitic relationship induces a systemic response in *T. guianensis*. Finally, high contents of flavonoids at the gall region could indicate a mechanism of ROS quenching.

Keywords Parasitic plant · Host-parasite interface · Tannins · Carbohydrates · Santalaceae · Santalales.

Introduction

Mistletoes are a polyphyletic group of plants that parasitize the stems and branches of host trees and shrubs (Mathiasen et al. 2008). Similar to other parasitic plants, mistletoes penetrate the living tissue of the host to obtain the resources that are necessary for their survival (Heide-Jørgensen 2008). A specialized organ called a haustorium acts as a physiological and morphological bridge between the parasite and the host and mediates the transfer of substances between them (Press and Graves 1995).

The current understanding of mistletoe physiology shows that these plants are often capable of deriving part of their carbon requirements from the host xylem (Těšitel 2016). Studies have also shown that for some mistletoe species there may be a flow of nitrogen compounds, either organic or inorganic, from the host xylem to the parasitic plant (Escher et al. 2004; Escher and Rennenberg 2006). Thus, parasitic plant infestation disrupts the usual carbon/nitrogen balance of the host. According to Knutson (1979), such disruption activates the primary mechanism by which parasitic plants induce disease symptoms in the host. In fact, nutrient uptake by some mistletoe species has been noted to cause severe damage to the host, affecting growth, reproduction, and survival rates (reviewed by Press and Phoenix 2005; Walters 2011). Additionally, these plants may have an impact on the structure and functioning of the xylem (Ehleringer et al. 1985; Teixeira-Costa and Ceccantini 2015) and phloem (Cocoletzi et al. 2016) tissues of their hosts.

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✉ Fernanda Anselmo-Moreira
fernanda.anselmo@usp.br

[1] Department of Botany, Institute of Biosciences, University of São Paulo, Rua do Matão, 277, 05508-090 São Paulo, SP, Brazil

In general, host plants are capable of activating defense mechanisms when parasitic plants infest them (Neumann et al. 1999). However, little is known about the chemical ecology (i.e. biological interactions mediated by molecules) (Meinwald and Eisner 2008) of host-parasitic plant interactions (Pennings and Callaway 2002; Cuevas-Reyes et al. 2017) and the defense mechanisms that participate in such interactions (Smith et al. 2009). According to Lozano-Baena et al. (2007), this lack of knowledge is due to the complexity of the host-parasite relationship because both plants share biochemical, physiological, and morphological characters.

Studies evaluating plant defenses against parasitic plants have shown that hosts respond by activating similar mechanisms to those used in defenses against herbivores and pathogens (Smith et al. 2009; Runyon et al. 2010a), namely local and systemic defense responses (Buchanan et al. 2015). Pathogens can induce a systemic defense response called systemic acquired resistance (SAR), which depends on salicylic acid signaling. SAR is characterized by increased resistance (i.e. formation of defenses) at locations that are distant from the attacked plant tissue (Conrath 2006; Hammerschmidt 2009) to help prevent additional infections in those regions (Baxter et al. 2014).

Secondary metabolism produces molecules with a wide structural diversity (Aharoni and Galili 2011) and is traditionally related to plant defense, especially regarding phenolic compounds, which play an important role in plant growth, reproduction, and general survival (Caretto et al. 2015). Still, few studies have addressed secondary metabolism in parasitized host plants. In these studies, the usual approach is to compare different cultivars to analyze defense mechanisms that could result in resistance and/or susceptibility (Hariri et al. 1991; Goldwasser et al. 1999; Echevarría-Zomeño et al. 2006; Lozano-Baena et al. 2007). In a similar manner, Tjiurutue et al. (2016) verified whether parasitism by dodder (*Cuscuta* spp., Convolvulaceae) induces changes in the contents of phenolic compounds and volatiles of cranberry cultivars (*Vaccinium macrocarpon*, Ericaceae). The authors observed increased levels of phenolic acids and flavonols in the leaves of this host plant.

Studies have reported major defense mechanisms against parasitic plants, including a reduction in the production of germination stimulants (Jamil et al. 2011); hypersensitive responses, characterized by the production of phytoalexins and lignification (Bringmann et al. 1999; Mohamed et al. 2003; Runyon et al. 2010b); suberization (Echevarría-Zomeño et al. 2006); and secondary metabolite production, especially phenolic compounds (Khanna et al. 1968; Hariri et al. 1991; Goldwasser et al. 1999; Echevarría-Zomeño et al. 2006; Lozano-Baena et al. 2007; Tjiurutue et al. 2016). Therefore, the aim of this study was to compare the leaves and branches of parasitized and non-parasitized *Tapirira guianensis* to gain insights into how the presence of the mistletoe *Phoradendron perrottetii* affects host primary metabolites and phenolic compounds. We currently know that (a) secondary metabolites act as plant chemical defense against biotic and abiotic factors, being enhanced during exposure to stress, in this case the mistletoe's presence and (b) parasitic plants, including some mistletoe species, are able to extract organic nutrients from their host, contributing to a carbon reallocation in host plant. Therefore, our initial hypothesis was that either the host's chemical profile determines mistletoe infestation, or the mistletoe infestation leads to fundamental changes in the metabolite profile of the host.

Materials and methods

Parasite and host species

Phoradendron Nutt. (Santalaceae) is the largest mistletoe genus and is exclusively distributed in the New World (Heide-Jørgensen 2008; Souza and Lorenzi 2008). It comprises 234 species (Kuijt 2003), among which 64 occur in Brazil (Arruda et al. 2012). *Phoradendron perrottetii* (DC.) Eichler is a xylem-tapping mistletoe that can be found in all Brazilian regions (Forzza et al. 2010), as well as in other South American countries (Kuijt 2003). This mistletoe species can be classified as a generalist that is capable of parasitizing a wide range of host species and plant families, such as Fabaceae, Sapindaceae, Sapotaceae, and Anacardiaceae. No form of host preference or specificity (Okubamichael et al. 2016) has been reported for this species. It attaches to host branches and grows into huge shrubs (up to 1.5 m long) that bear green to yellowish leaves throughout the year.

Personal observations in the field and during ex situ cultivation in greenhouses suggest that the initial growth of *P. perrottetii* after germination is slow, as it takes nearly 2 years from seed to seedling. This long germination process hampered our attempts to perform a temporal study in which it would be possible to analyze host phytochemical responses before and after the parasite infestation. As in several mistletoe species, *P. perrottetii* develops only a primary haustorium following germination, which originates from an adhesive disc at the base of the seedling. Usually, these plants lack root apical meristems. During the parasite's growth, the primary haustorium forms a localized swollen area in the host branch, which Mani (1964) referred to as a "woody gall". Following a detailed anatomical study of the interface formed by *P. perrottetii* and its host, Teixeira-Costa and Ceccantini (2016) observed that the swelling was caused by mild to severe hypertrophy and/or hyperplasia of the host's vascular tissue and the development of parasitic tissue within the host bark (endophyte). Therefore, the woody gall formed by the parasite when infesting host branches comprises both parasitic and host vascular tissue.

The selected host for this study was *Tapirira guianensis* Aubl. (Anacardiaceae), which is an arboreal species found in many Brazilian forest formations, especially in gallery forests and gleysols, a type of soil formed under waterlogged conditions (Souza and Lorenzi 2008; IUSS Working Group WRB 2015; dos Santos et al. 2017). It has been reported to have medicinal (Roumy et al. 2009) and commercial (Alvino et al. 2005; Costa et al. 2006; Cunha and Albuquerque 2006) applications. Additionally, it is commonly used for environment restoration due to its moderate to high tolerance of wind, high temperatures, and anthropic interference (Zamith and Scarano 2006; Villacís et al. 2016). Thus, unlike most studies about defense mechanisms against parasitic plants, the *T. guianensis*-*P. perrottetii* model (host plant-mistletoe) involves a tropical arboreal species of economic and ecological interest and a mistletoe. For these reasons this model was chosen for the present study.

T. guianensis was highly abundant in the studied area, which was a pasture field near a small river in Minas Gerais state within the Mata Atlântica in the southeast region of Brazil, which is a global hotspot of biodiversity (S 21°49′20.06″W 45°25′03.1′, see Online Resource 1). Several plants that had been parasitized by the mistletoe *P. perrottetii* were found in the studied population. In addition, few non-parasitized trees were found.

A voucher of the host-parasite interaction (G. Ceccantini 3501) was deposited in the herbarium of the Institute of Biosciences of the University of São Paulo (SPF), as well as in the wood and spirit collections at the same institute (Xylarium SPFw). Based on the work by Sangüesa-Barreda et al. (2012), we classified the population as severely infested by mistletoe (*P. perrottetii*), which was present throughout the host crown (Online Resource 1). A few plants of two other parasitic species, *Phoradendron crassifolium* and *Tripodanthus acutifolius* (Loranthaceae), were found in the population, which are both mistletoes and both were infesting *T. guianensis*. Host trees bearing more than one mistletoe species were not sampled.

Although *T. guianensis* usually forms annual growth rings (Terrazas and Wendt 1995), trees in the studied population formed only poorly marked rings, probably due to their proximity to a perennial water source. For this reason, neither the precise age of the host trees nor the age of each parasitic infestation was obtained (as proposed by Scharpf and Parmeter 1966). It is also important to highlight that the parasite's growth does not show an annual branching pattern, as occurs in other species such as *Phoradendron juniperinum* (Dawson et al. 1990). Therefore, we chose to sample host trees with similar height and branches with similar diameter, which can also be used as a fair proxy for the age of mistletoe infection (Norton et al. 1997).

Field sampling

Plant material was collected during the rainy season in January 2014. Leaves (L) and branches (B) of six non-parasitized (NP) *T. guianensis* trees were sampled (groups NP-L and NP-B). Nine parasitized (P) *T. guianensis* trees were also sampled. Although the degree of infestation was severe, not all of the branches of the parasitized trees bore a *P. perrottetii* plant. Thus, 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). The leaves of all sampled branches were fully expanded and sun exposed (i.e. not shaded).

Considering that the studied host-parasite interaction includes the formation a woody gall, the infested branches were further divided into two regions, the gall region (IB-G) and the proximal region (IB-P), which corresponded to the portion of host branch closer to the node. Samples from proximal regions were cut immediately before the gall region, making sure they did not contain parasitic tissue. In branches not bearing mistletoe plants—both non-parasitized trees (NP) and non-infested branches of otherwise infested trees (NIB)—proximal regions were cut at areas with a similar diameter when compared to proximal regions of infested branches. Figure 1 outlines the sampling scheme for both parasitized and non-parasitized trees and the groups of samples that were created.

Chemical analysis

Following field sampling, all wood and leaf material was frozen, freeze-dried, ground in a ball mill and kept at room temperature until it was chemically analyzed.

Carbon/nitrogen ratio

Two milligrams of plant material from each sample were analyzed by an elemental analyzer (Perkin-Elmer 2400) to determine the percentage of carbon and nitrogen in the material. These results were used to calculate the C/N ratio of each sample group.

Carbohydrates

Ten milligrams of plant material were extracted with 1.5 mL of 80% ethanol in a water bath at 80 °C for 20 min. The extract was centrifuged, and the supernatant was collected. The precipitate was dried at 40 °C for 24 h while the supernatant was dried under a vacuum. The supernatant was taken up in 1 mL of ultrapure water, and 0.5 mL of chloroform ($CHCl_3$) was added to remove non-polar constituents. After centrifugation, the aqueous extract was separated (de Souza et al. 2013).

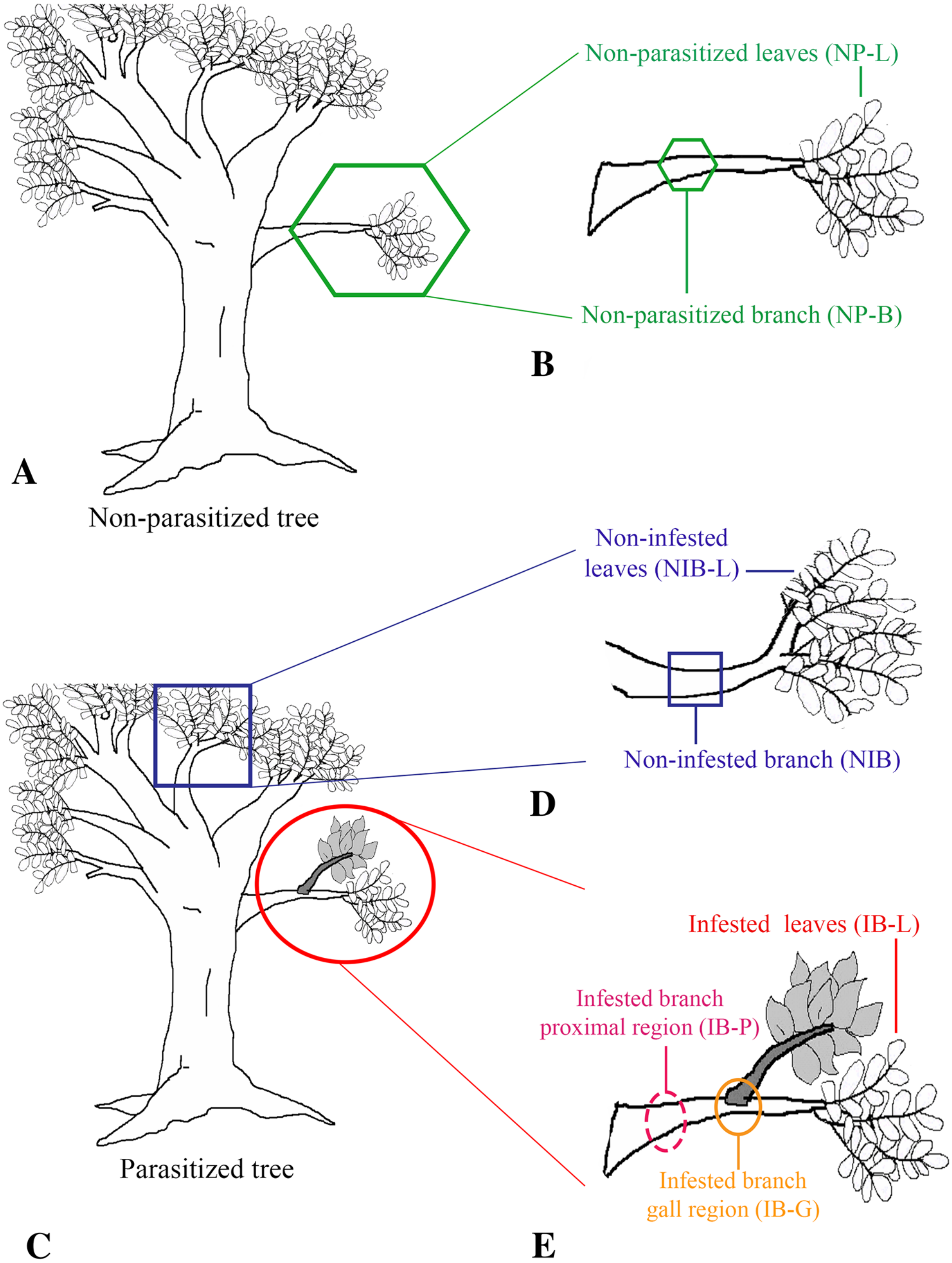

Fig. 1 Schematic drawing of the sampling groups selected from the host-parasite interaction between the host tree *Tapirira guianensis* and the mistletoe *Phoradendron perrottetii*. **a** Non-parasitized tree; **b** Non-parasitized branch (NP-B) and its leaves (NP-L); **c** Parasitized tree; **d** Non-infested branch (NIB) and its leaves (NIB-L) from otherwise parasitized tree; **e** Proximal and gall regions of infested branch (IB-P and IB-G), and its leaves (IB-L)

The soluble carbohydrate content was determined according to the phenol–sulfuric acid method using a 96-well microplate (Rezende et al. 2015). Briefly, the aqueous extract (water was used as negative control) was mixed with concentrated sulfuric acid and 5% phenol. The solution was homogenized and incubated for 5 min at 75 °C, plus 5 min in an ice bath. As a positive control, a standard curve of glucose (from 100 to 1000 µg mL^{-1}) was prepared, and the soluble carbohydrate content was determined using a microplate reader at 490 nm. All samples and the negative and positive controls were analyzed in triplicate.

Starch content was determined following the procedures of Amaral et al. (2007) with modifications. The precipitate obtained during carbohydrate extraction was digested using α-amilase (120 U mL^{-1}) from *Bacillus licheniformis* and amiloglucosidase (30 U mL^{-1}) from *Aspergillus niger*. To stop the reaction, the enzymatic extracts were kept in boiling water for 10 min, then immediately cooled. The enzymatic extract (water was used as negative control) was combined with a glucose PAP Liquiform reagent (glucose oxidase (~ 11,000 U mL^{-1}), peroxidase (~ 700 U mL^{-1}), 4-aminoantipyrine (290 µmol L^{-1}), and phenol (50 mM, pH 7.5), Labtest). The material was homogenized, and the plate was incubated for 15 min at 37 °C. As a positive control, a standard curve of glucose (25–350 µg mL^{-1}) was prepared and the starch content was determined using a microplate reader at 490 nm. All samples and the negative and positive controls were analyzed in triplicate.

Lipids

The lipid content was determined using the procedures described by Ahmad et al. (1981). One gram of the freeze-dried and powdered plant material was extracted with hexane in Soxhlet for 6 h. The solvent was recovered by rotary evaporation under reduced pressure at 40 °C. The material was dried under a nitrogen stream and kept in a desiccator until a constant weight to calculate the yield.

Phenolic compounds

To quantify the phenolic compounds, 100 mg of freeze-dried and powdered plant material was extracted for 1 h using 80% methanol at 70 °C in a dry bath. The extract was filtered, and the volume was adjusted to 25 mL. All samples and the negative and positive controls were analyzed in triplicate.

The total phenolic content was determined according to the Folin–Ciocalteu method. Ultrapure water, Folin–Ciocalteu reagent (Sigma–Aldrich), 80% methanol extract (80% methanol was used as negative control), and 10% sodium carbonate was added to each well of a 96-well microplate. The material was homogenized, and the plate was incubated for 30 min at 40 °C. The total phenolic content was determined using a microplate reader at 760 nm (Furlan et al. 2015). The results were compared to a standard curve for gallic acid (5–80 µg mL^{-1}) and were expressed as a percentage.

The flavonoid content was determined according to the aluminum chloride method, which was modified from Santos and Furlan (2013). Eighty percent methanol extract (80% methanol was used as negative control) and 5% aluminum chloride were added to each well of a 96-well microplate. The material was homogenized, and the flavonoid content was determined at 420 nm. The results were compared to a standard curve for quercetin (3.6–84 µg mL^{-1}) and were expressed as a percentage.

The total tannin content was determined using the protein precipitation-BSA method described by Waterman and Mole (1994), with modifications. One hundred seventy-five microliters of BSA solution (bovine serum albumin 1 mg mL^{-1} in acetate buffer 0.2 M, pH 5.0) and 50 µL of extract (80% methanol was used as negative control) were transferred into 0.5 mL microtubes. The material was homogenized, and after 10 min it was centrifuged at 10,621 g for ten more minutes. The supernatant was then discarded. Two hundred microliters of 1% SDS reagent (sodium dodecyl sulphate) and 75 µL of 0.01 M ferric chloride reagent were added to the precipitate. After homogenization, the material was transferred to a 96-well microplate and was incubated for 15 min at room temperature. The total tannin content was determined at 520 nm. The results were compared to a standard curve for tannic acid (0.2–1.6 mg mL^{-1}) and were expressed as a percentage.

The proanthocyanidin content was determined according to the butanol method described by Waterman and Mole (1994), with modifications. Two hundred microliters of butanol/hydrochloric acid reagent (0.018 g of ferrous sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$) and 1.25 mL concentrated hydrochloric acid (HCl) added to 25 mL of *n*-butanol) and 40 µL of extract (80% methanol was used as negative control) were added to 0.5 mL microtubes. The material was incubated at 95 °C for 30 min, then it was transferred to a 96-well microplate and the proanthocyanidin content was determined at 550 nm. The results were compared to a standard curve for quebracho tannin (0.6–2.4 mg mL^{-1}) and were expressed as a percentage. All samples and positive controls were analyzed in duplicate. For each sample and negative control, a blank sample of 200 µL of butanol reagent without hydrochloric acid (0.018 g of $FeSO_4{\cdot}7H_2O$ and 1.25 mL water added to 25 mL of *n*-butanol) was also analyzed.

Statistical analysis

To investigate differences among group means, an initial assessment of data normality was carried out using JMP SAS software (version 14). When a normal distribution and homoscedasticity were observed, we applied a one-way ANOVA followed by a Tukey post-hoc test. When an absence of homoscedasticity was detected, we performed a nonparametric ranking test (Wilcoxon/Kruskal–Wallis) followed by a nonparametric post-hoc test (Steel–Dwass).

Additionally, a statistical tool frequently used in meta-analytical studies known as effect size was applied to evaluate the intensity of the effect caused by *P. perrottetii* on *T. guianensis*. This approach was chosen because the *p*-values obtained with an ANOVA (or any other hypothesis test) are designed to inform only whether there is a statistical difference among the analyzed groups, but do not actually indicate the magnitudes of the detected differences (Sullivan and Feinn 2012; Gerstner et al. 2017). On the other hand, effect size provides a standardized, directional measure of an effect (i.e. the magnitude of a difference or an effect) (Harrison 2011). It is noteworthy that effect size is not an additional hypothesis test or a post-hoc test. Considering that statistical significance is dependent on sample size, the analysis of large enough samples will almost certainly result in statistically significant differences. Reporting effect sizes helps the reader understand the intensity of a detected difference (Gerstner et al. 2017).

To calculate the effect size for each class of primary and secondary metabolites evaluated in this study, we considered leaves (L) and branches (B) of non-parasitized (NP) trees as "control" groups, while all leaves (L) and branches (B) of infested branches (IB) and non-infested branches (NIB) of otherwise parasitized trees were considered different "treatment" groups. Based on the sample sizes, mean values, and standard deviations, we calculated the effect size known as Hedges' *d* using a web-based effect-size calculator (Wilson 2018) through the following equations:

$$d = \frac{m_2 - m_1}{S_{\text{pooled}}}$$

$$S_{\text{pooled}} = \sqrt{\frac{(n_2 - 1)s_2^2 + (n_1 - 1)s_1^2}{n_1 - n_2 - 2}}$$

where, m_1 and m_2 are the means of the control and treatment groups, respectively; s_{pooled} is the pooled standard deviation; n_1 and n_2 are the sample sizes of the control and treatment groups, respectively; and s^2 is the variance (Nakagawa and Cuthill 2007).

Following Nakagawa and Cuthill (2007), the effect size was significant when its confidence interval did not include zero (i.e. confidence intervals did not cross zero) (Online Resource 2).

Table 1 Contents of primary and secondary metabolites (%), plus C/N ratio (mean ± standard deviation) in leaves of *Tapirira guianensis*

Parameters	Groups		
	NP-L	NIB-L	IB-L
Nitrogen	1.29 ± 0.37	1.28 ± 0.05	1.26 ± 0.22
Carbon	46.45 ± 1.14	46.99 ± 0.74	47.11 ± 0.94
C/N ratio	38.40 ± 10.07	36.74 ± 1.31	38.19 ± 6.33
Soluble carbohydrates*	24.07 ± 2.81 *c*	30.79 ± 5.44a	28.55 ± 3.66b
Starch$^{\text{np}}$	0.17 ± 0.29	0.62 ± 0.90	0.18 ± 0.12
Lipid	5.25 ± 2.22	4.78 ± 1.37	5.21 ± 1.73
Total phenolic	16.88 ± 2.28	17.01 ± 3.49	17.46 ± 2.02
Flavonoid	3.60 ± 1.33	3.48 ± 0.66	3.46 ± 0.76
Total tannin	14.26 ± 2.63	16.40 ± 3.88	15.53 ± 2.66
Proanthocyanidin	25.17 ± 5.37	27.56 ± 5.10	26.04 ± 3.82

Leaves from non-parasitized trees (NP-L, $n = 6$) were compared to leaves from branches infested by the mistletoe *Phoradendron perrottetii* (IB-L, $n = 9$), and to leaves from non-infested branches from otherwise parasitized trees (NIB-L, $n = 9$). Parameters indicated with a superscript *np* ($^{\text{np}}$) indicate non-parametrical distributions. Parameters indicated with an asterisk (*) and mean values followed by different letters represent statistically significant differences ($p < 0.05$, post-hoc Tukey or Steel–Dwass tests)

Results

Primary metabolites

Analyses of the primary metabolite contents of mistletoe-infected trees indicate that leaves seem to be the less-affected organ. Statistically significant differences among groups were observed only for the contents of soluble carbohydrates, with the highest levels found in leaves from non-infested branches (NIB-L), followed by leaves from infested branches (IB-L) (Table 1). The analysis of effect size values showed a similar result, suggesting a strong positive effect of the mistletoe *Phoradendron perrottetii* on the content of soluble carbohydrates in the leaves from parasitized trees (Fig. 2F).

On the other hand, the comparison of primary metabolite contents of branch samples reveals strong differences among groups, except for carbon contents (Table 2). Nevertheless, the effect size analysis showed that the parasite had a positive effect on the proximal region of the infested branches (IB-P) regarding carbon contents (Fig. 3d).

In general, significant differences were observed among infested (IB) and non-infested branches (NIB and NP-B). In fact, non-infested branches (NIB) and branches from non-parasitized trees (NP-B) do not differ from each other, suggesting that NIB and NP-B are chemically similar in relation to the content of primary metabolites (Table 2). The effect size data also showed that the parasite did not have a significant effect on NIB (Fig. 3c–h) as was expected, considering that no statistically significant difference was detected between these groups.

Regarding nitrogen contents, only IB-P was significantly different from the other groups, presenting a lower level of this nutrient (Fig. 3c). Furthermore, nitrogen contents in IB-P also influenced the C/N ratio. This group differed from the others because of its higher C/N ratio (Table 2; Fig. 3e).

Infested branches (IB-P and IB-G) also had lower levels of soluble carbohydrates and lipids than non-infested branches (NP-B and NIB) (Table 2), as well as a significant negative effect of the parasite (Fig. 3f). However, only IB-P was statistically significant for lipids (Fig. 3h).

Regarding starch, a significant difference was only observed between IB-P and IB-G; the latter group had a higher content of this polymer (Table 2). Although IB-P did not differ significantly from non-infested branches (NP-B and NIB), this group was still negatively affected by the presence of mistletoe (Fig. 3g).

Secondary metabolites

Analyses of the content of phenolic compounds in *Tapirira guianensis* leaves revealed no statistically significant differences among groups (Table 1). Results of the effect size analyses also did not reveal significant differences, demonstrating that the mistletoe had no effect on the leaves of the host (Fig. 2i–l).

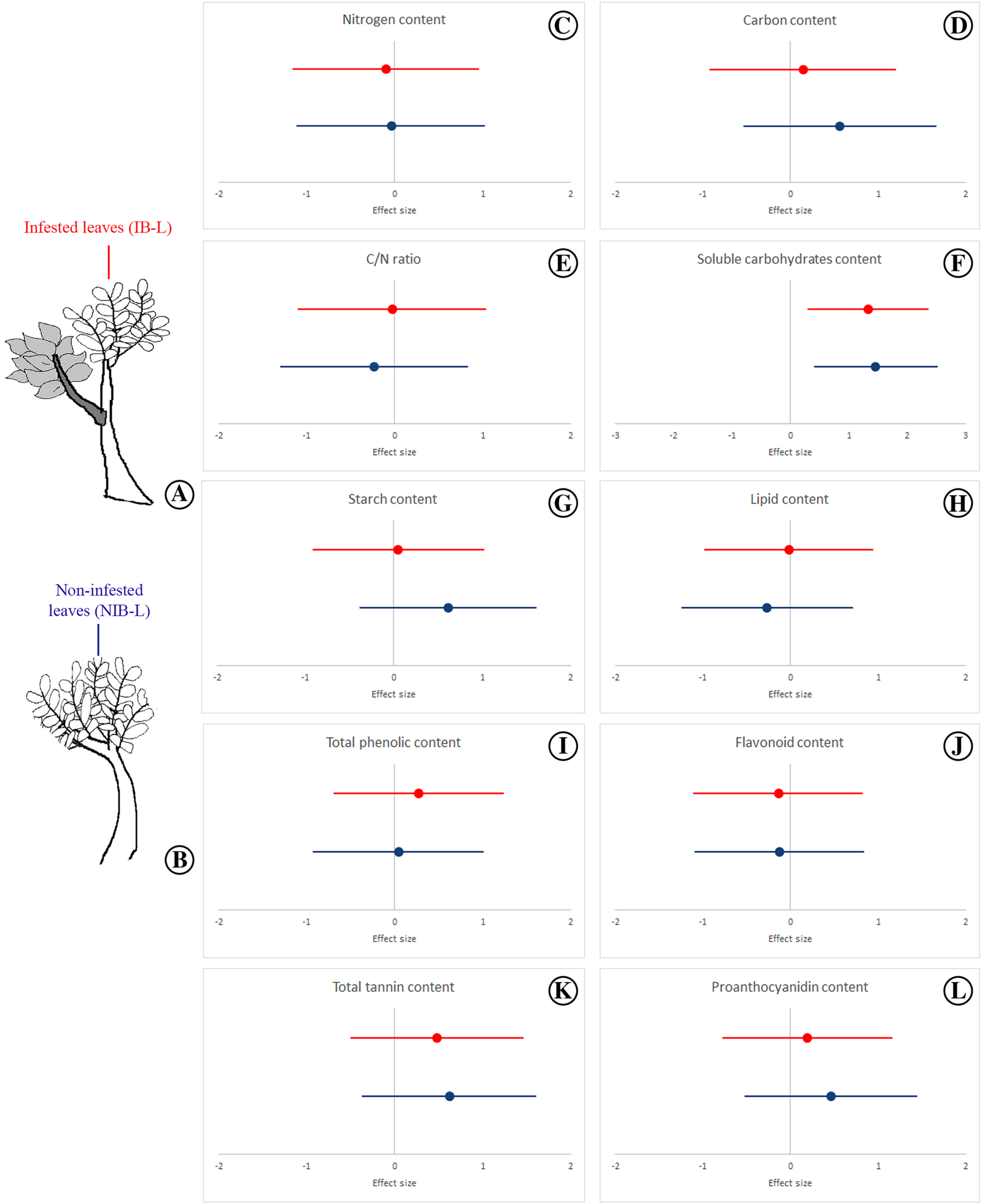

Fig. 2 Effect size values and their 95% confidence intervals for primary and secondary metabolites analyzed in leaves from infested (IB-L) and non-infested (NIB-L) branches. **a** Schematic drawing of leaves from infested branches (IB-L); **b** schematic drawing of leaves from non-infested branches (NIB-L); **c** nitrogen content; **d** carbon content; **e** C/N ratio, **f** soluble carbohydrates content; **g** starch content; **h** lipid content; **i** total phenolic content; **j** flavonoid content; **k** total tannin content; **l** proanthocyanidin content

Table 2 Contents of primary and secondary metabolites (%), plus C/N ratio (mean ± standard deviation) in branches of *Tapirira guianensis*

Parameters	Groups			
	NP-B	NIB	IB-P	IB-G
Nitrogen*	0.51 ± 0.17a	0.58 ± 0.10a	0.19 ± 0.07b	0.49 ± 0.09a
Carbon	44.98 ± 1.01	45.77 ± 0.91	46.22 ± 0.75	45.86 ± 0.38
C/N ratio^{np}*	95.17 ± 27.55b	81.85 ± 15.65b	269.89 ± 92.76a	95.54 ± 18.10b
Soluble carbohydrates*	19.47 ± 2.43a	18.96 ± 3.97a	13.58 ± 1.84b	13.61 ± 2.69b
Starch^{np}*	2.58 ± 2.24ab	2.20 ± 3.02ab	0.64 ± 0.95b	3.38 ± 1.51a
Lipid^{np}*	1.15 ± 0.32a	1.22 ± 0.27a	0.51 ± 0.13c	1.01 ± 0.51b
Total phenolic^{np}*	6.71 ± 0.72a	7.95 ± 1.41a	4.59 ± 0.83b	4.72 ± 1.11b
Flavonoid^{np}*	0.27 ± 0.03b	0.32 ± 0.08ab	0.09 ± 0.01c	0.40 ± 0.12a
Total tannin*	4.08 ± 0.40a	4.85 ± 0.66a	3.16 ± 0.53b	2.66 ± 0.70c
Proanthocyanidin*	20.16 ± 5.24ab	26.07 ± 4.89a	14.07 ± 3.80bc	12.15 ± 3.85c

Branches from non-parasitized trees (NP-B, $n=6$) were compared to non-infested branches from otherwise parasitized trees (NIB, $n=9$), the proximal region (IB-P, $n=9$), and the gall (host-parasite interface) region of branches infested by the mistletoe *Phoradendron perrottetii* (IB-G, $n=9$). Parameters indicated with a superscript *np* (^{np}) indicate non-parametrical distributions. Parameters indicated with an asterisk (*) and mean values followed by different letters represent statistically significant differences ($p < 0.05$, post-hoc Tukey or Steel–Dwass tests)

Regarding host branches, significant differences were detected among groups. For total phenolic compounds, total tannin, and proanthocyanidin, all regions of the infested branches (IB-P and IB-G) showed significantly lower amounts of these phenolic groups than those of non-infested branches, especially when compared to those from parasitized *T. guianensis* (NIB) (Table 2). The analysis of effect size values yielded similar results, showing a strong negative effect of the parasite on the proximal and gall regions of infested branches (IB-P and IB-G) for total phenolic compounds (Fig. 3i), total tannin (Fig. 3k), and proanthocyanidin (Fig. 3l). Interestingly, the same pattern was not observed for flavonoid content. The proximal region of infested branches (IB-P) had a significantly lower flavonoid content than the other groups, including the gall region (Fig. 3j). On the other hand, IB-G presented the highest amount of flavonoid, differing significantly from non-parasitized branches (NP-B) (Table 2; Fig. 3j).

In accordance with primary metabolite data, phenolic compound contents did not differ significantly between non-parasitized branches (NP-B) and non-infested branches from parasitized *T. guianensis* (NIB) (Table 2). Still, the effect size analysis showed a strong positive effect on the production of total tannins and proanthocyanidins in NIB (Fig. 3k, l), showing that *P. perrottetii* has significant effects in this group.

Discussion

Research on mistletoe phytochemistry and medicinal potential has increased substantially during the past decade (Watson 2017). However, the chemical ecology of the interaction between these parasites and their hosts has been the subject of only a few studies so far (Cuevas-Reyes et al. 2017). The scarcity of studies is even more pronounced for host secondary metabolism. The under-representation of mistletoes in chemical ecology studies can be attributed to the slow establishment and distribution rates often reported for many species of these parasites (Lamont 1983). Precisely for this reason, the present study reports a stationary comparison between parasitized and non-parasitized host trees instead of comparing host trees before and after mistletoe infestation. This before/after approach is often replicated on short-lived parasites that infest crops, such as Orobanchaceae. Such a stationary study design raises the issue of whether mistletoe infestation can be explained as a cause or a consequence of the different chemical profiles detected between parasitized and non-parasitized host trees.

Host quality may play an important role in mistletoe distribution in space, often explaining why only a subset of the hosts that are theoretically suitable are actually infested within a population (Watson 2009). Nitrogen and water content are usually considered to be limiting nutrients for mistletoes, thus being the most common proxies for host quality (Ehleringer et al. 1985; Marshall et al. 1993). However, recent studies (Scalon and Wright 2015; Griffiths et al. 2016) suggest that the nitrogen content may not play such a critical role in host quality for mistletoes (Okubamichael et al. 2016). Our analysis detected no statistical differences among groups regarding leaf nitrogen content, which agrees with what has been proposed by other studies (Logan et al. 2002; Bowie and Ward 2004; Hosseini et al. 2008; Sangüesa-Barreda et al. 2012; Cuevas-Reyes et al. 2017). However, we did detect a negative effect at the proximal region of

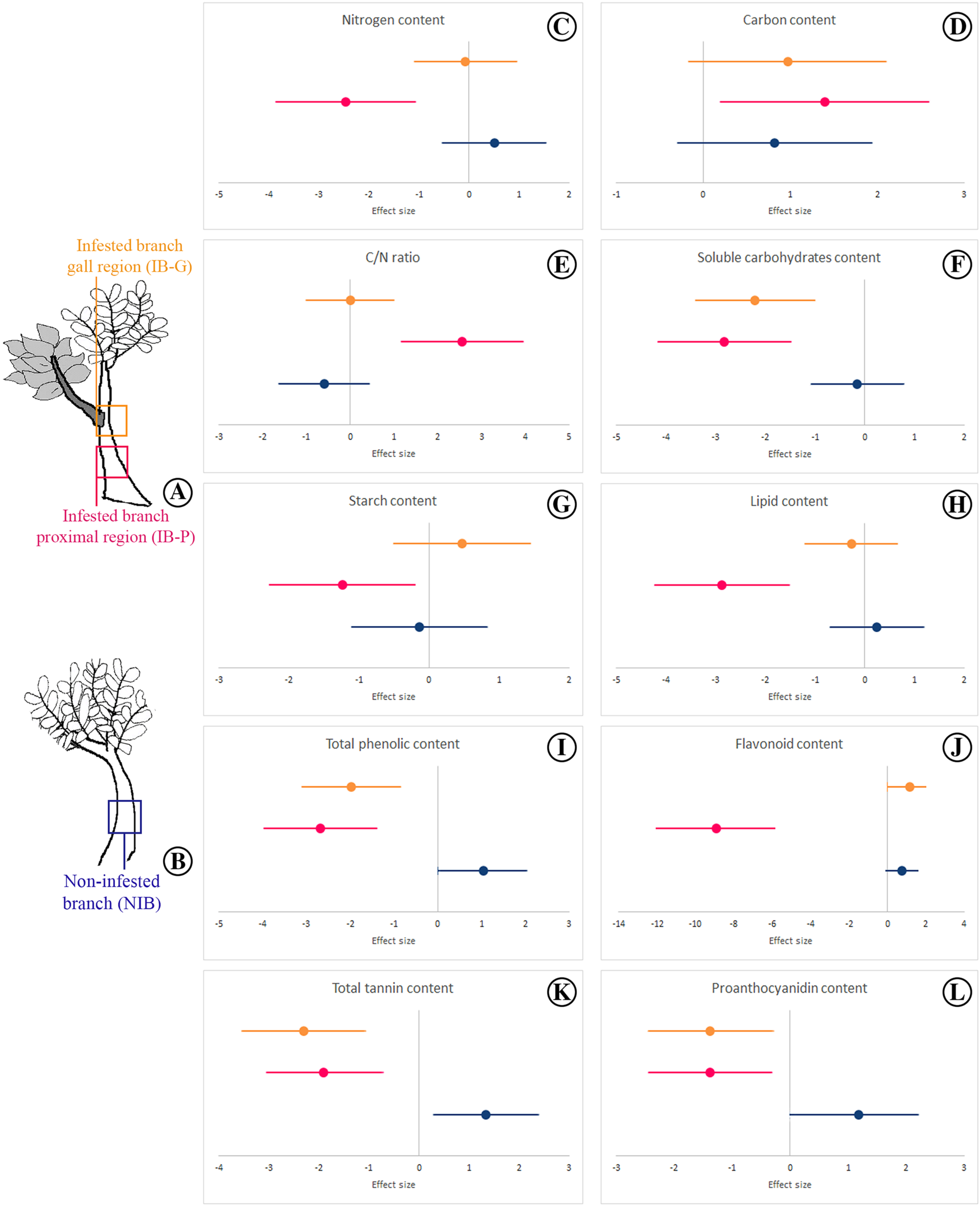

Fig. 3 Effect size values and their 95% confidence intervals for primary and secondary metabolites analyzed in samples from non-infested branches (NIB), proximal (IB-P) and gall (IB-G) regions of infested branches. **a** Schematic drawing of proximal (IB-P) and gall (IB-G) regions of infested branches; **b** schematic drawing of non-infested branches (NIB); **c** nitrogen content; **d** carbon content; **e** C/N ratio, **f** soluble carbohydrates content; **g** starch content; **h** lipid content; **i** total phenolic content; **j** flavonoid content; **k** total tannin content; **l** proanthocyanidin content

infested branches, which is more likely to be a consequence rather than a cause for mistletoe infestation.

Under this new paradigm, water can be seen as the most important proxy for host quality, which leads to host quality being dependent on site quality (underground water availability). Although we did not measure host water status, all sampled trees were rooted in humid soils located on a riverbank, as is usual for the selected host species (IUSS Working Group WRB 2015; dos Santos et al. 2017). This would explain the frequent occurrence of *Phoradendron perrottetii* for *Tapirira guianensis* host trees in populations found in Cerrado (Brazilian savannas) areas, indicating a case of local host preference (i.e. a form of host specificity in which there is a hierarchical ranking of host use by the parasite) (Okubamichael et al. 2016). Therefore, we consider that, at least for the studied population, there was no difference among individual *T. guianensis* trees regarding their nutritional quality. This leads to the assumption that detected differences in primary and secondary metabolite contents of *T. guianensis* are a consequence of mistletoe parasitism.

In the studied model, the only difference detected in leaf samples was higher soluble carbohydrate contents in leaves from both infested branches (IB-L) and non-infested branches (NIB-L). Cuevas-Reyes et al. (2017) obtained a similar result and considered it to be an indication of parasitized trees showing a better quality than non-parasitized trees. On the other hand, Yan et al. (2016) reported an increase in the soluble sugar content in the leaves of the host *Pinus sylvestris* only after mistletoe removal, thus showing the usual negative impact mistletoes have on host photosynthesis and consequent carbon gain (Glatzel and Geils 2009). Rather than an indicator of host quality, we hypothesize that the high soluble sugar content in parasitized tree leaves could be the result of mistletoe's effect on host carbon distribution.

Teixeira-Costa and Ceccantini (2015) also analyzed *T. guianensis* parasitized by *P. perrottetii* (cited as *P. crassifolium*) and proposed that host penetration by parasitic plant can cause ruptures in the phloem tissue. Indeed, anatomical studies carried out by Cocoletzi et al. (2016) reported systemic effects of another large-sized mistletoe species, *Psittacanthus schiedeanus* (Loranthaceae), on the phloem of its hosts. Thus, it seems plausible that some mistletoe species can compromise basipetal nutrient flow in host branches. Consequently, part of the carbohydrates' flow from the leaves (source organ) of infested branches downwards (sink areas) would be blocked, causing soluble carbohydrates to be accumulated in leaves and depleted at the proximal and gall regions of infested branches (IB-P and IB-G). At the same time, a high content of soluble sugars in the leaves of non-infested branches of the same trees (NIB-L) could indicate an attempt of the host to compensate carbon distribution affected by the parasite.

Additionally, the negative effect of the parasite at the gall region of infested branches (IB-G) regarding soluble carbohydrates could indicate a partially heterotrophic carbon acquisition by *P. perrottetii*. This phenomenon has been reported for other species of the genus (Marshall and Ehleringer 1990), as well as for other mistletoe species (Schulze et al. 1984; Pate et al. 1991; Richter and Popp 1992; Marshall et al. 1994; Richter et al. 1995). However, a confirmation of this partial heterotrophic nutrition in *P. perrottetii*, which is a mistletoe species that is capable of photosynthesis, can only be obtained via carbon isotopic studies (either stable or radioactive).

The proximal region of infested branches (IB-P) was also negatively affected by the presence of the parasite regarding starch, lipid, and nitrogen contents. The negative effect on the nitrogen content led to a consequent positive effect on the C/N ratio.

Host-mistletoe nitrogen flow has already been reported for several parasitic plant interactions (reviewed by Popp and Richter 1998; Pate 2001). With respect to starch, studies carried out by Sangüesa-Barreda et al. (2012) detected no significant differences between infested and non-infested branches in the association between *Pinus sylvestris* and *Viscum album*, while Broshot and Tinnin (1986) detected increased starch contents in *Pinus contorta* branches parasitized by *Arceuthobium americanum*. This pool of contrasting observations reinforces the diversity of host-parasite associations, as proposed by Scalon and Wright (2015).

As previously mentioned, mistletoe infection negatively affects hosts' nutrient contents in infested branches, either due to the partial heterotrophic nutrition of the mistletoe or by the interruption of the flow of nutrients (soluble carbohydrates) from source to sink organs. We hypothesized that this depletion of soluble carbohydrates observed in the proximal and gall regions of the infested branches (IB-P and IB-G) may be associated with the reduction of phenolic compounds in infested branches. Arnold et al. (2004) analyzed the source-to-sink carbohydrate flow and observed that there is a positive correlation between the import rate of soluble carbohydrates and the production of phenolic compounds.

On the other hand, the transfer of host metabolites into parasitic plants such as alkaloids (Stermitz et al. 1989; Mead et al. 1992; Adler et al. 2000), phenylpropanoids (Boros et al. 1991), and glucosinolates (Smith et al. 2016) has been shown for witchweeds and broomrapes (Orobanchaceae) and dodder species (*Cuscuta*, Convolvulaceae). This topic has also been reviewed by Pennings and Callaway (2002), and by Bouwmeester et al. (2003).

However, a secondary hypothesis could help explain the positive effect observed in the total tannin and proanthocyanidin contents of non-infested branches of parasitized *T. guianensis* trees (NIB). Teixeira-Costa and Ceccantini (2015) proposed that mistletoes' haustoria penetration into host tissues could be seen as a mechanical injury. This could lead to the production of tannins, such as proanthocyanidins (Arnold et al. 2004), and in turn induce systemic defense responses (Schilmiller and Howe 2005), as has been reported for other parasitic plants (Runyon et al. 2010a).

Simulation of pathogens penetrating plant tissues led Yahraus et al. (1995) to the conclusion that plant cells were able to detect this mechanical disturbance and respond to it by triggering an oxidative burst. The oxidative burst is characterized by a marked increase in reactive oxygen species (ROS) (Apel and Hirt 2004; Foyer and Noctor 2005; Dinakar et al. 2012). ROS are important signaling molecules that help to control several processes in plant metabolism, including defense responses against biotic and abiotic stresses (Dinakar et al. 2012; Sharma et al. 2012; Baxter et al. 2014). Moreover, ROS are known to damage plant cells (Hernández et al. 2009; Dinakar et al. 2012; Sharma et al. 2012), which in this case would affect both host and parasitic plants. Considering that flavonoids have high antioxidant capacity and protect cells from damage caused by oxidative stress, these compounds could also be concentrated at the host-parasite interface due to the stress factor it represents to both plants (Hernández et al. 2009; Agati et al. 2012; Sharma et al. 2012).

Branches of *P. perrottetii* showed about five times more flavonoids than those of *T. guianensis* (data not shown). In this way, the parasitic plant is most likely to be the agent that is responsible for the higher content of flavonoids detected at the gall region (IB-G). This could be a mechanism for quenching ROS during haustorium penetration, gall formation, and its maintenance. Still, considering mechanical injury formation, the higher content of flavonoids in the gall region compared to other regions of infested branches may corroborate the injury hypothesis.

Conclusions

The mistletoe infection by *Phoradendron perrottetii* mainly had a negative effect on primary and secondary metabolites. Host trees' primary and secondary metabolite profiles do not significantly differ among branches from non-parasitized trees and not-infested branches from parasitized trees, although both do differ from the metabolite profiles of infested branches. To some extent our data could indicate carbon withdrawn by the parasitic plant, which would be in accordance with previous data for the genus.

Contrary to the idea that stressful situations favor the production of phenolic compounds, *P. perrottetii* infection did not positively affect the phenolic compound contents of infested branches of *T. guianensis*. However, we detected a positive effect of the mistletoe on non-infested branches of parasitized trees, especially tannin content. Moreover, the positive effect on flavonoid content at the host-parasite interface region could indicate a mechanism of quenching reactive oxygen species produced during haustorium penetration and woody gall formation and maintenance.

In conclusion, parasitism by the mistletoe *P. perrottetii* fundamentally affects the metabolite profile of *T. guianensis*, its host tree. The reduction of primary and secondary metabolites in infested branches seems to be a local effect, while changes in the host carbon distribution and the positive effect on the production of tannins in non-infested branches from parasitized trees indicates a systemic effect. Both local and systemic effects are a consequence of mistletoe parasitism in the studied model.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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