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Extrafloral nectaries in *Nephelium lappaceum* (Sapindaceae)

Nectarios extraflorales en *Nephelium lappaceum* (Sapindaceae)

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Abstract
Background: There is no previous evidence in the literature that documents the presence of extrafloral nectaries (EFNs) or the exomorphology of domatia in *Nephelium lappaceum* (rambutan), a fruit tree native to Malaysia introduced in various American countries.
Question: It is the presence of EFNs responsible for the frequent visit of ants to young leaves of *Nephelium lappaceum*?
Study site and dates: This study was conducted in a commercial rambutan orchard in the town of Francisco I. Madero, municipality of Frontera Hidalgo (Chiapas, Mexico) during the period September 2020-February 2021.
Methods: External morphology of EFNs and domatia were studied using scanning electron microscopy. Nectar sugar concentration produced by EFNs was determined by refractometry. EFNs location on the plant and the frequency of visiting ants were quantified by visual counting.
Results: A description of the external morphology of EFNs and domatia, as well as their location on rambutan leaflets are presented. A volume of 0.3 ± 0.2 µL/EFN of a nectar (9.3 ± 0.6 °Bx) was collected each day. Ten species of ants feeding on the nectar excreted by the EFNs were identified.
Conclusion: Evidence of the presence of NEFs on rambutan leaflets is presented, which have an external morphology and location different from that observed in domatia, producing a sweet nectar used as food by a large community of ants.
Key words: Ants, domatia, leaflet, nectar, rambutan.

Resumen
Antecedentes: No hay evidencia previa en la literatura que documente la presencia de nectarios extraflorales (NEFs) o la exomorfología de domacios en *Nephelium lappaceum* (rambután), un árbol frutal nativo de Malasia introducido a varios países de América.
Pregunta: ¿Es la presencia de NEFs responsable de la frecuente visita de hormigas a hojas jóvenes de *Nephelium lappaceum*?
Sitio y fecha del estudio: Este estudio fue conducido en un huerto comercial de rambután en la comunidad de Francisco I. Madero, municipio de Frontera Hidalgo (Chiapas, México) durante el período septiembre 2020-febrebro 2021.
Métodos: La morfología externa de NEFs y domacios fue descrita usando microscopía electrónica de barrido. La concentración de azúcar del néctar producido por los NEFs fue determinada por refractometría. La localización de NEFs sobre la planta y la frecuencia de hormigas visitadoras fueron cuantificadas visualmente.
Resultados: Una descripción de la morfología externa de NEFs y domacios, así como de su localización sobre los foliolos de rambután son presentadas. Un volumen de 0.33 ± 0.2 µL/ NEF de un néctar (9.3 ± 0.6 °Bx) fue recolectado cada día. Diez especies de hormigas alimentándose del néctar excretado por los NEFs fueron identificadas.
Conclusión: Se presentan evidencias de la presencia de NEFs sobre folíolos de rambután, que poseen una morfología externa y localización diferente de la observada en domacios, produciendo un néctar dulce usado como alimento por una amplia comunidad de hormigas.
Palabras clave: Domacio, folíolo, hormigas, néctar, rambután.

Many plants have developed some defensive strategies against herbivory. Some of them possess themselves structures such as extrafloral nectaries (EFNs), food bodies and domatia, used by insects as food or refuge (Janzen 1969, Del Val & Dirzo 2004). EFNs are nectar-secreting glands not directly involved with pollination and may eventually mediate the establishment of insect-plant mutualism (Gonzalez 2011, Chomicki *et al*. 2015, Dáttilo *et al*. 2015). Plants with EFN are more ecologically successful, since these glands confer a coevolutionary advantage in the form of defense against herbivory (Escala & de Enrech 1991, Horvitz & Schemske 1994, Chomicki *et al*. 2015, Dinda & Mondal 2015). EFN mediated mutualism with plants has been documented for a wide variety of arthropods mainly spiders and insects (Ruhren & Handel 1999, Marazzi *et al*. 2013). For example, the EFNs of *Eriotheca gracilipes* (K. Schum.) A. Robyns (Malvaceae), are visited by spiders that protects the plant from herbivores and allows it to produce fruit and viable seeds (Stefani *et al*. 2015). EFNs might also play a prominent role in shaping arthropod communities, which include predators, parasitoids, as well as herbivores (Bentley 1977, Heil 2008). For instance, *Exoplectra miniata* Germar (Coccinellidae), a predator reported feeding on the extrafloral nectar of *Inga edulis* Mart. (Almeida *et al*. 2011). The protection of *Solanum adhaerens* Willd. ex Rhoem. & Schult. (Solanaceae) by certain lepidopteran parasitoids against herbivory can be cited as an example of EFN-mediated facultative protection mutualism (Gentry 2003).

Hymenopterans are the most common insects that exploit plant nectar in plant-animal mutualistic interactions (Del-Claro *et al*. 2016). Ants stand out from this large group of insects as the herbivores commonly associated with plants with which they maintain an EFN-mediated facultative mutualistic relation. Ants are very effective in safeguarding plants against herbivory. Their aggressive stance, their social behaviour, and -for some species- in particular their venom, are the tools of interchange offered by ants for a nectar rich in sugars and essential amino acids (Parker & Kronauer 2021). *Vicia sativa* L. var. *angustifolia* L. (Fabaceae) is one of many plants that successfully carry out this exchange, whose EFNs attract ants which then protect the plant against herbivory, and consequently allow rapid growth (Katayama & Suzuki 2011). The frequent visits made by ants and other arthropods to rambutan foliage suggested the probable presence of ENFs in this Sapindaceae species. The Sapindaceae family contains approximately 1,630 species from more than 140 genera. Yet, a mere 14 species from eight genera have been reported to possess EFNs (Weber *et al*. 2015). Particularity, *N. lappaceum* leaves have domatia used as a taxonomic feature to identify species belonging to *Nephelium* genus (Leenhouts 1986) and that are also found in species of other genera belonging to the family Sapindaceae (van Welzen 1997). Domatia are natural cavity present in some plant species, which are used as a refuge site by arthropods, as predatory and fungivores mites (Pemberton & Turner 1989). However, to our knowledge, the occurrence of EFNs in rambutan has not been previously documented and there is no information about exomorphology of domatia in rambutan, aspects that could be useful to discuss about the ecological importance of these structures in their natural habitat. We hypothesize that the presence of EFNs attract ants to *N. lappaceum* leaves. Therefore, this work aimed to demonstrate the occurrence of nectaries in rambutan leaflets based on the following evidence: i) comparison of its external morphology, frequency, and location on the plant in relation to domatia, ii) quantification of the volume and sweetness of the nectar excreted, and iii) description of the community of ants visiting EFN.

Materials and methods

Study site. Field observations were conducted in a commercial rambutan orchard (14° 45’ 7.47” N, 92° 11’ 23.53” W; 52 m asl) in the town of Francisco I. Madero, municipality of Frontera Hidalgo, Chiapas, Mexico. The orchard covers an area of 2 ha and is planted with grafted trees (cultivar Jitlee, 180 trees per ha, tree height range 2.70 - 3.30 m). Orchard management practices include pruning, weeding, fertilizing, micro-sprinkler irrigation, and refraining from the use of pesticides.

Frequency and location of extrafloral nectaries and domatia. Leaf samples were taken for the purpose of counting and analysing the location of extrafloral nectaries and domatia on leaflets. To this effect, leaves free of herbivore damage were gathered from a randomly-chosen tree in the orchard. Four or five leaflets per tree were collected and transported to the laboratory in HDPE bags at 25 ºC for observation under stereomicroscope (Wild M5A, Wild Heerbrugg, Heerbrugg, Switzerland). A leaflet was defined as one of the separate segments of the blade of a compound leaf of rambutan (Ash 1999). In total, 90 young and 86 mature leaflets were collected from 15 trees. The number of nectaries and domatia located on the leaves underside (abaxial leaf surface) was determined in three parts of each leaflet: (i) in the apex, (ii) near the midrib, and (iii) in the rest of the leaflet, over the collected leaves. The frequency and distribution were also compared between young (apical) and old (basal) leaflets of the same samples.

External morphology of extrafloral nectaries and domatia. The external morphology of EFNs and domatia located on the leaf surface of rambutan was studied analysing eight nectaries, five domatia and seven nectaries associated with domatia by scanning electron microscope (SEM). The external characteristics were compared fresh between young and mature leaves. Plant material was prepared by fixation in FAA for 30 min (formalin, acetic acid, and alcohol 70 %, 5:5:90), followed by dehydration in an alcohol series (80, 90, 96 % for 30 min each), critical point drying and coating with gold/palladium. The processed samples were examined under a Topcon SEM (SM-510, Topcon, Tokyo, Japan) at Laboratorio de Microscopía Electrónica de Barrido of El Colegio de la Frontera Sur in Tapachula, Chiapas, Mexico. The average diameter of these structures was calculated from five measurements.

Volume and sugar concentration of extrafloral nectar. We estimated the number of EFNs that had to be sampled to obtain three µL of nectar (minimum volume required by the refractometer), taken from the same tree. Extrafloral nectar was drawn into a five µL micropipette tip (Blaubrand, Brand GmbH & Co KG, Wertheim, Germany) by capillary. The estimated volume and sugar concentration of secreted nectar was compared between nectaries on the leaf apex (n = 11) and those on vein bifurcations (n = 10), as well as between young (n = 10) and mature (n = 10) leaflets. All samples were collected in the dry season (January-February 2021) to prevent interference from the rain on the composition of the nectar and were taking overnight (21:00 - 6:00 h) to avoid the evaporation of the content of the nectaries. Nectar sugar concentrations (three µL sample volume) were measured using a handheld refractometer (Atago, Tokyo, Japan). The results were expressed as degrees Brix (°Bx) and converted to mass concentration (µg/µL) as described by Weast (1984).

Ant visitation of extrafloral nectaries. Ant's visitors of the EFNs to feeding on their nectar were collected in the rainy season (September-October 2020), which coincides with the vegetative growth phase of rambutan tree. They were collected at the four cardinal points of a tree using an insect aspirator and a paintbrush. Ant sampling was conducted three times, each time on 10 randomly chosen trees and between 7:00-12:00 h (every 15 days, total of 30 samples). The ants were preserved in 70 % alcohol until taxonomic identification. The average visit time (from arrival to departure) was recorded for 40 ants.

Results

Frequency and location of extrafloral nectaries. The average (± standard error) EFN frequency was 8.2 ± 0.4 per leaflet. All EFNs were located on the abaxial leaf surface and distributed over the three leaflet parts as follows: i) EFNs in the apical part (68 %) (Figure 1A), assembled in groups of 2-13 glands (average of 5.6 ± 0.2 per leaflet); ii) individual EFNs along the midrib (31 %, average of 2.6 ± 0.2 per leaflet; Figure 1B), which may be associated with domatia or not; iii) isolated EFNs scattered over other areas of the abaxial surface (1 %, average of 0.08 ± 0.02 per leaflet; Figure 1C). The leaflet apex exhibits several separate EFNs which together form a glandular field (Figure 1A). Individual EFNs located near the midrib can be found adjacent to, partially inside, or distant from a domatium (Figure 2A, B, and C, respectively). The average number of EFNs on young and mature leaves was 9.3 ± 0.3 and 7.0 ± 0.3, respectively.

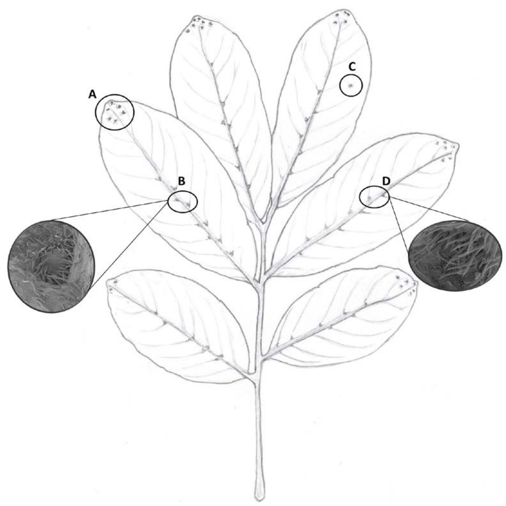

Figure 1. Abaxial side of rambutan leaf with the three EFN-distribution areas indicated. A) glandular field near the leaf apex. B) EFNs distributed along the midrib, which may be associated with domatia or not. C) Isolated EFNs scattered over other areas of the abaxial surface. D) Domatia localized in the axils formed by the midrib and the secondary veins.

Frequency and location of domatia. Like EFNs, domatia are distributed across the abaxial leaf surface. They are, however, only found in the axils between the midrib and the secondary veins (Figure 1D). Domatia are invariably located near the leaflet's midrib and occur either on one side or on alternate sides of it. They are not present in every axil and can be either solitary (Figure 1D) or associated with EFNs (Figure 1B). An average of 8.3 ± 0.2 domatia was detected per leaflet (7.4 ± 0.4 in young and 9.1 ± 0.3 in mature leaves).

External morphology of extrafloral nectaries. EFNs have a circular or elliptical shape and a smooth surface that is surrounded by simple trichomes (Figure 2A and B). In young leaves, these glands are elevated yellow structures (Figure 3A), whereas they have a withered brown appearance in mature leaves (Figure 3B). This morphology is the same for all nectaries regardless of their location on the leaf. The gland diameter ranges from 285 to 541 µm [average size (± SE): 406.4 ± 34.4 µm], randomly distributed on the abaxial face of the leaflet.

External morphology of domatia. These structures are form of funnel-shaped cavities (Figure 2A, B and D). A group of simple trichomes protrudes from the domatium cavity and it cover the triangular area formed by the vein bifurcation. The size of domatia range from 295.5 to 520.7 µm (387 ± 39.4 µm).

Volume and sugar concentration of extrafloral nectar. The nectar of on average 9 ± 0.6 EFNs from young leaflets was pooled to obtain a sample volume of three µL. Mature leaflets, in contrast, did not produce nectar. These EFNs secrete droplets (Figure 3C and D) with an average volume of 0.33 ± 0.2 µL and an average sugar concentration of 9.3 ± 0.6 °Bx (corresponding to 290.8 ± 19.5 µg total sugar per sample, or 32.3 ± 6 µg per gland). Nectar samples collected from the leaflet apex and midrib contained, respectively, 288.8 ± 26.8 µg and 293.1 ± 29.9 µg total sugar on average.

Ant visitation of extrafloral nectaries. Ten species belonging to five ant subfamilies were observed visiting the EFNs on young leaflets. Formicinae was the subfamily with highest representation (three species, see Table 1). Ants visited the leaflets to feed on the extrafloral nectar either individually or in group. *Solenopsis geminata* Fabricius was frequently seen to visit EFNs and feed on their nectar in group (Figure 4A). *Forelius pruinosus* Cole was the most common ant species (107 worker ants collected from EFNs on young leaflets) (Figure 4B), followed by *Crematogaster rochai* Forel (19 worker ants) (Figure 4C). The highest number of ants observed feeding simultaneously on EFNs on the same leaflet was six (*C. rochai*). Larger ant species, such as *Camponotus* sp. and *Ectatomma ruidum* Roger were also observed feeding on extrafloral rambutan nectar (Figure 4D and E). The different ant species did not associate with each other to simultaneously share the same EFN or leaflet. However, some species (*Camponotus* Mayr, *C. rochai*, *F. priunosus* and *Pseudomyrmex* Lund) were found foraging on the same rambutan tree. The average EFN visit time was 1.2 ± 0.01 min for 40 ants of different species that visited the nectaries. A variety of other arthropods was noticed to feed on the extrafloral nectar as well adult specimens of various species belonging to the Hymenoptera (families Encyrtidae, Eucharitidae, Braconidae and Vespidae), flies (family Stratiomyidae) and phytophagous bugs (family Miridae), but also larvae of Chrysopidae, mites, and spiders.

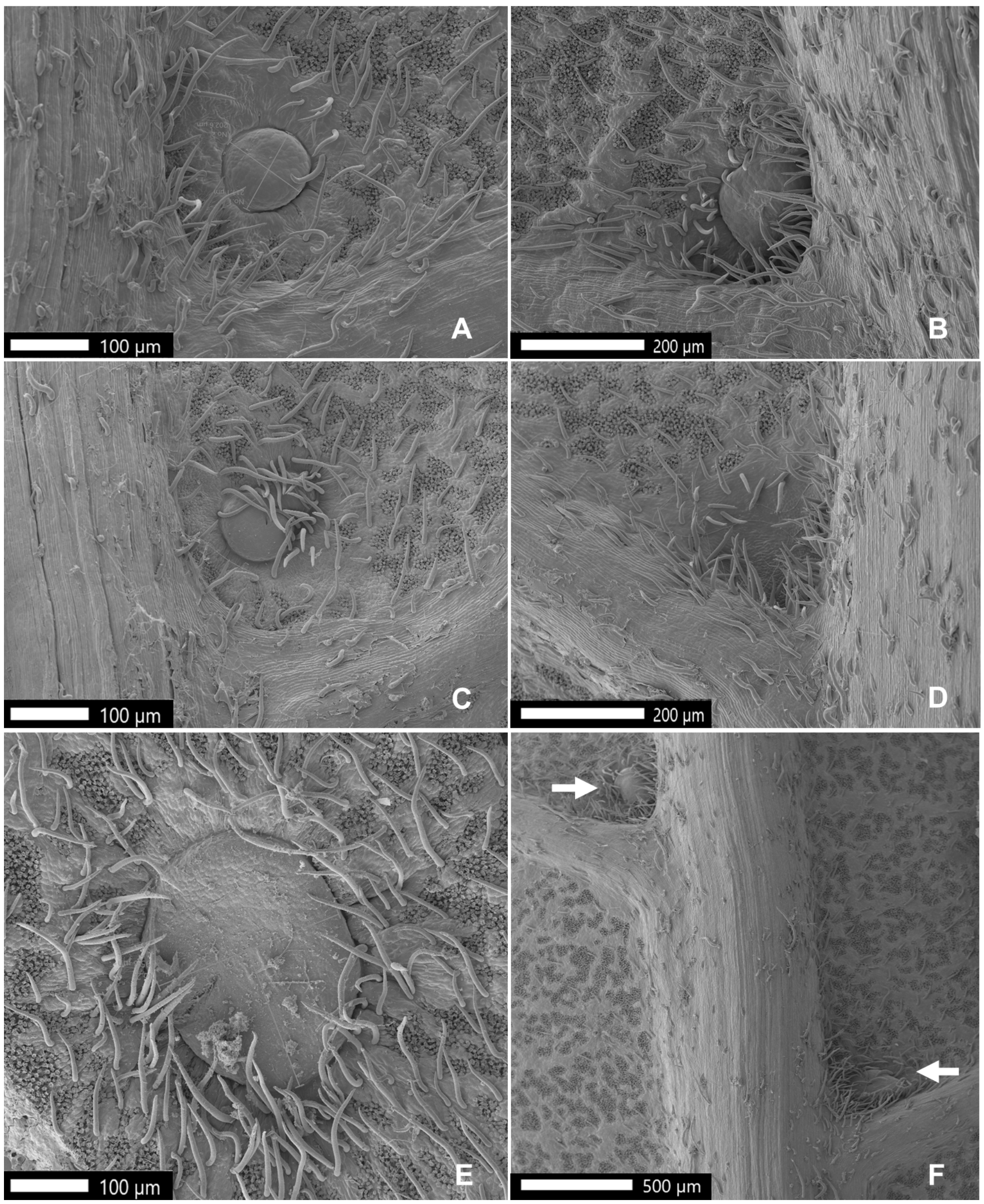

Figure 2. Extrafloral nectaries (EFNs) and domatium located in different parts of the abaxial leaf surface of rambutan. EFN near domatium (A), EFN partially inside domatium (B), EFN without domatium (C), domatium without EFN (D), EFN located near the leaf apex (E), and EFN between midrib and secondary vein (F). The arrows point towards extrafloral glands in the axil.

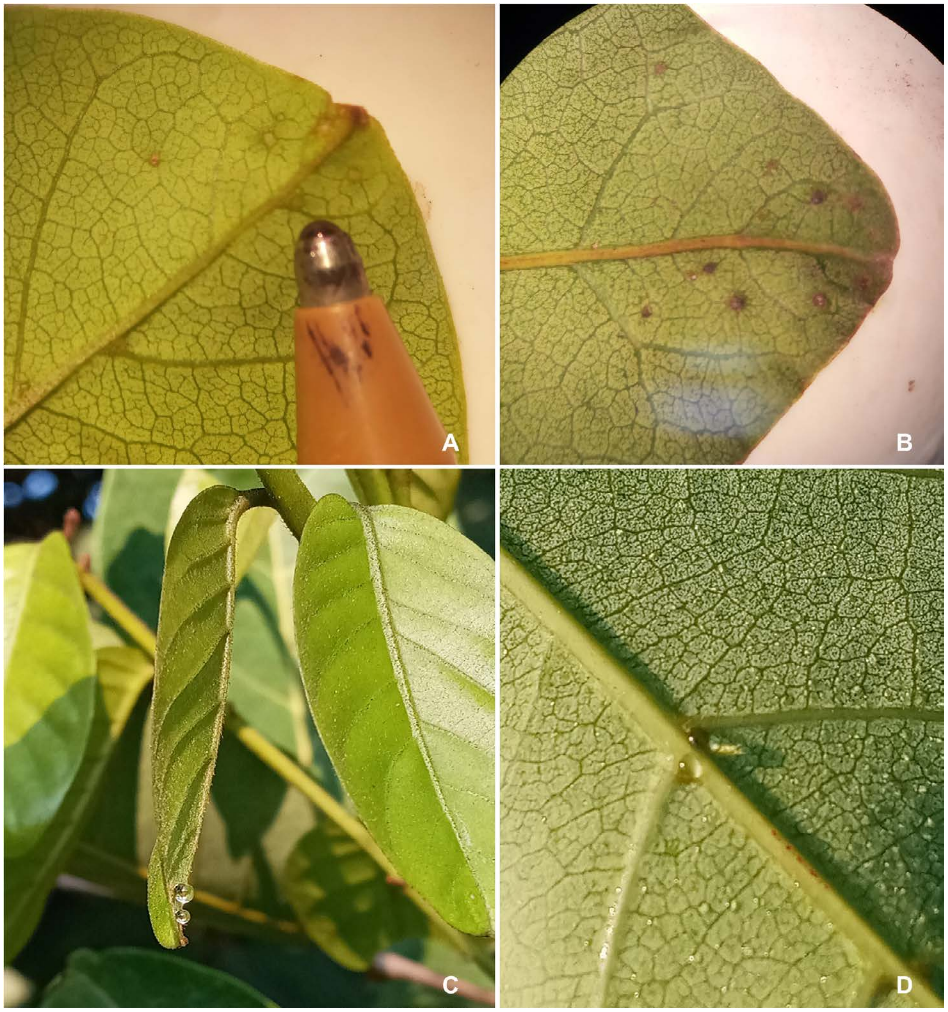

Figure 3. Rambutan leaflet showing young (A) and mature (B) EFNs. Nectar droplets excreted by EFNs near the leaf apex (C) and near the veins (D).

Table 1. Frequency of different ant species (Formicidae) that visited extrafloral nectaries on the leaves of rambutan (*Nephelium lappaceum*).

Subfamily	Species	Number of Individual
Formicinae	*Brachymyrmex minutus* Forel, 1893	15
	Camponotus sp.	1
	Paratrechina longicornis Latreille, 1802	14
Dolichoderinae	*Dorymyrmex sp.*	2
	Forelius pruinosus Roger, 1863	107
Pseudomyrmecinae	*Pseudomymex sp.* 1	1
	Pseudomymex sp. 2	1
Myrmicinae	*Solenopsis geminata* Fabricius, 1804	10
	Crematogaster rochai Forel, 1903	19
Ectatomminae	*Ectatomma ruidum* Roger, 1860	10

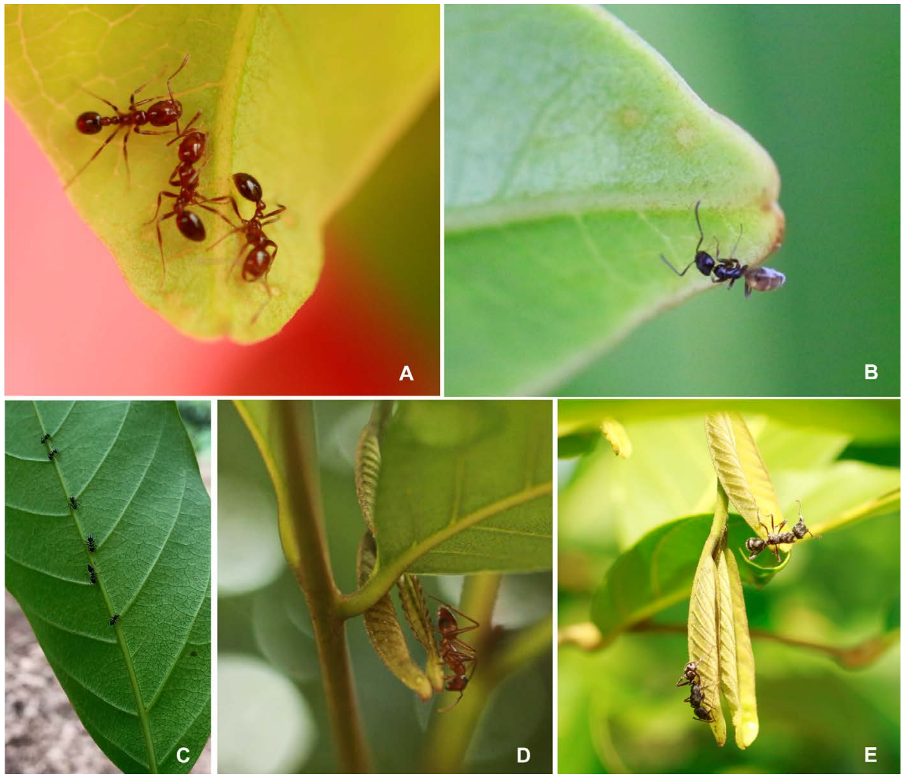

Figure 4. Ants *Solenopsis geminata* (A), *Crematogaster rochai* (B), *Forelius pruinosus* (C), *Camponotus* sp. (D) and *Ectatomma ruidum* (E) on a rambutan leaflet feeding on the nectar excreted by EFNs.

Discussion

This study reports for the first time the occurrence of extrafloral nectaries in *Nephelium lappaceum* by the characterization of its external morphology and location on leaflets in relation with domatia, as well as by the detection of a sweet excretion used as food by a community of ants. Although the occurrence of EFNs has been documented in Sapindaceae, such as *Chimborazoa lachnocarpa* Benth. ex Radlk. (Beck 1992), there are so far no reports of this type of secretory structure in other species of *Nephelium*. On the other hand, it is difficult to associate the external morphology of EFN between species of this family, since few studies have explored the anatomy and morphology of these glands in Sapindaceae (Tölke *et al*. 2022). Consequently, EFNs described in this work have an external morphology similar to the nectaries described in Bignoniaceae. In several species of the latter family have been reported nectaries as circular or elliptical structures, surrounded by simple trichomes that protrude slightly from the leaf surface (Gonzalez 2011, 2013). The location, size, and form of EFNs among the Sapindaceae are diverse and may possibly play a mutualistic role with ants in plant defense. For example, *Pometia pinnata* J. R. Forst. & G. Forst., possesses three EFN groups with a form and location distinct from those of rambutan (Moog *et al.* 2008). The largest EFNs in *P. pinnata* are located at the base of the leaflets close to the midrib, small nectaries on the underside or edge of the leaflets, and smaller nectaries along both sides of the midrib (Moog *et al.* 2008). However, the nectaries in the Sapindaceae have in common that they mainly attract ants to protect themselves against herbivores. This function contrast with floral nectaries, which only are involved in pollination (Bentley 1977, Elías & Prance 1978, Heil 2008, Weber & Keeler 2013, Marazzi *et al*. 2013, Rodríguez-Morales *et al*. 2016). Therefore, this first report of the presence of EFN's in rambutan offers broad expectations for future studies related to its biological interactions.

The presence of domatia on the leaflets of *N. lappaceum* has been previously documented as a taxonomic characteristic of this species (Leenhouts 1986). In accordance with the classification by Wilkinson (1979), who proposed the categorization of domatia into five types, rambutan was observed to possess pouch-shaped domatia in the axils of primary and secondary veins. While rambutan domatia are shaped like cavities, the EFN´s are flattened glands (Flachnektarien) according to the Zimmermann classification (Zimmermann 1932). The association between EFNs and domatia observed in this work suggests that, even though rambutan domatia and EFNs in rambutan are morphologically and functionally different from one another, they seem to provide food and permanent refuge simultaneously, as has been described for other plants as well (Gonzalez 2011). However, there is a need for continuing studies to determine which organisms occupy the domatia. This is also relevant for mature leaves, where these structures could be used by ants, mites, wasps and other invertebrates as a nest or refuge site in exchange for protection against fungi and herbivores (Krombein *et al*. 1999, Moog *et al*. 2008).

We noticed that EFNs from young leaves excrete nectar but did not excrete nectar on mature leaves, which could be useful potentially to investigate the possible protective role of ants to rambutan. It is possible that EFNs represent a constitutive defence of new foliage against herbivores, which is also consistent with the Optimal Defence Theory (Rhoades 1979). The latter predicts that a plant's fitness is negatively impacted by damage to young growth, which hence will be better protected against herbivores (Dáttilo *et al*. 2015). Rico-Gray *et al*. (2008) also recorded an increased extrafloral nectar production in the young leaves of other plant species. Although our data were derived from trees that were already producing, training pruning applied to trees after harvest encourages the appearance of young foliage, which explains the intense ant activity on apical leaflets.

Extrafloral nectar attracts a more limited set of insects than the floral nectar, mainly ants that keep herbivores away (Pacini & Nicolson 2007). *Nephelium lappaceum* is an exotic species in a neotropical environment interacting with local arthropods, so the ants recorded in this work probably does not have any mutualistic interactions with the plant. Probably, several of this species recorded in this work are merely opportunistic visitors of EFNs. In this context, it is worth noting that except for the Ectatomminae subfamily, the species documented in our study belong to the same subfamilies as the EFN-visiting ants described in a Brazilian report (Oliveira & Pie 1998). In another publication from Brazil, three species of the genus *Camponotus* (Formicinae) were identified as protectors of the EFN-bearing tree *Qualea grandiflora* Mart. against termites (Oliveira *et al*. 1987). However, according to other reports, the presence of ants does not always reduce the abundance of herbivores. In this respect, the ant *Crematogaster peringueyi* Emery has been documented to prey on eggs of certain herbivores without affecting the number of larvae (Rashbrook *et al*. 1992). Hence, further work is needed to investigate the role of the ENF in rambutan-ants interactions.

Our results show that EFNs rambutan excrete a sweet nectar attractive to a diverse crop-associated neotropical fauna, in addition to ants. Our results are unprecedented since there are no previous studies regarding the concentration of sugar and volume of nectar produced by EFNs in Sapindaceae; although it is known that generally the quality of extrafloral nectar no vary between species and each nectary produce only few microliters per day (Pacini & Nicolson 2007). Probably EFNs in rambutan produce nectar as result of a plant-animal mutualistic interaction in its original geographic region, being a valuable resource for ants and other EFN-visiting insects (Moog *et al*. 2008, Mathews *et al*. 2009, Meng *et al*. 2012). The extrafloral nectar of other species contains sugars (mainly fructose, glucose and sucrose), essential amino acids, mineral ions, tricarboxylic acid, and water (Luttge & Schnepf 1976, Ruffner & Clark 1986, Grasso *et al*. 2015). It is recognized that nectar quality and composition can temporally vary in relation to plant reproductive phenology (Ruffner & Clark 1986). Therefore, a more precise evaluation of the chemical composition of extrafloral rambutan nectar is necessary.

The discovery of EFNs in rambutan may have broad and important implications for the success of rambutan plantations. The manipulation of mutualistic interaction structures (*i.e.* the protection mutualism in ant-plant interactions) has been put forward as an alternative to reduce herbivory in commercial plantations (Mathews *et al*. 2009, Jones *et al*. 2017, Heil & McKey 2003). However, the presence of extrafloral nectaries might also facilitate the establishment of unwanted species in commercial rambutan plantations, such the fire ant *S. geminata,* an invasive species originating from this Neotropical region. The distribution of plantations of *N. lappaceum* in tropical countries, makes it necessary to study the interactions between the rambutan and the native fauna of the Neotropics, as well as the analysis of the unanticipated ecological consequences of rambutan's introduction and growing expansion of this species in the Americas.

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