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Post-seminal structure and development of the hemiparasitic plant *Escobedia grandiflora* (Orobanchaceae)

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ABSTRACT. *Escobedia grandiflora* (L.f.) Kuntze is a wild hemiparasitic plant with orange roots. Little is known about the development of initial parasitism with the host, despite the significant value of roots for Central and South American communities. Therefore, this study aimed to characterize post-seminal structure and development of *E. grandiflora* in *Pennisetum purpureum* host. To analyze the structure and development of *E. grandiflora*, seedlings, stems and roots samples were processed and examined under light, confocal and scanning electron microscopy. *Escobedia grandiflora* seeds are composed of seed coat, perisperm, and embryo. Emergence of the radicle began eleven days after imbibition. Seedlings showed a root hair collar encircling the axis at the root-hypocotyl junction with elongation of internal cortical cells. Seedlings formed haustoria and successfully reached of the host roots 22 days following root emergence. In the root many starch grains were observed, albeit more scarce in the hypocotyl. After 43 days of root emergence, the seedling stage was finished with the formation of the definitive leaves, and star of the plant stage. After 64 days, root ramification, amount of starch, and orange pigmentation increased with formation of haustoria. The developmental pattern of *E. grandiflora* plants was slow, but the roots grew faster than the stem. *Escobedia grandiflora* seeds were not endospermic and have limited nutritional value. After root emergence, the young seedling must develop roots and starch storage towards to haustorium formation and attachment to host roots.

Keywords: parasitism; seed; seedling survival; orange roots; haustorium.

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Introduction

Parasitic plants comprise an intriguing group characterized by the invasion of host tissues with an organ called the haustorium, which draws water and both inorganic and organic elements from the host (Heide-Jørgensen, 2008; Pielach, Leroux, Domozych, Knox, & Popper, 2014). A large body of knowledge supports the importance of parasitic plants of the Orobanchaceae, a family which includes the largest number of angiosperm root parasitic plant species (Bennett & Mathews, 2006). Some Orobanchaceae genera, such as *Striga* and *Alectra* have a negative impact on crops, like maize, cowpea, and sorghum (Kokla & Melnyk, 2018). Other species are important in natural communities, while a few species are used in medicine and food processing (Ren, Guan, Li, Hu, & Zhang, 2010; Muriel, Cardona, Arias, & Gómez, 2015). The Chibcha civilization was the first culture to recognize the use of hemiparasitic *Escobedia grandiflora* (L.f.) Kuntze (Pennell, 1931), which has orange-colored roots. The roots of this species have been used as a food coloring and as a treatment for liver disorders (Pennell, 1931; Muriel et al., 2015).

Evidence suggests that seedlings represent the most susceptible stage in the parasitic life cycle (Press, 1995; Phoenix & Press, 2005), because those seeds have a low amount of resources, limiting seedling growth; endangering the haustoria formation, and penetration in host tissue, and thus putting seedling survival at risk (Heide-Jørgensen, 2008; Joel & Bar, 2013). For their establishment into hosts, *Alectra vogelii* and *Striga gesneroides,* these plants must concentrate all resources toward growth of the radical system, which is necessary in order to establish a rapid contact with a host tissue for removal of water and nutrients (Okonkwo & Raghavan, 1982). Other studies reporting on parasitic plants belonging to different genera of Orobanchaceae, such as *Alectra*, *Striga*, and *Orobanche*, reported that those seeds require germination stimulants (Cardoso, Ruyter-Spira, & Bouwmeester, 2011; Joel, Chaudhuri, Plakhine, Ziadna, & Steffens, 2011).

Plant-parasitic researches are focused on parasitic weeds owing to impact on agriculture; yet, the studies into other areas of this issue are few. Cardona-Medina and Muriel (2015) investigated aspects of seed germination, seedlings and development of *E. grandiflora* plants, stating that plants need a host for survival. However, the investigation founded a low survival rate, even in the presence of the host, which points to gaps in our knowledge about the initial development of *E. grandiflora*.

This genetic resource has significant value, yet, little is known about its initial development. Therefore, it is necessary to advance in basic scientific understanding of this critical initial development stage of parasitic plant. Therefore, based on analyses with light, confocal and electron microscopy, the present study aimed to characterize the post-seminal structure and development of the hemiparasitic *E. grandiflora*, during the establishment of parasitism on the *Pennisetum purpureum* roots; in order to identify aspects that might influence the survival of initial development for parasitic seedlings and plants.

Material and methods

Plant material: *Escobedia grandiflora* fruits were collected in two natural populations in Campos Novos (27° 18.414´ S, 051° 11.728´ W) and Água Doce (26° 36.944´ S, 051° 29.847´ W), both located in Santa Catarina State, Brazil. Naturally grown rootstocks of *Pennisetum purpureum* Schumach. (Poaceae) were collected in the *Centro de Ciências Agrárias*, Florianópolis, and used as a host based on its rapid growth and strong attachment of *E. grandiflora* haustoria (Cardona-Medina & Muriel, 2015).

Assays: We sowed *E. grandiflora* seeds on to moistened absorbent paper at a temperature of 25°C and 12 hours light-1 photoperiod within a growth chamber (Cardona-Medina & Muriel, 2015). To characterize general aspects of seed and penetration of the radicle through the surrounding seed tissues (Assay 1), *E. grandiflora* seeds were collected at six, ten, eleven and fifteen days after imbibition, according to germination *sensu stricto*, as detailed by Bewley, Bradford, Hilhorst, and Nonogaki (2013). To determinate seed structures, sections were compared with the structures of other seeds in Orobanchaceae, reported by Joel and Bar (2013) and Joel et al. (2012). To describe *E. grandiflora* post-seminal structure and ultrastructure with *P. purpureum* host (Assay 2), the rhizome of host plants was grown over a period of 30 days in 338 cm3 (8×6.5×6.5 cm) pots before sowing parasite seeds in a greenhouse. The pots were filled with a mixture of vermiculite and commercial substrate (Tropsustrato HA-Hortaliças®, Mogi Mirim, Brazil) (1:1). Four seeds, previously imbibed in water for 5 days, were individually sown in pots containing a host plant in each pot. For characterization of stems, leaves, and roots samples were collected at 15, 22 and 64 days after the emergence of the seedling root.

Histology: Samples were fixed using a solution containing 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer. After fixation, samples were dehydrated in an ethanolic series according to Ruzin (1999). Samples were infiltrated and then embedded with Historesin® (Leica, Heidelberg, Germany), following a protocol suggested by Gerrits and Smid (1983). Cross and longitudinal 5 μm sections were cut with an RM 2125RT rotating microtome (Leica®, Nussloch, Germany) and stained with toluidine blue (O'Brien, Feder, & McCully, 1964) for characterization and with lugol in order to observe the presence of starch grains (Johansen, 1940). Sections were examined with a BX-40 microscope (Olympus, Tokyo, Japan), and images were taken with a DP71 digital camera (Olympus, Tokyo, Japan).

Scanning electron microscopy (SEM): Samples were fixed and dehydrated, as described above, followed by critical point drying with CO_2 in an EM CPD030 (Leica®, Heidelberg, Germany), according to Horridge and Tamm (1969). Dried samples were fastened to aluminum supports using double-sided carbon tape and covered with 30 nm gold-palladium film in an high vacuum sputter coater EMSCD500 (Leica®, Vienna, Austria). Evaluations were carried out with the JEOL XL 30 scanning electron microscope (JEOL, Tokyo, Japan).

Confocal laser scanning microscopy: Five days after radicle emergence, seedlings were fixed using a solution containing 2.5% paraformaldehyde in 0.1 M sodium phosphate buffer and then evaluated using a DMI600B TCS SP-5 confocal laser scanning microscope (Leica®, Germany). Excitation and emission wavelengths were set to 488 and 412 to 501 nm, respectively. Image processing was performed with LAS-AF Lite software (Leica®, Mannheim, Germany).

Results

Seed and seedling: The mature seeds were composed by three recognizable regions (Figure 1A-D): the seed coat, including seminal tegument, perisperm and the embryo. The embryo occupied the central part of the seed and was composed of a hypocotyl-radicular axis and two cotyledons. The apex of the radicle was not yet well developed, and densely cytoplasmic cells were observed in the cotyledons (Figure 1B). In the perisperm, we found a simple layer of densely cytoplasmic cells with thick exterior wall (Figure 1C-D). Endosperm traces were verified, but some seed regions were imperceptible (Figure 1C-D). *Escobedia grandiflora* root emergence began eleven days after imbibition (Figure 1E-G), ending the germination phase and giving rise to seedling growth (G-I). In principal root, root apex had slow development (Figure 1G). In the proximal region of root there developed an annular area densely covered by hairs was verified (Figure 1H-I). However, other hairs were present along the root, as well (Figure 1J). In the rootcap, mature xylem elements were noted near the promeristem region (Figure 1J).]

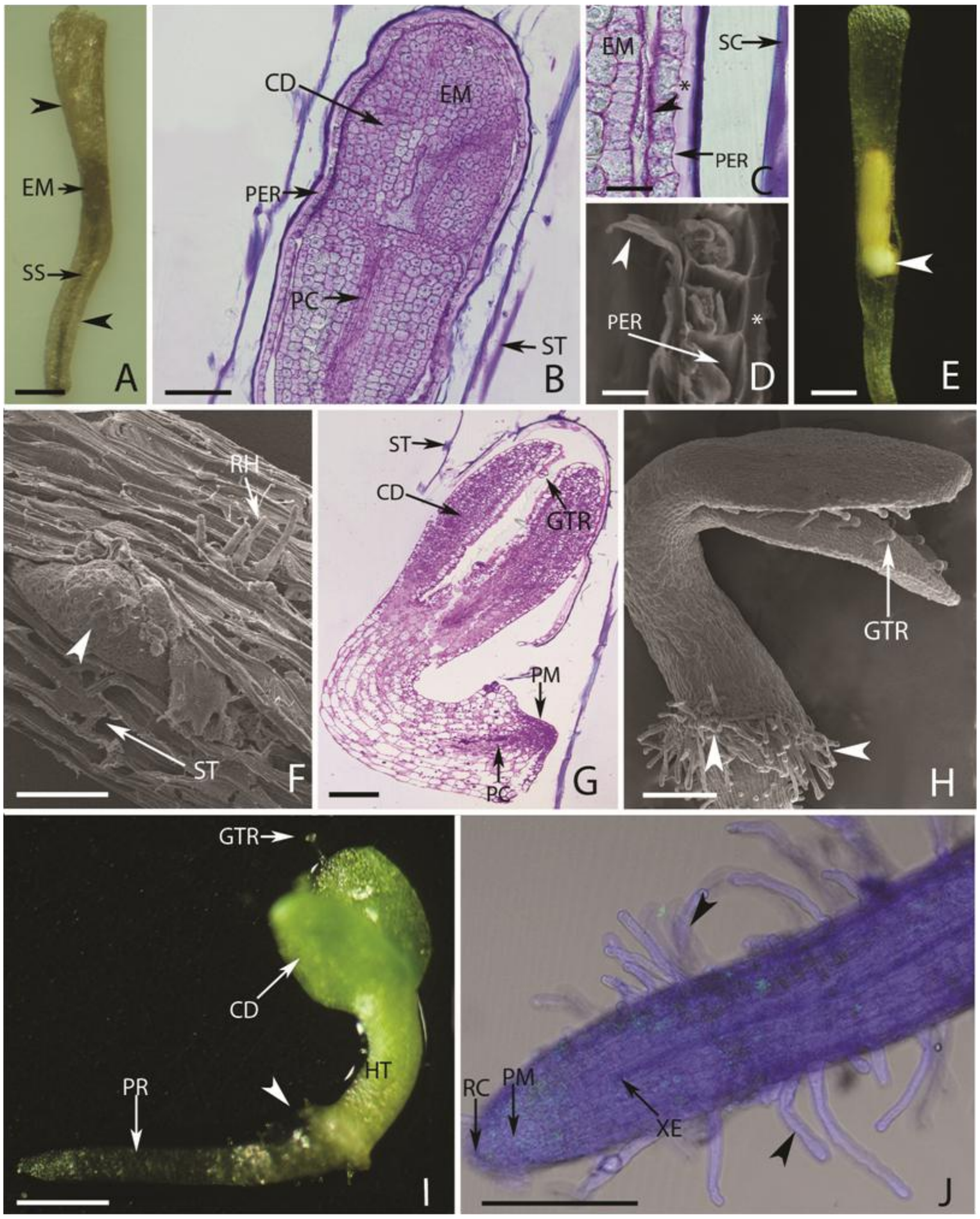

Figure 1. Seed (A-D) and seedling (G-J) morphoanatomy of *Escobedia grandiflora*. Scanning electron micrograph (D, F, H), longitudinal sections in light microscopy (B, C, G); stereoscopic (A) overview of seed, embryo and suspensor observed for transparency and the seminal tegument involving the entire structure (short arrow); (B) internal embryo structure; (C-D) seminal tegument and perisperm detail, including the presence of endosperm traces(short arrow); (E) seed with root emergence (short arrow) (F) root emergence detail (short arrow), showing the seminal tegument and root hairs; (G) seedling emergence detail; (H-I) seedling with glandular trichomes in the adaxial surface of cotyledons and root hairs in root proximal zone (short arrows); (J) seedling root detail with root hairs (short arrow). Abbreviations: CD = Cotyledons; EM = Embryo; GTR = Glandular trichome; HT = Hypocotyl; RH = Root Hair; PR = Parasite root; PC = Procambium; PER = Perisperm; PM = Promeristem; RC = Rootcap; ST = Seminal tegument; SS = Seed suspensor; XE = Xylem elements. Scale: (A, E, I) = 500; (B, F) = 100; (C) = 20; (D) = 10; and (G, H, J) = 200 µm;

Seedling and initial parasitism: Soon after the emergence of the radicle (Figure 1E, I) the root lacked pigmentation. Fifteen days after the beginning of root emergence, we detected initial root pigmentation (Figure 2A). In a number of cross sections, the hypocotyl tissues were homogeneously distributed, with the vascular cylinder, cortex and epidermis. The vascular cylinder was composed of pericycle, formed by a ring of cells, smaller in size than the cells of the endoderm, and vascular elements arranged in a form similar to that of the root, with two protoxylem strands (diarch) alternating with phloem strands (Figure 2B). In the root proximal zone (Figure 2C), the vascular cylinder showed a structure similar to that described for the hypocotyls. We noted peripheral swelling, a manifestation of the elongated cortex, parenchyma and endoderm cells. In the internal structure, the protoxylem was observed to alternate with phloem, and root branching also occurred (Figure 2A, D).

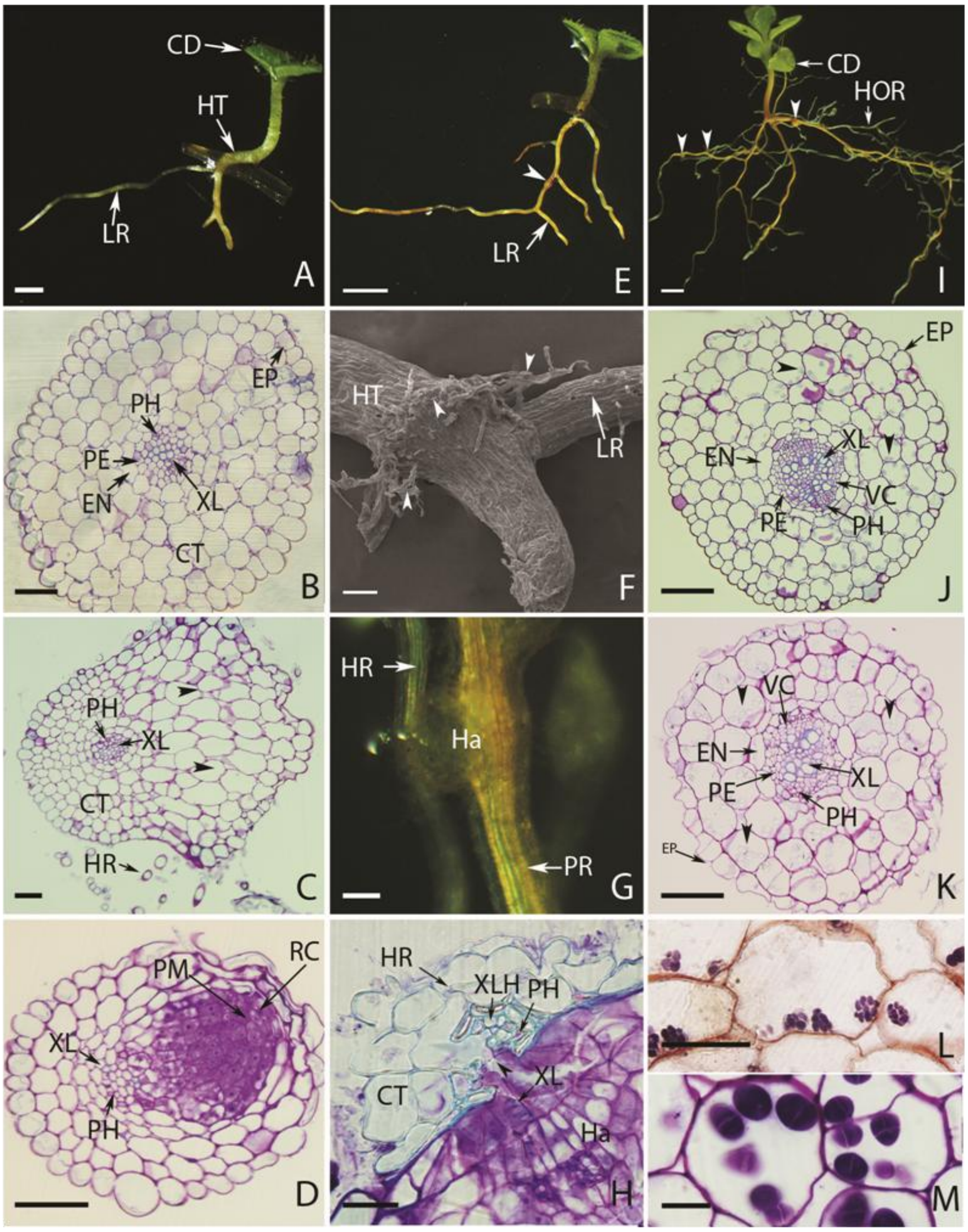

Figure 2. *Escobedia grandiflora* morphoanatomy in the presence of host *Pennisetum purpureum* for 15 (A-D), 22 (E-H), and 64 days (I-M). Cross sections in light microscopy (B-D, H, J-M), stereoscopic microscopy (A, E, G, I), and scanning electron microscopy (F). *Escobedia grandiflora* seedlings (A) and plants with haustoria attached on roots of the host (short arrows) (E, I), hypocotyl internal structure in the seedling (B); root proximal zone structure of seedling (C); root structure with initial root branching in the seedling, consisting of cells with visible nucleus and dense cytoplasmic content (D); root hairs in root proximal zone (short arrow) (F); *E. grandiflora* haustoria attached to *P. purpureum* root (G); vascular connection between haustoria and host root (short arrow) (H); stem structure (J); root structure with starch grains in cortical cells (short arrow) (K); detail of starch grains in cortical stem cells, (L) and in cortical root cells (M), stained with toluidine blue and lugol. Abbreviations: CD = Cotyledon; CT = Cortex; EM = Endoderm; HR = Host root; HT = Hypocotyl; LR = Lateral root; PE = Pericycle; PH = Phloem; PM = Promeristem; PR = Parasite root; RC = Rootcap; VC = Vascular cambium cells; XL = Xylem elements. Scale: (A, E, I) = 2 mm; (B-D) = 50; (F, J-K) = 100; (G) = 200; and (H, L-M) = 20 µm.

At 22 days after root emergence, the seedlings showed secondary haustoria only in principal root, and was not developed primary haustoria (Figure 2E). Some developed haustoria began to attach to *P. purpureum* roots (Figure 2E-G), and haustorium endophyte penetrated within the host´s tissue connecting to host vascular elements and causing fragmentation of the surface (Figure 2H). Therefore, *E. grandiflora* parasitism on *P. purpureum* appeared to begin at 22 days post-root emergence.

On day 64 after radicle emergence, the parasitic root system grew faster with more ramification compared to previous developmental stages and more haustoria attached to host root. Roots, mainly older, showed strong orange pigmentation (Figure 2I). As shown in Figure 2J-K, we saw evidence of vascular cambium cells, even though no evidence of secondary xylem and phloem elements was found through histological analysis. We observed the accumulation of starch in the stem and root cortex of *E. grandiflora* (Figure 2L-M).

Presence of the primary definitive leaves was verified about 43 days after root emergence (Figure 3). This stage was indicative of the transition from seedling to plant, and it was possible to detect three foliar types; cotyledons, eophylls, and definitive leaves (Figure 3A-B). The three foliar types presented both glandular and non-glandular trichomes (Figure 3A, C-D) on both adaxial and abaxial surface. Glandular trichomes were visible after radicle and cotyledon emergence (Figure 1I-J). Non-glandular trichomes had a sharp apex and were visible 15 days after radicle emergence.

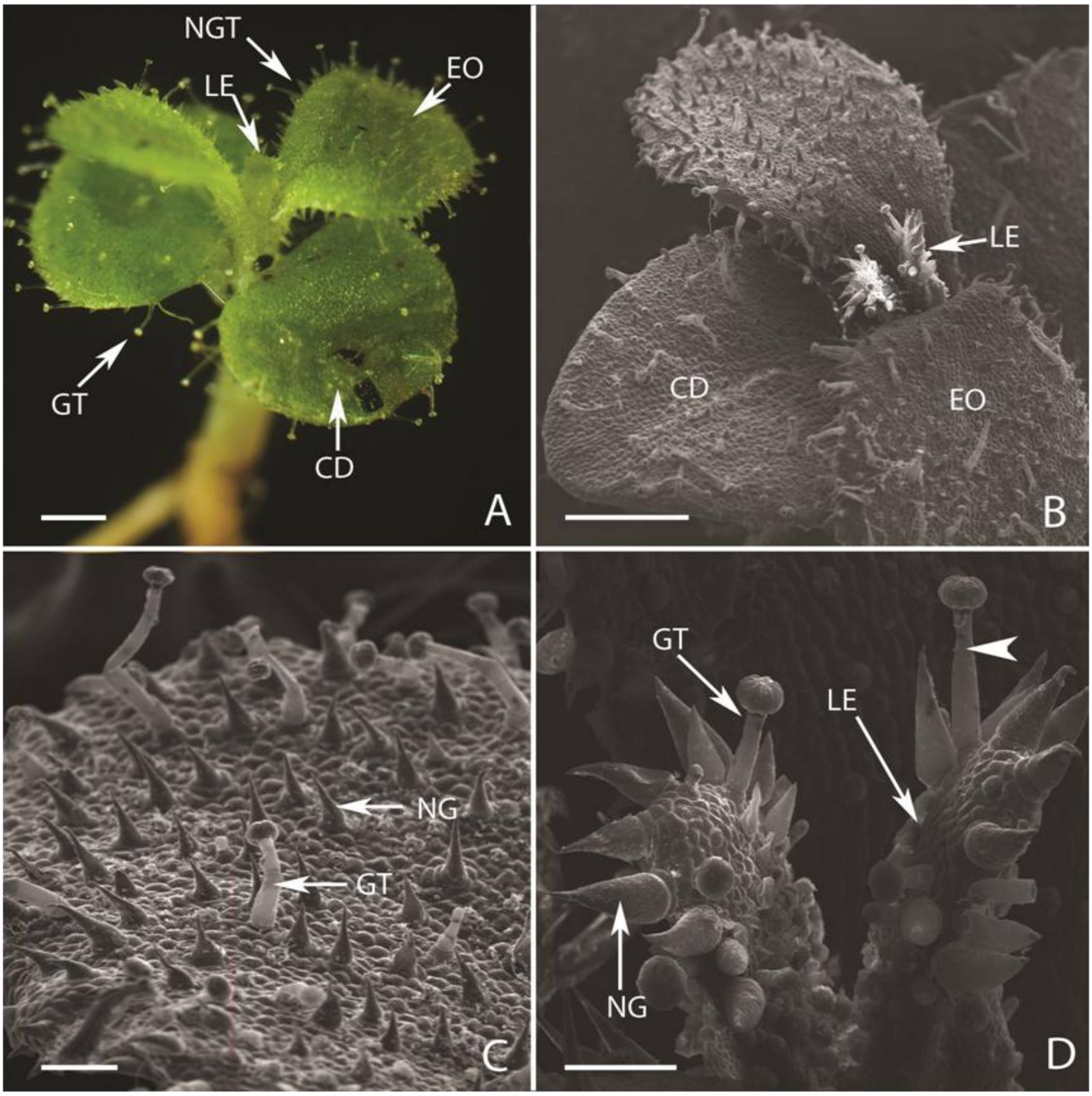

Figure 3. Leaf surface of *Escobedia grandiflora*. Stereoscopic microscopy (A); scanning electron microscopy (B-D). *Escobedia grandiflora* plant with evidence of definitive first leaves developed about 43 days after radicle emergence (A); adaxial surface of the three foliar types (B); adaxial surface of the eophyll with glandular and non-glandular trichome details (C); definitive first leaves developed (D). Abbreviations: CD = Cotyledons; EO = Eophyll; GT = Glandular trichome; LE = leaf; NG = Non-glandular trichome. Scale: (A-B) = 500; and (C-D) = 100 µm.

Discussion

The research elucidated the post-seminal development of *E. grandiflora*. We founded a presence of a reserve for nutrients in the perisperm and cotyledons. Perisperm is also an important reserve tissue in other Orobanchaceae seeds, including such genera as *Striga*, *Alectra*, *Pheliphanche*, *Orobanche*, and *Aeginetia* (Joel et al., 2012; Joel & Bar, 2013). Seed reserve tissues accumulate carbohydrates, proteins and lipids, during germination these reserves are degraded and mobilized to different parts of the embryo (Sert, Bonato, & Souza, 2009). Perisperm cells are involved in the transfer of reserve nutrients to the embryo during germination (Joel et al., 2012). The cotyledons, the first leaves of the embryonic axis, have a role in photosynthesis, but also function as nutrient reserves (Souza, 2009). For germination *E. grandiflora* seeds only have reserves in the perisperm and cotyledons. However, according to Joel and Bar (2013), the low amount of seed resources limits the parasitic seedling growth. After germination, therefore, the survival of *E. grandiflora* seedlings depends on their own root absorption and host parasitism.

Escobedia grandiflora seedling root showed mature xylem elements near the promeristematic region. It can be argued that this feature contributes to the slow growth of the roots, as claimed earlier by Esau (1959). This interpretation is in agreement with other studies. For example, in the principal root of *A. vogelii* and *S. gesnerioides* seedlings, mature xylem elements were far from the promeristem region (Okonkwo & Raghavan, 1982), resulting in relatively fast root growth (Dörr, 1997). Despite the slow growth of *E. grandiflora*, the trend is toward faster root growth, compared to that of stem, since the parasite's root must develop haustoria for attachment to the host.

In addition, since *E. grandiflora* forms only secondary haustoria after the formation of the main root, it needs more time to reach the parasitism phase in the host, when compared to parasite species that form primary haustoria. Also, because of the need to use its own resources to form the main root first, growth and the seedling survival could be affected. Conversely, after seed emergence, *Striga* seedlings develop primary haustoria. This haustorium type is developed in the root apex, allowing fast parasitism and increased seedling survival (Dörr, 1997; Hood, Condon, Timko, & Riopel, 1998).

The faster root growth compared to that of stem is related to the presence of starch grains, which is characterized by a higher proportion in the root and haustoria cortex than stem. Starch storage in the root and haustoria, as founded in roots of *E. grandiflora*, is key to parasite survival because it supports plant growth, flowering, and seed production, even after the death of the host (Joel & Bar, 2013).

Parasitism takes place after seedlings have formed cotyledons, and leaf formation occurs after development of the many haustoria and its attachment to host root. In the present study, the development of first leaves occurred 43 days after root emergence. According to Souza (2009), leaf development indicates the conclusion of the seedling stage and the beginning of the plant stage. Therefore, this developmental time would be the most critical stage for *E. grandiflora* conforming to the definitions of Press (1995); Phoenix and Press (2005); Těšitel, Lepš, Vráblová, and Cameron (2011).

Trichomes of cotyledons, eophylls and definitive leaves of *E. grandiflora* present morphological features similar to the leaf trichomes of the parasitic plant *Orobanche ramose* L (Orobanchaceae) (Sacchetti, Ballero, Serafini, Muzzoli, & Tosi, 2003). According to the authors, *O. ramose* trichomes showed such chemical compounds as terpene and flavonoids. The trichomes are important for protection against pathogens and herbivorous insects (Glas et al., 2012).

Conclusion

This is the first research to describe the structures of the initial development and successful parasitism of the hemiparasitic plant *E. grandiflora* on the host *P. purpureum*. The main features observed during parasitism were poor seed reserve, fast growth of roots and poor stem development, accumulation of starch grains in the roots and formation of haustoria. The information here in should contribute to better understanding of the development of parasitic plants relative to their establishment in their hosts. In addition, with such basic knowledge, it will be possible to develop strategies of conservation and cultivation of the specie, especially considering the value of this genetic resource.

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