Exploring the Hidden Connections: Endophytic System and Flower Development of *Pilostyles berteroi* (Apodanthaceae) and Their Interactions with the Host *Adesmia trijuga* (Fabaceae)

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Article

Exploring the Hidden Connections: Endophytic System and Flower Development of *Pilostyles berteroi* (Apodanthaceae) and Their Interactions with the Host *Adesmia trijuga* (Fabaceae)

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Abstract: *Pilostyles*, an endoparasitic genus within the Apodanthaceae family, grows inside host stems, with flowers and fruits being the only external manifestations. Previous studies of *P. berteroi* growing on *Adesmia trijug*a provided limited details of the endophyte and omitted the origin of flowers and synker structure. This study, using classical methods of optical microscopy applied to the analysis with scanning electron microscopy and confocal laser scanning microscopy, expands the understanding of the *P. berteroi*/*A. trijuga* complex. We find that *P. berteroi* develops isophasically with its host, forming meristematic patches between secondary phloem cells. The parasitized *Adesmia* stem's cambium primarily produces inward parenchyma cells, with limited vessel production and ceased fiber formation. The polarization of meristematic patches led to the formation of floral meristems. Flowers develop endogenously and emerge by the breakthrough of the host stem. Flowers connect to the host cambium via chimeric synkers, combining *P. berteroi* parenchyma and tracheoids with *Adesmia* vessels. Unlike previous studies that show uniformity among *Pilostyles* species, our analysis reveals new insights into the structural interaction between *P. berteroi* and *A. trijuga*. Further ultrastructural investigations are needed to explore potential direct or indirect connections between the vascular systems of the two taxa.

Keywords: CLSM; chimera; endoparasites; holoparasitic; SEM; sinkers; tracheoids; wood

1. Introduction

Researchers have always been fascinated by parasitic plants, their life cycles, complex and bizarre structures, physiology, and interaction with their hosts [1,2]. Due to the strange and cryptic nature of this life form, they have been the subject of multiple studies, especially in recent years with researchers contributing to special issues of botanical journals [3–12].

Traditional classifications divide parasitic plants into hemiparasites, capable of photosynthesis, and holoparasites, achlorophyllous plants, totally dependent on their host [1]. Other classifications present functional groups, taking into account, in addition to the traditional characters, others such as type of haustorium, place of connection with the host, growth form, and type of germination, among others [5,6,8,13]. Thus, Tesitel [13] proposes four functional groups: root hemiparasitism, root holoparasitism, stem parasitism, and endophytic parasitism. Recently, Teixeira-Costa and Davis [5] identified five functional groups of parasitic plants focused on life history: euphytoids, mistletoes, parasitic vines, obligate root parasites, and endoparasites.

Among the variants of parasitism among plants, endoparasites represent the most advanced and specialized form of holoparasitism [1,2,5,13,14]. Endoparasites are emphasized as a distinct functional group due to the "*predominance of an endophytic phase in their life cycle*" [13]. These organisms

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have a unique vegetative structure, as they are completely enclosed in the stems of their host plants, where they grow as fungi [6,13,15,16]. The only visible signs of the parasite are the flowers and fruits that emerge from the host stem through the bark.

Endoparasites are present in families such as Apodanthaceae, Cytinaceae, Mitrastemonaceae and Rafflesiaceae [5,16,17]. Alternatively, they may appear as shoots in various endophytic mistletoe genera within the Santalales [5,13].

The family Apodanthaceae Tiegh. ex Takht., belonging to the order Cucurbitales, consists of endoparasites, including two genera and 13 species [17,18]. The genus *Apodanthes* Poit. is monospecific and can only be found in the tropical rainforests of the New World. On the other hand, the genus *Pilostyles* Guill. consists of 12 species and has a broad distribution that spans arid regions in North, Central, and South America, Iran, Iraq, Syria, subtropical East Africa, and southwestern Australia [17,19–22]. *Pilostyles* species grow inside the stems of species in the Fabaceae and Salicaceae families [17,23]. *Pilostyles berteroi* Guill. was collected from *Adesmia trijuga* Gillies ex Hook. & Arn. during field trips in NW Argentina.

The endophytic body of *Pilostyles* is simple, without typical organs, such as roots, stems, and leaves, presenting only a mycelial-like system formed of parenchymatous cells and tracheary elements that invade the host stem [14,23–32]. The development of morphology, anatomy, and flowers has been documented in various species within the Apodanthaceae family [18,25,28,30,35–38]. Kummerow [28] made the only study of *P. berteroi* growing on *A. trijuga*, focusing on the general structure of the staminate and pistillate flowers and proposing a mechanism of emergence through the cortex of the legume. Based on previous descriptions by Endriss [27] and Solms-Laubach [25], who studied *P. blanchetii* [Gardner) R. Br. and *P. haussknechtii* Boiss. respectively, Kummerow [28] provides general details of the endophytic body. Their descriptions do not take into account the development of the flowers. A comprehensive morphological and anatomical analysis of the sterile and fertile cycles in staminate and pistillate flowers of *P. berteroi*, including gamete formation, fertilization, and seed development, has been undertaken by Romero, Sato & Gonzalez [38].

The host of *P. berteroi* is *Adesmia trijuga*, a spiny legume native to Argentina and Chile that grows in semi-arid or arid mountainous regions at elevations of 1100-3000 m [39–41]. Anatomical studies of *A. trijuga* have focused on the leaves [42] and some secretory structures [43]. The wood anatomy has only been described in *A. boronioides* Hook [44], *A. rahmer*i Phil., and *A. spinosissima* Vogel [45]. Within the Apodanthaceae family, the impact of holoparasite growth inside the host has been studied in *P. blanchetii* [Gardner) R. Br. in various species of *Mimosa* [23].

Given the scarce knowledge of this particular species of endoparasitic plant, the objectives of this study are: 1) Provide a comprehensive description of the anatomical differences between healthy *A. trijuga* stems and those parasitized by *P. berteroi*; 2) Re-evaluate the characteristics of the endophyte; 3) Elucidate the process of flower primordia formation; and 4) Detail the structure and cellular composition of the sinkers, comparing these characteristics with those of other *Pilostyles* species analyzed so far.

2. Results

2.1. Pilostyles Berteroi - Adesmia Trijuga System

Adesmia trijuga is a spiny, microphyllous shrub (Figure 1A,B). Its stems are characterized by the rapid development of secondary growth, which appears only a couple of centimeters from the apex of the branches (Figure 1C). External signs of parasitism by *P. berteroi* on the stems of *A. trijuga* are the appearance of flowers and the scars left by fallen staminate flowers or fruits from previous seasons (Figure 1C–F).

The flowers break through the bark on stems with secondary growth, approximately 30-40 cm from the apex of the branch (Figure 1C,D). These unisexual flowers are distributed across different branches of the same *A. trijuga* specimen. They emerge gregariously through the bark in longitudinal rows (Figure 1D–F). The flowers of *P. berteroi* appear acropetally on the apical branches of *A. trijuga*. In the upper half of the infested area, flower buds are intermixed with blooming flowers (Figure

 doi:10.20944/preprints202409.1881.v

1D,E). On the other hand, at the base of the infested area, the senescent flowers or fruits and the scars from the flowers of the previous season are visible (Figure 1F). Thus, each year, the appearance of flowers progresses towards the apex of the branches of *A. trijuga*.

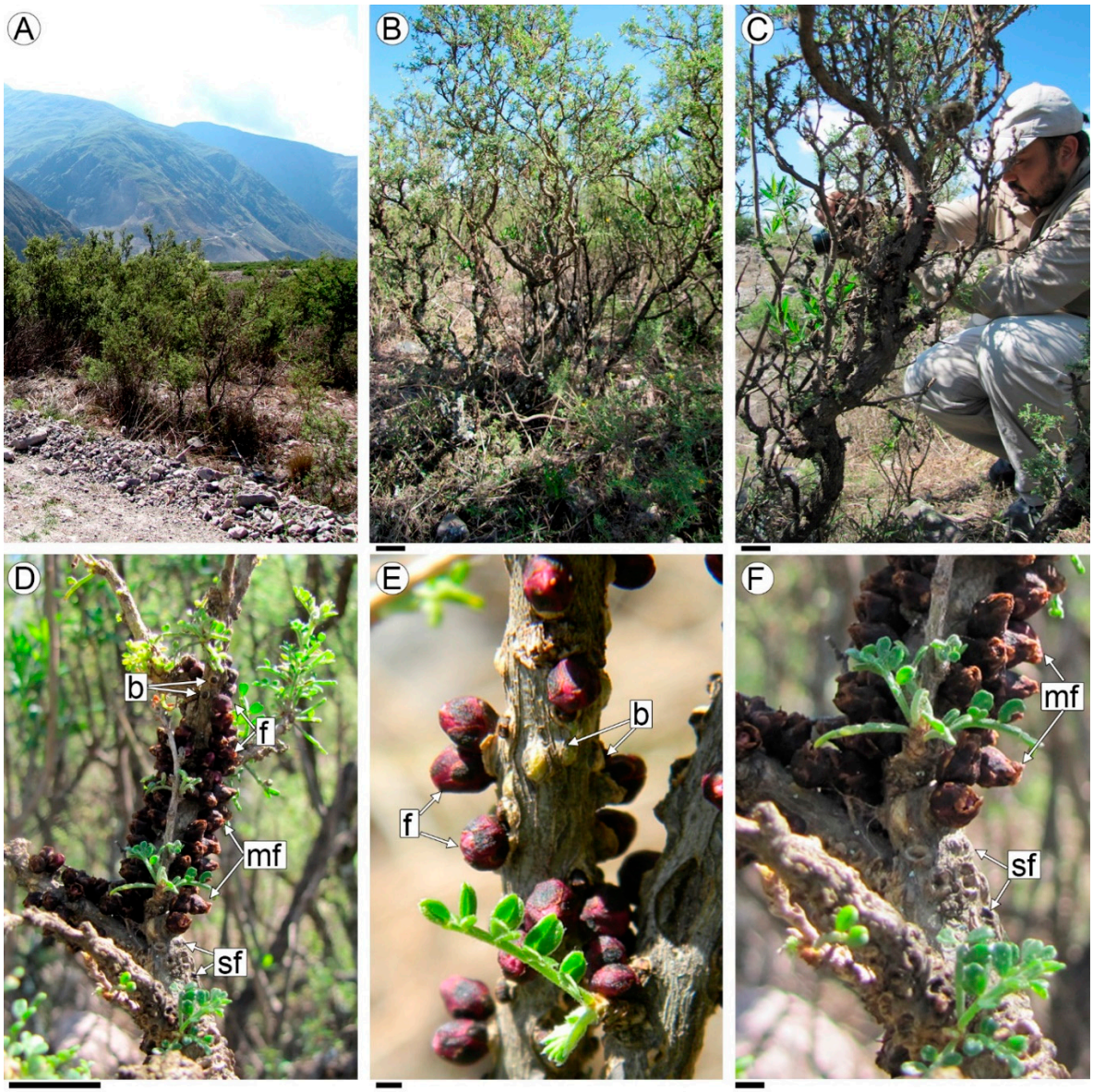

Figure 1. Specimens of *A. trijuga* parasitized by *P. berteroi*. (**A**) General aspect of the landscape with *A. trijuga* plants; (**B-C**) *Adesmia trijuga* plants with flowers of *P. berteroi*; (**D-F**) *Pilostyles berteroi* flowers emerging from host stems; (**E**) Detail of buds and flowers at anthesis; (**F**) Basal region with older staminate flowers and scar from previous year's flowers. Abbreviations: b: flower buds, f: flowers in blossom, mf: mature flowers; sf: scars of flower or fruits. Scales: B-C: 10 cm; D: 1.5 cm; E-F: 0.5 cm.

2.2. Anatomy of Non-Parasitized Stems of Adesmia Trijuga

The stems with secondary growth are characterized by the presence of a continuous ring-shaped periderm, composed of phellogen or the cork cambium, phellem or "cork" to the outside, and phelloderm to the inside (Figure 2A,B). The phellogen and phelloderm are unistratified, and the suber varies from 3 to more than 10 cell layers (Figure 2A,B). All periderm cells form very ordered stacks aligned in radial brick-like columns with no intercellular spaces (Figure 2B). Lenticels are abundant and large relative to the stem diameter (Figure 2C).

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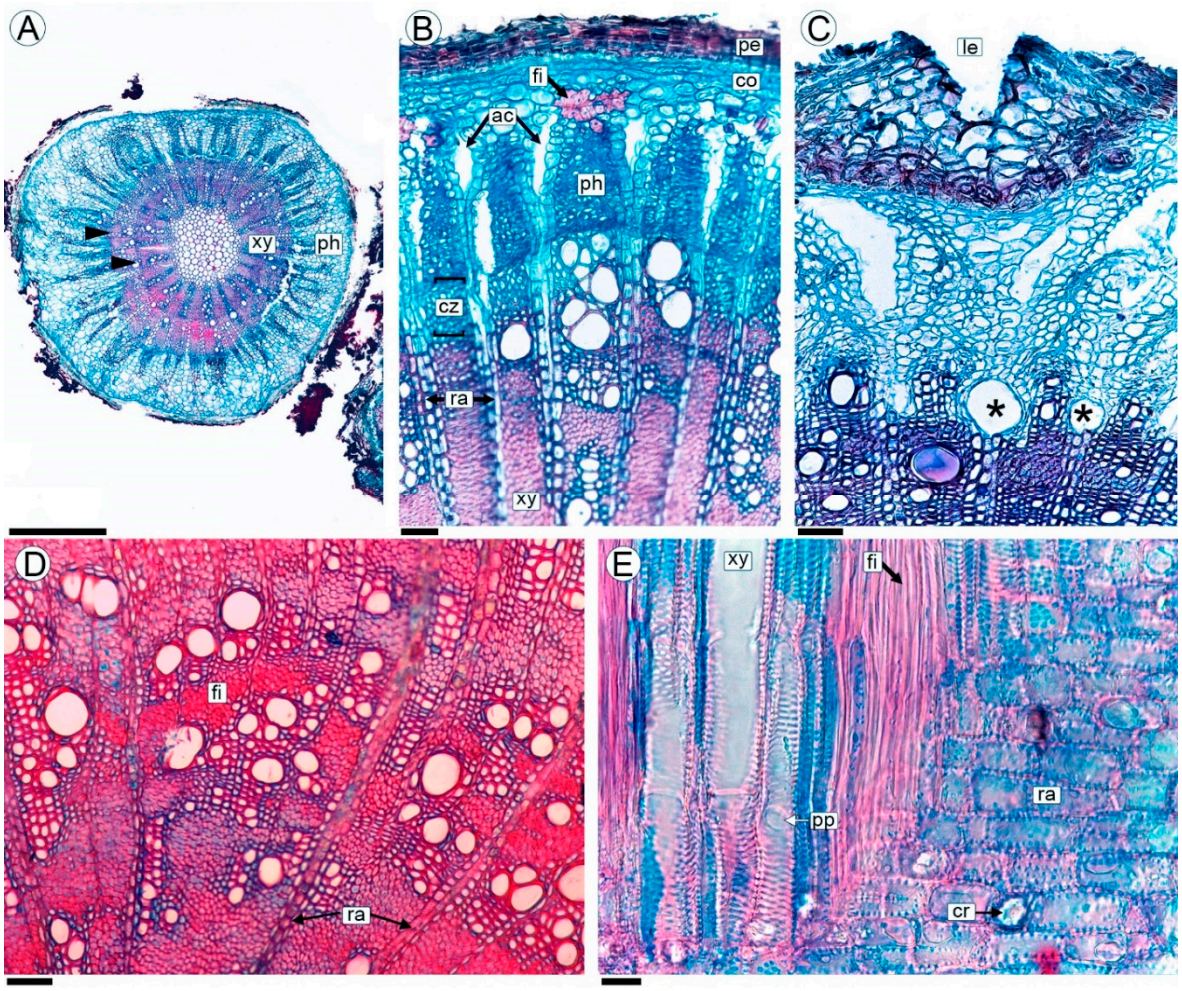

Figure 2. Anatomy of the non-parasitized stem of *A. trijuga* in transverse (**A-D**) and radial longitudinal sections (**E**), analyzed with LM. (**A**) Transection of the non-parasitized secondary stem; (**B-C**) Detail of phloem and cambial zone in a stem with vessels in the process of formation (*); (**D**) Secondary xylem showing diffuse porosity; (**E**) Radial longitudinal section of the xylem with vessels, fibers, and rays. Abbreviations: ac: air chambers, co: cortex, cr: prismatic crystals, cz: cambial zone, fi: fibers, le: lenticels, pe: periderm, ph: secondary phloem, pp: perforated plates, ra: rays, xy: secondary xylem. Scales: A: 0.5 mm; B-D: 50 μm; E: 20 μm.

The cortex, located in the transition zone between the periderm and the secondary phloem, consists of parenchymatous cells with very thin cellulose walls. The primary phloem has small caps of thickened-walled fibers. The secondary phloem is characterized by sieve elements and thin-walled fibers, with wide phloem rays that are 2-3 cells thick at the cambium level and widen towards the periphery, forming long triangular extensions (Figure 2 B). In young stems, ray cells separate to form large radially arranged air chambers (Figure 2B,C).

The secondary xylem has poorly defined annual rings. Thick-walled fibers characterize the growth ring limit (Figure 2A,B). Porosity is diffuse, vessels can be either mostly solitary or arranged in clusters. The vessels range in diameter from 13.8 to 34.2 μm and have simple perforated plates and vestured pits (Figure 2B,C,E). Xylem fibers have very thick walls and simple to minutely bordered pits. Axial parenchyma is paratracheal. Rays are 1-3-seriate with thickened secondary walls, conspicuous simple pits, and abundant prismatic crystals (Figure 2B,D,E).

2.3. Parasitized Stems

The first evidence of *P. berteroi* cells appears in secondary growth stems with at least 1-2 growth rings (Figures 3A and S1A). The endophytes grow vegetatively as more or less rounded and compact

 doi:10.20944/preprints202409.1881.v

clusters or meristematic patches (mp) interspersed among the elements of the secondary phloem of *A. trijuga* (Figure 3B,C). All *P. berteroi* cells in mp are parenchymatous, isodiametric, with uniformly thin walls, and no intercellular spaces (Figure 3C,D). Endophytic cells have a prominent, more or less centrally located nucleus (6.15-8.25 µm), often with two nucleoli. They have a dense cytoplasm and a marked absence of vacuoles, which distinguishes them from the host tissue (Figure 3C). These cells have no visible starch granules but react positively to various dyes (Lugol, Sudan black, and ferric chloride, Figure 3E–G). There is no cell differentiation or vascular elements within the mp (Figure 3D).

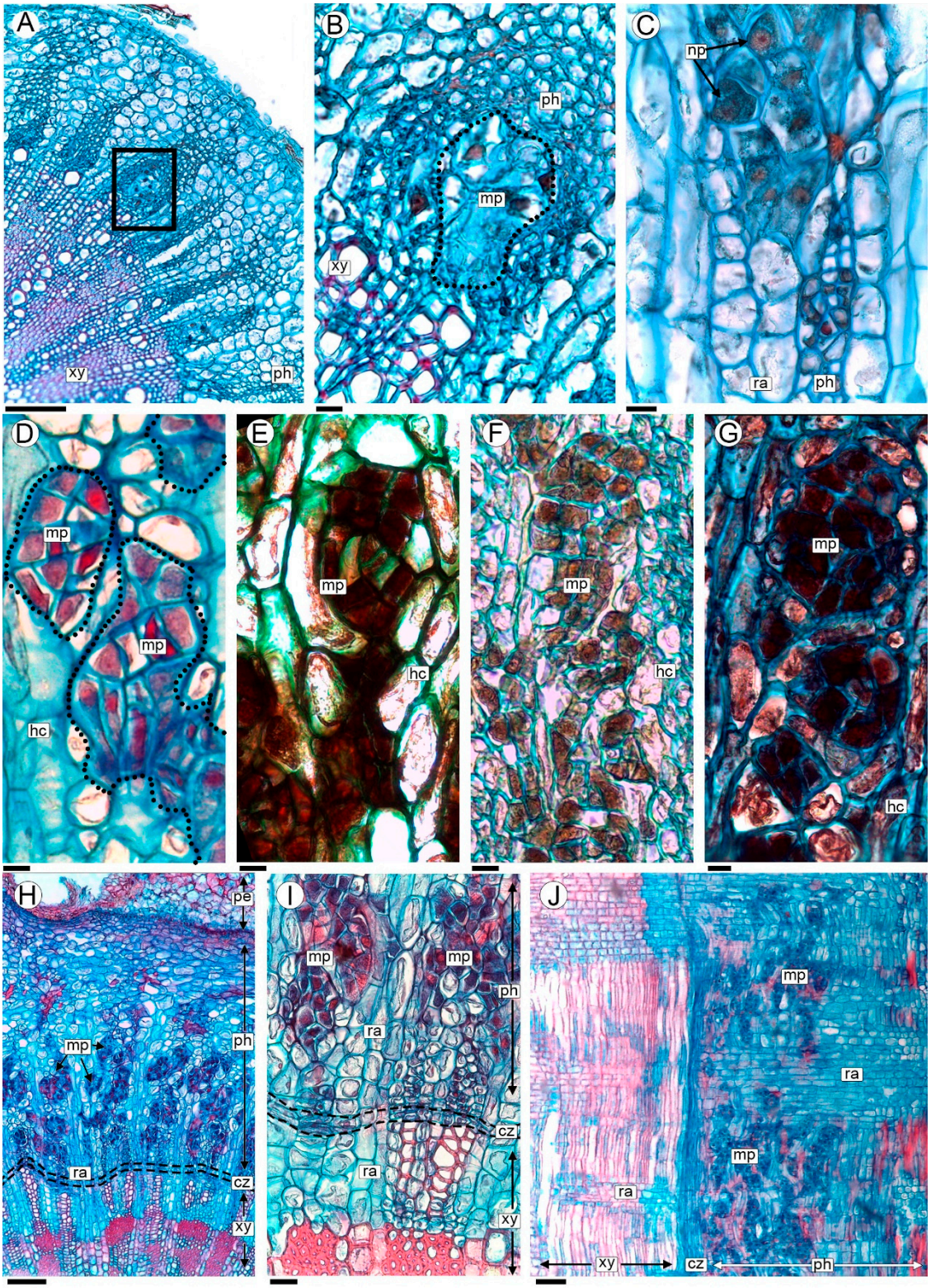

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Figure 3. Stems of *A. trijuga* parasitized by *P. berteroi* in transverse (**A-I**) and radial longitudinal section (**J**), analyzed with LM. (**A**) Stem of *A. trijuga* with early stages of parasite development, the first meristematic patches (mp) of *P. berteroi* cells are seen in the phloem (inset); (**B**) Detail of a mp of *P. berteroi* corresponding to the box in photo A; (**C**) Detail of a mp of *P. berteroi* in secondary phloem; (**D-G**) Details of a mp of *P. berteroi* subjected to different histochemical tests; (**D**) Safranin-astra blue, (**E**) Lugol, (**F**) Ferric chloride, (**G**) Sudan black; (**H-J**) Parasitized stems showing the presence of numerous parasite nodules that occupied most of the secondary phloem. Abbreviations: cz: cambial zone, hc: host cells, mp: meristematic patches, np: parasite nuclei, pe: periderm, ph: secondary phloem, ra: rays, xy: secondary xylem. Scales: A, J: 100 µm; B-F: 10 µm; G, I: 20 µm; H: 50 µm.

As the host stem grows, mp cells divide and proliferate throughout the secondary phloem, forming a mycelial-like structure representing *P. berteroi*'s vegetative body (Figures 3H–J and S1). When the cambium of a stem invaded by an endoparasite is forming new elements, the mps cells penetrate the cambial region, at the level of the rays. However, they were never observed penetrating xylem vessels (Figure 3H–J).

The cambium of *A. trijuga* shows abnormal growth as *P. berteroi* invades the stems. The cambium produces mostly parenchyma cells towards the interior, vessel production is limited to some sectors and fiber formation ceases (Figures 3H–J and S1). In addition to the proliferation of mps, the secondary phloem cells that are formed by the cambium during the *P. berteroi* infestation do not show any signs of variation (Figures 3H–J and S1).

2.4. Origin of Pilostyles' Flowers

Pilostyles berteroi flower differentiation occurs entirely within the mp, i.e., it is completely endogenous (Figures 4A–F and S2). The development of floral meristems is preceded by an increase in the mass of parasitic cells in the mp (Figure 4A). These mp become polarized, with the inner group of cells (oriented towards the center of the *A. trijuga* stem) retaining the previously described characteristics. Meanwhile, histological differentiation occurs in the outer cells (oriented towards the periderm of the host stem), where the cells slightly expand in size, and there is an increase in vacuolization, with small nuclei relative to the size of the cells (Figure 4B). Parenchymatisation and cell elongation proceed acropetally. This group of new cells grows and develops into a floral meristem (Figure 4B). Almost immediately, and before crossing the peridermis, the floral organs are formed (Figure S2). Development is so rapid that no mitotic figures are observed.

Host cells contacting floral primordium collapse, gradually forming a small chamber where floral meristem and flowers develop (Figures 4B,C and S2). The floral meristem develops either staminate or pistillate flowers (Figure 4E,F). They are distributed on different branches of the same individual of *A. trijuga*. The flower develops and grows through the periderm and emerges from the stem of the *A. trijuga* plant (Figures 4D–F and S2). Flowers emerge perpendicular to the length of *A. trijuga* branch (Figure 4D–F). New floral primordia are formed as the parasite mps invade new host tissues, always toward the apex of the host branches (Figure 4E,F).

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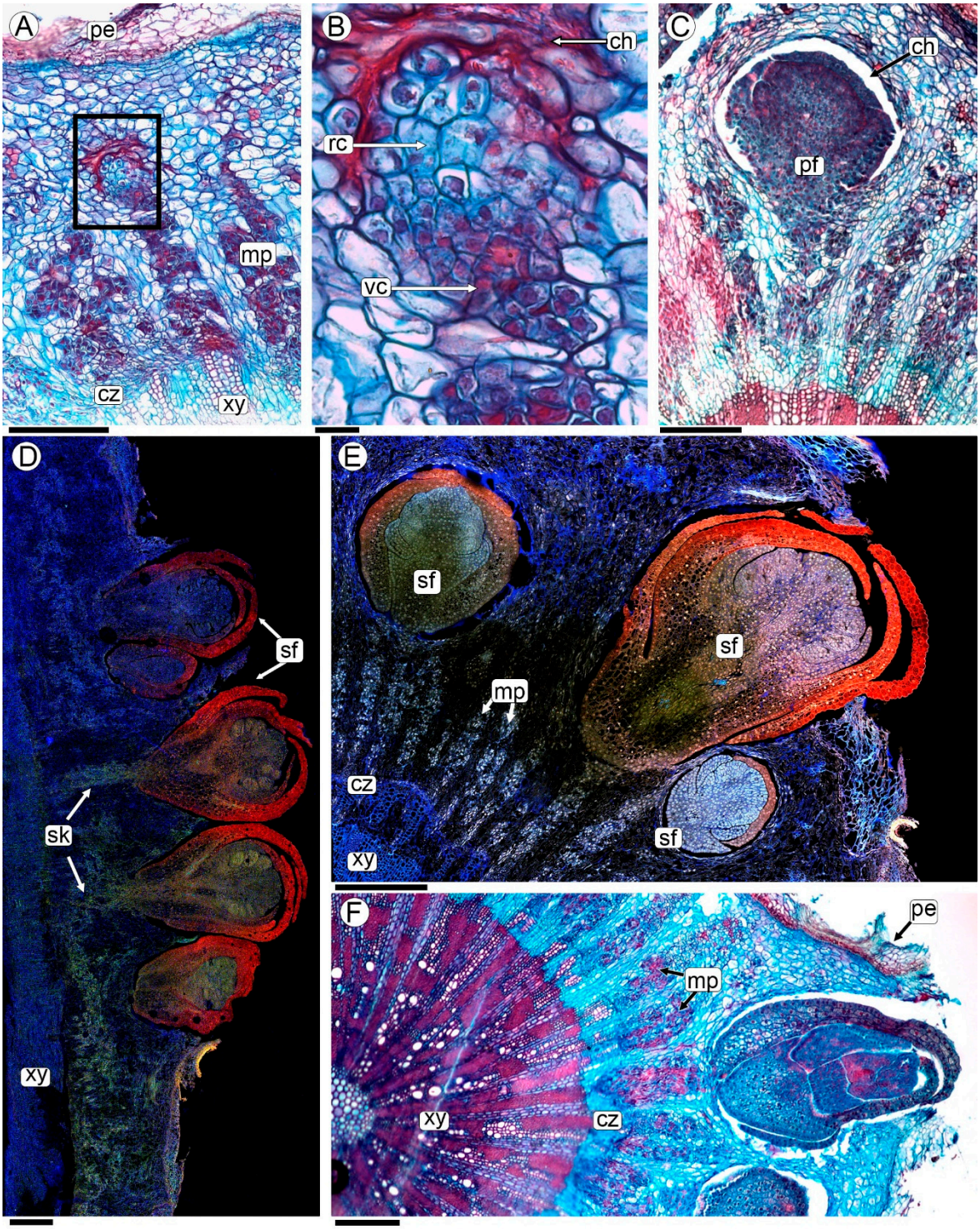

Figure 4. Flower development of *P. berteroi* in stems of *A. trijuga*, analyzed with LM (**A-C, F**), and CLSM merged three channels (ex/em, blue: 405/425–489nm, green: 503/508–633nm, red: 627/635-750nm (**D-E**). (**A-C, F**) Cross-sections of infested *A. trijuga* branches showing the origin of flowers; (**D**) Longitudinal section of *A. trijuga* branch; (**A**) Origin of the *P. berteroi* flower from a mp; (**B**) Close-up of inset in (**A**) showing the polarized mp with vegetative and reproductive parasitic cells surrounded by collapsed host cells; (**C**) Young parasite flower, still included in the host phloem; (**D-F**) *P. berteroi* staminate flowers at various stages of development, some already emerging from the *A. trijuga* branch. Abbreviations: ch: collapsed host cells, cz: cambial zone, fp: flower primordium, mp: meristematic patches, pe: periderm, rc: reproductive cells, sf: staminate flower, sk: sinker, vc: vegetative cells, xy: xylem. Scales: A: 200 μm; B: 20 μm; C: 100 μm; D-E: 0.2 mm; F: 1 mm.

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2.5. Sinkers of Flowers

The formation of a peg-like extension, the sinker, can be observed at the base of each flower. The sinkers connect the flowers of *P. berteroi* to the cambial region of the host (Figures 4D, 5A–F, S2 and S3). In flowers already formed, the sinker has a greater volume, consisting of abundant parasite parenchyma and intermingled xylem elements belonging to both species (Figures 5B–F and S2F). The xylem elements of *P. berteroi* are tracheoids, that are almost always oriented with their long axis perpendicular to the sinker of the flower (Figure 5B–F). Tracheoids are short, thick-walled cells with scalariform, non-vesiculated pits. They lack perforating plates (Figure 6A–D).

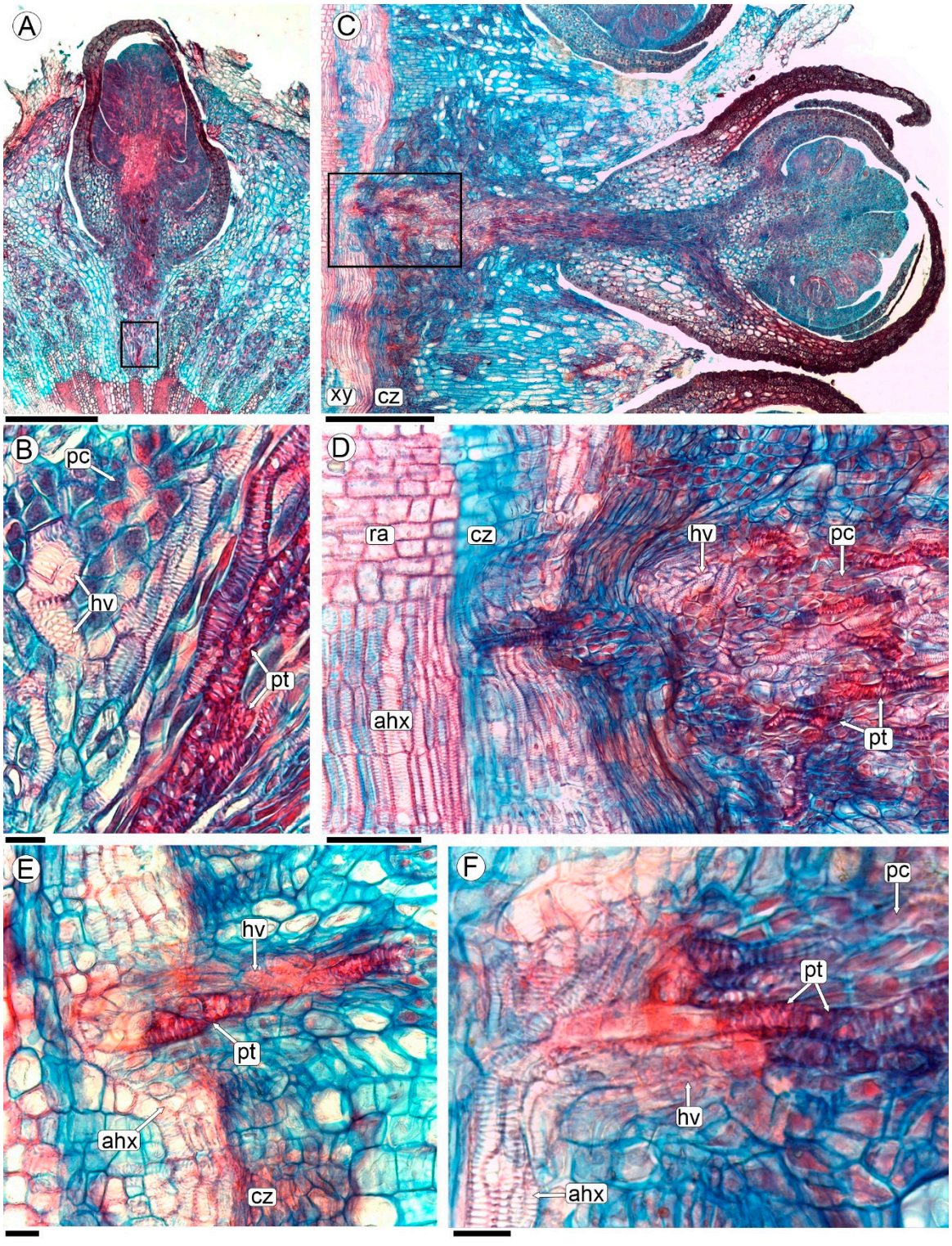

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Figure 5. Sinker formation in *P. berteroi* flowers in transverse (**A-B**) and longitudinal radial section (**C-F**), analyzed with LM. (**A-D**) Flowers of *P. berteroi* and their sinkers in contact with the cambium of the host plant; (**B**) Detail of the sinker showing parasite and host xylem elements separated by parenchymal cells, corresponding to the region indicated in photo (**A**); (**D**) Close-up of the plate in the radial longitudinal section of the stem, corresponding to the boxed area in the photo (**C**); (**E**) Detail of the cambial zone showing the sinker with the *A. trijuga* vessels and the tracheoids of *P. berteroi*; (**F**) Another detail showing the *A. trijuga* vessels of the axial system and those entering the sinker. The vascular elements of *A. trijuga* in the secondary xylem are not invaded by the parasite. Abbreviations: ahx: axial host xylem, cz: cambial zone, hx: host vessels, pc: parenchyma cell, pt: parasite tracheoid, ra: rays, xy: xylem. Scales: A: 200 μm; B, E-F: 20 μm; C: 500 μm; D: 100 μm.

Conversely, *A. trijuga* xylem elements that form the sinker are vessels with simple perforated plates. (Figure 6E). Using MO, *P. berteroi* tracheoids can be distinguished from *A. trijuga* vessels by their more intensely stained walls (Figure 5B–F). The *A. trijuga* vessels in the interior of the sinkers are almost always oriented with their longitudinal axis parallel to the sinkers, as are the tracheoids of the *P. berteroi* (Figure 5B,E,F). This means the *A. trijuga* xylem elements inside the sinkers are perpendicular to those of the secondary xylem of the stem.

The sinkers do not penetrate the xylem of the *A. trijuga* stem, remaining confined to the cambial zone (Figures 5D,E and S3). Vessel production from the cambium of *A. trijuga* into the sinker is evident, although sinkers are entirely formed within the secondary phloem region of the *A. trijuga* stem (Figure 5B,D–F). The vascular elements of *A. trijuga* in the secondary xylem are not invaded by the parasite, exhibit a larger diameter, and are perfectly aligned longitudinally with the stem (Figure 5E,F).

The xylem elements of the two species in the synkers were never observed to be in direct contact, there is always at least one layer of elongated parenchyma cells between them (Figures 6A,B and S3). These parenchymatous cells have a small diameter and an elongated shape, parallel to the vascular elements. However, the presence of a dense cytoplasm allows us to assume that they belong to *A. trijuga*.

In the tracheoids of *P. berteroi*, the wall is thickened in an annular or helical pattern with smooth ridges (Figures 6C,D,G and S3). The elements of the xylem of *P. berteroi* are always tracheoid (Figure 6G,H), both those that are present in the sinker and those that are found inside the flowers. The walls of the vessel of *A. trijuga* located inside the sinkers have scalariform inter-vessel pits, which are arranged in a ladder-like series (Figure 6E–G). Vestures are present in the pits and helical thickenings of the vessel walls. They are tiny protuberances on the secondary wall of the vessel. These ingrowths are only visible in the SEM (Figure 6E,F). The xylem vessels of the axial system of the stem of *A. trijuga* are longer than those of the sinkers and have numerous intervascular pits, alternate or opposite (Figure 6I).

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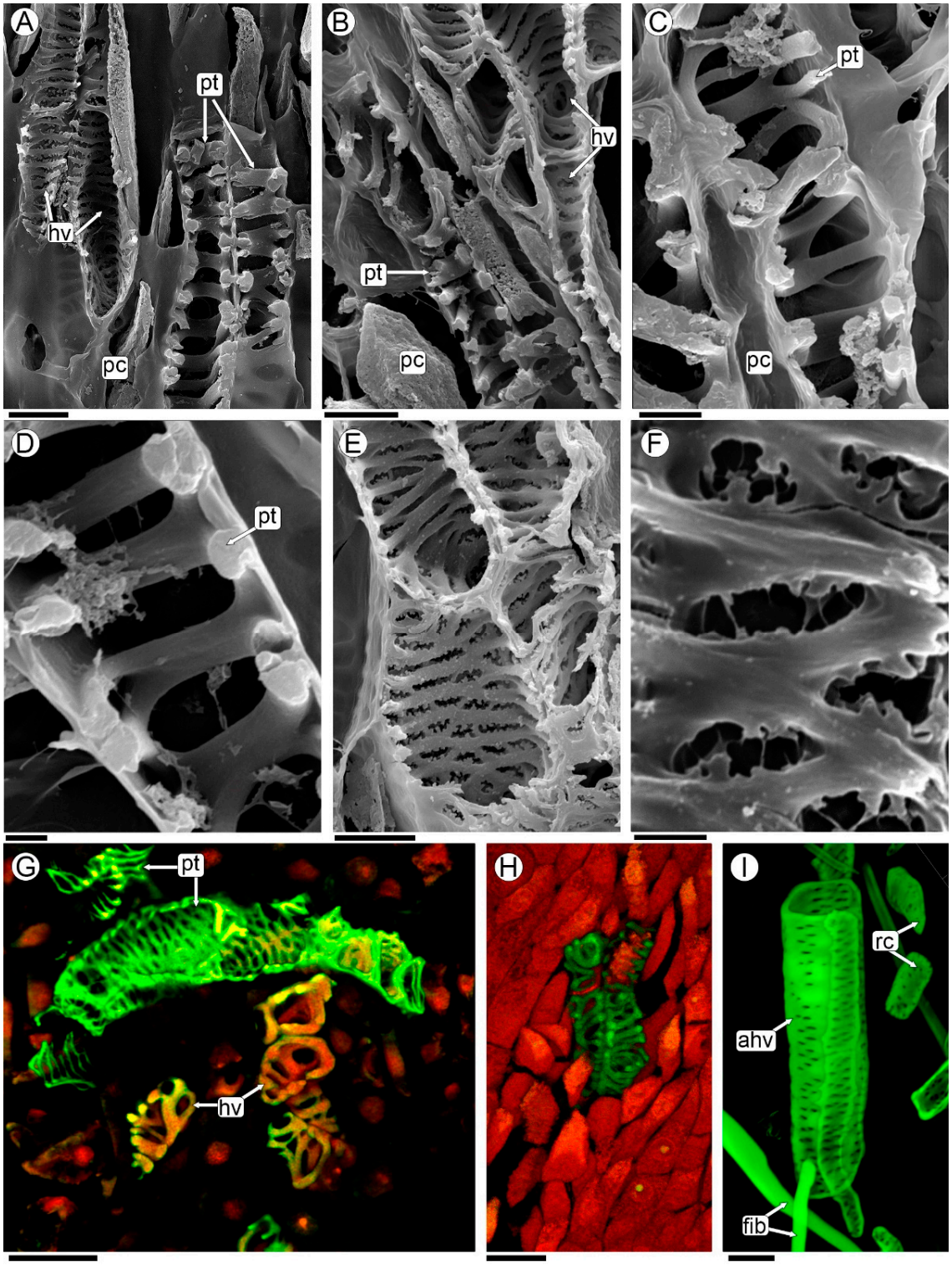

Figure 6. Host vessels and parasite tracheoids of sinkers analyzed with SEM (**A-F**) and CLSM maximum images projection of 30 optical sections at 0.95-µm intervals (**G-I**). (**A-B**) Parasite tracheoids and host vessels separated by parenchymatic cells; (**C**) Parasite tracheoids and parenchymatic cells; (**D**) Detail of tracheoid with smooth ridges; (**E**) Host vessels with vestured pits; (**F**) Detail of vessels with vesture. (**G-H**) CLSM merged two channels images, green: ex/em 503/508–633; Red: 627/635-750; (**G**) Detail of sinker highlighting elements of the parasite and host xylem; (**H**) Tracheoids from the flower of *P. berteroi*; (**I**) Detail of macerated host stem elements from a region not invaded by the parasite, CLSM Green: 503/508–633nm. Abbreviations: ahx: axial host xylem, fib: fibers, hv: host vessel, pc: parenchyma cell, pt: parasite tracheoids, rc: rays' cells, Scales: A-B, E: 10 µm; C: 5 µm; D, F: 2 µm; G-I: 20 µm.

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3. Discussion

The family Apodanthaceae, together with the Cytinaceae, Mitrastemonaceae, and Rafflesiaceae are classified as endoparasitic plants, so named because their vegetative body is reduced to filaments of hyphal-like parenchyma cells, which grow inside their host [6,8,13]. This mode of vegetative body development was named the "endophytic system" by Thoday and Johnson [46], to describe the body of the endoparasite *Arceuthobium pusillum* Peck (Santalaceae). It is only the appearance of the flowers or the fruit scars that makes the presence of the parasite visible [2,47]. Given the peculiarity of this vegetative development, several authors have dealt with the subject in depth [6,15,16].

The endoparasitic *P. berteroi* are notable for invading only those parts already in secondary development. The endophyte's presence and the emergence of flowers are consistently observed at the same distance from the apex of *A. trijuga* branches. This phenomenon was initially described in dwarf mistletoes (*Arceuthobium*) as "isophasic parasitism", meaning it grows at the same rate as its host [48]. This pattern aligns with observations in other species of the *Pilostyles* genus [14,25,26,29]. Despite this isophasic growth, in *P. berteroi* the floral primordia and flowers at anthesis are observed together in the apical region of the infested zone. This phenomenon could be explained by the structure of the endophyte, characterized by multiple meristematic patches capable of generating floral meristems, or even by the presence of multiple individuals of *Pilostyles* parasitizing the same branch of *Adesmia*. However, this last hypothesis cannot be corroborated with the methodology used in the present study.

In general terms, the anatomy of healthy wood of *A. trijuga* agrees with that described for other species (*A. boronioides*, [44], *A. rahmeri* and *A. spinosissima* [45]). None of these studies included electron microscopy observations, nor the structure of the cambium or phloem. Roig [49] described the presence of vessels with ventured pits in *A. horrida* Hook. & Arn. that are similar to those described in other Fabaceae, and also the deformation of the cambium and wood due to environmental variations. Amaral and Ceccantini [23] studied the modifications in the wood of *Mimosa* species caused by *P. blanchetii* and reported that parasitized plants had smaller vessels and characteristics associated with reduced total hydraulic conductivity. Other authors analyse the effects of *P. blanchetii* on *M. maguirei*, included host architecture and reproductive performance [50,51]. In *A. trijuga* parasitized by *P. berteroi,* we describe changes in the cambium leading to increased parenchyma formation at the expense of vessel and fiber production. To verify whether the presence of the parasite affects the life and normal development of *A. trijuga* both structurally and physiologically, new collections and in-depth studies are needed.

In the genus *Pilostyles*, the anatomical features of the endophytic system have been examined in several species, including *P. blanchetii* [23,27], *P. hamiltonii* C.A. Gardner [31], *P. haussknechtii* Boiss. [25] and *P. thurberi* A. Gray [29,30]. Although various species of *Pilostyles* parasitize several genera within the Fabaceae family, the studies referenced above highlight their distinct common characteristics in terms of endophytic structures. First, parasite cells are easily recognised by their large nuclei, usually with two nucleoli, and dense cytoplasm, which resemble meristematic cells. Second, these cells invade only the parts of the plants that have undergone secondary growth. Finally, the cells that form the vegetative body of *Pilostyles* are organized into an endophytic system consisting of clusters, nodules or meristematic patches (mp) of cells growing exclusively between the elements of the secondary phloem of its host, these mps are composed exclusively of parenchyma cells without xylem or phloem elements.

The only previous study of *P. berteroi* growing on *A. trijuga* has been published by Kummerow [28], who briefly describes the endophyte restricted to the cortex of the host, consisting of cells rich in cytoplasm and with large nuclei. Kummerow [28] states that these descriptions are based on the results of previous studies of *P. blanchetii* and *P. hausknechtii* by Endriss [27] and Solms-Laubach [25,26] respectively. Our observations of the endophytic body of *P. berteroi* are consistent with previous studies on other species of this genus. The cells of the endophytic system of *P. berteroi* are extremely similar to a meristematic one, given their large nucleus, which occupies a large part of the cell volume, and the absence of a developed vacuolar system. These traits have been observed and discussed for Rafflesiaceae [16] and endoparasitic plants in general [6]. Even it was possible to

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appreciate the presence of double nucleoli in the cells of *P. berteroi*, a characteristic previously mentioned for *P. thurberi* [30].

The origin of flowers in all *Pilostyles* species studied is endogenous. Solms-Laubach [25] described the mass of tissue embedded in the host phloem that gives rise to flowers as the "floral cushion." Contrary to earlier reports [25,27,31], our observations of *P. berteroi* revealed the polarization of meristematic patches and the subsequent formation of floral meristems from these patches. Kuijt [30], studying *P. thurberi*, was the only author to show some degree of specialization of the endophyte. He recognized three cell types in larger cortical strands and radial sinkers using transmission microscopy. Two cell types are differentiated by the density of cytoplasm and degree of vacuolization, while a sieve element identifies the third cell type. This author did not observe xylem elements in sinkers, nor did he recognize the histology of those portions of the endophyte that give rise to floral meristems. We were unable to identify such cell types in the mps or sinkers of *P. berteroi* with the techniques used in the present study. However, our observations have shown the same difference in vacuolization that identifies two cell types in the mp that give rise to floral meristems. The study carried out here did not allow differentiation of the sieve elements described, so ultrastructural analysis by transmission microscopy would be required.

It is only after the floral meristem is formed and develops whorl primordia that the connection with the xylem of the host is established. These connections were named sinkers [52,53]. Kuijt [53], defined them as "*a portion of an endophytic system which extends radially into host tissues*" and cited them, especially for the genus *Pilostyles* following Rutherford [29]. The formation of sinkers is a common feature of *Pilostyles* parasitism, previously described for other species [23,25,27–29,31]. In the *Pilostyles* species examined in the studies cited above, it was possible to describe that the sinkers penetrate the xylem of the host, often following a ray. In the analysis of *P. berteroi* presented here, the sinkers only reach the cambium, without penetrating the xylem of Adesmia. Kummerow [28] states that he has not been able to prove the presence of sinkers, only that he has seen individual cell threads that have broken through the cambium and penetrated the xylem or ray, without describing their histology.

The sinkers of *P. berteroi* have a peculiar structure: they are composed of parenchyma and tracheoids of *P. berteroi* mixed with vessels of *Adesmia*. These vascular elements are not in direct contact, they are separated by parenchymal cells. Several authors have reported the presence of vascular elements in sinkers of *Pilostyles*, always discussing whether they are tracheids or modified vessels [21,23,25–27,29]. In his study of *P. berteroi*, Kummerow [28] mentions the presence of so-called tracheids (in german: *tracheiden*] only for the "large haustorial complexes", i.e. sinkers. According to the current bibliography, these tracheary elements are classified as tracheoids, resembling tracheids in their pitted or spirally thickened walls but differing in their form, size, and general topography [54,55].

Tracheoids are common elements at vein endings, either in foliar leaf laminae or vascular terminals of nectaries [55–57]. These cells are also described associated with parenchyma in transfusion tissue in several organs, such as staminodes and petals [58], hilum of seeds of papilionoid legumes [59], stylopodium in *Bulbostylis* [Cyperaceae) fruits [60] and they even occur outside the vascular bundles in diverse tissues of epiphytic orchids [61] and ferns [62]. Despite being found in various locations, it has been hypothesized that tracheoids function as water storage cells, or to maximize apoplastic transport, like in Orchidaceae [63]. Considering that the holoparasite requires both nutrients from the host phloem with which it is in intimate contact and water, it is hypothesized that tracheoids here play the role of increasing apoplastic transport.

This is the first time that tracheoids are described together with vessels in the haustorial connection between a *Pilostyles* species and its host. Using SEM and CLSM it was possible and easy to differentiate the tracheoids of the parasite and vessel of host. Although the tracheal elements of *Adesmia* within the sinkers are somewhat irregular compared to those in the uninfested wood, the presence of perforation plates identifies them as vessel elements. The presence of vestured pits in the vessels of *Adesmia* is another typical characteristic of legumes [64,65], which allows them to become fully recognized within the sinker.

 doi:10.20944/preprints202409.1881.v

The vascular cells in the synkers of the *P. berteroi*/*A. trijuga* system (tracheoids and vessels) and the parenchyma cells between them form a special type of transfusion tissue. This structure is consistent with the historical and histological definition of a "chimera" [66]. Chimeras have been previously described to exist in other holoparasites [4,67]. The interface between host and parasite in the tubers of the Balanophoraceae shows two main patterns, although there are also intermediate forms. In some species, the interface is discrete. The boundaries of the two individuals are well defined, as in *Helosis* and *Lophophytum* [68,69]. In other genera, such as *Balanophora* and *Langsdorffia*, the host/parasite interface is diffuse, and the vascular tissues of the host penetrate the tuber of the parasite, forming chimeric vascular filaments composed of parasite and host cells [70–73]. In *P. berteroi*, the sinkers are a highly modified chimeric structure, similar to that described in the tuberous organ of Balanophoraceae. However, there is no tissue hypertrophy in the *P. berteroi*/*A.trijuga* system leading to the formation of the typical galls or woodrose of Balanophoraceae.

The flowers of *P. berteroi* obtain their nutrients and water through these chimeric sinkers, as they have greater needs than the meristematic patches of the vegetative endophyte. Only further ultrastructural analysis will reveal whether there is a direct or indirect connection (via parenchyma) between the vascular elements of both taxa. Such analysis would also allow us to clarify whether the parenchymal cells described here between vessels and tracheoid are transfer cells as reported in other parasites [74,75].

4. Materials and Methods

4.1. Plant (Parasite and Host) Material

Branches from two different populations of *A. trijuga* parasitized by *P. berteroi* were selected and fixed in FAA (formaldehyde, 70% alcohol, acetic acid, 5:90:5). Plants of *A. trijuga* without holoparasites were also collected for anatomical comparison. The material studied, localities, voucher data, and herbaria are detailed in Appendix A.

4.2. Light Microscopy (LM)

To assess the degree of colonization by *Pilostyles*, *Adesmia* branches with flowers of endoparasites were selected and samples were collected up to the apex of the *Adesmia* stems. Both healthy and parasitized *Adesmia* branches, were subjected to histological dehydration followed by a tertiary butanol series [76] and then to paraffin infiltration [77 Johansen [77]. Serial transverse and longitudinal sections were cut with a rotary microtome to a thickness of 10-12 µm (MICROM). All sections were stained with Safranin-Astra blue [78] and mounted in synthetic Canada balsam. A Leica DM LB2 microscope equipped with a digital camera was used for observation and photography.

The histochemical tests used were Lugol (starch), Nile Blue, Sudan III and Sudan Black (oils, suberin, wax, cutin), Cresyl Blue 1% (mucilage), Coomassie Brilliant Blue and Xylidin Ponceau 2R (proteins), Toluidine (phenols and pectins), and ferric chloride, (soluble polyphenols) [77–80].

4.3. Scanning Electron Microscopy (SEM)

Paraffin-embedded sections of parasitized *Adesmia* stems were also processed for SEM. Serial sections of 30 µm thickness were cut and mounted on glass slides using Haupt's adhesive [81] and dried for 48h. Sections were deparaffinized in xylol, dehydrated in an ascending series of acetone, and dried to the critical point in CO2. To perform critical point drying, the slides used were pre-cut (4 cm in length) so that they could be placed in the chamber of the critical point drying unit (Denton Desk II). The specimens were gold-palladium coated and observed at the Electron Microscopy Service of the UNNE, Corrientes, using a Jeol LV 5800 SEM at 20 kV.

4.4. Confocal Laser Scanning Microscope (CLSM)

For the analysis by CLSM, two types of preparations were carried out. Paraffin-embedded material cut at 12 µm thickness (see technical LM) was deparaffinized and mounted in pure glycerol

 doi:10.20944/preprints202409.1881.v

without staining. Additionally, samples of the interface between the host and the base of the parasite flowers were extracted under stereoscopic microscopy (avg. 5 mm × 5 mm × 5 mm) and subjected to maceration with a solution of hydrogen peroxide (30%), distilled water (DW) and glacial acetic acid (>99.8%, V: V: V = 1:4:5) and kept in the oven (60 °C) for 48hs according to Chatelet et al. [82]. The macerated samples were rinsed three times with DW, stained with 1% Safranin O in DW for 20 minutes, and then washed with DW until the wash solution was clear. After staining, small portions of cells were put on a glass slide on a drop of pure glycerol, the cells were separated with a needle under a stereomicroscope and covered by a cover glass.

Confocal imaging was acquired with a Stellaris 8 White Light Laser (Leica Microsystems) inverted confocal microscope using HC PL APO 10× dry (NA 0.4), and 63×oil immersion (NA 1.4) objectives. Excitation/emission wavelengths were set: Blue: 405/413–489, Green: 503/508–633, Red: 627/635-750nm. The LAS Navigator software is used in conjunction with a 63x objective to view and merge tiles into a single image. Images and three-dimensional (3D) reconstructions were performed with Leica LAS X Stellaris Compass software. Serial optical sections were taken in the xyz mode to image the specimens in three dimensions (3D), from the surface down to the required depths.

5. Conclusions

This study provides an in-depth analysis of the micromorphological characteristics of the endophyte, the origin of the flowers, and the structure of the chimeric sinkers of *P. berteroi* growing inside *A. trijuga* stem. These characteristics have not been studied in such detail before. This is in contrast to the earlier work by Kummerow [28], which only gave a brief overview of these features. There is considerable uniformity of characters among the various species of the genus that have been studied, as noted in a study of the flowers of *P. berteroi* (Romero MF, Sato H & Gonzalez AM, unpublished manuscript]. The different species subject to the most extensive study (*P. boyacensis, P. blanchetii, and P. berteroi*) show a striking convergence of floral and embryological characteristics. The extensive reduction in holoparasites results in a lack of consistent morphological characters for differentiation [21,27,37]. Consequently, the host taxon has been included as a criterion for distinguishing *Pilostyles* species [14]. In this study, several previously undescribed differences were identified, prompting the question: ¿Does the host determine the structure of the parasite? It appears that the host species indeed influences the structure of the endophyte. The data collected so far suggest new research directions and a future comparative study of all hosts and the different *Pilostyles* species that parasitize them could help answer the questions raised by these fascinating plants.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Stems of *A. trijuga* parasitized by *P. berteroi.* (A) Longitudinal section of initial stages of infestation. (B-F) cross-section showing changes in the cambial zone and mp. Abbreviations: cz: cambial zone, fi: fibers, mp: meristematic patches, pe: periderm, ph: phloem, ra: rays, ve: host vessels, xy: secondary xylem. Scales: A-B, E-F: 100 µm; C-D: 10 µm. Figure S2: Stems of *A. trijuga* showing floral primordia and flowers at various stages of development. Abbreviations: ch: collapsed host cells, fp: flower primordium, mp: meristematic patches, pe: periderm, sk: sinker, xy: xylem. Scales: A: 20 µm; B-C: 100 µm; D-F: 0.5 mm. Figure S3: Host vessels and parasite tracheoids. (**A-C**) Tracheoids from the flower of *P. berteroi*, CLSM images of maximum projection of 70 optical sections at 0.45-µm intervals (Green: 503/508–633, Red: 627/635-750nm, (**A**) image digitally colored in red, (**B**) image digitally colored in green, (**C**) Overlaid images from Figures (A) and (B). (**D-F**) LM images of sinkers showing parasite tracheoids and host vessels intermixed, separated by parenchymatic cells. Abbreviations: ca: cambium, hv: host vessel, pc: parenchyma cell, pt: parasite tracheoids. Scales: A-E: 20 µm; F: 50 µm.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Material studied, localities, data of the vouchers, and herbaria. Pilostyles berteroi: Argentina. Jujuy. Tilcara. 23°33'48.7"S 65°23'59.7"W 2562 msm. 9/05/2015. Sato HA 444 (JUA). Idem 4/12/2015. Gonzalez AM & Sato H 497 (CTES). Idem. Gonzalez AM & Sato H 498 (CTES). Idem. Tumbaya. Volcán. 23 56' 18"S 65° 27' 47,5"W 2021 msm. 4/12/2015. Gonzalez AM & Sato H 496 (CTES). Idem. 20/11/2021. Sato HA 480 (JUA). Idem. 20/11/2022. Sato HA 497 (JUA).

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