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A New Extrafloral Nectary-Bearing Plant Species in the Brazilian Savanna and its Associated Ant Community: Nectary Structure, Nectar Production and Ecological Interactions

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Abstract

Brazilian Savanna stands out for the large number of species with extrafloral nectaries (EFNs) with high morphological diversity. In *Smilax polyantha* (Smilacaceae), the base of the petiole showed a slight secretion and great visitation by ants suggesting the presence of an EFN. In this way, we aimed to determine the ant community associated with this plant, as well as to identify and characterize this unsuspected structure and determine the phenology and liquid production of this tissue. The study was carried out in a Cerrado area, in Uberlândia, Brazil. Ten individuals were used for the anatomical analysis and histochemical tests and the EFNs secretory activity was monitored, being categorized into active and non-active EFN. In addition, the volume and sucrose concentration were determined from three nectaries of ten individuals, and individuals of ants found foraging on these nectary were collected, day and night. Results showed a large amount of extrafloral nectar secretion and the EFN tissue is composed of a few cell layers that showed positive reactions for proteins and reducing sugars. We recorded a significantly correlation between percentage of EFNs activity and abundance of ants. The secretory activity is concentrated in September and ten ant species, of five subfamilies, were identified foraging on the EFNs.

Introduction

Tropical regions show a high diversity and distribution of plants bearing extrafloral nectaries (EFNs) (Oliveira & Leitão-Filho, 1987; Koptur 1992; Rico-Gray & Oliveira, 2007) and many of them are morphologically simple and composed of only a few layers of secretory cells (Machado et al., 2008). Escalante-Pérez and Heil (2012) have characterized the anatomy and ultrastructure of nectariferous tissue as a secretory epidermis and specialized nectariferous parenchyma that produces or stores the pre-nectar; the cells commonly have dense cytoplasm and a well-developed membrane system (Fahn, 1979; Nepi, 2007). This secretory tissue can be connected to the phloem, xylem or both; however, in some cases, the nectaries do not show any vascular connections (Fahn, 1988; Wist & Davis, 2006). Thus, anatomical and histochemical studies can aid the identification and characterization of inconspicuous nectaries, such as what we will present here.

EFNs are structures that produce a liquid substance, the extrafloral nectar, rich in carbohydrates andother diluted compounds (e.g. lipids, amino acids, etc.) (Baker & Baker, 1983; Koptur, 1994; González-Teuber & Heil, 2009), which attracts several arthropods for feeding (Marazzi et al., 2013). These arthropods, such as ants, can play an important role as biotic defenders of plants against herbivore attack (Rico-Gray & Oliveira, 2007; Heil, 2015).In return, there is a significant gain in survival and reproduction by extrafloral nectar feeding (Byk & Del-Claro, 2011).

The EFNs are observed in a wide variety of species from different plant families (Koptur, 1992; Weber & Keeler, 2013; Weber et al., 2015). In addition, they exhibit great morphological diversity and can be found in different sites on the plant (see Elias, 1983; Díaz-Castelazo et al., 2005; Aguirre et al., 2013). Some studies have been conducted in Cerrado to identify the species of EFNs-bearing plants (Oliveira & Leitão-Filho, 1987; Oliveira & Oliveira-Filho, 1991; Machado et al., 2008) and more recently the extrafloral nectar production and quality (Lange et al., 2017). Most of these plants have prominent, hollow and flat EFNs, which are easily observed in the field (Oliveira & Leitão-Filho,1987; Machado et al., 2008). However, we suspect that many inconspicuous EFNs have been overlooked in Cerrado plants and they are more common than was previously thought. These secretory tissues can frequently be identified by a visible secretion and by intensive visitation by ants and even wasps and spiders (Stefani et al., 2015).

Smilax L. is the largest genus of Smilacaceae, with species found in Temperate, Subtropical (Koptur, 1992) and especially in Tropical regions (Andreata, 2009). Koptur (1992) found four species of *Smilax* containing EFNs: *S. auriculata* Walt., *S. bona-nox* L., *S. havanensis* Jacq., *S. laurifolia* L. Four other species are mentioned in the taxonomic distribution database of species with EFNs (see Weber et al., 2015): *S.*cf. *australis* R.Br. (Blüthgen & Reifenrath, 2003), *S. bracteata* C. Presl, *S. lunglingensis* F.T. Wang and Tangand *S. perfoliata* Lour. (Liu & Chen, 2008). None of these species have been recorded in Brazil (Andreata, 1997). However, *S. polyantha* is recorded in Brazil and the base of the petiole is unsuspected for the presence of EFNs, which shows a viscous secretion that attracts intense visitation by ants.

The focus of this study was to determine the ant community associated with this plant, as well as to identify and characterize this unsuspected structure of *Smilax polyantha* and determine the phenology and liquid production of this tissue. In this way, our, main hypothesis is *S. polyantha* has EFNs at the base of petiole.

Material and Methods

Study area

The study was carried out in Clube Caça e Pesca Itororó de Uberlândia (CCPIU), Uberlândia, Minas Gerais, Brazil (18°56′21″ S, 48°16′14″ W). The climate in this region is characterized by two distinct seasons: a dry season (from May to September) and a rainy season (from October to April) (Ferreira & Torezan-Silingardi, 2013). Studies were carried out between October 2013 and October 2014.

EFN structure and histochemical analyses

The base of one petiole was collected only from branches with fully expanded leaves in ten individuals of *S. polyantha* Griseb. (voucher in *Herbarium Uberlandensis*-HUFU 68459). The anatomical study was performed with material fixed in FAA50 for 48 hours which was dehydrated in a graded butanol series and embedded in Paraplast® (Johansen, 1940; Kraus & Arduim, 1997). The samples were sectioned using a rotary microtome (12 - 14μm thick), stained with Astra Blue and Safranin (Bukatsch, 1972 modified to 0.5% v/v) and mounted with Vitral varnish.

Histochemical analyses were performed with fresh material that was free hand sectioned. For the detection of reducing sugars, Fehling's reagent was used, and for lipids,Sudan III (Sass, 1951).Proteins were detected with bromophenol blue (Baker, 1958), starch with Lugol reagent (Johansen, 1940), and pectins and mucilage with Ruthenium red (Jensen, 1962). All histochemical tests were compared with the white samples (control), fresh and sectioned material without reaction. The histological and histochemical slides were photographed on a Leica DM500 microscope coupled to HCHD50 digital camera and analysis software.

EFN phenology

Observations were made on three leaves every two weeks for one year to record EFNs activity (N = 10 plants of equal size and phenology). Nectaries were classified as 'active' when the nectarines were shiny and visited by ants, and 'not active' when the nectarines had necrotic secretory tissue and did not have ant visitation.Circular statistical analyses were done using the variable, 'active' and 'not active' nectary. For this, the interval remarks were converted into angles, first half of October 2013 (0°) to September 2014 (345°) (see Vilela et al., 2014). The percentage of individuals with active EFNs was utilized for the calculation of parameters: vector (m), mean vector length (r), median, standard deviation, Rayleigh test (z) and Rayleigh test (p). The Rayleigh test indicates the seasonality of the data, in which a p-value less than 0.05 means that the data are not evenly distributed throughout the year, suggesting a seasonality of vector r, i.e., concentration around a average. Data were subjected to the statistical software Oriana 4.0.

EFN production

For extrafloral nectar production analyses, the volume and sucrose concentration was measured from three nectarines of ten different plants. The nectar was collected with 5 μL graduated microcapillary tube sand a portable Eclipse refractometer (50% brix) was used to measure the sucrose concentration. Before collection, each EFN was washed with distilled water and dried with filter paper and covered with a voile bag for 24 hours, preventing the nectar from being removed by animals and/or diluted by rain and dew (see Blüthgen et al., 2004; Bixenmann et al., 2011). The samples were conducted in September, between 6 am and 8 am, because only in this period is observed enough secretions to collect.

The concentration of the sugar solution (sucrose equivalent) was calculated using the volume data (µL) and nectar concentration (% brix). The calculation was performed according to the method described by (Dafni et al., 2005), following the equation: $y = 0{,}00226 + (0{,}00937\ x) + (0{,}0000585\ x^2)$, x being the concentration (refractometer reading) and y the amount of total sugars in 1 µl, resulting in a sugar concentration corresponding to the number of milligrams (mg) of sugar per microliter (µL). The amount of sugar found in each nectary was subsequently converted into calories, each mg of sugar equivalent to four calories corresponding to the energy value of the solution (see Lange et al., 2017; Byk & Del-Claro, 2011).

Ants associated

The ants found foraging in the EFNs were collected monthly for one year, through active searches in the daytime and nighttime. We used a Spearman correlation to observe if abundance of ants is correlated with the percentage of EFNs activity throughout the year. One individual of each species was collected, with the aid of forceps, stored in eppendorfs tubes, fixed in 70% alcohol and identified to species level at the Federal University of Parana. We classified as morphospecies using the term sp., when we have not reached the species level.

Results

The base of the petiole is wrapped by an arc-shaped structure that has secretory tissue secreting nectar and intense visitation by ants (Fig 1A-B).This arc-shaped structure (Fig 1C) has a uniseriate adaxial epidermis displaying features typical of secretory cells, such as a thin cuticle and dense cytoplasm (Fig 1D). Adjacent to the adaxial epidermis of the arc-shaped nectariferous tissue, we can note a secretory parenchyma with cells containing dense cytoplasm and conspicuous nucleus. Phenolic-idioblasts are distributed randomly in the secretory tissue. Collateral vascular bundles with large quantities of phloem are distributed along the arc-shaped nectar secretory tissue (Figs 1C, 2A). Perivascular fibers can be observed surrounding the vascular bundles.

Reducing sugars were detected in the adaxial epidermis and secretory parenchyma (Fig 2B-C). Lipids were detected in the cuticle and as small droplets in the protoplasts of the

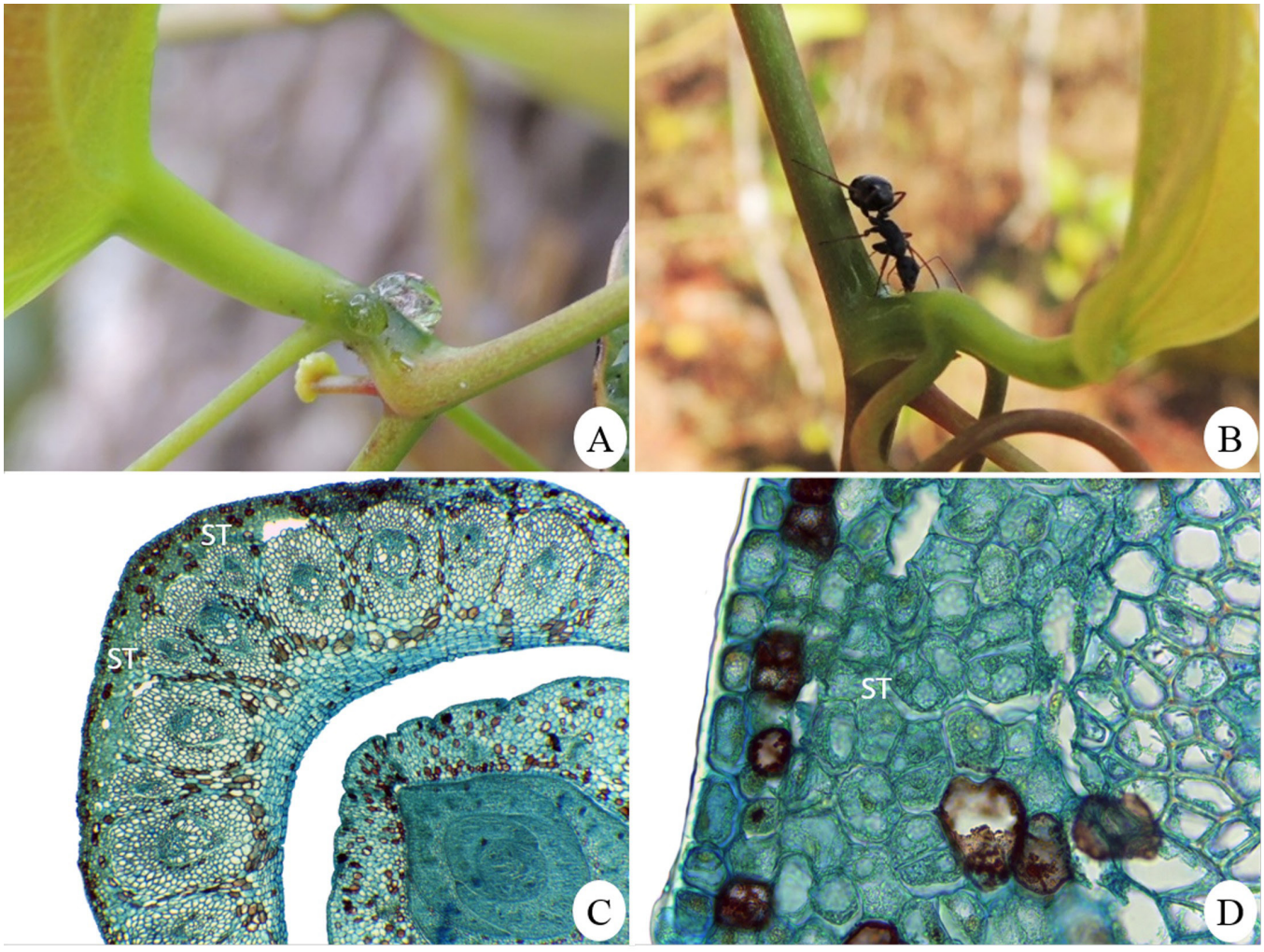

Fig 1. Extrafloral nectary of *Smilax polyantha*. (A) nectar secretion; (B) *Camponotus crassus* foraging in a nectary; (C) cross section. Scale: 500µm; (D) cells of the secreting tissue with dense cytoplasm and nuclei conspicuous. Scale: 50µm.

secretory adaxial epidermis (Fig 2D). Starch was detected just around the vascular bundles (Fig 2E-F), in cells adjacent to the secreting epidermis, as well as in the secretory parenchyma. Pectins were stained most intensely in the outer periclinal walls of the secretory adaxial epidermis. Intense staining for proteins was detected in all the secretory parenchyma and secretory adaxial epidermis.

The average angle of EFNs activity was seasonally significant (Rayleigh test $p < 0.001$; $Z = 72.159$), that is, the EFN activity is concentrated in September. However, the mean vector length (R), which can vary between 0 and 1 (see Vilela et al., 2014), was 0.31, indicating that few individuals have EFNs active in other periods of the year (Fig 3). The EFNs of all sampled individuals produced 6.51 ±2.78 uL (mean ± standard deviation) of volume and 32.13 ± 14.68 calories.

Ten species of ants were identified feeding on the nectar (Table 1). Among the subfamilies, Formicinae was the most representative, with four species, followed by Myrmicinae with three species and Ectatomminae, Ponerinae and Pseudomyrmecinae with one species each. *Camponotus atriceps*, *C. crassus*, *Ectatomma tuberculattum*, *Cephalotes pusillus*, *Crematogaster crinosa*, *Pheidole* sp.1,

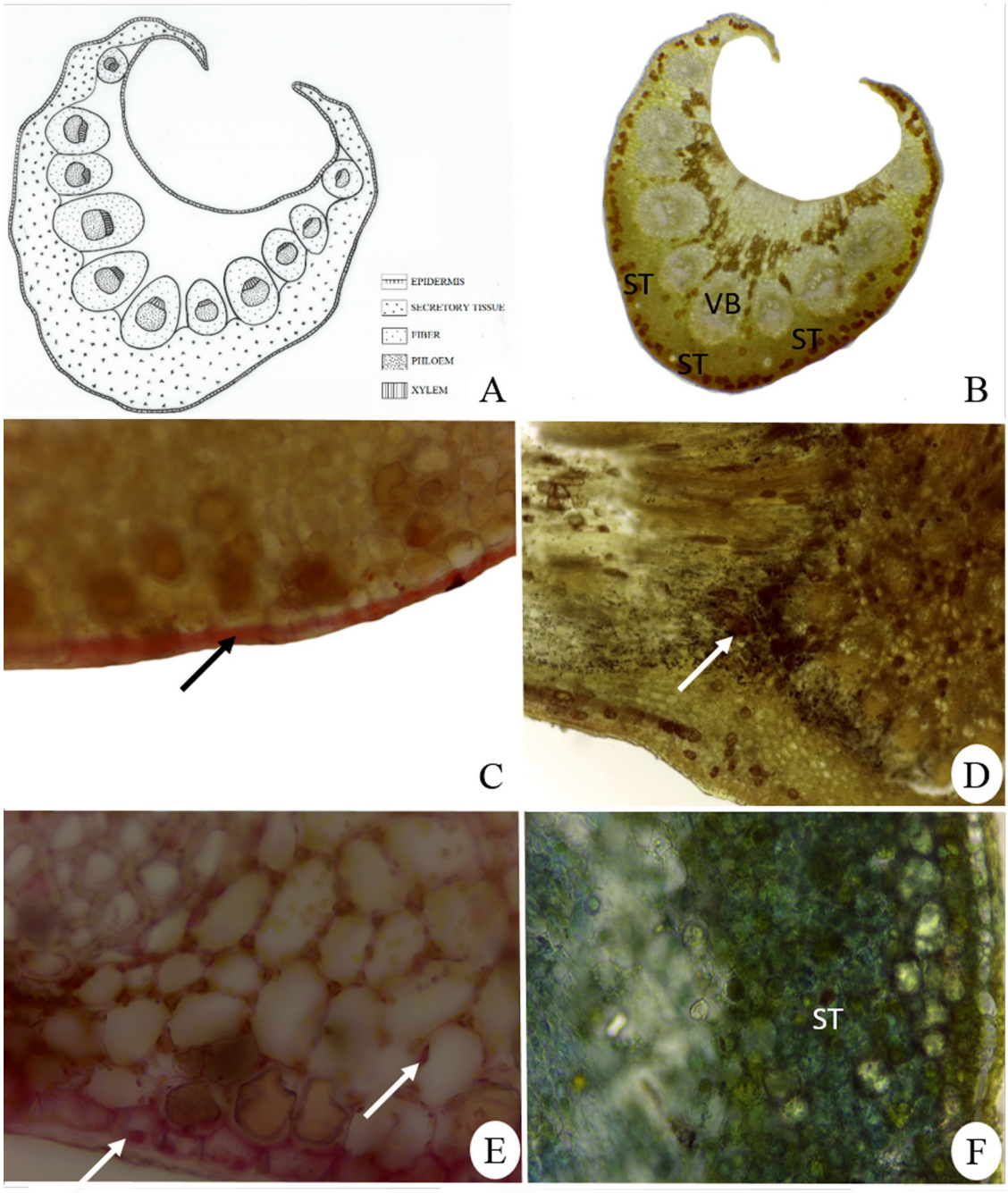

Fig 2. Structure extrafloral nectary. (A) Representative scheme of the different tissues encountered on the extrafloral nectary. Histochemical analyzes of extrafloral nectary of *Smilax polyantha*; (B, C) Reducing sugars (brown) in the secretory parenchymal cells. Scale: (B) 500µm, (C) 200 µm; (D) Lipid (pink) in the cuticle of the epidermal cells. Scale: 50µm; (E, F) Starch (black) in cells near the epidermis. Scale: (E) 200µm, (F) 50 µm.

Pseudomyrmex gr. elongatus sp.1, were collected only during the day. *Camponotus substitutus* and *Neoponera villosa* were collected exclusively at night. *Brachymyrmex* sp.1 was observed foraging in EFNs in both periods, day and night. The most frequent and abundant ant in *S. polyantha* was *C. crassus*. This ant species was observed 8 times, which means that over a period of one year we observed it in 8 months, while other species were observed only one time (see Table 1). Furthermore, this species represented 28% of the total number of ants observed in *S. polyantha*.

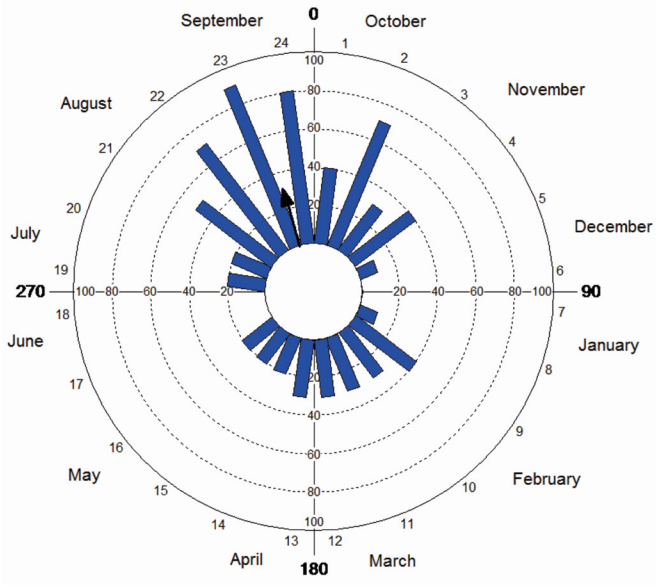

Fig 3. Circular activity representation of extrafloral nectary of *Smilax polyantha*, indicating a peak of activity in September (p <0.001, Rayleigh test).

Spearman's correlation showed that abundance of ants is significantly correlated with EFNs activity (rs = 0.657, p < 0.05). Our results showed that EFNs are active throughout the year, including during dry season, where we can observe foraging ants, even if in lower number. More detailed data about ant abundance and EFNs activity throughout the year are shown in Figure 4.

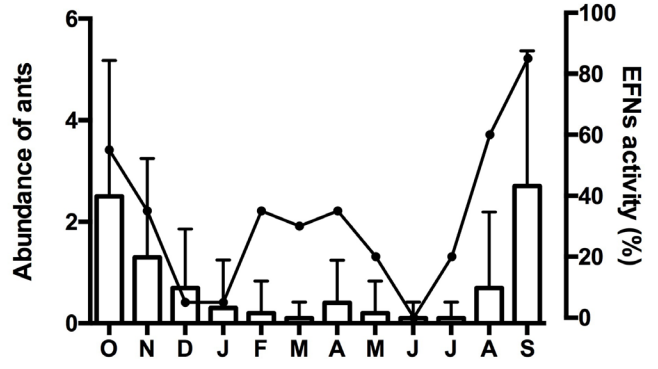

Fig 4. Abundance of ants (bars: mean and standard deviation) and percentage of EFNs activity (lines) in *Smilax polyantha* throughout the year.

Table 1. Ant species found foraging in EFNs of *Smilax polyantha* in the Cerrado area of Minas Gerais, Brazil, from October 2013 to September 2014.

Ant Species	Activity period	No. of records	Abundance*
Formicinae			
Brachymyrmex sp.1	Day, Night	4	12(13)
Camponotus atriceps (Smith, F., 1858)	Day	2	4(4)
Camponotus crassus Mayr, 1862	Day	8	26(28)
Camponotus substitutus Emery, 1894	Night	1	2(2)
Ectatomminae			
Ectatomma tuberculattum (Olivier, 1792)	Day	4	7(8)
Myrmicinae			
Cephalotes pusillus (Klug, 1824)	Day	5	14(15)
Crematogaster crinosa Mayr, 1862	Day	4	22(24)
Pheidole sp.1	Day	1	3(3)
Ponerinae			
Neoponera villosa (Fabricius, 1804)	Night	1	2(2)
Pseudomyrmecinae			
Pseudomyrmex gr. Elongatus sp.1	Day	1	1(1)

*Numbers represent absolute abundance (relative abundance in %).

Discussion

Our main hypothesis was confirmed, that *Smilax polyantha* possesses EFNs at the base of the petioles. The presence of an arc-shaped structure composed of adaxial epidermal cells with secretory features such as a thin cuticle and dense cytoplasm and underlying parenchyma with dense cytoplasm and conspicuous nucleus, together with the presence of sugars and proteins in these tissues and nectar-collecting activity exhibited by ants, show that the structure located in the petiole is an EFN. These glands effectively attract ants, which abundance on plants depends on EFNs activity.

In the Cerrado, most of the known EFNs are elevated (Oliveira & Leitão-Filho, 1987; Machado et al., 2008), thus differing morphologically from those observed in *S. polyantha*. However, anatomical features such as a secretory epidermis and underlying parenchyma and vascular bundles connected to secretory tissue were also shown in EFNs of other botanical families (see Melo et al., 2010; Paiva & Machado, 2006).

Starch grains, as in the secretory parenchyma of *S. polyantha*, have been observed in the parenchyma of the extrafloral nectary of *Rodriguezia venusta* (Orchidaceae) (Leitão

et al., 2014), *Hibiscus pernambucensis* (Malvaceae) (Rocha & Machado, 2009), *Catalpa syringaefolia* (Bignoniaceae), *Vicia sepium* (Fabaceae) (Gaffal, 2012), among others. There are reports that starch grains are broken in small sugars (sucrose) during extrafloral nectar secretion and senescence in the genera *Passiflora* (Durkee, 1982) and *Cuscuta* (Schaffner, 1979), which may indicate that starch is utilized when the nectary is active (Stpiczynska & Davies, 2006). Furthermore, according to Fahn (1979; 2000) and Nepi (2007), plastids, especially amyloplasts, can play an important role in the production of nectar, fully or partially hydrolyzing starch grains during the process of secretory activity. The presence of reduced sugar in the secretory epidermis of the EFNs of *S. polyantha* is a strong indication that the starch could have been hydrolyzed into smaller sugars and exudated in the form of nectar. Thus, the degradation of starch may contribute, in part, to the production of extrafloral nectar (Galetto et al., 1997). However, we have not directly investigated the role of starch on EFNs cells in the nectar secretion.

Lipids were detected mainly in the cuticle of the EFNs and as droplets in the secretory epidermis. Similarly, Stone et al. (1985) and Rochaand Machado (2009) showed the presence of lipids in EFNs of *Gossypium hirsutum* (Malvaceae) and *Hibiscus pernambucensisi* (Malvaceae), respectively. Some studies have shown that lipids in the nectar may also be considered to be a food resource to attract ants (Koptur et al., 1998; Heil & McKey, 2003). Furthermore, pectins were also observed in the epidermis of the EFNs of *S. polyantha*. Coutinho et al., 2012 observed the presence of pectins in the EFNs of *Chamaecrista* and *Sapium biglandulosum* (Euphorbiaceae). These structures can increase the cell wall porosity (Albersheim et al., 2010) and facilitate the exudation of the nectar, which could explain the presence of these substances in the external periclinal wall of the secretory epidermis in the EFNs.

Proteins found in the secretory tissue of *S. polyantha* may be hydrolysed into amino acids and form part of the secreted nectar, as observed in some species of the genera *Chamaecrista* (Coutinho et al., 2012) and *Acacia* (González-Teuber & Heil, 2009; Orona-Tamayo et al., 2013). Enzymes with proteolytic activities are common in plants and play multiple roles, including defense against herbivores and pathogens, mobilization of protein reserves, release of amino acids and protein degradation (Muntz et al., 2001; Schaller, 2004). Some studies have shown that ants foraging on plants with EFNs have a preference for nectar with amino acids as compared to solutions only composed of sugars (Wagner & Kay, 2002; González-Teuber & Heil, 2009; Wilder & Eubanks, 2009). In addition, the histolocalization of proteins are associated with cells that showed high metabolism (Held & Piechulla, 2011), essential for secretory activity (Escalante-Pérez & Heil, 2012).

Extrafloral nectaries of *S. polyantha* produce the most nectar during September, at the end of the dry season, a time when the majority of EFN species show secretory activity (Lange et al., 2013). In the Cerrado, in general, a different pattern was observed, where the activity of EFNsis greatest during the rainy season, attracting several ant species, mainly from *Camponotus*, *Ectatomma* and *Cephalotes* genera (Melo et al., 2010; Lange & Del-Claro, 2014;Vilela et al., 2014). Many Cerrado plants have raised EFN with a cavity that accumulates nectar (Thadeo et al., 2008), allowing greater nectar secretion (Oliveira & Leitão-Filho, 1987; Díaz-Castelazo et al.,2005). However, the EFNs of *S. polyantha* secrete large amounts of nectar (see Lange et al., 2017 to compare), even if it presents inconspicuous. Therefore, the extrafloral nectar produced by *S. polyantha* likely is a valuable source of energy for ants in the Cerrado, especially in the dry season. We know that a diet rich in extrafloral nectar can increase ant colony size, survivorship and reproduction (Byk & Del-Claro, 2011; Del-Claro et al., 2016).

In this biome, Cerrado, ants are frequently observed foraging on plants seeking a variety of resources. The presence and quality of extrafloral nectar can result in an increase in the abundance and richness of these organisms on plants (Rico-Gray & Oliveira, 2007). Some studies have shown that EFNs-bearing plants present a greater assembly of ants when compared to neighboring plants without these glands (Lange & Del-Claro, 2014; Stefani et al., 2015). Thus, extrafloral nectar in *S. polyantha* may play a fundamental role in plant protection against herbivores, since itattracts various species of ants that can act as biotic defenders. Three species of the genus *Camponotus* were observed on the EFNs of *S. polyantha*. Two of these were found only during the day and the other only during the night, thus displaying resource partitioning. This genus of ants occurs most frequently on the plants in the Cerrado (e.g. Lange et al., 2017) and is the main defender in ant-plant interactions (Del-Claro & Marquis, 2015). *Camponotus crassus*, for example, shows aggressive behavior, attacking and removing possible plant herbivores, which consequently causes reduction in leaf herbivores (Nascimento & Del-Claro, 2010).

Our results showed a significantly correlation between percentage of EFNs activity and abundance of ants. We can explain this from the EFNs productivity. If a greater number of EFNs is active, it means that a greater amount of resource is available. In this way, Lange et al. (2017) observed that the frequency and richness of ants is higherin periods with greater resource availability. Similar, Bixemann et al. (2011) showed that the increase in the reward of extrafloral nectar directly influences the abundance of ants. Therefore, we can observe that EFNs productivity of *S. polyantha* can vary over time and directly influences the abundance of ants.

Smilax polyantha is a Cerrado plant that provides a source of food for ants and other arthropods, even during times when food sources are limited, for example, during the dry season. The discovery of EFNs in *S. polyantha*may lead to new studies of interactions between plants and insects mediated by EFNs, which are necessary to understand the community structure in the Cerrado biome.

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