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A macro- and micromorphological survey of floral and extrafloral nectaries in the epiphytic cactus *Rhipsalis teres* (Cactoideae: Rhipsalideae)

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ABSTRACT

Floral and extrafloral nectaries in plants favor pollination and defense against herbivory. Despite their wide distribution in plants and differences in position, structure, and topography, their biological and systematic significance has been underutilized. This study investigated the macro- and micromorphology of floral and extrafloral nectaries in the epiphytic cactus *Rhipsalis teres* and reports unusual bristle-like structures (bracteoles) functioning as extrafloral nectaries in the cactus family. The floral nectary is disc-shaped embedded in the hypanthial floral cup with anomocytic stomata as secreting structures present on the epidermal nectarial tissue. Small multicellular bristle-like extrafloral nectar-secreting structures, homologues to bracts, were observed on the plants' stems and function as bracteolar nectaries having a relatively long and continuous secretory activity throughout several stages of the reproductive structures. Both the floral and bracteolar nectaries are functional. It is possible that in the latter nectar discharge occurs though epidermal cells, which build up pressure inside as nectar accumulates, thereby ending with rupture of the cuticle to release the liquid. The nectar in both secreting structures is scentless and colorless, and the concentration from floral nectaries is slightly lower than that of the bracteolar nectaries, 70.6% and 76.4%, respectively. The relatively higher concentration in the latter might be correlated with exposure, relative humidity and water evaporation, leading to crystallization of sugars on the stem surface in a short period of time.

Introduction

Nectaries are specialized structures present in plant parts and are referred to as floral and extrafloral nectaries. The position, type of nectary and nectar produced are often correlated with reproductive efficiency (Richards, 1986). At present, two types of nectar-secreting structures have been recognized in plants since Bonnier (1879) first described these structures: floral nectaries (FNs), usually located in the perianth, androecium, gynoecium, and floral axis (receptacle) but also in association with interstaminal, intrastaminal, extrastaminal, hypanthium, tepal, sepal, petal, stamen, staminode, stigma, style, ring, septal and pistillode nectary parts (Bernardello, 2007), and extrafloral nectaries (EFNs), situated in plant parts outside the flowers (Leins and Erbar, 2010). In addition to different locations in the plant, FNs and EFNs vary in anatomical structure, nectar composition, and mode of nectar presentation (Davis et al., 1988; Fahn, 1979; Pacini and Nicolson, 2007). Despite their wide distribution in plants and differences in position, structure, and topography, their biological and systematic significance has been underutilized (Bernardello, 2007; Fahn, 1979).

It is known that FNs play a direct role in pollination and provide nectar rewards for diverse animal visitors. Conversely, EFNs are not directly involved in pollination; these structures play a vital role in maintaining a mutually beneficial relationship between plants and insects. Among plant–insect interactions, the ant–plant relationship is a mutualistic partnership in which ants are attracted to EFNs in search of sugar resources, offering in return anti-herbivore protection (Beattie, 1985; do Nascimento and Del-Claro, 2010; Heil and McKey, 2003). The lack of mobility in plants restricts their ability to disperse pollen and seeds and to defend themselves from herbivorous predators, but the lack of motion is in part compensated by FNs and EFNs, which produce energy-rich exudates that plants trade for physical defense, mostly with insects (Pacini and Nicolson, 2007). Although EFNs are known in ca. 70 families of flowering plants (Bentley, 1977; Elias, 1983), FNs have been more extensively investigated and are reported in ca. 220 families (Bernardello, 2007). The reason for this unbalanced knowledge is that the wide array of morphological and structural floral diversity in conjunction with the different breeding systems has long intrigued biologists, who have devoted more attention to the study of flowers and their FNs.

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Also, nectar from FNs is an important food source for honeybees and the pollination and reproduction of numerous plants of economic significance. Extrafloral nectaries are more widespread in tropical and subtropical plants (Bentley, 1977), but notwithstanding their ecological and evolutionary role, they have limited economic implications (Pacini and Nicolson, 2007). EFNs are common in non-reproductive structures, such as leaves (petiole, stipule, blade), in the form of small to medium-sized protuberances covered or not by protective non-secretory trichomes, and may be associated with flowers and fruits of certain Bignoniaceae (Thomas and Dave, 1992).

References to EFNs nectaries in the cactus family have been made since the late 1800s. Irmisch (1876) first described the secretion of sugar in the stems of *Rhipsalis cassytha* Gaertn., Förster and Rümpler (1886) reported EFNs as glands ("Drüsen"), and Goebel (1889) observed them in *Mammillaria* Haw. and *Rhipsalis* Gaertn. Other early records reporting EFNs in the family include those of Ganong (1894), Lloyd (1908), and Lloyd and Ridgway (1912). In fact, Lloyd (1908, p. 138) indicated that "cacti are to be numbered among the plants which possess nectaries other than those which are found in the flower, and it appears that in many more cacti than would be expected". Support for this statement includes reports of FNs in *Epiphyllum* Haw. (reported as *Phyllocactus* Link; Beutler, 1930), in over 29 taxa of cacti (Buxbaum, 1953), *Selenicereus wittii* (K. Schum.) G.D. Rowley (Barthlott et al., 1997), species of *Stenocereus* (A. Berger) Riccob., *Pilosocereus* Byles & G.D. Rowley, and *Subpilocereus* Backeb. (Nassar et al., 1997), *Weberocereus tunilla* (F.A.C. Weber) Britton & Rose (Tschapka et al., 1999), *Peniocereus* (A. Berger) Britton & Rose (Raguso et al., 2003), species of *Opuntia* Mill. (Fuentes-Perez et al., 2009), and *Epiphyllum phyllanthus* Haw. (Almeida et al., 2010), to name a few. Alternatively, studies dealing with EFNs in cacti are more limited, except those previously indicated and those of Weingart (1920a,b), Pickett and Clark (1979), Blom and Clark (1980), Mauseth (1982), Ruffner and Clark (1986), Oliveira et al. (1999), among a few others.

The location of EFNs in cacti varies among species. For instance, in *Opuntia acanthocarpa* Engelm. & Bigelow var. *major* (Engelm. & Bigelow) Benson, they are present as secretory glands (Pickett and Clark, 1979); in *Ferocactus gracilis* H.E. Gates, the glandular spines secrete droplets of sweet concentrated nectar on the upper side of the areoles (Blom and Clark, 1980); in *Ancistrocactus scheeri* (Salm-Dyck) Britton & Rose, they occur on the tubercles and never mix with the spines (Mauseth, 1982); in *Echinocactus polycephalus* Engelm. & Bigelow, they are located above the areoles, and in *Carnegiea gigantea* (Engelm.) Britton & Rose (=*Cereus giganteus* Engelm.) in the floral bracts (Elias, 1983).

The understanding of the structure, function, ecological, and evolutionary role of secretory structures in plants provides significant information to understand the different types of plant–insect interaction and floral anatomy in relation to reproductive biology. To date, detailed morphological analyses or the usage of histochemical procedures to investigate the function of FNs and EFNs or the manner of nectar secretion in the Cactaceae, and specifically *Rhipsalis teres* (Vell.) Steud., are lacking. Within this scope, the present study investigated the macro- and micromorphology of FNs and EFNs in this species. In addition, this paper describes modified bracts functioning as EFNs in the cactus family.

Materials and methods

The study plant

The genus *Rhipsalis* includes 35 species circumscribed within the Rhipsalideae, a mainly South American tribe of the subfamily Cactoideae (Anderson, 2001). The species investigated, *Rhipsalis teres*, is an endemic, threatened epiphytic cactus distributed in southern and southeastern Brazil (Taylor, 1997). According to Barthlott and Taylor (1995), the species includes four formas, namely *R. teres* f. *teres*, *R. teres* f. *capilliformis* (F.A.C. Weber) Barthlott & N.P. Taylor, *R. teres* f. *heteroclada* (Britton & Rose) Barthlott & N.P. Taylor, and *R. teres* f. *prismatica* (Lemaire) Barthlott & N.P. Taylor. Since all these taxa have the same type of FNs and EFNs, the morphological observations of these secreting structures are based on the type of this species, i.e., *R. teres* f. *teres*, with additional observations in *R. teres* f. *capilliformis* and *R. teres* f. *prismatica.*

Morphological analyses

Macromorphological analyses of FNs and EFNs were performed using fresh flowers collected from plants originally obtained from the living collection of the Montreal Botanic Garden (MBG) and propagated in the greenhouses of the Department of Biology at the University of Saskatchewan. Our analyses of FNs and EFNs are mainly based on *Rhipsalis teres* f. *teres* (MBG 993-1995/SASK Acc. No. 160,582), from which 15 flowers/plant were collected from two stem branches per individual. Additional observations of FNs and EFNs of this species were made using material from *R. teres* f. *capilliformis* (MBG 1190-1989/SASK Acc. No. 160,583) and *R. teres* f. *prismatica* (MBG 161-2001/SASK Acc. No. 160,584), from which five stem segments and three flowers per forma were collected. Voucher specimens of the plants investigated were deposited at the W.P. Fraser Herbarium (SASK) of the University of Saskatchewan. Photographs were taken with a Nikon D100 digital camera, lenses 28, 60 and 70 mm and a Carl Zeiss Tessovar Photomicrographic Zoom System. The terminology used to describe the flower and nectary morphology was adapted from that of Buxbaum (1953), Bernardello (2007), and Leins and Erbar (2010).

Scanning electron microscopy (SEM)

For micromorphological analyses of fresh vegetative and reproductive features, flowers and stem portions with EFNs were fixed in 2.5% glutaraldehyde (in buffer 0.05M phosphatate, pH 7.2) for 48 h, dehydrated in a graded acetone series to 100%, critical-point dried with liquid CO_2 (Polaron Instruments E3000), affixed to aluminum stubs, and gold-coated with an Edwards Sputter Coater S150B. The nectaries and surrounding tissues were examined with a Philips SEM 505 at 29.0 kV and microphotographed using Polaroid 665 positive/negative film. Whenever possible, three or more flowers of the same accession were observed for comparative purposes.

Nectar sugar concentration

Nectar collection from FNs and EFNs took place after a preliminary inspection of the stem segments and the flowers to locate the nectaries. Sixteen (eight flowers/individual) out of the 30 flowers collected from *R. teres* f. *teres* were used to determine the nectar solute concentration in FNs immediately after collection. In addition, 12 samples (six stem segments/individual) were used to evaluate the nectar concentration in EFNs. Three stem segments and three flowers per specimen were used in the other formas, namely, *R. teres* f. *capilliformis* and *R. teres* f. *prismatica.* The nectar was collected by gently touching the floral nectary with micropipettes of known volume and/or Drummond Scientific Microcaps (1.0 μL) and, whenever possible, at different times and different days, always in virgin flowers. The nectar was immediately expelled onto the prismatic surface of a hand refractometer (0–50%, 40–85%; Bellingham and Stanley, Tunbridge Wells, Kent) to determine nectar solute concentrations, measured as percent nectar concentration by weight (% NCW). Sugar scales are based on % (w/w) sucrose in water. The sugar content of nectar was calculated

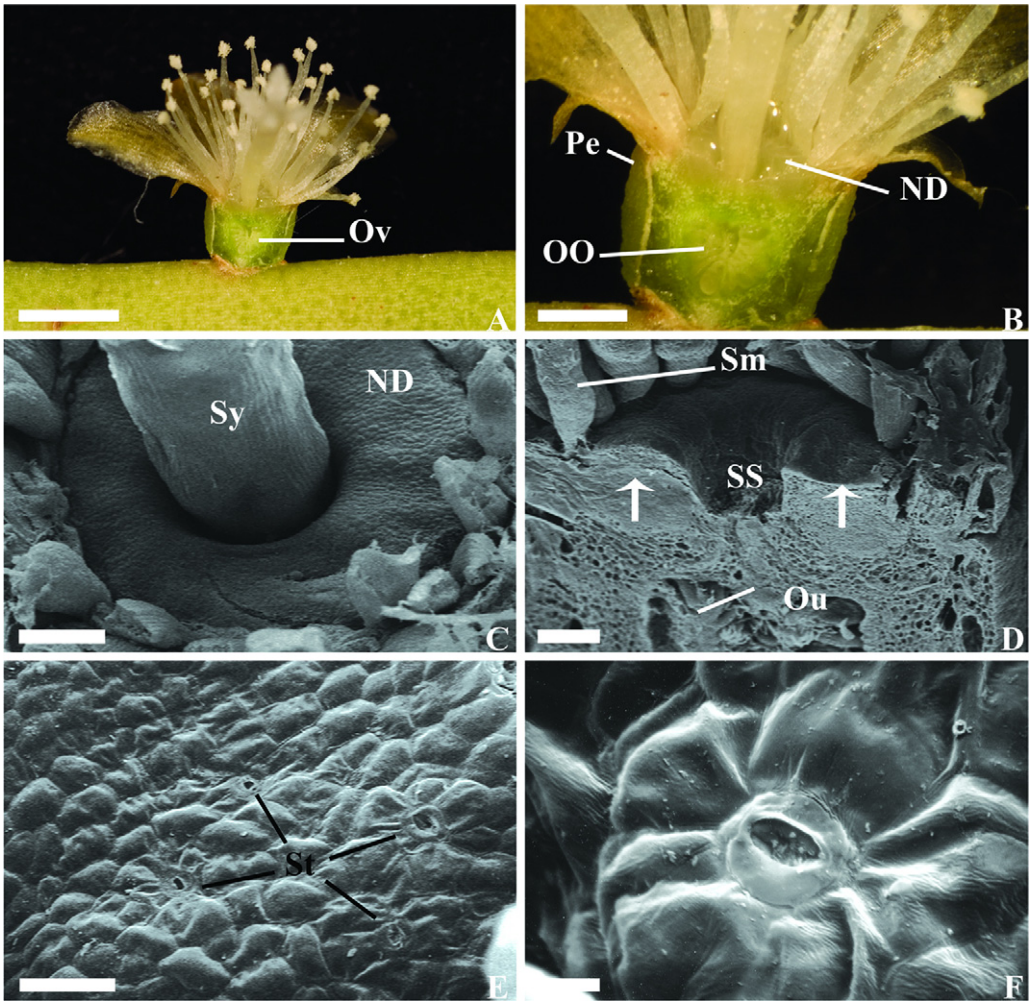

Fig. 1. Flower and floral nectary (FN) structure in *Rhipsalis teres* f. *teres*. (A) Flower in longitudinal section. (B) Flower in longitudinal section showing detail of nectary and ovary. (C) SEM view of the disc-shape FN. (D) Detailed SEM view of FN (arrows) in longitudinal section. (E) SEM view of the FN epidermis with stomata. (F) Detailed SEM view of nectary's epidermis and anomocytic stomata. ND, nectary disc; OO, ovary with ovules; Ou, ovule; Ov, ovary; Pe, pericarpel; Sm, stamen; SS, style scar; St, stomata; Sy, style. Scale bars: 3 mm (A), 1 mm (B), 250 μm (C and D), 50 μm (E), and 10 μm (F).

from the volume and the solute concentration of the nectar sample from each flower.

Results

Flower morphology and floral nectary

The flowers of *Rhipsalis teres* f. *teres* are borne laterally, usually in young shoots and at the base of fuzzy gray areoles. The flowers are diurnal, lasting one day, whitish, small (6.2–6.5 mm in diameter and 4.6–4.8 mm in length), scentless and sessile, with numerous stamens and with a marked greenish pericarpel. The flower is epigynous with the ovary sunken in the pericarpel (Figs. 1A, B and 2I), a feature of the cactus family. At the base of a short tube, the hypanthium, there is an annular secretory tissue surrounding the style. This tissue forms a disc-shaped floral nectary of the hypanthial type (Fig. 1B–D) with anomocytic stomata on the epidermis (Fig. 1E and F). The disc-shaped nectary is embedded in the hypanthial floral cup, an arrangement matching the morphological descriptions documented in *Rhipsalis* by Buxbaum (1953), Barthlott and Hunt (1993), and Anderson (2001). Although these reports make no reference to the presence of stomata associated with the nectary, our survey revealed the presence of anomocytic stomata on the epidermal tissue of the nectary (Fig. 1E and F) forming slits from which nectar is released.

Extrafloral nectaries

Our survey indicates that the stems of the three entities of *R. teres* investigated, namely *R. teres* f. *capilliformis* (Fig. 2A), *R. teres* f. *prismatica* (Fig. 2B and C), and *R. teres* f. *teres* (Fig. 2D–F), have bristle-like bracteolar structures functioning as EFNs. These specialized appendages are arched with a hood-like shape and are located on the shoot meristem of the stem (Figs. 2D and 3A) and at the base of the flowers and fruits on the areolar region (Figs. 2A–C, E, F and 3D). These minute structures, referred hereafter as bracteolar nectaries (BNs), have a rather long and continuous secretory activity throughout several stages of the reproductive structures. The peak activity of nectar secretion occurs prior to flower development and before the growth of shoot meristems. The appendages (Fig. 2E) release nectar before the floral bud develops (Fig. 2F), continuing during pre-anthesis (Figs. 2G, H and 3E), anthesis (Fig. 2I), and throughout fruit growth (Fig. 2J) and even after fruit abscission (Fig. 2K). Sometimes the nectar crystallizes on the BN (Fig. 2H). This lengthy secreting activity stops with the

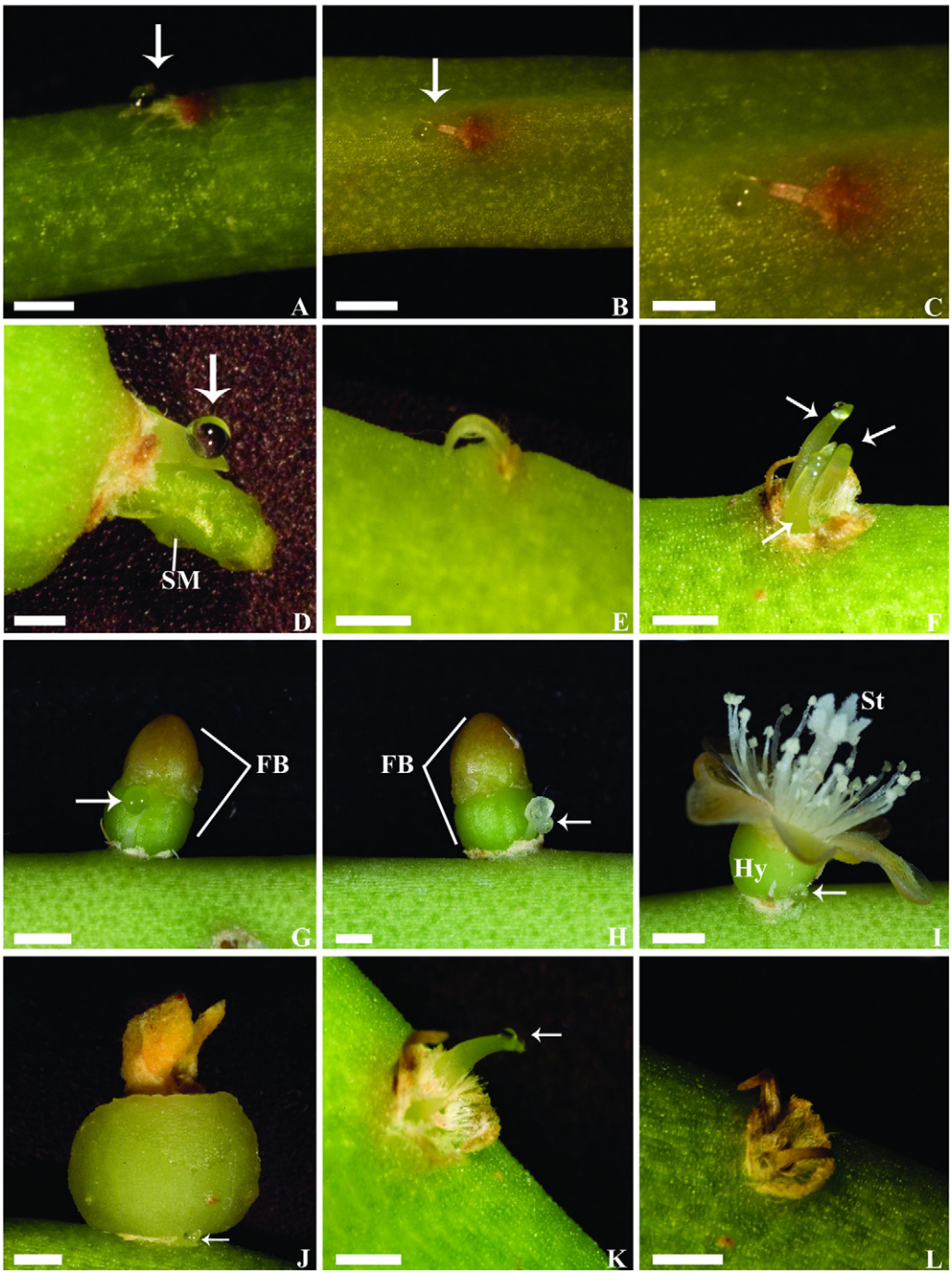

Fig. 2. Extrafloral secretory structures on shoot meristem and areoles during floral development in *Rhipsalis teres*. (A) Hook-like bracteolar nectary (BN) secreting nectar (arrow) in *R. teres* f. *capilliformis*. (B) Hood-like BN in *R. teres* f. *prismatica* (arrow). (C) Detail of BN secreting nectar in *R. teres* f. *prismatica*. (D–L) *R. teres* f. *teres*. (D) Shoot meristem. (E) Areole showing (arrow) the first hook-like BN. (F) Areole with at least three secretory bracts (BNs) prior to floral bud development (arrows). (G) Floral bud with hood-like BN secreting nectar (arrow). (H) Floral bud and hood-like BN with crystallized nectar (arrow). (I) Flower in anthesis with hood-like BN (arrow) secreting nectar. (J) Immature fruit with nectar secreting EFN (arrow). (K) Areole with active EFN (arrow) at the onset of senescence, after the fruit abscission. (L) Areole with dry BNs. FB, floral bud; Hy, hypanthium; SM, shoot meristem; St, stigma. Scale bars: 0.5 mm (A–C), 1 mm (E, F, J, K and L), and 2 mm (D, G, H and I).

senescence of the BNs as these dry out (Fig. 2L). Similar secretory structures with comparable nectar-producing activity were observed in areoles without flowers in the shoot meristematic region (Figs. 2D and 3A).

Nectar sugar concentration

The nectar of *R. teres* is colorless, and the amount secreted by FNs was very scarce (about 1 μL per flower) but always viscous, indicating relatively high sugar content. The floral nectar had a comparatively high solute concentration with a mean value of 70.6 ± 2.2%. In turn, the nectar exuded by the BNs varied from 0.33 to 1 μL with a mean solute concentration of 76.4 ± 1.7%.

Discussion

According to Fahn (1979), exudation of nectar via stomata occurs in FNs of numerous plants. Whereas stomatal pores can facilitate nectar release, the real secretory structures are the parenchyma cells of the nectary disk. Stomata (guard cells) are

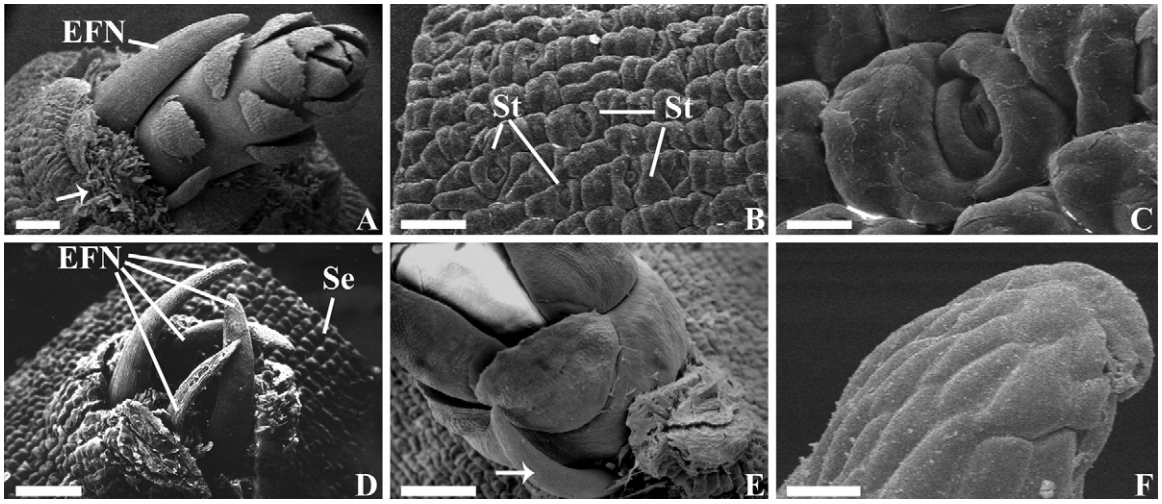

Fig. 3. Structural details of bracteolar nectary (BN) in *Rhipsalis teres* f. *teres* as viewed in scanning electron microscopy. (A) Shoot meristem with EFN and bracts (arrow). (B) Epidermal stomata from stem segment. (C) Detail of parallelocytic stomata on the stem. (D) Areole with BNs prior to flower bud development. (E) Flower bud with EFN (arrow). (F) Detail of apical region of BN. EFN, extra floral nectary; Se, stem; St, stomata. Scale bars: 250 μm (A), 200 μm (B and F), 50 μm (C), and 500 μm (D and E).

not known to be secretory structures on nectaries, but rather their pores serve as passive exit for nectar flow (Fahn, 1979). The floral nectar of *Rhipsalis teres* is seemingly secreted through the epidermal anomocytic stomata (with more than two subsidiary cells), see Fig. 1E and F, quite likely in association with vascular bundles, and is accumulated at the base of the short tube and nectary disc (Fig. 1B–D). Similar patterns of epidermal stomata and guard cells have been observed in FNs of other *Rhipsalis* and *Lepismium* Pfeiff. species (O.J.G. Almeida and J.H. Cota-Sánchez, unpub. data). Similarly, in *Portulaca grandiflora* Hook. the aperture of the stomata is surrounded by more than two subsidiary cells (Fahn, 1979, and references therein). As members of the ACPT clade (Anacampserotaceae, Cactaceae, Portulacaceae, and Talinaceae; Stevens, 2001 onwards), the shared pattern of morphological arrangement of stomata in the FNs represents another example of convergence in these succulent plant families.

This study documents the first report of nectar secreting through bristle-like structures or BNs in the Cactaceae. We believe that these unusual nectar-secreting multicellular structures (Fig. 3F) are modified bracts functioning as EFNs. Vogel (1977) characterized three types of nectaries in terms of histological features, specifically epithelial, mesophyllary, and trichomatic. Although no histological analyses were performed in the EFNs of *R. teres*, we hypothesize that these structures are composed of mesophyll tissue and storage cells rather than epithelial tissue because of their position on the areoles. This is a pluripotent tissue, which can produce both vegetative as well as reproductive complex organs.

The role of BNs in *Rhipsalis teres* is unknown, but it is feasible that the nectar secreted by these specialized structures attracts ants, which can potentially develop a mutualistic association with the plant because the secretion is relatively abundant and available for a lengthy duration. The active secretory process starts in the meristematic regions of the plant and continues during the developmental stages of different organs, such as floral buds and shoots, which are the plant's vital parts with soft tissue more vulnerable to herbivory. Hence, frequent visits by ants feeding on nectar may provide protection against herbivory. Similar cases of EFNs in association with defense against herbivorous animals have been reported in other cacti. For example, early reports of extranuptial nectaries on young shoots of *Hariota salicornioides* DC. var. *gracilis* Web. to protect the rudimentary buds support the mutualistic association with ants since nectaries occur only when young shoots are attacked by herbivores (Weingart, 1920b). Similarly, the EFNs of *Opuntia acanthocarpa* var. *major*, located in the areoles of new reproductive and vegetative structures, exude nectar that attracts ants (*Crematogaster opuntiae*) feeding on the sugary fluid and acting as guardians against cactus-feeding insects, such as the nymph *Chelididea vittiger* (Pickett and Clark, 1979). Also, several species of ants collecting nectar in the EFNs of the barrel cactus *Ferocactus gracilis* have been reported (Blom and Clark, 1980).

The slightly lower nectar concentration in the FNs of *R. teres* is likely due to the protective effect of perianth parts, whereas the relatively higher concentration in the secretion of BNs is correlated with exposure, as water tends to evaporate from the nectar, which eventually crystallizes on the stem surface (Fig. 2H). Nonetheless, the concentration of the nectar solution in both FNs and EFNs depends on other factors, such as immediate connection with phloem sieve elements, proportion of xylem in the vascular trace, and photosynthetic activity (reviewed in Bentley, 1977). Although there is a wide range in total sugar concentration in nectar among plant species (from 5% to 87%, but normally from 25% to 75%: Leins and Erbar, 2010), it is noteworthy that the FNs and BNs of *R. teres* are at the high end of this spectrum. Thus, the high concentration in this species may represent an adaptation leading to the attraction of several pollinators and visitors favoring pollen transfer and protection against herbivory. It has been suggested that bracteolar nectaries protect flowers from nectar robbers (Inouye, 1983; Wäckers and Bonifay, 2004) or promote outcrossing by reducing the time pollinators spend visiting flowers (Altshuler, 1999; Wäckers and Bonifay, 2004). It is probable that the FN and BNs promote the interaction of *Rhipsalis teres* with at least two different types of visitors: one group representing the pollinators, feeding on floral nectar, and the second group being ants in a protectionist role against herbivory while using extrafloral nectar as reward. It should be noted that ants can also be detrimental rather than beneficial because they may rob floral rewards and/or damage flowers, generally without contributing to pollination (Beattie, 1985). Nonetheless, extrafloral nectar may serve to prevent these problems by distracting ants away from the delicate flower structures. Considering that small to medium-sized bees have preference for flowers with high nectar solute concentration ranging from 50% to 65% or higher (Nicolson and Thornburg, 2007; Roubik and Buchmann, 1984), it makes sense to hypothesize that a 70% (w/w) concentration in the nectar in

the generalistic flowers of *R. teres* would attract a wide array of bees.

Unlike the epidermal tissue of the floral nectary bearing anomocytic stomata, the stem epidermis of *R. teres* has parallelocytic stomata (Fig. 3B and C). However, the BNs have no stomata on the surface of the nectar secreting bracts (Fig. 3F), as found in other cases of EFNs (e.g. Paiva, 2011). Consequently, the mechanism of nectar release in the BNs is difficult to explain because these secreting structures have neither openings nor stomata through which the fluid could be discharged, nor do we know whether or not these structures are vascularized. Considering that in the nectar glands of EFNs in *Echinocactus* Link & Otto, *Mammillaria*, and *Opuntia* nectar secretion is preceded by the digestion of the epidermal cells and subsequent disorganization of their walls and contents, ending with the rupture of the cuticle (Lloyd and Ridgway, 1912), we believe that release in the BNs of *R. teres* occurs though the epidermal cells with concomitant accumulation of nectar. This should build up pressure inside the cells, ending with rupture of the cuticle which enables subsequent secretion of the liquid. In fact, Nepi (2007) indicated that this mechanism of nectar release in EFNs by means of cuticular or epidermal rupture takes place in other plant groups.

In conclusion, our survey indicates that both structures (FNs and EFNs) are present in *R. teres* and produce nectar with similar concentrations. But there is great structural disparity and level of complexity between these two nectar secreting structures. Other discordant morphological (and cytological) patterns have been reported in FNs and EFNs of *Vicia faba* L. (Davis et al., 1988) and *Campsis radicans* (L.) Seem. (Elias and Gelband, 1976), and between different EFNs in the Gentianaceae genus *Calolisianthus* (Griseb.) Gilg (Delgado et al., 2011). Forthcoming studies in pollination biology and internal anatomy, in particular concerning vascular supply, in both EFNs and the BNs will be instrumental to characterize the conductive system of these secreting structures in relation with the plant's vascular system and the associated pollinating agents.

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