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Anatomical and histochemical characterization of extrafloral nectaries of *Prockia crucis* (Salicaceae)[1]

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Besides being vital tools in taxonomic evaluation, the anatomy of plant secretory structures and the chemical composition of their secretions may contribute to a more thorough understanding of the roles and functions of these secretory structures. Here we used standard techniques for plant anatomy and histochemistry to examine secretory structures on leaves at different stages of development of *Prockia crucis*, to evaluate the origin and development of the structures, and to identify the disaccharides and monosaccharides in the exudates. Fructose, glucose, and sucrose constituted up to 49.6% of the entire secretion. The glands were confirmed to be extrafloral nectaries (EFNs); this is the first report of their presence in the genus *Prockia*. These EFNs are globular, sessile glands, with a central concavity occurring on the basal and marginal regions of the leaf. The epidermis surrounding the concavity is secretory, forming a single-layered palisade that strongly reacts with periodic acid–Schiff's reagent (PAS) and xylidine Ponceau, indicators of total polysaccharides and total proteins, respectively, in the exudate. On the basis of the similarity of these glands to the salicoid teeth in *Populus* and *Salix*, we suggest that these three taxa are phylogenetically close.

Key words: extrafloral nectaries; Flacourtiaceae; histochemistry; ontogeny; *Prockia crucis*; Salicaceae; secretion chemistry.

According to Cronquist (1968), the family Flacourtiaceae has the most basal characteristics within the order Violales. Under the phylogenetic classification system of the Angiosperm Phylogeny Group (Stevens, 2001 onward; Chase et al., 2002), the family Flacourtiaceae no longer exists, and its member species have been assigned to two other families, Salicaceae and Achariaceae, with most of the members in Salicaceae, within the Eurosideas I, order Malpighiales. This group contains about 300 neotropical species (Klein and Sleumer, 1984), with 19 genera and approximately 90 species occurring in Brazil (Barroso et al., 2002). The unifying characteristics of members of the Salicaceae s.l. are single foliar teeth, called salicoids, and salicin phenolic-like compounds (Chase et al., 2002). The purpose was to show any similarities or differences between the glands in these two "families" and to reevaluate the term salicoid tooth.

Prockia comprises two species. *Prockia flava* Karsten is found only in Venezuela. *Prockia crucis* P. Browne ex. L., distributed throughout tropical and subtropical regions of South and Central America, is a shrub or small tree, and its leaves have glandular-serrate margins, generally with two basal glands located at different levels (Klein and Sleumer, 1984).

The secreting structures vary considerably in morphology, anatomy, function, position, and the type of secreted substance. Knowledge of the chemical composition of the secreted substances along with detailed descriptions of the anatomy of these structures may contribute to an enhanced understanding of the role and function of the secreted product (Fahn, 1979). This approach will also serve as an important tool for taxonomic evaluations (Solereder, 1908; Metcalfe and Chalk, 1979). The secreting structures thus far recorded in Salicaceae are highly diverse, and in many cases the term gland is adopted for structures not well characterized. Terms such as salicoid teeth and glandular margins (Sleumer, 1980; Klein and Sleumer, 1984; Chase et al., 2002) are used community-wide for taxonomic description. A detailed characterization of such structures is of utmost importance because the relation between morphological and structural observations is not always described (Lersten and Curtis, 1996).

Glands in the leaves of *P. crucis* are topographically and morphologically similar to extrafloral nectaries and salicoid teeth. Nectaries are structures that secrete nectar, a fluid consisting predominantly of glucose, fructose, and sucrose at various concentrations. Nectar may also contain maltose, raffinose, melobiose, mucilage, amino acids, proteins, organic acids, mineral ions, phosphates, vitamins, phenolic compounds, oxidases, and tirosinases (Fahn, 1979; Baker and Baker, 1983; Nicolson and Thornburg, 2007). Nectar composition varies substantially depending on the type and position of the nectary (Roshchina and Roshchina, 1993).

Depending on the location, nectaries are classified as floral if they occur in the flower and classified as extrafloral if they occur in vegetative organs of the plant (Schmid, 1988). The morphologies of floral and extrafloral nectaries, however, may be extremely similar (Leitão et al., 2005).

Extrafloral nectaries (EFNs) were initially suggested to be responsible for secretion of surplus photoassimilate and were not necessarily associated with any explicitly adaptive benefit (Mound, 1962). This hypothesis has since fallen out of favor with the scientific community in light of mounting evidence

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suggesting that EFNs offer a protective function. Nectar may serve as a resource for visitors to a plant, particularly for aggressive ants that aid and protect the plant's foliage. The visitors would act as effective agents against herbivores, allowing for greater plant adaptive success (Roshchina and Roshchina, 1993; Morellato and Oliveira, 1994; Heil, 2008).

This work aimed to describe the structure and development of glands on leaves of *P. crucis* and to characterize the monosaccharides and disaccharides in their exudates. Anatomical characterization may assist in establishing relationships between *Prockia*, *Populus*, and *Salix* and will contribute to a rigorous phylogenetic classification system analysis.

MATERIALS AND METHODS

Leaves of *Prockia crucis* were collected from three native plants in a montane, semideciduous, seasonal forest (Veloso et al., 1991) located at Sítio Bom Sucesso (Viçosa, MG, Brazil). The geographic region is private property located about 5 km from the Universidade Federal de Viçosa (UFV). This forest is 750 m a.s.l. and is located near Mata do Paraíso, the largest forest fragment of the Viçosa region. Voucher material is kept at the Herbarium of the Universidade Federal de Viçosa under number VIC 17.167.

To study the ontogeny of *P. crucis* glands, we collected samples at various developmental stages of the leaf: stage I (leaf primordium at the shoot apical meristem), stage II (first node leaf), stage III (second node leaf), stage IV (third node leaf), stage V (fourth node leaf), and stage VI (fifth node leaf). The material was either fixed in FAA_{50} (formalin-acetic acid-50% alcohol, 1:1:18) for 24 h or in a solution of 10%ferrous sulfate (w/v) in 4% formalin to detect phenolic compounds for 48 h, then stored in 70% ethanol (Johansen, 1940). The FAA_{50}-fixed samples were dehydrated in an ethanol series and embedded in methacrylate (Historesin, Leica Instruments, Heidelberg, Germany). Cross and longitudinal sections of 6–8 μm were obtained with an automatic advance rotary microtome (model RM2155, Leica Microsystems, Deerfield, Illinois, USA) with disposable steel blades. The sections then were stained with toluidine blue pH 4.0 (O'Brien and McCully, 1981) for structural characterization, xylidine Ponceau (XP) (O'Brien and McCully, 1981) and Coomassie brilliant blue (Fisher, 1968) for total proteins, ruthenium red for pectic substances (Johansen, 1940), and periodic acid–Schiff's reagent (PAS) for total polysaccharides (Maia, 1979).

Fresh samples of adult leaf glands (stages V and VI) were cut transversally using a cryomicrotome (CM1850, Leica Microsystems) or table microtome (model LPC, Rolemberg and Bhering Comércio e Importação LTDA, Belo Horizonte, Brazil). These sections were used in histochemical tests to detect total lipids with Sudan IV (Pearse, 1980), terpenoids using Nadi reagent (David and Carde, 1964), lignin using phloroglucinol (Johansen, 1940), alkaloids using Dragendorff reagents (Svendsen and Verpoorte, 1983), and starch using Lugol's iodine solution (Jensen, 1962); control sections were performed simultaneously.

The fixed sections were mounted on slides using synthetic resin (Permount) and the fresh ones in glycerinated gelatin. Observations and photographs were done using a light microscope (model AX70TRF, Olympus Optical, Tokyo, Japan) equipped with a U-Photo system (Olympus Optical).

Fragments of adult leaves (V and VI stages) were diaphanized using 10% sodium hydroxide solution and 20% hypochlorite (Johansen, 1940, modified), stained with safranin (1% alcoholic solution), and mounted in glycerinated gelatin. Leaf border samples were fixed in a solution of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) (Karnovsky, 1965, modified) for 48 h, and postfixed for 18 h in1% osmium tetroxide in the same buffer, buffer washed, then dehydrated, and stored in 70% ethanol. After dehydration in an ethanol series, samples were to critical-point dried (model CPD020, Bal-Tec, Balzers, Liechtenstein) and coated with a thin layer of gold (Bozzola and Russel, 1992). Samples were examined at the Microscopy and Microanalysis Center of the Universidade Federal de Viçosa with a scanning electron microscope (JSMT200, JEOL, Tokyo, Japan) at an accelerating voltage of 15 kV.

For exudate collection and sugar detection, branches were taken to the laboratory and kept in buckets of running water to enhance exudate secretion. Exudates were collected directly from the leaf glands into a capillary tube, then transferred to eppendorf tubes at −15°C until analysis at the Chemistry Laboratory in the Chemistry Department at the UFV. The entire secreted solution was mixed with distilled and purified water by Millipore system making a final volume of 300 μm that was then subjected to HPLC (model LC-6AD, Shimadzu, Osaka, Japan) with a Techsphere column (NH_2, 5 μm, 250 × 4.6 mm id, HPLC Technology, Herts, UK) NH_2 precolumn (20 mm × 4.6 mm inner diameter [id]; 5 μm), a Techsphere NH_2 column (250 mm × 4.6 mm id; 5 μm), and a refractive index detector (RID). The mobile phase was acetonitrilec : purified water (70 : 30 v/v), with a flux of 1 mL/min. A calibration curve for each sugars standard was obtained using 10, 20, and 30 μL of a solution containing fructose, glucose, and sucrose standards at 1.2 mg/mL. Finally, 2 μL of the solution (300 μL) obtained by diluting the secretion (23 mg) in water (Meyer, 1994) was injected into the HPLC.

The calibration curve for each sugar was determined by the relationship between the mass of the injected sample, expressed in μg (10 μL = 12 μg), and the area of the respective peak related to the pattern (glucose, fructose, or sucrose). Distilled water was used to determine the control peak and control area. Thus, the linear equation for each peak was based on the standard data as follows: (1) Fructose—0 μg, area 0; 12 μg, area 876543; 24 μg, area 1509481; 36 μg, area 2223781; linear equation, $y = 60869x + 56809$, $R^2 = 0.9956$. (2) Glucose—0 μg, area 0; 12 μg, area 744222; 24 μg, area 1326124; 36 μg, area 2267784 linear equation, $y = 61544x - 23256$, $R^2 = 0.9915$. (3) Sucrose—0 μg, area 0; 12 μg, area 294222; 24 μg, area 698258; 36 μg, area 1043284; linear equation, $y = 29449x - 21142$, $R^2 = 0.9967$.

The nectar was classified by its sugar composition as proposed by Baker and Baker (1983).

RESULTS

The sugar content in *Prockia crucis* secretion comprised 49.6% of the whole secretion, with 12.4% fructose, 16.2% glucose, and 21% sucrose. When the area related to fructose in the secretion examined (1 213 320) was used with the appropriate linear equation, we obtained a value corresponding of 19.0 μg fructose. Because the total volume of the nectar sample is 150 times greater than the injected volume, the total amount of fructose in the nectar is 150 times greater than 19 μg, or 2.85 mg, constituting 12.4% of the nectar (23 mg). For glucose, with an area of 1 507 138, the amount in the injected sample was 24.9 μg, which makes 3.73 mg or 16.2% of the total nectar. Finally, sucrose yielded an area of 927 276, and the amount in the sample was 32.2 μg, making 4.83 mg or 21.0% of the total nectar. Because the secretion is clearly a nectar, the structure must be classified as an extrafloral nectary (EFN). The disaccharide to monosaccharide ratio [i.e., sucrose/(fructose + glucose)] was 0.73; therefore, the nectar could be classified as sucrose-rich.

The EFNs occur at different leaf positions. Where the petiole joins the leaf blade, a projection toward the abaxial surface was observed (basal EFN; Fig. 1A, B), as well as similar structures occurred throughout the leaf margin, located in the top position on the teeth (Fig. 1C, D). Such secretory structures were sessile and globular, forming a central concavity (Fig. 1F) and secreting a viscous, translucent liquid, with a brilliant dark green color. The basal EFNs were larger than the marginal ones and occasionally occurred in pairs.

EFNs were intensely vascularized by lateral veins converging on the margin (Fig. 1E), with a large amount of druse-like calcium oxalate crystals. The nectaries showed an irregular concavity surrounded by an elevated margin with nearby tector trichomes. The cuticle was smooth, both on the margin and cavity (Fig. 1F).

Anatomically, the EFNs were similar, though the basal nectaries were larger than those on the margins (Fig. 1B, D). The EFNs had a single-layered secretory, palisade-like epidermis, with high columnar cells and a dense cytoplasm (Fig. 2A–C), which strongly reacted with PAS, XP, and Coomassie brilliant blue, showing the presence of carbohydrates and proteins (Fig. 3A, B, E). Anticlinal and internal periclinal walls stained with

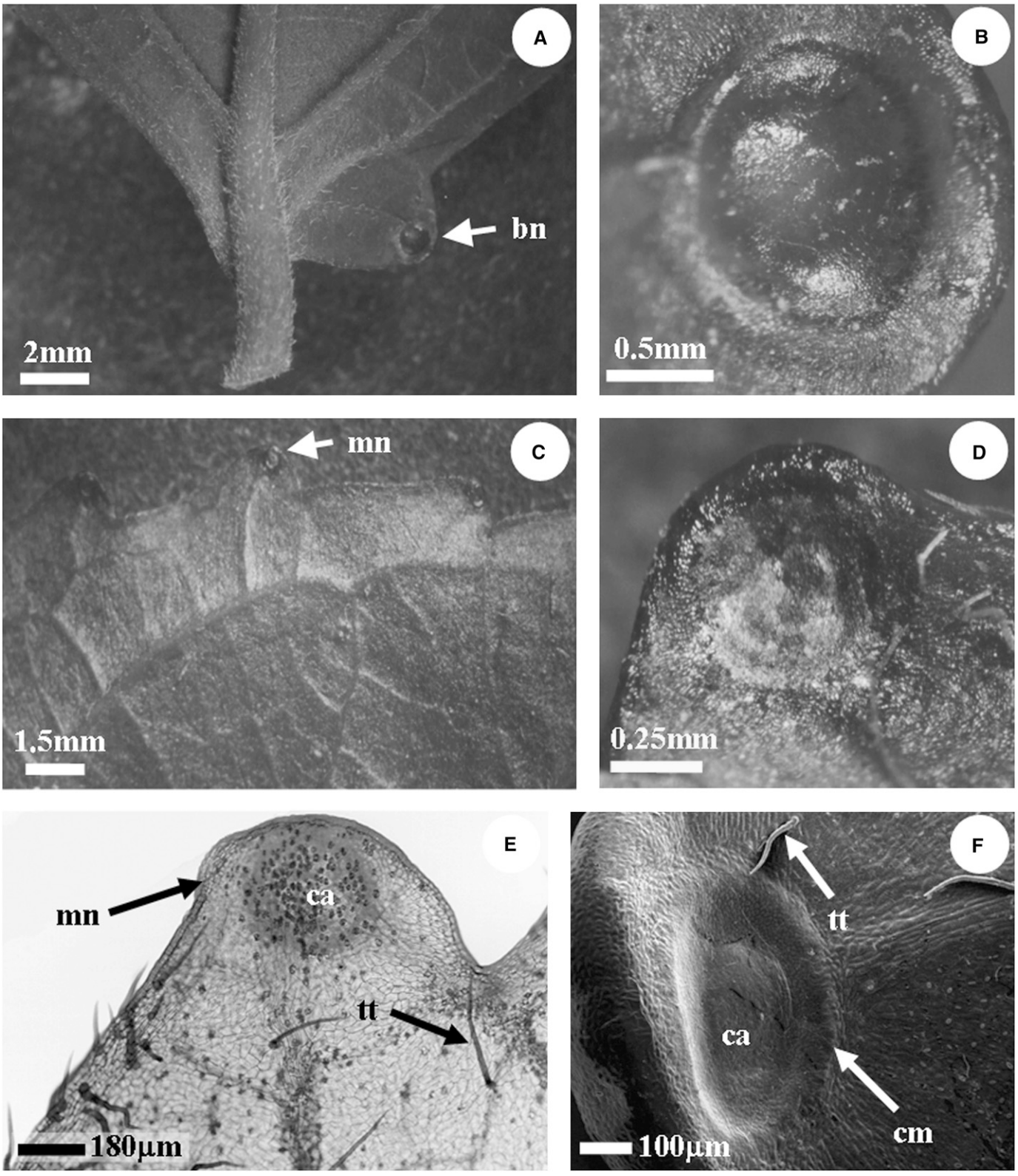

Fig. 1. Extrafloral nectaries of *Prockia crucis*. A–D. Observations with stereomicroscope. (A, B) Basal nectary, abaxial leaf surface. (C, D) Marginal nectaries, abaxial leaf surface. (E) Diaphanized leaf, highlighting marginal extrafloral nectary. (F) Scanning electron micrograph of basal nectary, with the cavity margin slightly elevated. bn = basal nectary; ca = cavity; cm = cavity margin; mn = marginal nectary; tt = tector trichome.

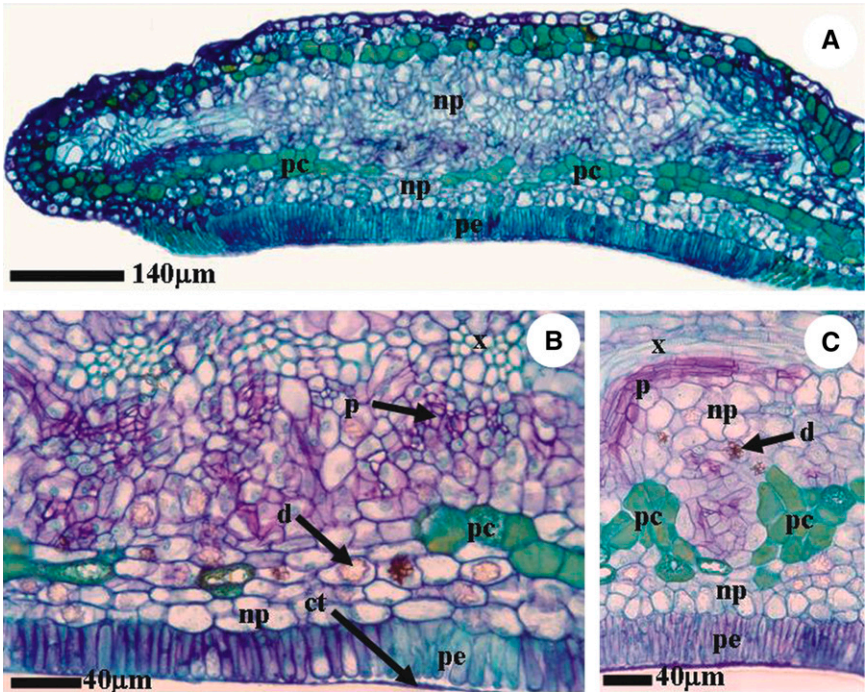

Fig. 2. Anatomy of the marginal extrafloral nectaries of the leaf of *Prockia crucis* (transverse sections). A–C. Toluidine blue. (A) Overall aspects. (B, C) Details of the secretory epidermis, nectariferous parenchyma, and vascularization. ct = cuticle; d = druse; np = nectariferous parenchyma; pc = phenolic compounds; pe = palisade-like epidermis; p = phloem; x = xylem.

ruthenium red had a thin pectocellulosic nature. The external periclinal wall was thicker and darkly stained by ruthenium red (Fig. 3C, D). The thick cuticle was stained by Sudan IV. In some sections, the cuticle was detached from the cells, forming small spaces where secretion is able to accumulate (Fig. 3A, B).

The subepidermic nectariferous parenchyma did not react to the histochemical tests applied (Fig. 3). This tissue was formed by layers of polygonal cells without chloroplasts, which have a peripheral nucleus, evident nucleolus and prominent vacuole (Fig. 2A–C). Druse-like crystalliferous inclusions (Fig. 2B, C) were abundant in this region. Scattered among these cells were other cells with granular cytoplasm that was stained deep green by toluidine blue (Fig. 2A–C), indicating the presence of phenolic compounds (Fig. 3F). Such cells were not always contiguous on the abaxial leaf surface; they were often interrupted by vascular extensions branching into the secretory epidermis. Vascular bundles composed of xylem and phloem reached the foliar margin where the nectary was located, nearly reaching the secretory epidermis (Fig. 2B, C).

Histochemical test results are summarized in Table 1. Hydrophilic (neutral polysaccharides, proteins, and phenolic compounds) and lipophilic (lipids) substances were detected. Alkaloids and starch were not detected. Granules present in the cytoplasm of the epidermis cells from the adaxial surface reacted with Sudan IV and Nadi reagent.

The development of the basal nectary was synchronized with the development of the nectaries along the margin, beginning very early in the development of the newly formed leaf primordium. The first developmental stage (stage I) was recognized as a dilated region on the primordium margin (Fig. 4A, B), with a single procambial bundle in its central portion surrounded by the ground meristem and protodermal cells. At this stage, the cells had dense cytoplasm, large nuclei, and intense cellular division. At the start of the differentiation, some cells of the future ground meristem stained dark green with toluidine blue. Nectary formation involved the activity of meristematic cells of the protoderm, primary meristem, and procambium.

After many consecutive anticlinal divisions (stage I), a group of protoderm cells in the leaf primordium margin were unique in having greater volumes and more prominent nuclei than the surrounding cells. These changes were characteristic of the initial stage of the EFN development. At later stages, these cells differentiated into columnar, secretory cells. The protoderm cells adjacent continued to divide anticlinally, and their derivatives underwent the same expansion (Fig. 4B–D). Subsequently, the protoderm cells of the leaf margin differentiated radially from the center to the periphery of the putative nectary. The external periclinal walls of the secretory epidermis were notably thick, particularly during the initial stages (I and II) of development (Fig. 4B, D).

At stage II (Fig. 4C, D), two distinct types of parenchymatic cells began to differentiate from the ground meristem. One group of these cells differentiates into polygonal and achlorophyllous cells with peripheral nuclei, an evident nucleolus, well-distinguished vacuoles, and dense cytoplasm. The second group of cells had granular cytoplasm and stained dark green with toluidine blue.

In the leaf primordium, the vascular system constituted a single procambial bundle that did not branch into the marginal portions. During the nectary development, no vascular system differentiated; regular foliar xylem and phloem vascularization after stage III, was observed all the way to the nectariferous parenchyma.

The leaf was coiled during the initial phases of development, and the nectary, though totally formed, was not yet exposed (Fig. 4E, F). Leaf blade expansion led to the uncurling of the leaf, and the nectary gained its typical ventral position

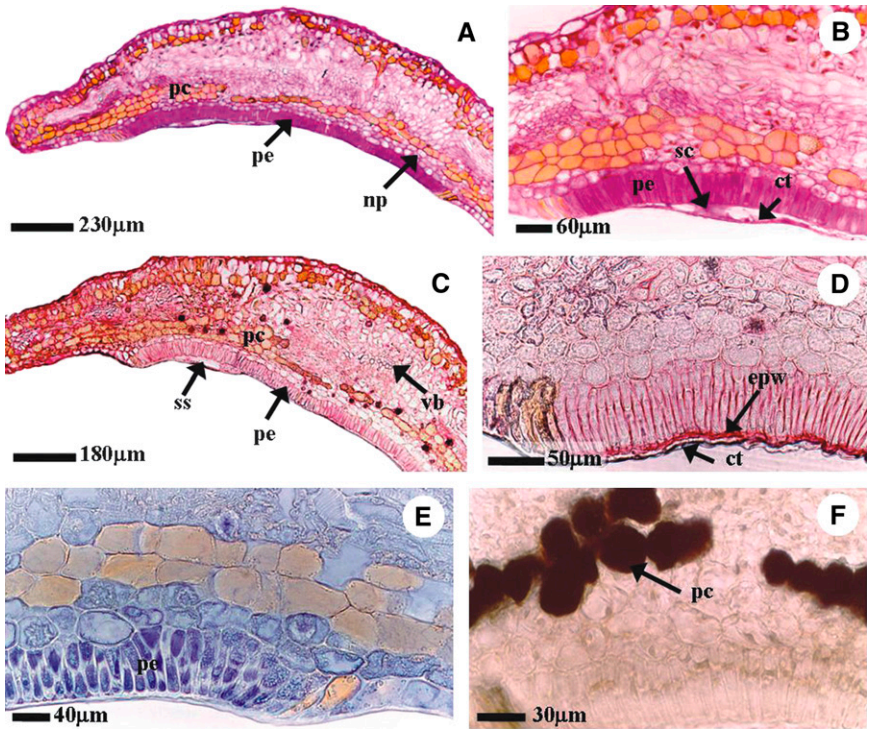

Fig. 3. Histochemistry of the marginal extrafloral leaf nectaries of *Prockia crucis* (transverse sections). (A, B) PAS highlighting a reaction with polysaccharides in the palisade-like epidermis and accumulation in the subcuticular space. (C, D) Ruthenium red staining highlights density of pectic contents in the external periclinal wall. (E) Detail of palisade-like epidermis, protein granules after staining with Coomassie brilliant blue. (F) Positive reaction to ferrous sulphate, highlighting the presence of some phenolic compounds in some cells of the nectariferous parenchyma. ct = cuticle; epw = external periclinal wall; np = nectariferous parenchyma; pc = phenolic compounds; pe = palisade-like epidermis; sc = secretion; ss = subcuticular space; vb = vascular bundle.

(Fig. 2A). At stage IV, the nectary was completely formed and could be identified by a palisade-like secretory epidermis, characteristic of the adult nectary (Fig. 4E, F).

DISCUSSION

The glucose, fructose, and sucrose in the glands of *P. crucis* characterized them as EFNs. As found in other plants such as cotton (with a 61.7% sugar nectar) and castor (74%), the high sugar concentration in the EFNs exudates may result from the the EFNs being more exposed than the floral nectaries and thus subject to more intense evaporation. The high sugar concentrations may also act as an indirect defense tool because they may increase ant visitation and permanence in the nectaries. Moreover, the concentrated extrafloral nectar dries into a viscous covering, which may exclude nonintended visitors such as lepidopterans, whose mouthpart morphologies require nectars with low sugar concentrations (Wäckers et al., 2001).

TABLE 1. Results of histochemical tests in extrafloral nectaries on leaves of *Prockia crucis*.

Metabolite	Test	Extrafloral nectary		
		Palisade-like epidermis	Nectariferous parenchyma	Adaxial epidermis
Total lipids	Sudan IV	–	–	+
Terpenoids (resin-oils)	Nadi reagent	–	–	+
Phenolic compounds	Ferrous sulphate*	–	–	–
	Phloroglucinol (lignin)	–	–	–
Alkaloids	Draggendorf reagent	–	–	–
Polysaccharides	PAS	+	–	–
Starch	Lugol	–	–	–
Pectins	Ruthenium red	–	–	–
Total proteins	Xilidine Ponceau, Coomassie brilliant blue	+	–	–

Notes: * Fixative; +, positive reaction; –, negative reaction

Although Klein and Sleumer (1984) observed the species to have two basal glands located at different levels, in this study, only one basal nectary was confirmed in many of the leaves observed. Morphological and micromorphological analyses of the margin and foliar base nectaries showed that they share similar structures, the sessile type and the flat type (Flachnektarien) described in the classification of Zimmermann (1932).

The *P. crucis* nectaries are highly structured. The anatomy of the foliar nectaries in Acanthaceae (McDade and Turner, 1997), Euphorbiaceae (Freitas et al., 2001), Rosaceae (Dorsey and Weiss, 1920), Salicaceae (Curtis and Lersten, 1974, 1978, 1980; Wilkinson, 2007), and Verbenaceae species (Padma Rao and Ramayya, 1992) were largely similar.

Under the Cronquist system (1968), Flacourtiaceae and Salicaceae are closely related families. This study is the first confirmation of EFN structures in *Prockia*. These structures were considered to be rare among the Flacourtiaceae (Elias, 1983), but a recent paper described them as present in six species of 'Flacourtiaceae' and also confirmed their presence in two

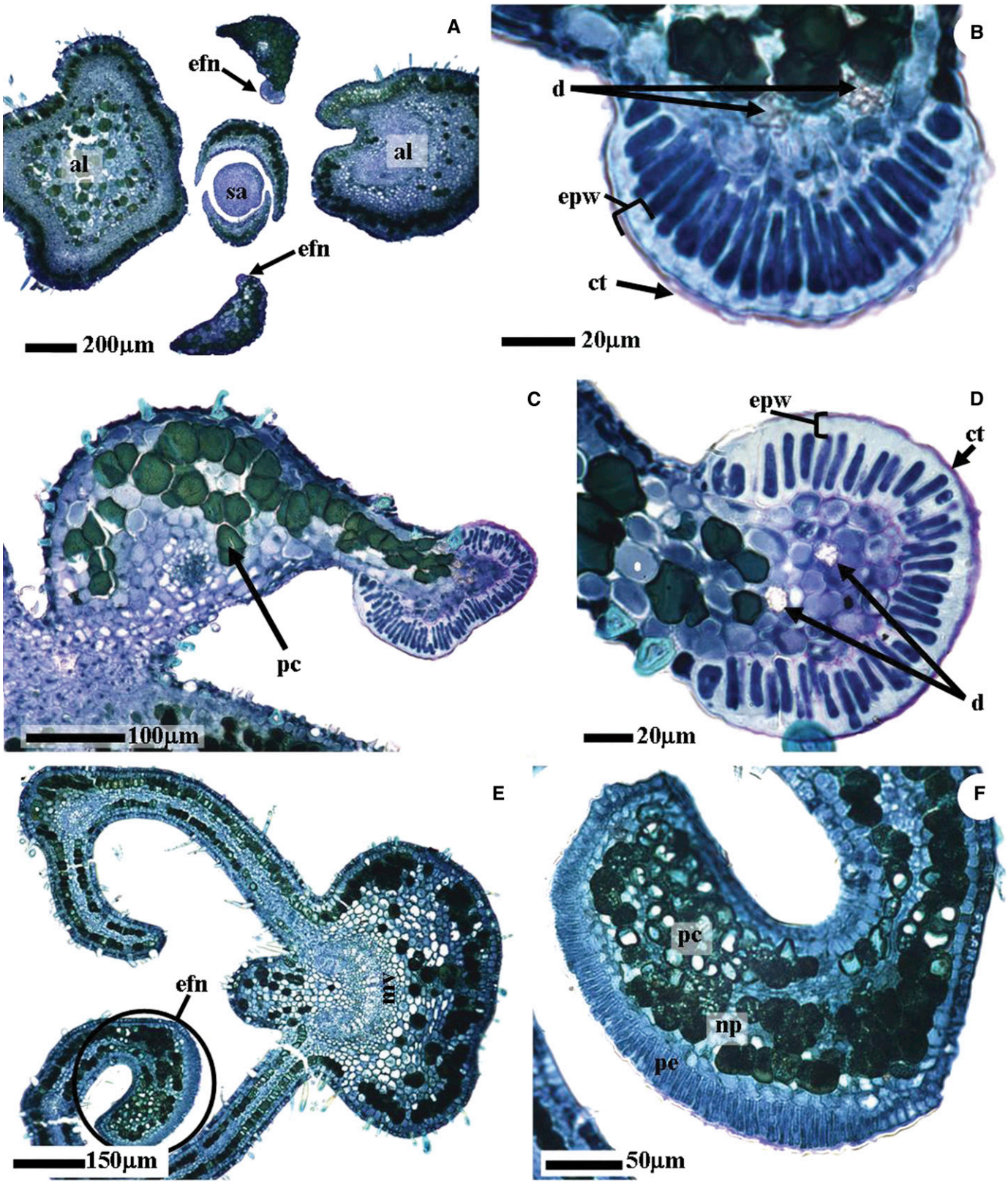

Fig. 4. Nectary on margin of *Prockia crucis* leaf at different stages of development (transverse sections stained with toluidine blue). (A) Shoot apical meristem, EFNs at stage I. (B) Leaf primordium with nectary at stage I. (C, D) Leaf with stem portion, highlighting stage II of nectary development; (D) detail of thickened epidermal cell walls. (E, F) Leaf with fully formed nectary (stage IV), located in the margin of expanding leaf. al = adult leaf; ct = cuticle; d = druse; efn = extrafloral nectary; epw = external periclinal wall; mv = middle vein; np = nectariferous parenchyma; pb = procambial bundle; pc = phenolic compounds; pe = palisade-like epidermis; sa = shoot axis.

genera of Salicaceae—*Populus* and *Salix* (Wilkinson, 2007). In *Populus* (Curtis and Lersten, 1974, 1978) and *Salix* (Curtis and Lersten, 1980), resin glands were reported with secretion of nectar, resin, or both, depending on the species. In *Populus*, a transition between resin and nectar secretion is likely (Curtis and Lersten, 1978). The anatomy of these structures in Salicaceae, the six taxa of 'Flacourtiaceae' (Wilkinson, 2007), and *P. crucis* is highly similar, contributing support to the phylogenetic proximity of these taxa.

The calcium oxalate crystals in extrafloral nectaries, observed here in *P. crucis*, are common in several taxonomic groups (Metcalfe and Chalk, 1979; Elias, 1983; Paiva and Machado, 2006). These crystals may be related to phloem assimilates (Elias and Gelband, 1977), the major source of nectar precursors (Fahn, 2000). Calcium immobilization in the phloem region may be explained by the fact that the transport of sucrose, the main component of *P. crucis* nectar, involves ATPase activity, which may be inhibited by Ca^{2+} (Leonard and Hodges, 1980).

Secretory fluids that accumulate in subcuticular spaces may be eliminated via cuticular pores, cuticle rupture, or in more permeable cuticular regions, as described for other types of nectaries and secretory trichomes (Fahn, 1979). In *P. crucis*, the secretor fluids accumulate in the subcuticular space, indicating that the nectar must be released through cuticle rupture. Further research may confirm this hypothesis.

Vascularized nectars, such as those of *P. crucis,* are highly specialized, and are only produced for several weeks, when the leaves are fully expanded (Elias et al., 1975). The nectaries of *P. crucis* remain protected throughout development and are exposed only when they are mature, after the secretion process has already been initiated.

Histochemical tests were positive for numerous substances including polysaccharides, proteins, and lipids in *P. crucis* nectaries. These compounds are considered nutritionally important to several classes of visitors (Baker and Baker, 1990), suggesing that ant species may play a mutualistic role in plant defense.

Phenolic compounds, as detected in the EFNs of *P. crucis*, are often found in the parenchymatous cells close to the nectary secretory cells (Elias, 1983). These compounds may inhibit herbivores and pathogens (Koptur, 1992), may protect the cells against excessive ultraviolet radiation, and may maintain protoplast integrity under water stress (Taiz and Zeiger, 2004).

This report is the first of EFNs in the genus *Prockia*. The sucrose-rich nectar was found to be of high energetic value. Nectaries developed early; they were fully formed and active in the expanding leaves. The EFNs of *Prockia crucis* are highly similar to the salicoid teeth of *Populus* and *Salix* species, lending strong support to the phylogenetic proximity of these clades.

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