PONTIFICIA UNIVERSIDAD CATÓLICA DEL ECUADOR

FACULTAD DE CIENCIAS EXACTAS Y NATURALES

ESCUELA DE CIENCIAS BIOLÓGICAS

CARRERA DE BIOLOGÍA

Defense strategies of a myrmecophyte: survival implications for *Duroia hirsuta* in mutualism with *Myrmelachista schumanni* and *Azteca* spp. in the Yasuní Amazonian Forest

Disertación previa a la obtención del título de Licenciada en Biología

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Quito, 2023

Certifico que la Disertación de Licenciatura en Ciencias Biológicas de la candidata Camila Viviana Rodríguez Ortega ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

Rafael E. Cárdenas . PhD
Director de la Disertación
Quito, Febrero 2023

DEDICATORIA

A mi familia, que siempre ha estado y estará para mí.

A Mikela Muñoz y Dominike García, mis hermanas del alma.

AGRADECIMIENTOS

Agradezco a mis papás Viviana Ortega y Christian Rodríguez por siempre brindarme su apoyo a lo largo de todas las etapas de mi vida. Por ser mi ejemplo a seguir ya que ellos siempre me enseñaron la importancia de seguir adelante, no rendirme y trabajar muy duro por mis sueños. Les agradezco infinitamente por siempre confiar y creer en mí, dándome ánimos cuando más lo necesitaba. A toda mi familia por ser las personas que me mantienen a flote y que siempre me ha brindado su apoyo incondicional.

Agradezco a mi director de tesis Rafael Cárdenas por sus orientación y enseñanzas durante los últimos años de mi carrera, por su apoyo para realizar esta investigación y por su invaluable contribución a mi crecimiento académico y personal. A Doménica López por su paciencia en la fase de laboratorio y por su linda amistad. También agradezco a todos mis profesores en general, porque gracias a ellos he podido obtener todos los conocimientos necesarios para ser una gran profesional y me han enseñado el amor a la Biología.

Agradezco a los amigos que me ha dado la carrera, especialmente a José Castro, Galilea Orellana, Natalia Berrazueta y Andrés Guerrero quienes siempre me brindaron su ayuda, cariño y apoyo a lo largo de esta etapa; y siempre me motivan a dar lo mejor de mí.

Finalmente agradezco Dominike García y Mikela Muñoz, son las mejores amigas que me pudo haber dado la vida. Son quienes me ayudan a levantarme siempre que me caigo y siempre están ahí para celebrar mis logros junto a mí.

TABLA DE CONTENIDO

LISTA DE FIGURAS

MANUSCRITO PARA LA PUBLICACIÓN

Revista

Frontiers in Ecology and Evolution

Título

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El siguiente trabajo de investigación se presenta en el formato de la revista Frontiers in Ecology and Evolution a partir de la siguiente página.

Defense strategies of a mirmecophyte: *Duroia hirsuta* and its physical and chemical defenses in mutualism with *Myrmelachista schumanni* and *Azteca* spp. in Yasuní Amazonian forest

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Keywords: Ecuador, insect-plant interaction, herbivory, physico-chemical defenses, secondary metabolites, trade-offs (Min.5-Max. 8)

Abstract

Duroia hirsuta K. Schum. (Rubiaceae) is a myrmecophyte that associates with ant colonies from some species such as *Myrmelachista schumanni* or *Azteca* spp and three other Neotropical Genera. It is known that *M. schumanni* protects its host plants against herbivory and eliminates adjacent allospecific plants in the surroundings of its host tree, while *Azteca* seems to just provide indirect protection from herbivores. This study aims to determine whether physico-chemical defenses, 'leaf dry matter content, herbivory,' and secondary growth of *D. hirsuta* are influenced by the identity of the associated ants (after 12 years of uninterrupted mutualism) within an Amazonian 50 ha 'forest dynamic plot'. For this we studied *D. hirsuta* trees with (i) *M. schumanni* and (ii) *Azteca* spp as indirect defenses treatments for comparison. We hypothesize that the individuals of *D. hirsuta* in mutualism with *M. schumanni* exhibit a lower inversion in physical and chemical defenses due to the advantages this ant species provides showing a lower damage by herbivory, and a higher secondary growth rate in contrast to the individuals associated with *Azteca* m.spp. For the growth record we used a digital caliper to obtain the DBH of the trees in the year 2019 to compare it withthe measurements of previous years (i.e. 2007, 2013 and 2017). We collected mature leaves to evaluate the percentage of herbivory, shearing resistance and LDMC. The percentage of herbivory was calculated with a digital tool *in situ*. To measure shearing resistance, we used a handmade steel instrument of standardized movements within a dual-range digital force sensor screwed with a peg-like folded steel sheet that supported a razor blade. In the chemical analyses we used young leaves and performed an ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). We

found secondary growth rate and shearing resistance significant differences between our treatments with higher values for the trees in mutualism with *M. schumanni*. We also found no correlation between herbivory, secondary growth, LDMC, and shearing resistance, suggesting no evidence of trade-offs between these variables. Our results, based on our long-term data of ants' presence/absence and given that absence of ants involves the death of the tree suggest that *Duroia*'s trees present an obligate mutualism with ants for survival in the tropical forests.

1 Introduction

Mutualism is a reciprocally beneficial interaction between species which is often ecologically dominant and fundamentally important at all levels of biological organization (Shingleton et al., 2005). This type of interaction can be classified in two: facultative and obligate. Facultative mutualism involves the survival of species in the absence of their mutualist partner, whereas in obligate mutualism the absence of the mutualist partner entails the death of the interacting species (Holland & Bronstein, 2008). A representative model of mutualism corresponds to the association between ants and plants (Frederickson, 2005). It is known that facultative ant-plant interactions commonly exist in every terrestrial ecosystem, except for the artic regions and high mountains, while obligate ones are limited to tropical regions (Heil & McKey, 2003; Orona-Tamayo & Heil, 2013; González-Teuber & Heil, 2015). Notably, obligate interactions are often associated with domatia-bearing plants that house ant colonies (i.e. chambers specifically used for ant nesting; these plants are called myrmecophytes), whereas non-domatia-bearing plants do not house interacting ants (myrmecophiles) (Fredderickson, 2005; Chamberlain & Holland, 2009; Mayer et al., 2014).

Besides nesting space, it is common for myrmecophytes to provide rewards to their resident ants directly as alimentary bodies rich in nutrients, extrafloral nectar or both; and even in indirect form via hemipterans to strengthen the association with specialized ants (Frederickson, 2005; Heil, 2008; Chamberlain & Holland, 2009; Mayer et al., 2014). In exchange, some literature has suggested that ants defend myrmecophytes from herbivory, pathogens, and intrusive vegetation (Frederickson, 2005; Rosumek et al., 2009; Mayer et al., 2014). There has also been growing evidence that many myrmecophytes obtain nutrients from ant activities (e.g. food storage, discarding debris or defecation) by accumulation of organic matter in their nesting sites (Sagers et al., 2000; Fischer et al., 2003; Gegenbauer et al., 2012; Mayer et al., 2014; Müller et al., 2022). It has been quantified that the mutualistic interaction between ants and plants in the tropics include species of more than 100 genera of Angiosperms with around 40 ant genera (Heil & McKey, 2003; González-Teuber & Heil, 2015).

Duroia hirsuta K. Schum. (Rubiaceae) is a small understory, dioecious, tropical tree with a broad distribution in central and western Amazon whose ecology, evolution and natural history in general is still little understood (Pfannes & Baier, 2002; Báez et al., 2016). This is a host tree of the ants *Myrmelachista schumanni* and of some ant species from the genus *Azteca*, *Brachymyrmex*, *Pheidole*, and *Solenopsis* (Báez et al., 2016; pers. obs.). *D. hirsuta* forms monospecific stands, known as "Devil's Gardens" or *supay chakras* in Quichua, characteristically surrounded by anomalously clear

zones devoid of other plants (Pfannes & Baier, 2002; Frederickson & Gordon, 2007; Báez et al., 2016). Although by 1994, Page et al. suggested that the roots of *D. hirsuta* exuded venom to kill surrounding plants other than *D. hirsuta* (allelopathic organism) to form the "Devil's Gardens", a subsequent study proved that these are product of the injection of formic acid by the *M. schumanni*'s workers (Frederickson et al. 2005) and thus ruled out *D. hirsuta*'s allelopathy as a result to explain the death of surrounding vegetation.

A "Devil's Garden" is assisted by one polygynous colony of *M. schumanni* comprising as many as 3 million workers and 15,000 queens (Frederickson et al., 2005). Up to date, this is the only ant species known that creates "Devil's Gardens" by killing surrounding plants of its host plant (Báez et al., 2016; Salas-López et al., 2016). Their injection of formic acid into the base of the principal veins of an allospecific plant causes necrosis and leaves dehiscence, eventually killing it (Mayer et al., 2014; Davidson & McKey, 1993). The principal advantage of this behavior is that its host plant can avoid competition for the scarce light of the understory (1-2% of light reaching the canopy) (Davidson & McKey, 1993). Outside "Devil's Gardens", *D. hirsuta* trees in mutualism with *Azteca* are protected from the presence of herbivores, as well as with *M. schumanni*, but not from adjacent allospecific plants (Frederickson & Gordon, 2007).

Notably, plants have evolved a wide variety of direct and indirect defenses against herbivory. Direct defenses include physical or chemical resistance traits, amongst others, whereas indirect defenses depend on traits that attract the natural enemies of herbivores (Heil, 2008; War et al., 2012; Aljbory & Chen, 2018; Müller et al., 2022). The first line of defense against herbivory is composed of plant structural traits such as leaf surface wax, thorns or trichomes, and cell wall thickness/lignification (Hanley et al., 2007; War et al., 2012; Mostafa et al., 2022). Secondary metabolites act as toxins and affect growth and development, thus forming the next barrier that defends the plant from subsequent attack (War et al., 2012; Mostafa et al., 2022). We refer to indirect defenses when natural enemies of plant herbivores are attracted by characters already present in plants (e.g., food boodies, extrafloral nectars and/or shelter) (I.K. Singh & A. Singh, 2021). Both defense mechanisms (direct and indirect) may be present constitutively or induced after damage by the herbivores (War et al., 2012; Aljbory & Chen, 2018; Garcia et al., 2021). Constitutive defenses are always present in the plant and do not depend on the attack of herbivores, in other words they are constantly activated but not always needed, while induced defenses are activated only in the presence of the attacker (García et al., 2021).

In this context, we want to evaluate the difference of direct defenses between two treatments: *D. hirsuta* trees in mutualism with (i) *M. schumanni* and (ii) *Azteca* m.spp. Initially, it was planned to work with a third treatment: *D. hirsuta* trees with ant absence but there were not enough trees (only 2 adult individuals throughout the study area with broken or destroyed branches and with very scarce number of healthy leaves), hence we decided to eliminate it: implications of this observation will be covered further in the discussion. In order to investigate whether the alternative ant partners have different impacts on *D. hirsuta*'s physiology, we worked with the following variables: secondary growth (to evaluate the plant efficiency use of resources; Frederickson, 2005), herbivory damaged area (to compare the effect of each ant species presence; Bizerril & Vieira, 2002), shearing resistance (as a measure of toughness, Onoda et al., 2012), LDMC (Leaf Dry Matter Content; as a key variable explaining covariation patterns among leaf functional traits; Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2016), relative abundance of secondary metabolites (because of their defensive role against herbivory; Wiggins et al., 2016; Cobo-Quinche et al., 2019), and the percentage of dry weight used to produce them.

We hypothesize that: (H_1) The individuals of *D. hirsuta* in mutualism with *M. schumanni* exhibit a lower inversion in physical and chemical defenses due to the advantages proportioned by *M. schumanni* such as protection against herbivores and the lack of competition with allospecific plants, thus show a lower herbivory proportion, and a higher secondary growth in contrast to the individuals associated with *Azteca* m.spp.

2 Material and Methods

2.1 Study site and sample collection

Sampling was conducted for a period of 25 days during the months of November and December 2019 in the 50-ha "Yasuní Forest Dynamic Plot" (YFDP; coordinates belonging to the northeastern corner of the plot: 00°41'0.5"S; 76°23'58.9"W) in the Yasuní National Park (YNP), Ecuador (Fig.1). The YFDP is located at an average of 230 m above sea level. This plot is composed of three types of geographic formations typical of folds due to its proximity to the Andes Mountain range: valleys, slopes, and hills (Valencia et al., 2004a). YNP is one of the most biodiverse forests in the world where can be found on average 670 species of trees in one hectare (Romoleroux et al, 1997). Moreover, Valencia et al. (2004a) found a total of 1104 morphospecies in 25-ha of the YFPD. In addition, it is a non-seasonal, lowland rainforest presenting an average annual precipitation of 2,826

mm and a monthly temperature between 22ºC and 32ºC (min: 16.9ºC; máx: 38.9°C) (see Valencia et al. 2004a for more details; data obtained from YRS meteorological station).

The YFDP was established in 1995 as part of the global network of plots of permanent forest dynamics, thanks to the collaboration between the Pontifical Catholic University of Ecuador, the Smithsonian Tropical Research Institute (ForestGEO Network) and the University of Aarhus, Denmark, to describe in the long-term the demography of thousands of plant species and explain their dynamics with different ecological theories. Since then and for this to be made, all the trees ≥ 1cm of DBH (Diameter at Breast Height i.e., 1.3 m at ground level) have been labeled, mapped, and identified up to species level (Valencia et al., 2004b).

In this study, the data available from the plot allowed us to obtain the exact position of 258 *D. hirsuta* trees and verify that the association of each ant species and tree has been uninterrupted since 2007 until 2019. A total of sixty trees, 30 per treatment, have been evaluated during these 12 years of systematic observation of the ant colonies dynamics with *D. hirsuta* where, treatment 1: *D. hirsuta* with *M. schumanni*, and treatment 2: *D. hirsuta* with *Azteca* m.spp (Supplementary material, Appendix S1). To confirm the presence of the same ant species during the time period mentioned above, 10 ants were collected from every tree and preserved in Eppendorf tubes with 80% ethanol for its identification and subsequent conservation *in situ*. About the *Azteca* m.spp. genus, due to its identification complexity and because at least 4 species of this genus are known in Yasuní, ants of this group have been typified as 'm.spp.' (Salazar & Donoso, 2013).

Finally, to perform chemical analyses, two young leaves per tree were collected and dried at 45ºC for 48–72 hours. Samples of each tree were stored in paper envelopes with silica gel, labeled with their respective code, and put to refrigeration at the Universidad de las Américas, Quito, Ecuador.

2.2 *Duroia*'s secondary growth record

To register the secondary growth of each individual plant, we used a digital caliper (Fowler Tools of Canada, Ontario, Canadá) to obtain in mm the DBH with 0.01 mm of accuracy. In order to reduce the error bias for this procedure, the measurement was performed three times by way of triangulation at the same altitude (i.e. 1.30m). Hence, a single value corresponding to the average of the three measurements was obtained, which would serve as a record of the year 2019 to compare it with the measurements of previous years (i.e. previous DBH measurements made in 2007, 2013 and 2017).

2.3 Herbivory and physical leaf-trait analyses

2.3.1 Percentage of herbivory in leaves of *D. hirsuta*

Damage caused by herbivory was established by the proportion of consumed leaf area during the tree lifetime (Schuldt et al., 2012). Only mature leaves, but not those that showed galleries, got torn or had necrotic areas, were taken into account for these analyses (Cárdenas et al., 2014). To first obtain the percentage of herbivory, a total of 885 pictures (15 per tree) of leaves were processed and analyzed. Pictures were taken at the level of the first branch of the tree and on branches higher than 5 m, for which an accordion ladder was used (3.8 m tall). For the pictures, we used a white background with 4 dark tacks in each corner as reference points forming a 27 × 27 cm square, thus obtaining a standardized scale for the size of the leaves. To avoid tearing the leaves from the trees, we only exerted manual pressure from the base of the leaf against the white background. Using a tablet (iPad 4, model MR7C2LL/A, Apple Inc, Foxconn, Taiwan) and *Leafbyte*® application, the percentage of herbivory was calculated automatically in cm^2 by selecting the 'Background Removal' option.

2.3.2 Mechanical resistance of leaves

For each tree 10 mature leaves showing no physical damage, fungi presence or galleries were collected. Just after abscission, leaves were put in wet cloth bags to keep the maximum cells turgor (Cornelissen et al., 2003). We used a handmade steel instrument of standardized movements, previously used in a study made by Cárdenas et al. (2014), to analyze the mechanical resistance of leaves (Appendix 5, Utreras, 2022). To the steel instrument a dual-range digital force sensor (Vernier Software & Technology, Beaverton, Oregon, EE. UU) with a peg-like folded steel sheet that supported a razor blade razor blade (Procter & Gamble Co., Rio de Janeiro, Brazil) was screwed to measure shearing resistance (Apendix 5, Utreras, 2022) (see methods and details in Cárdenas et al., 2014). To determine whether plants modulate defense strategies against herbivore damage, Cárdenas et al. (2014) performed tests of punching, shearing, and tearing. In this study, we used the shearing test ($N \times s \times mm^{-1}$) since this was the one showing a significant negative relationship with herbivory.

The leaves from *D. hirsuta* were fixed between two pressure plates leaving a space of 2 cm for shearing (Cárdenas et al. 2014; Cobo-Quinche et al., 2019). Cuts were made at different levels: two at ¼ from the base and two at ¾ of the apex of each leaf on each side, always avoiding the primary rib. The force was measured in Newtons (N) at 0.01 N precision and the measurements were recorded as the force per unit of time (s × N; the area under the curve) (Appendix 6, Utreras, 2022). The length of each cut was measured with a digital caliper to normalize de result of force per fracture

unit N × s × mm^{-1} (Onoda et al., 2011). Blades were replaced every 30 measurements to avoid their wear from influencing the result.

2.3.3 Leaf Dry-Matter Content (LDMC)

To obtain the Leaf Dry-Matter Content (LDMC) we used the same leaves as in the mechanical resistance analysis. They were kept at saturated humidity for their cells to present 100% turgor and the data to be comparable within and between treatments. After weighing the fresh leaves for the first time, they were covered with newspaper and placed in a drying chamber at 60ºC for 72 hours following the protocol of Pérez-Harguindeguy et al., (2016). Leaves were weighed a second time to obtain the LDMC index defined as dry mass (mg) exposed to an oven or drying chamber divided by its fresh mass saturated with water (g) expressed in mg × g^{-1}. The calculation of this index is important because leaves with a high LDMC tend to be relatively harder which would presumably provide them more resistance against physical damage caused by herbivory (Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2016).

2.4 Chemical leaf-trait analyses

2.4.1 Secondary metabolites extraction

One leaf per individual tree was selected and cut in thin strips for about 125 mg weight. The strips were placed in labeled, screw-capped Eppendorf tubes with 4–6 stainless steel beads for grinding in a Mini-Beadbeater-16 grinder for 2 to 3 cycles of 2 minutes each, with 1 minute intervals between cycles where the samples were placed in a cold rack (for safety reasons). Then, the beads were removed from the tubes with the help of a magnet. The tubes containing the crushed sample without the beads were placed in a vacuum drying chamber for 24 hours to ensure that there was no moisture in the sample that could affect its weight. Each of the dried samples was weighed and verified to be no heavier than 100.9 mg.

Once the sample preparation was finished, we used the extraction protocol presented by Cobo-Quinche et al., (2019). The samples were mixed with extraction buffer (60% acetate - 40% acetonitrile) according to their respective weights, samples weighing between 98.5 mg and 100.9 mg were mixed with 1 ml of extraction buffer. To homogenize the buffer and sample, tubes were vortexed and put in a shaker for 10 minutes to finally centrifuge them 10 minutes at 13,000 rpm. Once the centrifugation was completed, the supernatant was extracted and placed in a new set of tubes. To the precipitate in each tube, 1 ml of extraction buffer was added and the process described

above was repeated. Thus, the total supernatant recovered and placed in the new set of tubes is the crude extract. In addition, the tubes with remaining precipitate were stored at -20ºC.

Finally, from the extract was obtained 200 µl per sample which were transferred with 1 µl of 40 mg/ml cortisol in acetonitrile for mass spectrometry analyses. These were performed at the Coley/Kursar Laboratory, University of Utah, Salt Lake City, UT, USA, consisting of ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) and were processed in XCMS and MetaboAnalyst web server 3.0 software.

2.4.2 Percentage of dry weight invested in secondary metabolites production

The tubes with the remaining precipitate were placed in a drying chamber for 2 weeks until their dry weight (final dry weight) was smaller than the dry weight before the extraction (initial dry weight; i.e. a difference of ~19 mg between initial and final dry weight). Each tube was weighed and the percentage of dry weight was obtained by first using the rule of three multiplying the final dry weight obtained by 100 and dividing this by the initial dry weight. Then, the percentage of dry weight value was obtained by subtracting the rule of three value from 100. Finally, these values were transformed using the logit transformation.

2.5 Statistical analyses

2.5.1 Trees secondary growth rate

To determine if trees secondary growth data residuals were adjusted to a normal distribution, Shapiro-Wilk test for normality was used in the statistics Software PAST v.4.04. With residuals normally distributed (*p-value*>0.05), a parametric analysis of covariance (ANCOVA) was performed. For models to be comparable, linear regressions are used and must be statistically significantly adjusted (Gotelli & Ellison, 2004).

2.5.2 Herbivory and physical leaf-traits

2.5.2.1 Herbivory percentage

The total area and the consumed area were used to calculate the herbivory percentage of each leaf. Then we calculated the average for each tree. As in secondary growth, a Shapiro-Wilk test was performed and it was determined that the residuals were normally distributed (*p-value*> 0.05). The following assumptions were considered before the ANOVA analysis was made: the variable must have three or more categorical and independent groups, residuals must be normal, independent observations, constant variance throughout the observations (determined by Levene's test: *p*-

value>0.05), and absence of influential outliers. Finally, we performed a Tukey's test as a post-hoc (*q*>0.05 for both treatments).

2.5.2.2 Mechanical shearing resistance and LDMC

In the analysis of mechanical resistance to leaf shearing, the residuals of the measured data were not normally distributed according to the Shapiro-Wilk test (*D. hirsuta* with *M.* shumanni: W=0.785 and *p-value*<0.05; *D. hirsuta* with *Azteca* m.spp: W=0.848 and *p-value*<0.05). The same happened with LDMC analysis (*D. hirsuta* with *M. shumanni*: W=0.919 and *p-value*<0.05; *D. hirsuta* with *Azteca* m.spp: W=0.588 and *p-value*<0.05). Hence, we used the Kruskal-Wallis test and obtained a *p-value*<0.05 for shearing resistance and *p-value*>0.05 for LDMC. Finally, the Mann Whitney post-hoc test was performed for both shearing resistance (*p-value*<0.05) and LDMC (*p-value*>0.05).

2.5.2.3 Correlation between herbivory, physical traits, and secondary growth

We performed a Pearson correlation and linear regressions to determine if there is any direct influence of one variable over another and its significance using PAST v4.04 software. The comparisons were made between: LDMC vs. shearing resistance, DBH vs. shearing resistance, herbivory percentage vs. shearing resistance, LDMC vs. herbivory percentage, LDMC vs. DBH, and herbivory percentage vs. DBH. These correlations were made to evaluate the existence of trade-offs between variables.

2.5.3 Chemical leaf-traits

2.5.3.1 Secondary metabolites and percentage of dry weight analyses

It was employed a compound-based molecular networking approach, where we first group related features into compounds and then we generate (a) an individual-by-compound abundance matrix and (b) a compound-by-compound MS/MS cosine similarity matrix. Then, these data were combined into a pairwise species similarity matrix, which accounts for both shared compounds between individuals and the MS/MS structural similarity of unshared compounds (Endara et al., 2021).

This similarity matrix of relative abundances of UPLC-MS metabolites was used to make a hierarchical cluster, Principal Coordinates Analysis (PCoA), and a Permutational Multivariate Analysis of Variance (PERMANOVA). The hierarchical cluster was performed in R with the "pvclust" package. PCoA and PERMANOVA were carried out in the PAST v4.04 software. For the PERMANOVA, the variables taken into account, in addition to the similarity matrix, were the type of

ant and the DBH difference over 12 years for each individual. For this analysis the age effect in the DBH of the trees was eliminated. For this, the quartiles and their mean were calculated with the DBH differences and then the following formula was used:

$$x = \frac{\log(DBH\ difference + 1)}{\log(quartile\ mean + 1)}$$

Finally, to obtain the percentage of dry weight invested in the production of secondary metabolites, we first observed whether the data is normally distributed with the Shapiro-Wilk test (W=0.9268 and *p-value*<0.05). Then, we used the Mann Whitney U test and a *p-value*>0.05 was obtained.

3 Results

3.1 DBH records of *D. hirsuta* trees (2007 - 2019)

We obtained a positive linear regression between trees DBH of both treatments and censuses made over the years 2007, 2013, 2017, and 2019. For trees associated with *M. schumanni* the equation was $y = 0.554x - 1060.2$, with $R^2 = 0.996$ and p = 0.002, while for the ones in mutualism with *Azteca* m.spp. was $y = 0.221x - 387.580$, with $R^2 = 0.830$ and p = 0.003. The slopes were also evaluated for both regressions where significant differences were found (*F* = 2.77E+08, *p-value*<0.001) (Fig. 2). The average DBH obtained in 2019 for trees associated with *M. schumanni* was 59.27 mm while for the ones associated with *Azteca* m.spp. was 58 mm. From 2007 to 2019 the average growth rates for each treatment were as follows: for *M. schumanni* 52.5–59.27 mm and for *Azteca* m.spp. 55.34–58 mm.

3.2 Herbivory percentages per treatment

Fifteen leaves were randomly taken at two different heights where it was observed that the average of all trees for total leaf area was 215.49 cm^2 and the average consumed area was 7.13 cm^2 (8.85%). Moreover, the average of trees in mutualism with *M. schumanni* for total leaf area was 344.5 cm^2 and 7.22 cm^2 (9.01%) for consumed area while for the ones with *Azteca* m.spp. were 87.67 cm^2 and 7.05 cm^2 (8.69%) respectively. According to the ANOVA (*p-value*=0.666 and F=0.188) and the Tukey's test (*q-value*>0.05 for both treatments)**,** the herbivory percentage is not significant between treatments (Fig. 3).

3.3 Shearing resistances, a comparison

Results of the shear resistance test, obtained from the averages of the cuts made to the leaves of the 59 individuals from both treatments, were 2.99E-5 ± 1,03E-3 and 7,36E-06 ± 5,13E-04 (expressed in $N \times s \times mm^{-1}$) for *D. hirsuta* trees with *M. schumanni* and *Azteca* m.spp. respectively. The Kruskal-Wallis test showed significance in the difference between treatments with *p-value*= 0.009 and chi-square (H)=6.801, showing that the leaves from trees in mutualism with *M. schumanni* have a significant superior damage resistance than leaves from the ones associated with *Azteca* m.spp. (Fig. 4).

3.4 LDMC comparison

Results of the averages in the LDMC values did not show significant differences between treatments (Fig. 5): Kruskal-Wallis test yielded the values of *p-value*=0.078 and H=3.093 (ranges of 205–424 $mg \times g^{-1}$ for *M. schumanni* and 217–385 $mg \times g^{-1}$ for *Azteca* m.spp) and the Mann-Whitney test returned a *p-value*>0.05.

3.5 Relationships between herbivory, secondary growth rate, and physical traits

This comparison was made between all means obtained both in trees with *M. schumanni* and *Azteca* m.spp. for the variables of herbivory, secondary growth rates, and physical traits. We found no correlation between the variables , with values of R^2 closer to 0 (i.e. there is no linear relationship between variables; Figs. 6, 7).

3.6 Secondary metabolites and percentage of dry weight invested

According to the hierarchical cluster the trees associated with *M. schumanni* and *Azteca* m.sp. do not show differences chemically (Fig. 8). This was also confirmed with the Principal Coordinates Analysis (PCoA) in which both treatments are overlapped (Fig. 9). In addition, the percentage of dry weight invested in producing secondary metabolites was not significant between treatments.

The PERMANOVA showed that the type of ant and DBH have not significant effects on the quantity of the produced secondary metabolites (*p-value*>0.05) between treatments. Also, that the interaction effect between these two variables is not significant (*p-value*>0.05).

4 Discussion

4.1 What is the importance of studying the impact of a 12 year monospecific mutualism between *D. hirsuta* and its myrmecophytic host?

A strong component of our study experimental design was to consider the time of which one ant species or potential complex of the same Genus (*M. schumanni* or *Azteca* m.spp.) lived in mutualism with one individual plant. This is the first time to our knowledge that a research on the ecology of *D. hirsuta*'s natural history considers such long term interaction, portraying the strong dependency between this plant and its ants. Heil and McKey (2003) state that the more constant, long-lived, and exclusive association allowed when ants are resident in plants has usually led to specialization of both partners. This is the case of myrmecophytes which offer ants pre-formed nesting sites, or domatia, in hollow stems (e.g. *Cecropia*, *Leonardoxa*, *Macaranga*, *Duroia*), thorns (*Acacia*), petioles (*Piper*), or leaf pouches (e.g., *Hirtella*, *Maieta*, *Scaphopetalum*, *Tococa*) thus leading to a more stable and specific association by hosting an entire ant colony for a prolonged time span (Heil and McKey, 2003; González-Teuber & Heil, 2015). Moreover, myrmecophytes produce domatia independently of the presence of ants; nevertheless, ants can enlarge the nesting space and, in most cases, must actively open an entrance hole to get access (González-Teuber & Heil, 2015).

Most myrmecophytes can be associated with several ant species (e.g. *D. hirsuta*), so the varying degrees to which they are protected against herbivores or the advantages they have for growth and reproduction illustrate differences in efficacy between potential associate ants (Dejean et al., 2006). Palmer et al. (2010) showed that for the myrmecophyte *Acacia drepanolobium* (Fabaceae) none of its symbiotic ant species is a "perfect" partner because each one has contrasting effects on their host survival and reproduction throughout ontogeny. In that study, the sterilizing ant *Crematogaster nigriceps* increases *A. drepanolobium* fitness because it usually colonizes young ones and strongly bolsters their survival; trees are later colonized by *Crematogaster mimosae*, which does not sterilize, so they set seed. Another example of the importance of the identity of associate ants is the one presented by González-Teuber et al. (2014) with the plant *Acacia hindsii* (Fabaceae). That study showed that *A. hindsii* inhabited by *Pseudomyrmex gracilis* (parasitic ant) showed a higher percentage of pathogen-inflicted leaf damage than plants inhabited by *Pseudomyrmex ferrugineus* (mutualistic ant). The latter provides evidence of a direct beneficial effect provided by the mutualistic ants against microbial pathogens. These few examples depict the fundamental role of ants in mutualism with myrmecophytes beyond herbivory. In addition, there has been growing evidence that the absence of ants in this obligate interaction has negative effects for the plant such as an increase of induced direct defenses or even its death (Frederickson et al., 2013; Baez et al., 2016).

4.2 The role of myrmecophytic mutualism in the secondary growth of *D. hirsuta*

Our results showed significant differences in the secondary growth of *D. hirsuta* trees in mutualism with *M. schumanni* compared to those associated with *Azteca* m.spp., where the former presented a higher growth than the latter. A similar result was found in the research made by Báez et al. (2016) conducted in the same plot (in our case, avoiding the ontogenetic effect), where in a census of 234 individuals the growth of *D. hirsuta* was analyzed in the short and long term (6 and 18 years). In addition, 56% of the individuals presented *M. shumanni* as host and the remaining 44% were hosting other ant species (Báez et al., 2016). In the long term, mutualism with ants was found to be beneficial since the growth of trees that were associated with *M. shumanni* grew twice as fast as the ones hosting other ants, including *Azteca depilis*. Furthermore, Frederickson and Gordon (2009) also demonstrated a significant difference in the growth of *D. hirsuta* hosting *M. shumanni* than those hosting *Azteca* m.spp. or other ant species. Again, it was evident that in the long term there are periods of acceleration in the growth of trees that show mutualism with *M. schumanni*, something that we were able to support with our results, especially during the first 5 years (between the first and second census) where we observed an acceleration in the growth of *D. hirsuta* trees harboring *M. schumanni*. This may be due to the fact that inside the forest, especially in closed sites because of the vegetation density, the amount of light is relatively low most of the day which would influence in the reduction of the photosynthetic capacity (Kersch & Fonseca, 2005) and therefore in the growth rates of the trees associated with *Azteca* m.spp. The opposite would be occurring in areas with higher luminosity where *D. hirsuta* trees in mutualism with *M. schumanni* inhabit (Báez et al., 2016; Frederickson & Gordon, 2009). This may be reinforced by the tendency of *M. schumanni* to favor fast-growing hosts (Frederickson & Gordon, 2009) and thus become rapidly established through colonization on their constitutive structures (domatia) and give rise to mutualism between these two species.

4.3 Herbivory and dry weight for secondary metabolites percentage

Based on our results, there were no significant differences in terms of herbivory and percentage of dry weight invested in the production of secondary metabolites between treatments. Given that herbivory was not significant between treatments we assumed that there should be no reason why individuals in mutualism with *M. schumanni* should invest less dry weight to produce secondary metabolites than the ones associated with *Azteca* m.spp. This can be said because in the study made by Frederickson and Gordon (2007) they state that herbivory is the result of the load of herbivores that plants are given rather than the existence of a poorer protective efficacy of *M. schumanni* ants relative to *A. depilis*. So, in this matter it is not significant whereas individuals are associated with *M.*

schumanni or *Azteca* m.spp. because they have a similar load of herbivory. Furthermore, in similar studies performed in the Madre Selva Biological Station and in the Allpahuayo-Mishana Reserved Zone in the department of Loreto in the Peruvian Amazon, where ant exclusion experiments were carried out in *D. hirsuta* trees to evaluate the level of herbivory present in plants, it was observed that the level of herbivory in this species in mutualism with *M. schumanni* forming "Devil's Gardens" is three times greater than that present with trees associated with *Azteca* m.sp. (Frederickson et al., 2005; Frederickson & Gordon, 2007). This suggests that the identity of herbivory pressure and biotic and abiotic particularities in similar ecosystems may play different responses of both ants and plants (Djean et al., 2006; Ramos et al., 2022).

It is worth mentioning that with the formation of these gardens, *M. schumanni* kills potential resource competitors, thus providing benefits to *D. hirsuta*, but at the same time the herbivory pressure increases for those trees inside the gardens (Frederickson & Gordon, 2007). This happens because as long as it exists a low tree diversity and a great number of *D. hirsuta* individuals per garden, the herbivory level will increase (Frederickson & Gordon, 2007). This can be explained by the Conspecific Negative Density Dependence (CNDD) effect which states that recruitment of seedlings is lower in areas of high density of individuals of conspecifics (Wright, 2002; Johnson et al., 2012). Such an event may occur due to intraspecific competition, herbivory, or parasitism from neighboring adults (Peeters et al., 2007; Gonzalez et al., 2010; Johnson et al., 2012) thus herbivores will act more on mostly aggregated species, reducing their chances of survival (Cobo-Quinche et al., 2019; Johnson et al., 2012; Kursar & Coley, 2003).

4.4 Direct defenses against herbivory

4.4.1 Leaf resistance against herbivores

There were significant differences in shearing resistance in the individuals of *D. hirsuta* associated with *M. schumanni* compared with those in mutualism with *Azteca* m.spp. The hardness of a leaf is the most general physical defense that can be compared among several plant taxa found within tropical forests (Choong et al., 1992; Dominy et al., 2003; Westbrook et al., 2011; Cárdenas et al., 2014). Leaf toughness is expected to prevent herbivory and thus reduce mortality due to physical damage (Alvarez-Clare & Kitajima, 2007; Dominy et al., 2008). Therefore, increased leaf toughness would be expected to increase leaf lifespan and overall survival of the whole plant, especially in shade-grown plants (Kitajima & Poorter, 2010). Structural traits can prevent the action of predators, particularly chewing invertebrates, considered the dominant herbivore guild in tropical rainforests, as

they would avoid feeding on hard vein tissue (Choong et al., 1992; Coley & Barone, 1996). Leaf hardness has also been shown to be negatively correlated with herbivory and positively correlated with leaf lifespan, seedling survival, and shade tolerance in 19 species in a study conducted in the Bolivian Amazon (Kitajima & Poorter, 2010).

In the forest, particularly in open areas (gaps), some trade-offs have been identified: it is common to find a negative correlation between relative growth rates and leaf toughness (the lower the relative growth, the tougher the leaves) (Poorter, 1999; Kursar & Coley, 2003). Although in these zones rapid leaf expansion and leaf production rates are negatively related to leaf toughness, they are important for rapid growth within gaps (Kursar & Coley, 2003). Specialists in growing within gaps with low leaf toughness, compared to other understory species, can survive herbivore attacks through other mechanisms, including spines, tolerance through rapid growth and, as in the case of *D. hirsuta*, defense through mutualism with ants (Poorter, 1999; Grubb et al., 2008).

In our results there was a significant difference in the shearing resistance of trees associated with *M. schumanni*. Although for this treatment the highest value is 0.005 ($N \times s \times mm^{-1}$) in contrast with the one in the *Azteca* m.spp. treatment 0.002 ($N \times s \times mm^{-1}$), this could be considered either as an inconsistency in the cuts made manually or as an unexpected increase in the force that made those cuts. We can affirm that these are expected results since the resistance to leaf shearing in *D. hirsuta* within “Devil’s Garden” is superior to the ones outside from this, a phenomenon that can also be attributed to the CNDD, since the lower herbivory could be due to the increased resistance of leaves to physical damage by herbivores.

4.4.2 Secondary metabolites

At a chemical level there were no significant differences between *D. hirsuta* trees associated with *M. schumanni* and those in mutualism with *Azteca* m.spp. To explain this result, it is important to first mention that plant defenses can be classified broadly as constitutive (permanent) or induced (temporary) (Karban & Baldwin, 1997; War et al., 2012; Bixenmann et al., 2016; Aljbory & Chen, 2018; Garcia et al., 2021). Constitutive defenses are always present in the plant and do not depend on external stimuli such as the attack of herbivores or other sources. These defenses are constantly activated but not always needed, which entails high costs for the plants. On the other hand, induced defenses are activated as a consequence of an external stimulus that causes damage to the plants’ organs. (García et al., 2021). It is known that plants deploy two basic types of induced defenses: (1) resistance traits to reduce subsequent herbivore damage, by generally increasing baseline levels of

physical and chemical defenses; and (2) tolerance traits that minimize the negative effects of damage on plant fitness (Quintero & Bowers, 2013). One possible explanation for this lack of chemical significant differences between treatments is the secondary metabolites' role in *D. hirsuta* as constitutive defenses. According to Frederickson et al. (2013) even when an ant colony is present on a myrmecophyte nothing assures that it will provide effective defense against herbivores (e.g. Cárdenas, 2019), so myrmecophytic plants may benefit from having other resistance traits when indirect defense fails. Although inducible defenses might have greater advantages in temperate than tropical regions, because they are less costly, it is suggested that selection favors constitutive defenses in tropical plants because herbivore pressure is higher and more constant (Frederickson et al., 2013; Bixenmann et al., 2016).

Bixenmann et al. (2016) states that plants with expanding young leaves in high herbivore conditions will use constitutive defenses rather than induced. This can reinforce our principal idea, being secondary metabolites' role in our study as constitutive defenses, given that we only performed biochemical analysis in young leaves. In addition, Frederickson et al. (2013) states that *Cordia nodosa* (Boraginaceae) presented greater values of leaf toughness with more trichomes and more total phenolics (direct defenses) when ants are absent than in the presence of ants. We were not able to perform a comparison between trees with and without ants because of the level of deterioration and lack of leaves showed by the ones without ants. Perhaps *D. hirsuta* trees without ants are capable to induce secondary metabolites but that is not enough for their survival showing the importance of their mutualism with ants, regardless of the species they are associated with. It is said that biotic defense, in the form of ant mutualists, is the most effective protection against herbivory, which corroborates numerous studies showing ants often tightly coevolved defense, which could be vital (Massad et al., 2011). Moreover, Müller et al. (2022) hypothesized that plants colonized by ants do not require inducible defense mechanisms but did find that insect feeding led to increases in jasmonate levels and the emission of HIPVs (Herbivore Induced Plant Volatiles) regardless of the status of colonization. This suggests that perhaps ant protection is not sufficiently reliable under all conditions.

It is said that myrmecophytes may have not been selected to invest in additional anti-herbivore traits because of their indirect defenses (ants) conferring an effective resistance (Müller et al., 2022). However, a general trade-off between ants and other defenses has not been observed in ant-plants. Mutualist ants have been studied not only for their roles as protectors but also for their function in

plant nutrient acquisition (Sagers et al., 2000; Fischer et al., 2003; Gegenbauer et al., 2012), especially in the nutrient-poor soils of Neotropical Amazonian rainforests (Irion, 1978) which may be an important aspect affecting the general performance of this plants (Müller et al., 2022). For example, Dejean et al. (2012) showed that in *Cecropia obtusa* (Cecropiaceae) the δ^{15} N values were significantly higher in trees sheltering colonies of *Azteca ovaticeps* and *Azteca alfari* than in those without ants. Thus, it is not known how the presence/absence of ants is affecting plant's metabolism and if poor plant performance is the solely result from increased herbivory owing to a lack of alternative plant defenses or if the plant misses other ant benefits, such as the provisioning of nutrients, which in turn impairs its metabolism and growth (Müller et al., 2022).

Another reason potentially explaining our chemical result, and assuming that it is linked with constitutive defenses rather than induced, can also be related to plant ontogeny given that in this study we only evaluated young leaves from mature trees. According to Quintero and Bowers (2013), it has been predicted that younger stages should be better at inducing chemical defenses while older ones are more likely to show decreased induced resistance capabilities. However, Cobo-Quinche et al. (2019) did not found physicochemical differences between ontogenetic stages for 12 tree species in Yasuní. It is also known that the induction of defenses only occurs in tissues that are actively growing and the relative proportion of actively growing vs. differentiated tissue is greater in young plants, then young plants are expected to be more inducible than mature ones (Boege & Marquis, 2005). In addition, mature plants are more capable to retain anti-herbivore defenses than juveniles which need to allocate limited resources to early growth (Elger et al., 2009). We must remember that *D. hirsuta* trees do not associate with ants until the domatia are produced, an event that does not occur in seedlings (Moog et al., 2002). It would be interesting for future studies to compare the relative abundance of secondary metabolites between juvenile and mature trees to better understand how indirect and direct defenses affect the physiology of *D. hirsuta*.

Apart from the importance of plant ontogeny and its influence in the production of secondary metabolites, it is also important to mention leaf ontogeny. Our results only portray the chemical similarity between treatments with young leaves, thus it would be important to also analyze mature leaves to better understand how leaf ontogeny is influencing our results. Young leaves of most species experience considerably higher rates of herbivory than mature leaves (Brenes-Arguedas et al., 2006). Specifically in tropical forests, young leaves exhibit an even greater diversity of defenses than mature leaves because they can suffer herbivore pressure throughout the year in contrast to temperate

529 forests where they can rely on early spring flushing to escape herbivores (Kursar & Coley, 2003;
530 Brenes-Arguedas et al., 2006). They can even have higher concentrations as well as compounds not
531 found in mature leaves (Kursar & Coley, 2003). There has also been evidence that, in general,
532 immature and unexpanded leaves have higher nutrient concentrations and higher secondary
533 metabolite concentrations, whereas fully expanded mature leaves are tougher and have higher levels
534 of indirect defenses (Barton et al., 2019). Hence, we can potentially attribute leaf ontogeny to the
535 similarity of secondary metabolites between our treatments but we cannot confirm this idea due to
536 the lack of information of secondary metabolites from mature leaves.

537 **4.5 Indirect defense through mutualism with ants**

538 Changes in plant fitness will be influenced by resource allocation trade-offs and constrained
539 architectural traits that occur as plants develop (Boege & Marquis, 2005). For example, structural and
540 resource constraints during development influence mutual interactions between myrmecophytic
541 plants and their associated ants, because none of these interactions can occur until the domatia are
542 produced (*Duroia*'s case). This event does not occur in juvenile stages, thus the temporal availability
543 of rewards would therefore limit the protective function of ants (Moog et al., 2002). According to
544 Fonseca-Romero et al. (2019), indirect defenses involving protection provided by ants appear to
545 increase as plants age. There are multiple factors acting in the protective efficiency that ants exhibit
546 in mutualism with trees such as patrolling behavior, the level of aggressiveness towards herbivores
547 and associated signs of herbivory as each may vary between plants and ants (Bruna et al., 2004;
548 Huamantupa et al., 2011). It is worth mentioning that there was a high mortality of individuals if we
549 take as a reference the study of Baez et al. (2016), where a record of 80 dead trees was obtained in a
550 period of 18 years while in our study we found at least 28 dead trees out of a total of 30 that were to
551 be used for a third treatment (trees of *D. hirsuta* without ants), in a span of 2 years: 100% of these
552 dead recruits recorded absence of ants.

553 It appears that in this type of forest, indirect defenses through association with ants is virtually
554 mandatory in the long term, since the 28 trees found without ant association died during the course of
555 the study and the remaining two were on the verge of dying, with few leaves and a very poor general
556 condition. It should be mentioned that *D. hirsuta* trees without ant association have been recorded
557 (Frederickson et al., 2005; Báez et al., 2016) but the ones that survived were known to be those that
558 were subsequently colonized again by ants. This may be due to evidence of increased herbivory
559 pressure towards the equator, especially if there is evidence of healthy adult *Duroia* trees without

associations for long periods of time with ants at latitudes such as in Peru, Northern or Southern Brazil (Amador-Vargas, 2012; Salazar & Marquis, 2012). There is a clear example in the Reserva Comunal El Sira, in the Peruvian Amazon, where *Tococa occidentalis* trees inhabited by ants of the genus *Myrmelachista* die when there is no defense by the ants in killing the surrounding vegetation, possibly due to the slight attenuation resulting from the overgrowth of plants found around them (Morawetz et al., 1992; Amador-Vargas, 2012). In addition, genus *Inga* also presents associations with ants and a study developed in the forest of Monteverde, Puntarenas province in Costa Rica determined that ants positively influence the survival of trees being highly efficient at the moment of defending their host against herbivores (Koptur, 1994). When ants were excluded, mortality increased up to 80% in plants that were almost entirely defoliated, suggesting that particularly in certain ecological environments the levels of dependence on this association may be higher than expected (Koptur, 1994; Frederickson et al. 2005). Staab et al. (2020) synthesizes and highlights the variety of ecosystem properties including latitude, type of trees, environmental trees and degradation, and the type of predators that exist in these types of climates that could explain this phenomenon. In that review, it is mentioned that even though herbivore density increases towards the equator, there is no consensus whether leaf damage is systematically related to latitude and predator activity correlates at broad spatial scales with latitude, with highest activity in the tropics. Staab et al. (2020) also explains that trees with extrafloral nectaries may increase ant density, with plants benefiting through reduced damage and increased reproduction, which also apply for myrmecophytes. However, in our results although the difference in the percentage of herbivory was greater in *D. hirsuta* trees associated with *M. schumanni* than those associated with *Azteca* m.sp., it was not significant.

4.6 LDMC, palatability of leaves and the relationship between herbivory, secondary growth, and physical traits

Our results showed no significant differences between leaf LDMC of *D. hirsuta* trees associated with *M. schumanni* and those associated with *Azteca* m.spp. These were in accordance with the range of values expected for leaf LDMC (50–700 mg × g^{-1}) (Pérez- Harguindeguy et al., 2016). Multiple traits affecting leaf toughness may have different relationships with growth and survival despite having similar effects on leaf fracture properties, in other words, the defensive properties of some toughness-related traits may go even further than providing leaf toughness (Westbrook et al., 2011). For example, cellulose, which contributes to leaf toughness, also reduces leaf digestibility and thus lowers herbivore preference for leaf consumption (Sanson, 2006). In fact, Cárdenas et al., (2014) showed that cellulose is positively related to herbivory considering that they performed their

measurements in young expanding leaves which have less abundant cellulose, lignin and other cell wall compounds than mature leaves. While it is clear that LDMC is a good indicator of resource availability to the plant, there are several positions regarding the relationship of LDMC with traits that could be beneficial to the host (Wilson et al., 1999). None of our results showed indications of a possible correlation between the variables studied, either positive or negative despite evidence of the existence of a positive relationship between shearing resistance and LDMC for example (Poorter et al., 2009; Lusk et al., 2010). Other studies have shown that LDMC correlates negatively with growth rate and positively with leaf lifespan since the higher the LDMC the higher leaf toughness (Pérez-Harguindeguy et al., 2016). Previous studies have shown that indeed, shearing resistance shows a significant negative relationship with leaf damage caused by herbivory, thus demonstrating that toughness is an effective defense against herbivory and it would make sense since at the level of the tropics most of the damage caused by herbivores is by invertebrates whose jaws would act like the razor blades used in this work when feeding (Lowell et al., 1991; Choong et al., 1992; Coley & Barone, 1996; Cárdenas et al., 2014). Furthermore, it has been proved that LDMC is negatively correlated with herbivory (Cornelissen et al., 2003; Pérez-Harguindeguy et al., 2016), which concurs with our results given that between our treatments there are not significant differences of LDMC and percentage of herbivory.

5 Conclusion

Plants have a wide variety of direct and indirect defenses which can be either constitutive or induced; however, these strategies can be influenced by plant and leaf ontogeny, load of herbivory, CNDD, etc. Therefore, an understanding of these factors and how they interact with each other is important to better comprehend their role in plant physiology. Although the only significant differences between our treatments were found in secondary growth and shearing resistance, we were able to depict *Duroia'*s dependency with ants, regardless of the species. In fact, leaf toughness and secondary growth had higher values inside "Devil's Gardens" attributed to the NDD theory. None of our results showed a correlation between herbivory, LDMC, secondary growth and physical traits, therefore we cannot assure the existence of trade-offs. As a consequence, this study portrays the intricacy of direct and indirect defenses of myrmecophytes. At the same time, it represents a preliminary study of a long-term interaction of two different ant species (*M. schumanni* and *Azteca* m.spp.) with *Duroia hirsuta*. Moreover, it lays the foundation for future research focused on the study of this myrmecophyte and how its traits are influenced by the association with different ants. Nevertheless, our research points out that larger studies, in terms of the number of samples as well as more

variables (plant and leaf ontogeny, VOCs, resources/nutrients availability (light, macro and micronutrients), soil respiration), should be considered to expand our knowledge of these mutualistic associations.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

RC conceived the idea, laid out the conceptual framework and hypotheses. CR, M-JE, DU and RC analyzed the data. DD, PA, SQ and SB contributed with ants and plants pivotal information. CR and RC wrote the paper. All authors contributed to revising the manuscript through comments and discussions.

8 Funding

9 Acknowledgements

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11 Figures

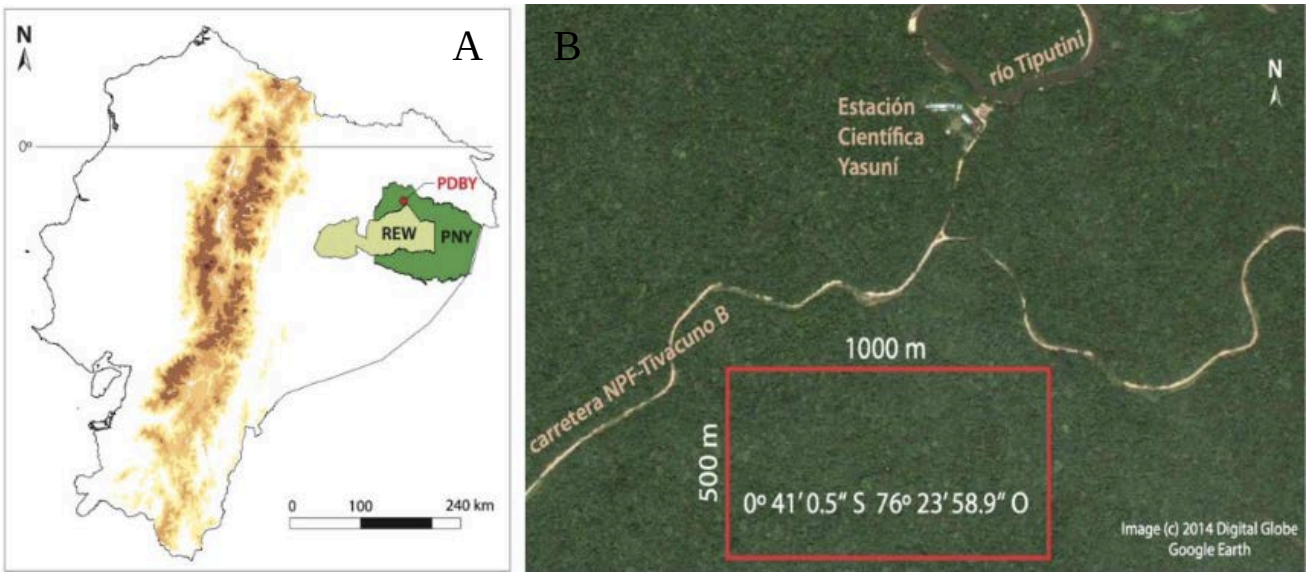
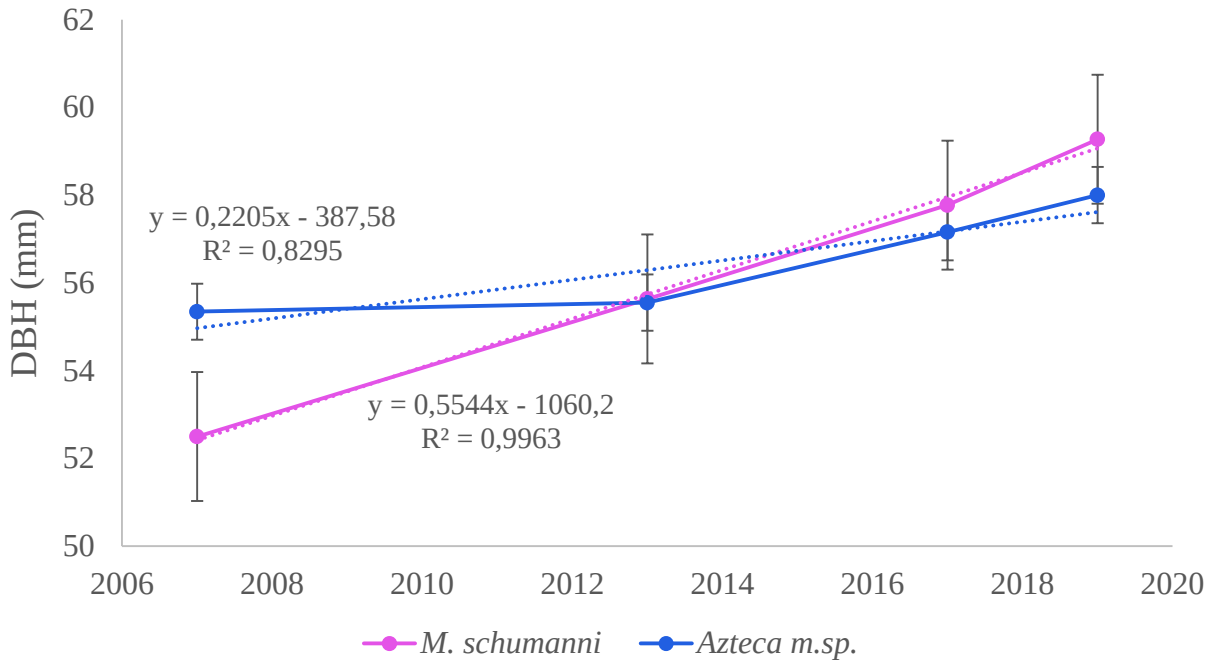


Figure 1. Study site location. (A) Ecuador continental map. Brown areas showing altitudes higher than 2000 m. Green areas show Yasuní National Park (YNP) and Waorani Ethnic Reserve (WER). The red dot marks the location of the "Yasuní Forest Dynamic Plot" (YFDP). (B) Location of the Yasuní Research Station (YRS). Plot of 50 ha represented with red rectangle. Extracted from Pérez et al. (2014) and Google Earth at 800 m above the ground.



907 **Figure 2. Average of the values of the DBH of 59 individuals of *D. hirsuta* over 12 years in a 50**
908 **ha plot in Yasuní National Park.** Host trees of *M. schumanni* and *Azteca* m.spp. throughout the
909 surveys (2007, 2013, 2017, 2019). The linear regression of each treatment is represented by dotted
910 lines with their respective equation and R^2 value. The graph shows the positive growth trend for *M.*
911 *schumanni*. The fit values for the linear regression of *M. schumanni* were F= 544.406 and *p-value*=
912 0.002 while for the linear regression of *Azteca* m.spp. was F= 9.729 and *p-value*= 0.089. The
913 ANCOVA showed values of *p-value*= 0.003 and F= 8.779.

914

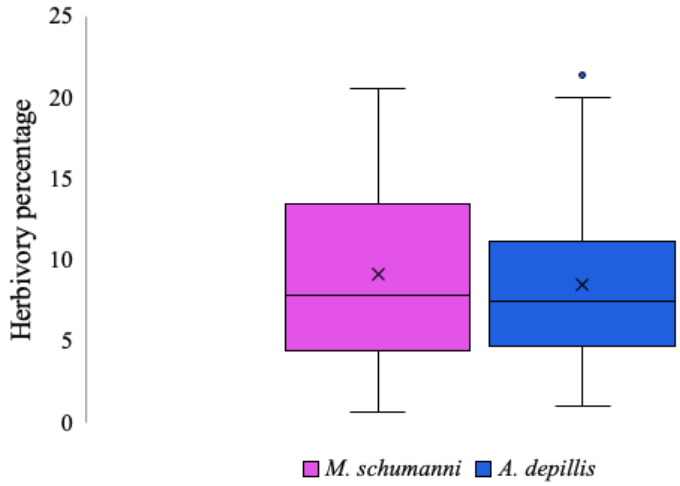


Figure 3. Box plot of the average of 59 individuals in the percentage of herbivory in the two observed treatments. There is no significant difference between treatments for the herbivory percentage.

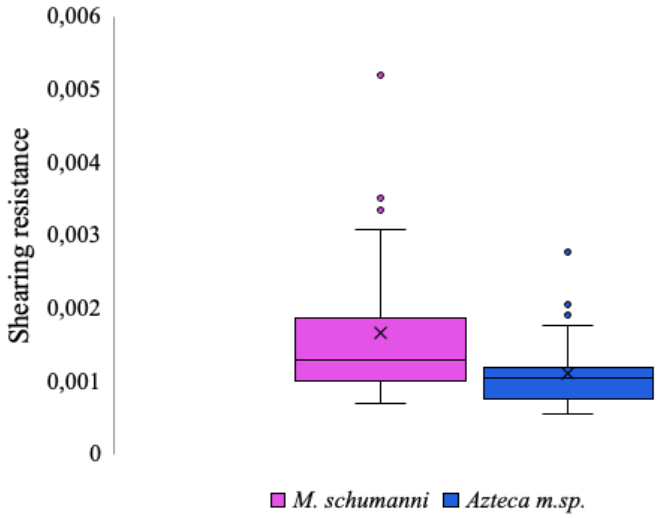
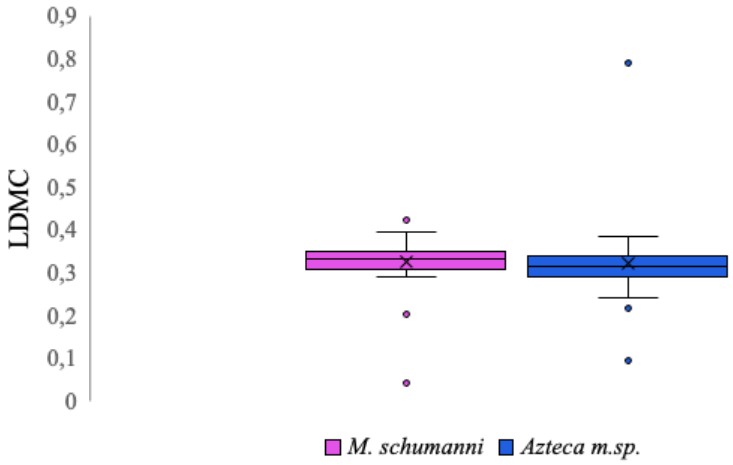
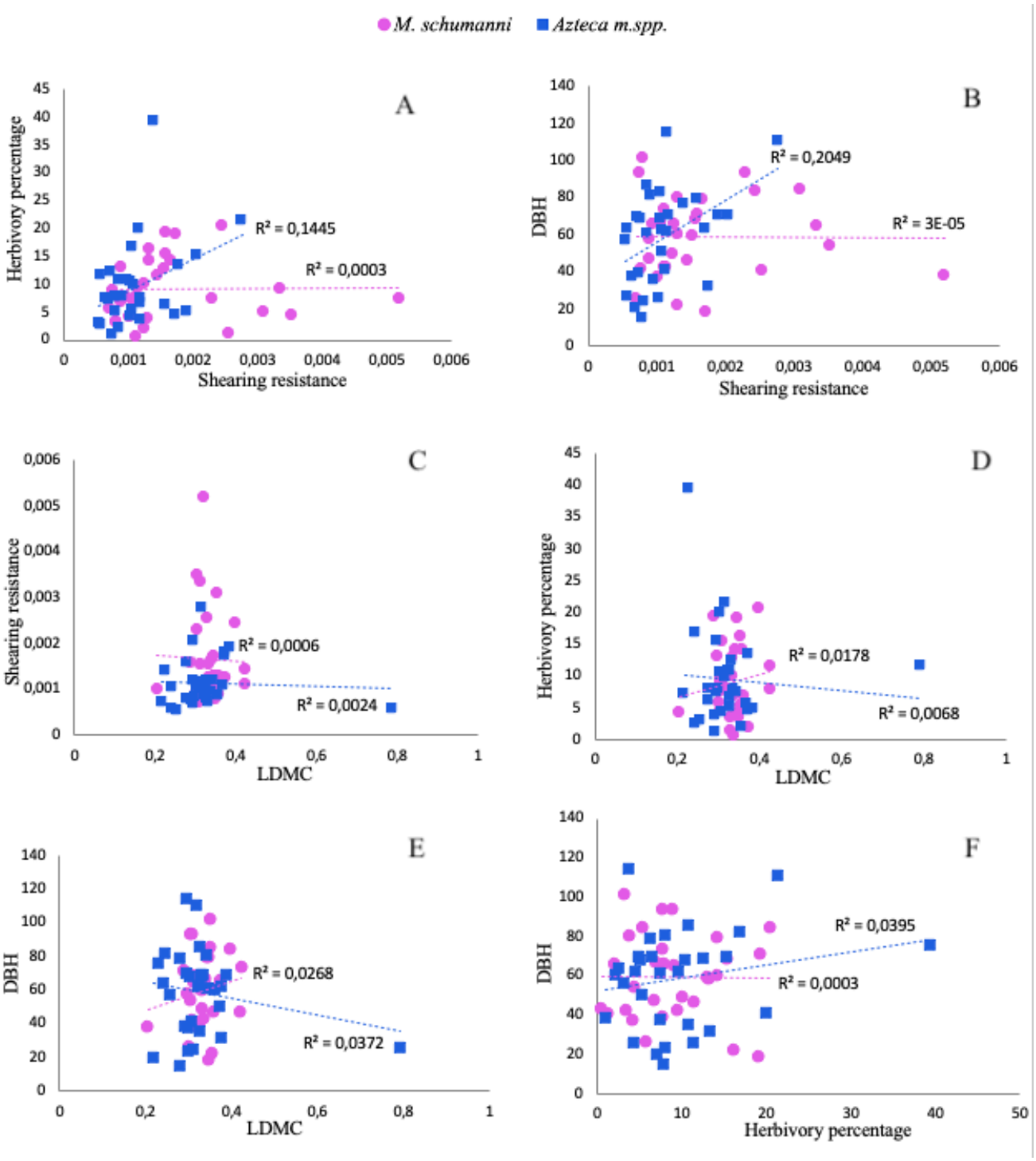


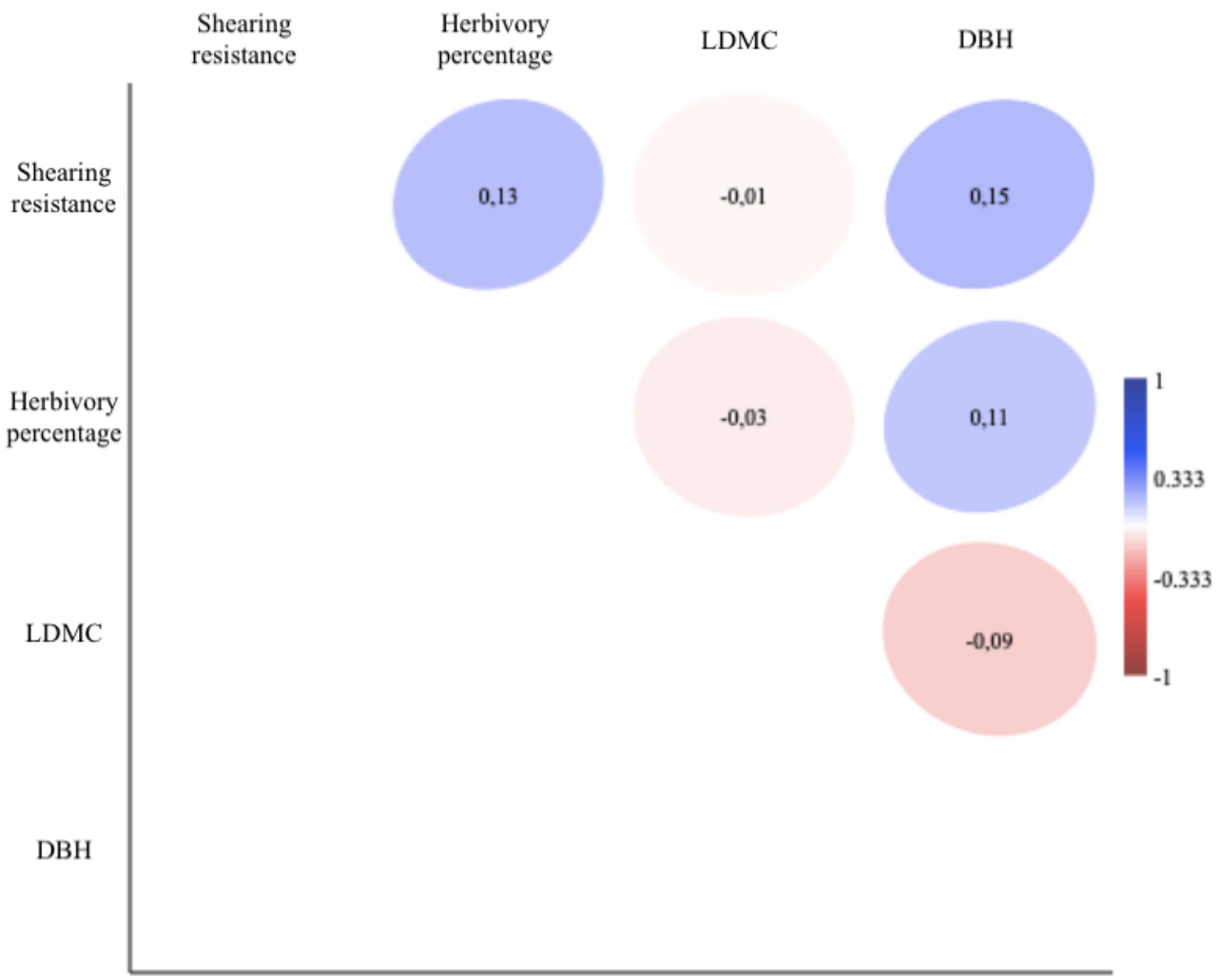
Figure 4. Box plot of the averages of the shearing resistance (N × s × mm^{-1}) obtained from all the cuts made on the leaves of each individual. A significantly higher resistance is observed in *M. schumanni* with respect to *Azteca* m.spp. The resistance is obtained by dividing the integral of the curve at the time of the cut (s × N) for the length of the cut in mm.



928 **Figure 5. Box plot of the average of the 59 individuals for both treatments with respect to (mg x**
929 **g-1).** The difference is minimal between the two treatments, the outer lines corresponding to the error
930 bars, the top one indicates the upper decile while the bottom one indicates the lower decile, the
931 transversal line indicates the median of the analyzed data and the "×" inside each rectangle indicates
932 the average of the data. The outer pink and blue dots belong to outliers in the sample.



933 **Figure 6. Linear regression between herbivory percentage, DBH, LDMC, and shearing**
934 **resistance.** Dotted lines show the tendency of the regressions of each treatment and R^2 shows the
935 correlation between variables for each treatment (R^2 closer to 0 means no correlation and $R^2 \geq 0.5$
936 means correlation). (A) Herbivory percentage vs. shearing resistance. (B) DBH vs. shearing
937 resistance. (C) Shearing resistance vs. LDMC. (D) Herbivory percentage vs. LDMC. (E) DBH vs.
938 LDMC. (F) DBH vs. herbivory percentage.



939 **Figure 7. Pairwise Pearson's correlation between herbivory percentage, DBH, LDMC, and**
940 **shearing resistance for both treatments.** Values indicate no correlation between variables (i.e.
941 values 0.5 < 0 means no correlation) for *D. hirsuta* in association with both *M. schumanni* and *Azteca*
942 m.spp.

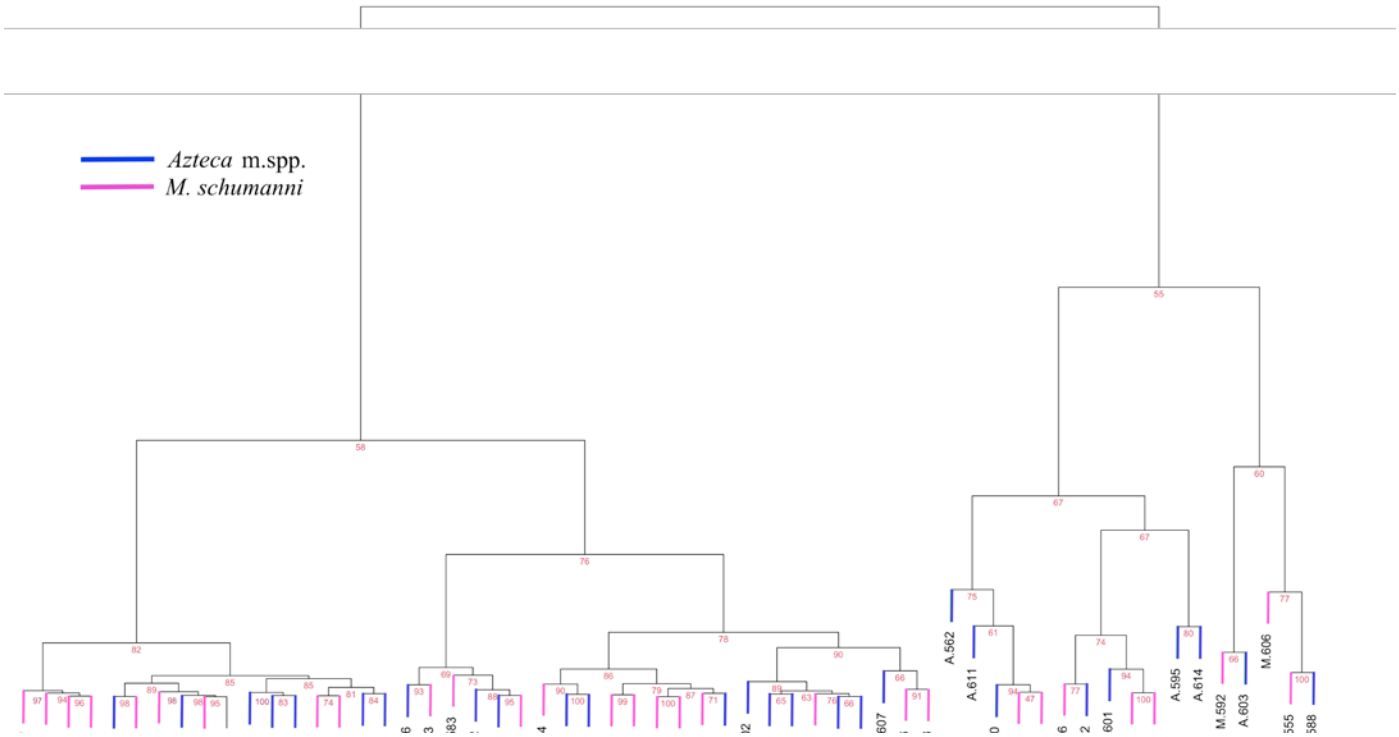
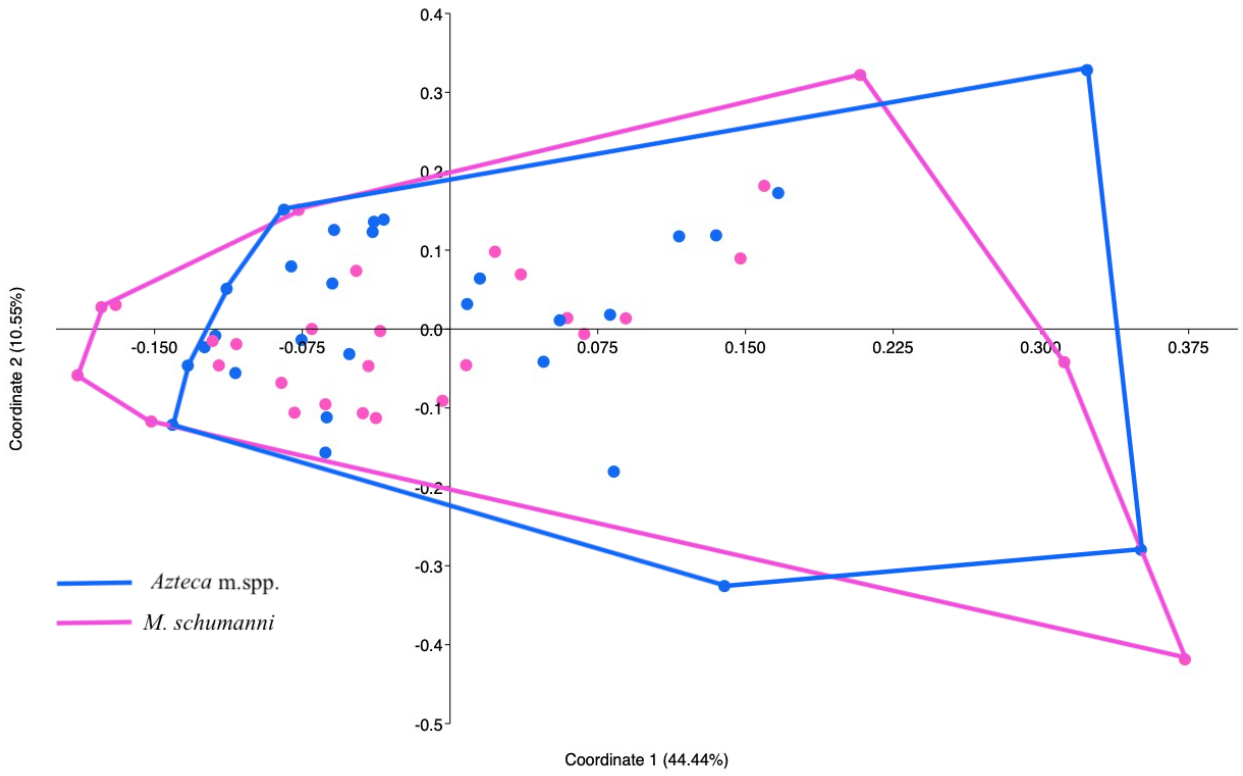


Figure 8. Hierarchical cluster dendrogram of *D. hirsuta* associated with *M. schumanni* and *Azteca* m.spp. The dendrogram is based on the similarity matrix of relative abundances of UPLC-MS metabolites and shows that both treatments are chemically similar because trees with *M. schumanni* and *Azteca* m.spp. are mixed in the clusters. Red numbers represent the Approximately Unbiased (AU) p-values; these indicate the probability that the samples below that point are a cluster. Clusters with values of 95 signify P = 0.05, indicating that these clusters are strongly supported by the data.



949 **Figure 9. Principal Coordinates Analysis (PCoA) of chemical similarity matrix.** Individuals of
950 *D. hirsuta* in mutualism with *M. schumanni* (pink) and *Azteca* m.spp. (blue) show an overlap in the
951 PCoA graph made from the chemical similarity matrix. This indicates that both treatments are
952 chemically similar.

953

Supplementary material

Appendix S1. Two trees of *D. hirsuta* in different microhabitats as a consequence of different ants associations.

Left: *D. hirsuta* tree in a “Devil's Garden” in mutualism with *M. schumanni*.

Right: *D. hirsuta* tree in the understory in mutualism with *Azteca* m.spp.

Images taken from Utreras (2022).

References

Utreras, D. (2022). Defensas físicas y crecimiento secundario de *Duroia hirsuta* (Rubiaceae) en contraste al mutualismo que presenta con hormigas en el Parque Nacional Yasuní. [B.S. dissertation thesis]. Pontificia Universidad Católica del Ecuador, Quito, Ecuador.